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Assessment of the performance of an ATP based rapid bacterial indicator test on potable water samples.

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Abstract

Introduction: There is an increasing need for more rapid detection methods for drinking water supplies; especially in developing areas. Hygiena International Ltd are a research and development company which have developed an ATP based technology called the MicroSnap (MS) system. MS is currently used in the food industries for the enumeration of bacteria in food samples. The purpose of this study was to determine whether this system could be applicable to the drinking water sector, by investigating the sensitivity and specificity of the product.

Methods and Results: There are four MicroSnap devices, MS Total count, Enterobacteriaceae (EB), Coliform, and *E. coli*. All four systems were analysed using pure bacterial cultures, as positive and negative control strains, to determine if the systems produced accurate and consistent results. Throughout initial testing, MS *E. coli* consistently produced relative light units (RLU) which were an underestimation of the bacterial load in the presented samples. These results formed the hypothesis that the lytic agent (extractant) in the detection devices was only allowing for a low percentage of bacterial cell lysis. The poor cell lysis meant that the detectable biomarker; β-Glucuronidase, was unlikely to be freed from cells in high enough numbers to be correctly quantifiable by the MS *E. coli* detection system.

Therefore, the original extractant was compared to one altered by Hygiena using plate count methods, and within the MS system. The results gained through these tests showed that the new extractant increased the percentage of cell lysis occurring and thus when analysed within the system, produced higher RLU results.

Conclusion: MS *E. coli* demonstrated that it is not yet applicable in the water sector as the WHO guidelines state that detection method must detect down to 1 CFU per 100ml of water. This investigation found that the lower detection limit for the MS system was around 10^1 and 10^2 CFU per ml of water. In order to increase the sensitivity two alterations to the system have been proposed to Hygiena International Ltd. With these suggestions, the system could reduce the current time taken to detect bacteria using traditional methods, down to 7 hours.

Section 1

Section 2

Introduction

Access to safe drinking water is without question, a basic human right, and yet millions of people go without what many of us believe to be a normal aspect of everyday life (World Health Organization, 2019, Griffiths, 2017). The presence of indicator bacteria such as *E. coli* is crucial in determining the quality of a body of water, and the risk to public health (Cabral, 2010). To ensure potable water, an adequate monitoring system is required (WHO, 2019). Conventional detection systems rely mainly on cultivation, which means they are time consuming. Therefore, there is a focus by current research to produce rapid, accurate, reliable and a low-cost detection system for the enumeration of bacterial load in a drinking water sample (Rajapaksha et al., 2019). With emerging technologies such as these, could this be the end to counting colonies?

Hygiena international ltd, have engineered a system called MicroSnap (MS) an ATP bioluminescence device to rapidly quantify microbial content in food samples. The aim of this project is to investigate whether this system could be applicable to the water sector, and if so, is there scope for it to be deployed in places where water monitoring infrastructure is lacking, such as low income and developing areas.

A critical review of the literature surrounding current potable water testing, and the need for a rapid, yet reliable detection method

3.1 Origins of epidemiology

The earliest evidence of water quality monitoring and epidemiology in Britain, was in the nineteenth century. John Snow, now considered one of the founding fathers of epidemiology, investigated an outbreak of cholera in Soho, London. In 1854, the miasma theory of disease persisted, and in a period spanning ten days, and in just one area, cholera had taken over 500 lives. Snow had previously speculated that cholera was not transmitted by air; which opposed scientific view of that time, but by the faecal oral route. The Soho outbreak, which was of unusual magnitude, was the opportunity that Snow needed to test his hypothesis (Brody et al., 2000). Snow's seminal work involved using a map created by Charles F. Cheffins and supplementing it with the locations of cholera deaths in the area (Figure 1), (Shiode et al., 2015). Due to Snow already theorising that cholera was transmitted by 'bad water', he deduced that the source of the outbreak was a local water supply (Bynum, 2013). From the meticulous investigations and plots on the map, Snow deduced that it was the pump on Broad Street that was to blame, as "nearly all the deaths had taken place within a short distance of the pump" (Snow, 1855). His work directly led to the subsequent removal of the pump's handle, and a reduction in cases of cholera. Snow presented his hypothesis on the spread of cholera, supported by his findings from the 1854 outbreak in the second edition of 'On The Mode of Communication of Cholera', this work would later be used as evidence in disproving the miasma theory in place of the germ theory (Halliday, 2001).

Figure 1: Johns Snow's map of the areas of London affected by the 1854 cholera outbreak, each dash represents a death in a household. Red circle: Broad Street Pump, Green dots: Other pumps in the area. (figures adapted from Snow, 1855).

The evolution of disease from the times of John Snow is palpable, including the prevalence of specific diseases, to what is classified as a disease. Up until the end of the 20th century infectious diseases were the largest contributor to mortality and disability globally (Bertozzi et al., 2017). In developed societies, the threat of infectious diseases such as cholera and TB, have lessened, and have almost been replaced by age-related non-communicable diseases including cancer and cardiovascular disease. It is speculated that this is due to a variety of factors including an ageing population, increased medical resources, improved sanitation and knowledge of diseases and vaccines (Preedy and Watson, 2010, Boutayeb, 2010). However, in other parts of the world such as developing countries the prevalence of infectious diseases is still potent, with children being most affected. 60% of disease burden in low income countries is caused by communicable diseases such as water-borne infections. In contrast only 5% of the burden in the developed world is attributed to communicable diseases (Roser and Ritchie, 2019). Communicable diseases are caused by bacteria and viruses and are spread through contact with either an infected individual, contaminated surfaces, food and water, bodily fluids and insect bites. A major contributor to infectious diseases or communicable disease are water and food borne pathogens (WHO, 2019, Edemekong and Huang, 2019).

3.2 Water quality and Water-borne diseases

The definition of water quality is difficult to pinpoint, as the term may differ depending on the use of the water, whether that be for drinking, food preparation, recreational use, agriculture or to support aquatic life. The definition of safe drinking water given by the WHO guidelines states that the water supply “does not represent any significant risk to health over a lifetime of consumption” (WHO, 2019). There are several factors which could decrease the quality of a water source, including inorganic and organic chemical pollution, radiological levels, and microbiological contaminants (WHO, 2018). The greatest microbial concerns to water are those associated with the ingestion of a water source that is contaminated with faecal matter, which include viruses, bacteria, and parasitic protozoa and helminths (Cabral, 2010, Pandey et al., 2014, Guidelines on sanitation and health, 2018). And

around 2 billion people around the world must use water sources that are contaminated by both human and animal faeces (WHO, 2019).
Unsafe drinking water is a vehicle of disease, the risks to public health are dependent on the pathogen type and the concentration in that body of water (Rodrigues and Cunha, 2017). Table 1 describes some common pathogenic microorganisms found in water sources and their associated diseases. Diarrhoea is the predominant symptom caused by a gastrointestinal infection, it is estimated that diarrhoea is the cause of around 4% of all deaths worldwide, and mostly affects people in developing countries (World Health Organization, 2019). In African and Asian countries, children under five are most at risk of diarrhoeal diseases caused by contaminated drinking water. Furthermore, if they are subjected to frequent infection, they are also likely to endure stunting and developmental issues (Seas et al., 2000, Saxena et al., 2015). It is estimated that around 95% of deaths associated with water-borne diseases could have been prevented with improved sanitation and access to safe water (Griffiths, 2017).

The current biological parameters of drinking water quality state that there should be 0 CFU of *E. coli* or coliforms per 100ml of water, and that these should be regularly monitored with standardised methods. (WHO, 2018). Regular and consistent monitoring is crucial in water quality control, spikes in coliform analysis could indicate the potential presence of pathogenic microorganisms, which may lead to an outbreak of water-borne diseases (Rajapaksha et al., 2019). These spikes can occur any time and could be caused by a wide range of events including, but not limited to, agricultural ‘run off’ also linked to rainfall, sewage leakage and defecation by recreational users. (Price and Wildeboer, 2017). Therefore, due to the sporadic, unpredictable nature of contamination, it is more valuable to perform relatively simple standard tests regularly, than a more complex test with much higher sensitivity and specificity, less regularly (Department of the Environment, 1982).

Even with regular monitoring, it may be too late to prevent a public health risk, as results are always retrospective. It is not practical however; especially in developing countries, to suggest an extreme measure such as every-day monitoring of all drinking water sources. As such there seems to be a fine line between the availability of monitoring, and how often it is required (Bartram, 2001). The control of water

quality is a multi-layered, complex approach which begins at the catchment area, moves on to the treatment procedures, and then to the distribution of the water. Therefore, attention is needed not just on the delivery of the water, but on the water supply system as a whole, such as the prevention of a contamination event. However, because contamination can originate from a wide range of sources and can occur at any time, it is difficult to control. Hence why a functional monitoring system is imperative, and the detection technique is fundamental to any monitoring scheme (UNESCO, 2019, Ramírez-Castillo et al., 2015, Bartram, 2001).

Group	Name	Disease
Bacteria	*Vibrio cholerae*	1. Cholera 2. Gastroenteritis
	Escherichia coli O157:H7	3. Severe bloody/non bloody diarrhoea 4. Cramps 5. Nausea
	Salmonella typhimurium	6. Typhoid fever 7. Gastroenteritis
	Legionella pneumphila	8. Legionnaires disease 9. Pneumonia
	Campylobacter spp.	10. Campylobacteriosis 11. Diarrhoea 12. Gastroenteritis
	Shigella dsyenteriae	13. Dysentery 14. Shigellosis
Viruses	*Hepatitis A and E virus*	15. Hepatitis
	Adenovirus	16. Diarrhoea
	Norovirus	17. Diarrhoea
Protozoa	*Cryptosporidium parvum*	18. Cryptosporidiosis: Diarrhoea, abdominal cramps and nausea
	Giardia lamblia	19. Giardiasis
	Entamoeba histolytica	20. Amoebic dysentery
Helminths	*Schistosoma spp.*	21. Schistosomiasis:Liver and kidney damage, Fever/chills
	Dracunculus medinensis	22. Guinea worm disease

Table 1 Common pathogens in water systems, Data obtained from: (Ramírez-Castillo et al., 2015, Johnson et al., 2017, Saxena et al., 2015, Cabral, 2010).

3.3 The 'Indicator' concept for assessing water quality

The presence of pathogenic bacteria in water can be difficult to detect, due to the generally low levels and sporadic nature of contamination (3.2). There are also difficulties associated with the culturing of pathogens. Therefore, standard microbiological analysis of water, tests for the presence of indicator organisms, or faecal indicator bacteria (FIB), as they are easier to isolate and culture. FIB exist in high numbers in the guts and faecal waste of warm-blooded animals, and are generally non-pathogenic (Cabral, 2010, Ramírez-Castillo et al., 2015). Pathogens are not a normal constitute of faecal microbiota and are only excreted by individuals who are infected with them, however the greater FIB count, the more likely it is that the water is also contaminated by pathogens (Ashbolt,N. J. et al 2001). Indicator bacteria generally includes three groups, Total coliforms, Thermotolerant coliforms and *Escherichia coli* (Cabral, 2010).

Total coliforms (TC) are defined as gram negative, rod shaped, non-spore forming bacteria which ferment lactose at 35-37^{o}C with gas production after 48 hours in the presence of bile salts, and are commonly found in the intestinal track of humans and animals (Cabral, 2010, WHO, 2019, Tallon et al., 2005). However, members of the TC group can sometimes be inaccurate indicators of faecal contamination (Ashbolt et al 2001). In a worldwide analysis from a variety of water types, 1017 strains belonging to the TC group were found, and 61% of strains were non-faecal (Gavini, Leclerc and Mossel, 1985). Members of the TC can be naturally found in the environment and therefore thermotolerant coliforms (or faecal coliforms) are a more suitable indicator.

Thermotolerant coliforms ferment lactose, with the production of gas and acid at around 44.5^{o}C, in the presence of bile salts (Tallon et al., 2005, Saxena et al., 2015). Members of the thermotolerant group include *Escherichia,* and some species belonging to the genus *Enterobacter*, *Citrobacter* and *Klebisella* (WHO, 2019). There is a positive correlation between faecal contamination and this group of coliforms, however the term faecal coliform is becoming discarded. Some species, which meet the criteria for this group have been found in environmental water samples, in absence of faecal contamination (e.g *Klebisella spp)* (Toranzos et al., 2007).

Members may even originate from the environment, for instance from decaying plant material (WHO, 2019). Although compared to TC, the probability of re-growth in the environment is less likely. And thus, the more scientifically accurate term is thermotolerant coliforms (Saxena et al., 2015). Therefore, usefulness of both TC and thermotolerant coliforms for indicator bacteria is somewhat limited (Tallon et al., 2005, Toranzos et al., 2007, WHO, 2019).

The use of the thermotolerant *E. coli,* is considered the most representative species of the thermotolerant coliforms to use in routine analysis of water (WHO, 2019). *E. coli* produces indole from tryptophan and is defined as the coliform which is able to produce β-glucuronidase (Ashbolt et al 2001). A study conducted by Wilkes et al 2009, using over 1600 water samples identified that *E. coli* was the most consistent indicator for pathogens, followed by faecal coliforms (thermotolerant). *E. coli* is found in both human and animal intestinal tracts and is released through excrement at around 10^9 per gram of faeces, and it is not normally found in the environment, without faecal contamination. Therefore, it is now considered the most reliable and accurate representation of faecal contamination (Department of the Environment, 1982, Edberg et al., 2000, WHO, 2004, Price and Wildeboer, 2017).

However, the question on the accuracy of using an indicator as a whole is still up for debate. There have been multiple studies which analyse the usefulness of FIB, as an indicator for the presence of pathogenic microbes, Wilkes et al 2009 found that it was very rare that a pathogenic microorganism was detected, in the absence of FIB. However, Thurman showed no correlation between indicator bacteria including *E. coli* and *Cryptosporidium* and *Giardia* found in creeks (Thurman et al., 1998). Although there may not be 100% correlation between FIB and the presence of pathogenic microorganisms, they are still a useful tool in the regular monitoring of water supplies (WHO, 2018).

3.4 Water quality in Africa

Communicable diseases are still the leading cause of child deaths in developing countries, and around 785 million people in Africa are still without access to basic drinking water services. The lack of access to safe available water sources could

also lead to people making the decision that hand washing is not of greatest importance, increasing the likelihood of the contraction and spread of other diarrhoeal diseases (WHO, 2019).

The responsibility of monitoring water supplies lies generally with a surveillance agency who are independent from the water provider. The water provider should routinely test that their systems are supplying safe drinking water. Both organizations should follow a framework of standards provided by a national or regional agency, and in theory these three agencies should work in unison to effectively monitor the adequacy of drinking sources (Guidelines for drinking-water quality, 2004). Although the reality may differ, in low income countries, water quality monitoring is less established, as a result of low resources and poor regulation. A study by Peletz et al demonstrated that out of the 72 institutions analysed across sub-Saharan Africa, most did not fulfil the number of water quality tests, set as standard by either the World Health Organisation or their national agency (Peletz et al., 2016).

The United Nations Sustainable Development Goals 2030 agenda (SDG), provides a framework of action. Agenda 6 is to “ensure availability and sustainable management of water and sanitation for all”, 6.1.1 aims to increase the population using safely managed drinking water services (Sustainabledevelopment.un.org, 2019). The WHO/UNICEF Joint Monitoring Programme for water supply, sanitation and hygiene (WASH), globally monitors the SDG in relation to the WASH targets. WASH data is usually collected at the household level and monitors the quality and availability of the main water source in a household (Washdata.org, 2019). The programs “Ladder drinking water” is used to describe the type water source (UN-Water, 2019):

1. **Safely managed** drinking water, is located on premises and is highly accessible. The water is also from an improved water source, improved water sources are classified as water that is absent of faecal and chemical contamination, this includes safe water delivered by, piped, boreholes, protected dug wells and springs, packaged or delivered water, and rainwater.
2. **Basic** drinking water is from an improved source but may take a round trip of up to 30 minutes to collect.

3. A **Limited** water source is also from an improved source, but collection times exceed 30 minutes.
4. **Unimproved** drinking water is taken from unprotected sources such as wells and springs.
5. **Surface water** as suggest is water which is located on the earth surfaces including rivers, dams, lakes, ponds and streams.

As figure 2 displays the percentage of the population who use safely managed water sources for drinking has increased, from the years 2000 to 2017, and an increase of 1.8 billion people gaining access to basic drinking water. Although global progression is clear, there is still more to be done, with 144 million still people dependent on surface water, those in Sub-Saharan Africa and rural communities being most afflicted (WHO, 2019) (Washdata.org, 2019). Many issues arise because of poor infrastructure and inadequate financing from governing bodies to monitor water resources (The Africa Water Vision for 2025, 2001).

Monitoring is a vital prerequisite for the control of water quality, and for the composition of strategies. As such, investments are needed in suitable monitoring technology, to aid Africa's water crisis (WHO, 2019). However, in areas of Sub-Saharan Africa national health laboratories and other laboratory services are deficient, which provides a difficulty in the testing of water samples (Alemnji et al., 2014). Therefore, it could be theorised that if communities can be provided with their own means of monitoring, and not rely on governing bodies, the number of water-borne diseases contracted may fall. However, in order for a method or piece of equipment to be successful it needs to be fit for purpose (see 3.6).

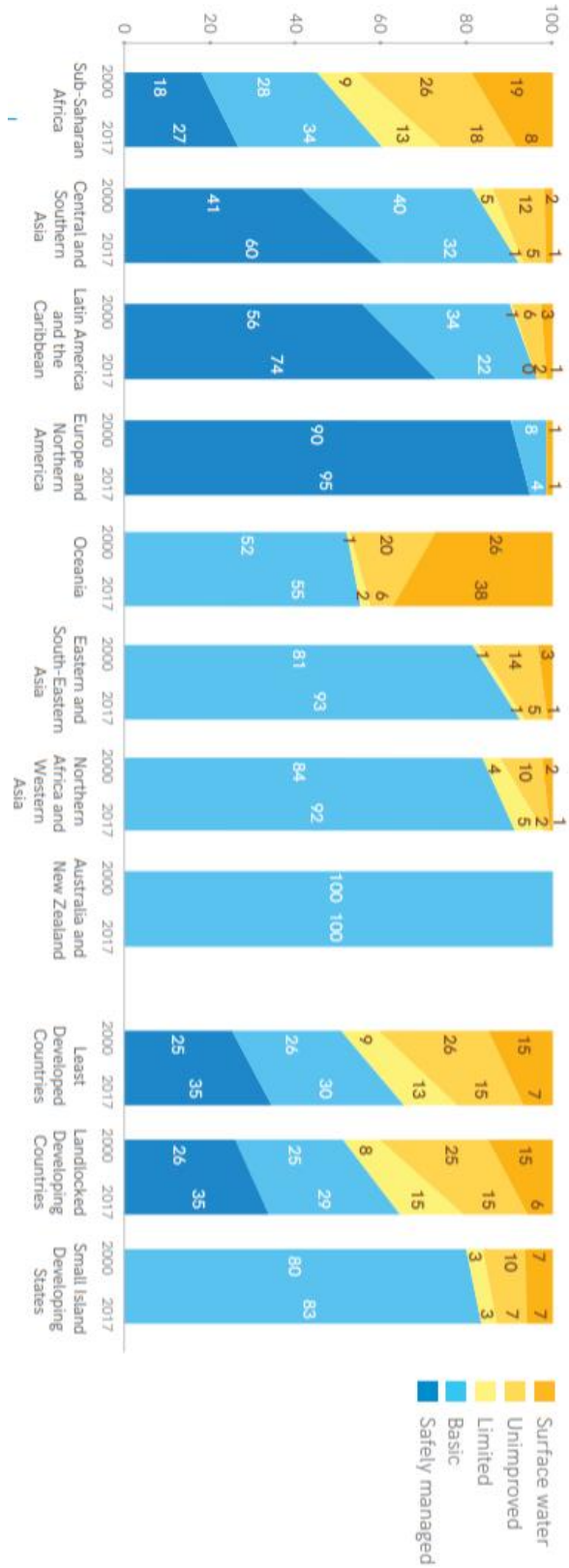

Figure 2 The % of people in various area, who use surface, unimproved, limited, basic and safely managed drinking water supplies, and the improvements made between 2010 and 2017 (Washdata.org, 2019)

3.5 Current methods used to evaluate water quality

Methods to assess the biological quality of water have remained constant due to their simplicity, range of applicability and widely accepted high levels of reliability. However, there is growing appeal for more rapid test methods (Rajapaksha et al., 2019). A comparison of the current commonly used methods in water analysis, is shown in Table 2.

3.5.1 Culture-based detection methods

Traditional methods have relied mainly on the cultivation of bacteria, or the detection of their metabolic reactions, to produce quantifiable evidence (Rajapaksha et al., 2019).

Membrane filtration (MF), is considered one of the gold standard detection methods, whereby the water sample is passed through a sterile 0.45µm filter upon which the bacteria become concentrated on a membrane (Rompré et al., 2002). The membrane is then transferred to the surface of a liquid or solid medium and incubated for 24 to 48 hours to allow the bacteria to grow, before enumeration. If a greater level of specificity for certain bacteria is required, the membrane can be placed upon a selective chromogenic agar. An example is Brilliance agar, *E. coli* colonies expressing the enzyme β-glucuronidase will form purple colonies due to the presence of the chromogenic agent X-Glu in the medium. Similarly, pink colonies are formed in the presence of β-galactosidase found in other coliforms, using the agent Rose-Gal. The medium also contains a selective agent to inhibit the growth of gram-positive microorganisms (Price and Wildeboer, 2017). MF is a highly sensitive technique, able to detect 1 cell in 1 litre of water, it is also easy to perform, which is why it is often the method of choice (Cabral, 2010).

Multiple tube fermentation using the Most probable number (MPN) method has been implemented for over 90 years in the water sector, as another widely approved technique (Rompré et al., 2002). This method involves a series of tubes containing a media, commonly lauryl tryptose broth, a series of dilutions of the given water sample are inoculated into the tubes and incubated for 24 - 48 hours. A positive presumptive test for coliforms occurs with gas production. After the allotted

incubation period, a confirmatory test can be carried out on the tubes with gas production, which requires a selective brilliant green lactose broth and another 24 - 48 hours. The number of tubes displaying positive for coliforms is used to determine the MPN, the statistical estimation of the number of microorganisms present in the water sample (WHO, 2019). This technique has an advantage over the MF method as it allows for the analysis of turbid waters. MPN provides a semi-quantitative result, but due to the possible irregular distribution of bacteria in a water sample, many tubes are required in order to yield true positive and true negative results. This method can be time consuming and require many consumables (Rompré et al., 2002).

The major limitation of current culture-based techniques is the inability to recover coliforms that are stressed or injured, which leads to an underestimation of the bacterial load. The first report in literature to observe the underestimation of bacterial load by means of plate counts, was Razumov in 1932, who noted disparity between viable plate counts and the total direct microscopic count, from samples taken from aquatic habitats (Razumov, 1932). He discovered numbers which were several orders of magnitude higher for the microscopic count than the plate count. This was verified in 1985, through the analysis of thousands of lake water samples, using the same two counting methods for enumeration. One of the theories behind the “great plate count anomaly” was that the bacterial cells were alive but unable to grow in the culture conditions provided (Staley and Konopka, 1985).

Currently, it is known that around 85 species of bacteria (67 of which are pathogenic) can enter what is now termed the ‘viable but non-culturable’ (VBNC) state (Zhao et al., 2017). VBNC cells are those that appear to have lost their ability to grow *in vitro* through current culturing techniques, but still display metabolic activity. As opposed to dead cells, the cell membranes of VBNC cells remain intact, and genetic material is undamaged (Robben et al., 2018, Li et al., 2014, Oliver, 2000). Microorganisms can enter this state, through exposure to stresses such as adverse environmental conditions, which induce this survival strategy (Robben et al., 2018). This state was first described in *E. coli* and *V. cholerae* in 1982 (Xu et al., 1982), and since then the research surrounding VBNC bacteria has not resolved whether they are a risk to human health (Pinto et al., 2013). Some research has implied that VBNC cells have

the ability to exit from this state under specific favourable conditions, as such the resuscitation of pathogenic bacteria would be a risk (Maalej et al., 2004, Cappelier et al., 2007). Although some studies have suggested that cells which are in the VBNC state remain infectious, such as the study by Jones et al who infected mice with apparent VBNC strains of *Campylobacter jejuni* (Jones et al., 1991). Others obtained data that did not support these findings (Smith et al., 2002). Due to this uncertainty, and lack of agreement among the scientific community concerning the risk of VBNC microorganisms, current routine detection techniques rarely focus on this (Committee on Indicators for waterborne pathogens, 2004). It is also important to point out that injured cells or stressed cells, which often prove difficult to culture, are not the same as VBNC cells, but both lead to the underestimations of microbial contamination through conventional culturing techniques (Pinto et al., 2013).

3.5.2 Molecular and immunological detection techniques

To overcome the limitations of culture-based techniques alternative methods have been developed. These methods have the ability to quantify microbial contamination through cellular properties such as genetic material or cell surface components (Committee on Indicators for waterborne pathogens, 2004). Molecular and immunological detection techniques usually are highly sensitive, and from a microbiological perspective, are more accurate than conventional methods (Rodrigues and Cunha, 2017).

Polymerase chain reaction (PCR) is a method used to amplify targeted DNA through thermocycling using oligonucleotide sequence primer. PCR involves cycles of denaturing the microorganism's DNA, annealing of the primers and then extension using a thermostable polymerase enzyme. Amplified PCR product are traditionally visualised under UV light after an agarose gel electrophoresis. The time taken from sample preparation to final results can be up to several hours (Rompré et al., 2002). PCR has been applied to the detection of diagnostic genes found in *E. coli* and other coliforms, such as the *lacZ* gene which encodes for β-galactosidase in coliforms and the *lamB* gene for maltose transport protein in *E. coli* (Babaie et al., 2017, Price and Wildeboer, 2017). Some PCR methods such as reverse transcription PCR (RT-PCR) can be used to evaluate viability as it indirectly detects

short lived messenger RNA (mRNA) that is only transcribed by viable cells (Li et al., 2014).

Enzyme-linked immunosorbent assay (ELISA) is an immunological detection method which permits identification of cells based on cell surface components such as antibodies or antigens. ELISA exploits the highly specific, high affinity antigen-antibody complex for detection of bacteria. The antibodies are conjugated to specific enzymes, and in the presence of a fluorogenic or chromogenic substrate, a colour change will be observed proceeding binding and enzymatic activity. The specificity of the ELISA method is high and can be increased by using monoclonal antibodies, although difficult and expensive to acquire (Verma et al, 2012). ELISA's can also yield high throughput results, as they are usually performed using a 96 well plate, which allows for multiple water samples to be analysed. However, a downside of an ELISA test is that they can take between 24 to 52 hours and thus are not considered rapid tests (Rodrigues and Cunha, 2017).

Molecular and immunological techniques are advantageous, producing sensitive results, faster than conventional culturing, however the difficulties present when there are very low number of bacteria, such as in a drinking water sample, and therefore the addition of a culture step is needed. Adding this step reduces the advantage of a quicker test and presents other issues associated with culturing as described previously (Rajapaksha et al., 2019). Disregarding this, these methods present their own challenges including the complex sample preparation and specialised equipment required (Table 2), meaning they are expensive for regular routine testing (Rodrigues and Cunha, 2017).

There is no doubt that there are many advantages to conventional methods, which is why, it seems, they have remained the standard methods for so long. However, the current methods are either too time consuming or too complex and expensive for routine testing or field deployment. There is a need for rapid, yet reliable and scientifically defensible detection methods. (Griffith and Weisberg, 2006).

Current methods	Advantages	Disadvantages
Multiple tube / MPM	- Easy interpretation of results - No specialised training needed	- Labour intensive and very time consuming - Gives a statistical estimate, not actual number of bacteria - Poor detection of stressed bacteria
Membrane filtrations	- Easy to preform and to identify results - Reliable - Can examine large volumes of water - Quantitative results - Selectivity can be increased with certain media	- Time consuming - Culturing difficulties - No detection of stressed or injured bacteria - Background bacteria may interfere with coliform growth
Molecular based methods	- Can be culture-independent - Fast - Sensitive and specific - Detection of VBNC	- Complex and specialised equipment and consumables needed - Specific training needed - Can be time consuming - May require cultivation step
Immunological based methods	- Highly specific - Very sensitive	- Time consuming around 24-52 hours

Table 2 Current methods to detect microbial contamination on water samples Data obtained from (Department of the Environment, 1982). (Rodrigues and Cunha, 2017) (Rajapaksha et al., 2019) (Ramírez-Castillo et al., 2015) (Ashbolt,N. J. et al 2001) (Rompré et al., 2002).

3.6 Emerging rapid testing techniques

These techniques have the potential to overcome the need for a more rapid detection method and delivery of results. However, in order for an emerging test method to become standardized, the methods need to be fit for purpose, and be capable of certain requirements. The requirements of a rapid detection technique for water contamination may differ depending on the user, developed countries may require the method to produce a high throughput of results, whereas developing countries may require a more user friendly approach to implement in local communities (Africa's Water Quality, 2010).

The requirements explained in the following text are mainly tailored towards the use of a rapid test method or device as standard, in developing areas, where infrastructure may be lacking. Although a proportion of these key requirements are also applicable to the implementation in the developed world.

1. **Time**: To be considered rapid, a test for the detection and enumeration of microbes must deliver reliable results within one day at the most, this includes the time taken to gather the sample, any sample preparation and then the generation of results. The test must also be able to perform multiple tests within that time frame, each of which produce consistent results for the same sample (Rajapaksha et al., 2019, Eijkelkamp, et al 2008).
2. **Sensitivity and Specificity**: The test must be able to detect and be selective for coliform bacteria, more specifically thermotolerant (faecal) coliforms and E. coli. The detection limit should be in accordance with the requirements set by legislation, as such should be able to detect 1 CFU in a 100ml water sample (WHO, 2019). However, the device may be used with the aid of a filtration technique to concentrate the sample, however the added filtration method must not increase the time of testing past 1 day. The results generated must also consistently lack the presence of false positives and false negatives (Rompré et al., 2002).
3. **Standardized**: The rapid test should be accompanied by a proven standardized method, the method should be compared against current standards to determine its validity. The new method must produce results

equal or improved in terms of accuracy and sensitivity (Rajapaksha et al., 2019).

4. **Simplicity**: In order for a new test method to be deployed in areas where readily available laboratory testing is lacking, the device must be user friendly, in that it does not require specialised scientific training, and can be used after a demonstration and the following of a manual. Simple sample preparation is also favourable, to minimise the risk of a human error interfering with the results generated (Committee on Indicators for waterborne pathogens, 2004). As well as the use of the device or method, the results must be easily presented and straightforward to analyse (Rajapaksha et al., 2019).
5. **Application and versatility**: Having the testing method or device in the form of a portable device or ready to use kit would be advantageous, often samples are taken from a site and regularly transported to specific accredited laboratories at different locations, the costs of this usually reaches thousands (Rajapaksha et al., 2019). Possessing the device on site, or even at household level would not only minimize costs but allow communities to be in control of their own water testing (Eijkelkamp et al., 2008, Africa's Water Quality, 2010).
6. **Low cost**: The device or method must incorporate low operational costs, including the reagents and consumables required, as testing needs to be at regular and consistent intervals to be most effective (Department of the Environment, 1982). The upkeep of the equipment should be minimal, notably in cases where there is a lack of engineers or trained personnel. The use and expenditure on resources should also be considered, such as some systems may require electricity, this may be difficult to sustain in some areas including rural communities (Africa's Water Quality, 2010).

3.6.1 ATP bioluminescence tests

Advances in microbial detection technologies have seen bioluminescence-based methods being developed to overcome some of the issues relating to current methods (Carrick et al., 2001). Bioluminescence is the process of light generation by living organisms and microorganisms (Xu et al., 2014). These methods used for the detection of microbial load, usually involves the observation of the luciferase/luciferin

reaction with adenosine triphosphate (ATP) (Hammes et al., 2010). ATP is utilised by all living organisms as an energy source, it is suggested that ATP can be used as a biomarker for cellular viability, as such it is as an indicator for microbial contamination (Karl, 1980, Venkateswaran et al., 2003, Hammes et al., 2010).

The luciferase reaction is a phenomenon occurring in nature, in a wide range of organisms from fireflies to fungi. The terms luciferin and luciferase are general, a luciferin is a substrate which is catalysed to produce light, and a luciferase is an enzyme which catalysis this reaction. The actual biochemistries of these systems are diverse, but what they all seem to have in common is the reaction involving oxygen and the release of an electronically exited molecule which emits light (Hastings, 1995, Baldwin, 1996). Firefly luciferase however involves ATP, which was determined by McElroy et al using *P. pyralis* (McElroy and Seliger, 1960). The first step of the methods which utilise this reaction is usually the extraction of ATP through the lysing of the bacterial cells, often first with some sort of removal of extracellular ATP. Therefore, the amount of light emitted (photons) is proportional to the amount of ATP present in the bacteria, the light is usually observed using a camera or luminometer (Carrick et al., 2001).

Studies have observed direct correlations between ATP detected in a sample and the number of CFUs detected by other means, such as standardized plate counts (Eed et al., 2016, Aycicek et al., 2006). These methods are usually faster than current microbial detection methods while remaining sensitive, therefore this area of research is now highly desirable (Deininger et al., 2011, Chollet and Ribault, 2012). As stated, ATP bioluminescence methods can be used to detect all viable cells, but an important question to ask is whether these methods can detect the presence of VBNC or stressed cells, unlike the conventional culturing methods. High ATP levels have been identified in both *Listeria monocytogenes* and *Campylobacter jejuni* after entering a VBNC state, it was detected in the *Listeria* up to one year after entering, hence ATP may also be a marker for cells in a VBNC state (Lindbäck et al., 2009, Beumer, 1992).

Although these technologies appear to have advantages over current methods, these methods often seem to have a detection limit ranging from 10^1 – 10^4 per ml of

water. It has been suggested that this is due to poor extraction of ATP from cells, therefore presently they are not applicable to the use of drinking water testing where bacterial levels are much lower, and more investigative research is needed (Lee et al., 2017).

3.6.2 New methods for Coliform detection

There are a few low cost and simple test methods commercially available which have been approved by the United States Environmental Protection Agency (EPA) for the analysis of drinking water. Some of these methods or devices are named Colilert (Idexx., 2019), and Coliscan (Micrologylabs., 2019), These methods are often assays which work on the basis of detecting an enzymatic reaction to identify the presence of β-galactosidase for coliforms and β-glucuronidase for *E. coli,* similar to that of the MS system (3.7). Most of these methods appear to an be easy, low-cost and yet reliable alternatives to current methods. However, some seem to require certain laboratory equipment and therefore may not be able to be deployed in field tests. Furthermore, what they all seem to have in common is the time which they consume, consequently they do not possess that advantage over current methods (Standard Methods, 2019, Gunda et al., 2014).

To my knowledge there appears to be only one method currently which is regarded as a rapid test method for the detection of indicator organisms in a water sample. The Mobile Water Kit determines total coliform count and *E. coli* contamination simultaneously within as little as 30 minutes (Gunda et al., 2014). A 100ml water sample is syringe filtered (0.45µm) to concentrate the sample, the product allows for the testing of three samples simultaneously. Four reagents A, B, C and D are then sequentially added to the filter unit, which is then incubated at 37ºC and monitored over 1 hour and up to 2 hours. A qualitative positive result for contamination is observed through a colour change, a red colour is produced through the enzymatic reaction with β-galactosidase and Red-Gal. A quantitative result can be gained by using a smartphone with a specific app and is said to detect down to 2 CFU/100ml in up to 1 hour.
The kit comprises a box in which the syringes are placed for the filtration step, four chemical reagents and 12 droppers to use with the reagents. The product is said to

be user friendly. To carry out this method, the user must dispense four chemical reagents sequentially which could prove somewhat challenging to persons with no scientific training, therefore the possible implementation in rural communities is up for question.

In the published work describing this method (Gunda et al., 2014), the researches do not specify which incubator is used for this test, assuming it is a general microbiological incubator, there would need to be sufficient and available energy resources in the low resource communities, to support this testing. Currently the red colour displayed is a representation of both total coliform and *E. coli* load, but as discussed previously (3.3) total coliform may not be a specific and accurate representation, and thus it is more consistent to focus on *E. coli* as the indicator. Although the researchers did state that their future work will try to incorporate the use of the MUG and β-glucuronidase reaction, as of yet the device does not have the capabilities to detect the blue fluorescent reaction for *E. coli* alone (Gunda et al., 2014).

3.7 The MicroSnap Detection Systems

Hygiena International Ltd are a company with headquarters in California, and offices globally. Their main objective is to provide rapid monitoring systems for a wide range of industries including, food and beverages, pharmaceuticals and health care. MicroSnap (MS) is a rapid bioluminogenic test for detection and enumeration of microbial content in samples. Using Hygiena's patented Snap-valve technology and a monitoring system quantitative results are obtained within 8 hours (Hygiena.com, 2019). There are four MicroSnap devices, Total count, Enterobacteriaceae (EB), Coliform, and *E. coli*, and the monitoring system. Hygiena's monitoring system is called the Ensure unit (Figure 3 C), which is a small handheld piece of equipment that has a self-check calibration at each start up. The Ensure unit is a luminometer which is said to detect down to 0.1 femtomoles of ATP in a given sample, within 15 seconds (Hygiena.com, 2019).

The MS system is a two-stage process, first a sample is collected and placed into the designated enrichment tubes, which contain a proprietary growth medium (Figure

3 A), and incubated for the allotted time (Appendix 1). The second stage is the detection stage, in which an aliquot is taken from the enrichment tubes (Figure 3 B) and placed into the corresponding detection tubes, then activated and slotted in to the Luminometer monitoring device. The monitoring device measures the amount of light produced by a luciferase reaction and gives a reading of Relative Light Units (RLU) as the unit of measure for ATP. The RLU is therefore directly related to light production, which is proportional to the ATP present, which is proportional to the number of Colony Forming Units (CFU) present. The CFU can then be determined using a RLU:CFU conversion table (Appendix 4-6). The conversion tables have been calculated by Hygiena International Ltd, based on the results of many experiments using comparisons to plate counts to generate standard curves.

MS is currently applied in the food industry, to be employed in the water sector this system must be able to detect the current water parameters. Water generally contains lower numbers of bacterial contamination than food, particularly drinking water. The European food regulations state that there are three levels set for *E. coli* contamination of minced meat for example, excellent meat will contain less than 50 CFU/g, the adequate level is between 50 and 500 CFU/g, and not fit for consumption is above 500 CFU/g (Commission regulation, 2005). Whereas previously stated, the levels for *E. coli* and other coliform contamination is 0 CFU/ml, and 1 CFU/ml is inadequate (WHO, 2018). Therefore, the MS may be used for food testing, but to be deployed in the water sector, the devices need to be sensitive enough to detect much lower levels.

As previously stated (3.5), current methods mainly rely on the culturing of microorganisms, in order to detect them. These include some limiting practicalities such as difficulties culturing some bacteria and long incubation periods. The MS system also requires a cultivation step, and therefore the issues previously stated may exist. Although the method is accelerated compared to current culture-based methods, it is designed so that the results are gained within one working shift. In order for a new system to be used in routine testing it must be comparable to standard methods in relaibilty and sensitivity and must also have advantages such as decreased time frame and low cost (Rajapaksha et al., 2019, Committee on Indicators for waterborne pathogens, 2004). It is also useful if the method is simple

and can be implemented in the field, even in low resource areas. MS is designed to be user friendly, the novel snap technology, allows the kit to contain all the reagents needed for the test. There is also no specific sample preparation, just place 1ml of sample directly into the enrichment tubes. The luminometer is powered by batteries which can power the device for up to several months, this means that the instrument can be easily transportable (Hygiena.com, 2019). The enrichment stage however requires and incubator capable of sustaining temperatures up to 37^{o}C, which could prove problematic is some areas. A dry block incubator is used, which requires less energy than a conventional microbiological incubator, and only needs to be turned on for around 10 minutes prior to usage. In theory if MS is found to be applicable to the water sector, the dry block could be set up in the field, which would reduce laboratory transport costs, but also allow communities to identify their own safe drinking water sources.

*Figure 3: Hygiena International Ltd.'s MicroSnap A) Total, EB, and Coliform/*E. coli *Enrichment tubes (Red snap valve), B) Detection tubes (Blue snap valve) and C) The EnSure luminometer device.*

3.7.1 MicroSnap Total and Enterobacteriaceae detection kits

MicroSnap (MS) Total is a non-specific test which gives a total viable count of all aerobic heterotrophic bacteria in a sample. MS EB detects all bacteria belonging to the large, Gram-negative Enterobacteriaceae family including S*almonella, Citrobacter, Shigella* and *E. coli*. The detection stage for both MicroSnap Total and EB is the same; the detection tubes contain Luciferase, Luciferin and buffer in the bulb, and Nucleotide releasing factor/ Extractant in the tubes. It is the enrichment stage however when the selectivity occurs, both the enrichment tubes contain a growth media consisting of buffered salt solution, vitamins, yeast extract and apyrase. The enzyme apyrase is an ATP-diphosphohydrolase, it catalyses the hydrolysis of adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and then into adenosine monophosphate (AMP) and inorganic phosphate (International.neb.com, 2019). This removes any environmental and extracellular ATP by converting it into AMP, which is then not detectable by MicroSnap, therefore apyrase acts as the first selective process, to produce RLU proportional to the actual number of bacteria present. MicroSnap EB enrichment contains another selective agent (antibiotic), which ensures that only Enterobacteria can grow during incubation.

MicroSnap Total and EB generate RLU's with the direct approach (Active ATP), when the enriched sample is added to the detection tubes, the extractant at the bottom of the tubes lyses the bacteria to release ATP as a biomarker, the ATP reacts with the luciferin catalysed by luciferase to generate light. The light produced is directly proportional to ATP from the bacterial cells. Therefore, the principle of the method of these MS systems depends on the growth of bacteria in the proprietary growth media in the enrichment stage, to produce ATP as a biomarker. At 7 hours incubation at 30^{o}C, the concentration of intracellular ATP should be directly proportional to the concentration of bacteria in the starting inoculum (Hygiena.com 2019).

3.7.2 MicroSnap Coliform and E. coli detection kits

MicroSnap Coliform detects all bacteria which are considered under the coliform groups; total coliforms, thermotolerant coliforms and *E. coli*, and as stated, MS *E. coli* detects *E. coli* species. The enrichment stage for these devices contains the same proprietary growth media, with the addition of enzyme inducers for β-galactosidase and β-glucuronidase (Figure 4). After a 6 hours incubation, the enzymes in the sample should accumulate and be proportional to the concentration of bacteria in the starting sample (Hygiena.com 2019).

The method of detection for MS Coliform and *E. coli* is an indirect assay, as opposed to a direct assay for MS Total and EB. There are two detection devices, which both contain, the lytic agent (extractant), ATP, luciferase and pro-luciferin, however the pro-luciferin differs between the MS *E. coli* and Coliform tubes (Figure 5). The Coliform detection devices contain pro-luciferin which has been manufactured to have a substrate for β-galactosidase, when the enzyme binds to the pro substrate, it becomes cleaved. Upon cleavage of the bond, the luciferin is free, to allow to bind with ATP in the presence of luciferase to produce light. In MS *E. coli*, the pro-luciferin is manufactured to recognise β-glucuronidase. Therefore, instead of the RLU being directly related to the ATP in bacteria, it is related to the amount of ATP and luciferin binding, which is related to how much enzyme is present. Other literature has shown that over 95% of *E. coli* strains express β-glucuronidase, therefore the enzyme provides specific identification of the presence of *E. coli* (Tallon et al., 2005, Price and Wildeboer, 2017).

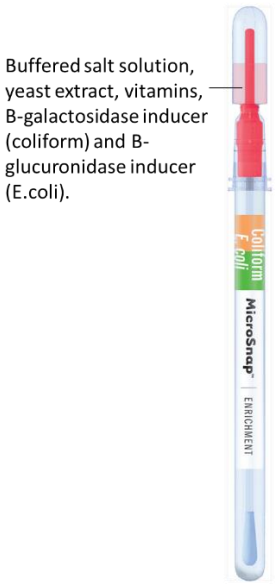

Figure 4 labelled photograph of E. coli and Coliform Enrichment tube

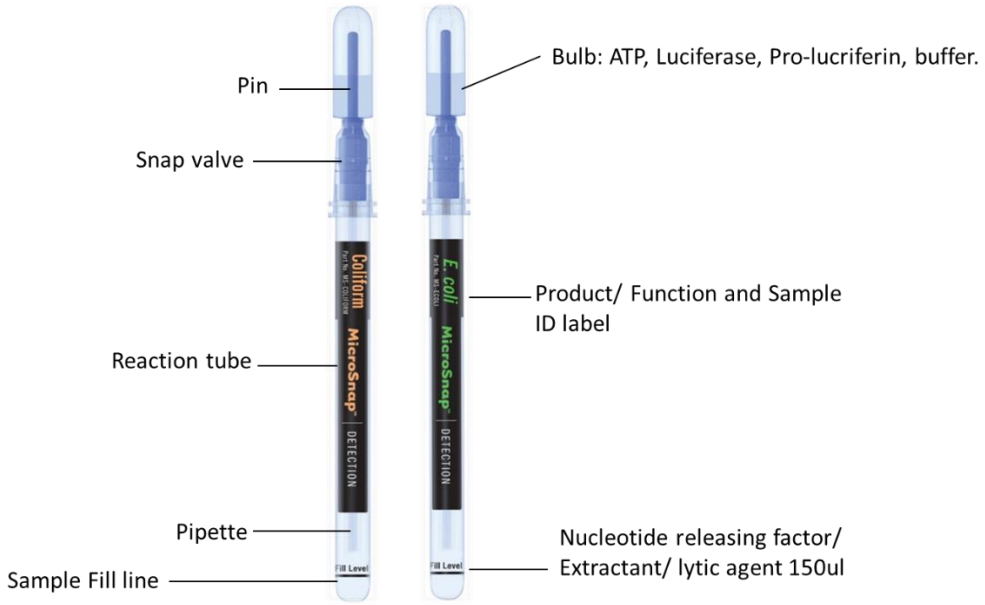

Figure 5: Labelled diagram of MicroSnap E. coli and Coliform detection tubes, detailing each component of the devices.

3.8 Moving forward

Water-borne pathogens are a leading water quality concern globally, and there is an obvious need for new technologies to detect microbial contamination. The current literature suggests that no device or method has been produced which conforms to

all the criteria stated in section 3.6, including the ability to detect 1 CFU per 100ml, as current WHO guidelines state. The aim and subject of this thesis was to determine if Hygiena's MicroSnap system could be applied to drinking water testing. More specifically, could it be used in low income areas where laboratory infrastructure is lacking. To determine the applicability of the system; the sensitivity, specific, accuracy and reliability will be investigated. The investigation is divided into three sections, chapters 1, 2 and 3 (section 4, 5 and 6). The three chapters are comprised of individual investigations, chapter 1 discusses the initial testing of the MS system and explores the sensitivity and specificity of all four MS devices. This chapter also aims to investigation the reproducibility of the system, whether the devices consistently produce accurate results. Chapter 2 addresses multiple hypothesis raised in regards to the underestimated bacterial level of samples, given by MS *E. coli*. Each methodology of this chapter explores a different hypothesis to determine where in the system lies the fault.

After determining the suspected cause of the results observed in chapters 1 and 2, chapter 3 aims to quantify the level of disruption to results, potentially caused by the hypothesis, and thus determine a course of action to correct this.

The initial testing of the MicroSnap system using pure cultures of known bacteria.

4.1 Introduction

Safe, uncontaminated water sources are vital for public health and economic stability. It is estimated that around 829,000 people die annually from diarrhoea caused by the use of unsafe drinking water and poor sanitation, however diarrhoea is preventable (WHO, 2019).

As previously stated (3.2), one of the most valuable aspects of controlling a potential water-borne disease outbreaks, is the continuation of regular monitoring of a water supply. The current gold standard methods such as Membrane filtration and MPN, have been so for many years due to their reliability, applicability and sensitivity (Rajapaksha et al., 2019). These methods however rely mainly on cultivation, requiring long incubation periods, which at present can take up to 72 hours, and usually require specialised laboratory equipment and trained personnel to interpret the results (Sidorowicz and Whitmore, 1995, Rajapaksha et al., 2019). Therefore, there is a need for more rapid, simple, reliable, sensitive and reproducible detection methods. Current research aims to find methods which shorten the time between sample extraction to the delivery of results, but still produce comparable results to established standard methods (Gunda et al., 2014). Promising rapid techniques are emerging, including the MicroSnap (MS) system, a quantitative test for the enumeration of microbial contamination, in which results are generated within 8 hours (Hygiena.com, 2019). The MicroSnap systems are currently used in the food industry to detect microbial load in food specimens.

4.1.1 Aims:

1. Sensitivity and specificity of the MS system: As previously explained (3.7) there are four MS systems: Total bacteria, Enterobacteriaceae (EB), Coliform and *E. coli*, an aim of this chapter was to determine if the systems are specific to their intended targets. Current WHO guidelines 2019 state that 1 CFU of *E.*

coli or another Coliform indicator organism in 100ml of water, is a positive test, as it should not exceed 0 CFU per 100ml. Therefore, it is important that whatever method is used to analyse a water sample, it must be sensitive enough to detect such low numbers.

2. The reproducibility of the MS system: to determine if the RLUs generated by the MS system are reproducible by consistently producing true positives and true negatives.

3. Whether the MS systems can detect coliforms and E. coli in environmental water samples, under different environmental conditions such as sediment load.

<u>Diagram depicting detection areas for the MS systems</u>

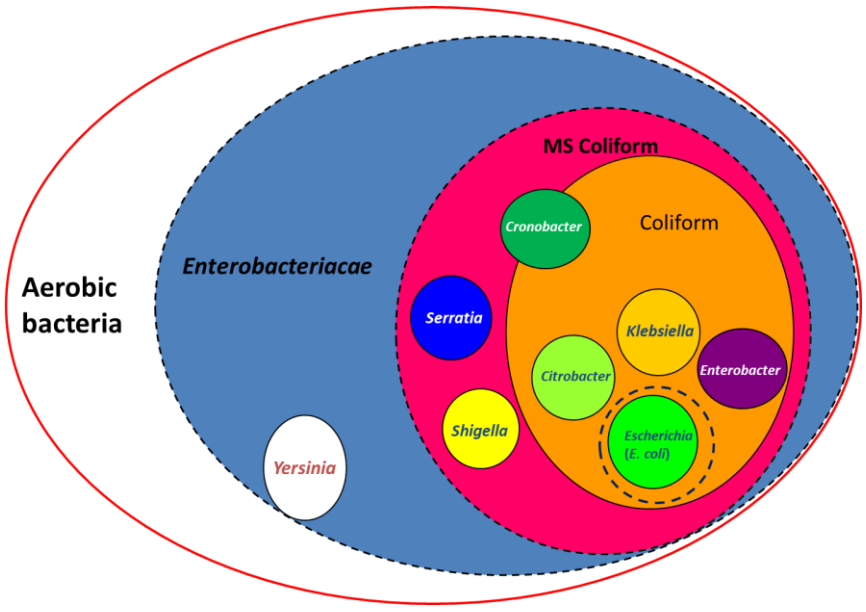

Figure 6: Schematic depicting the bacteria that each of the MicroSnap devices can detect, the red outer line shows those that MS Total can detect, the dashed blue line depict MS EB, the pink circle encapsulates the detection of MS Coliform and the small dashed line is MS E. coli*. (Image adapted from Hygiena.com, 2019)*

4.2 Materials and Methods

4.2.1 Bacterial strains and culturing

All Bacteria used are listed in Table 3, environmental strains were donated by Glenn Rhodes at the Centre for Ecology and Hydrology (CEH), Lancaster. The cultures were recovered from beads, that were kept frozen at -80^{o}C. *E. coli* NCIMB 12210, NTCT 12241 was chosen from Hygiena International Ltd positive control list, which had shown positive detection by MS at both 6 and 8 hours, the strain was ordered from Culture Collections England and recovered at 37^{o}C overnight in nutrient agar before testing. All strains were cultured for 24 hours at 37^{o}C on nutrient agar plates. The strains were chosen to give each MS test a target, for example *Citrobacter freundii* is a known coliform, and will act as a target for MS Coliform.

Table 3: Bacterial strains used in this study

Species	**Strain**	**Description**	**Source**
Escherichia coli	HB 101	K12 derivative strain Positive control test strain	G. Rhodes (CEH Lancaster)
Escherichia coli	12210	Recommended positive control	Culture collections England NCTC 12241
Citrobacter freundii	99	Windermere isolate	G. Rhodes (CEH Lancaster)
Pseudomonas syringae subsp. Phaseocola	Unknown	Plant pathogen Negative control test strain	G. Rhodes (CEH Lancaster)
Serratia rubidaea	Unknown	Negative control test strain	G. Rhodes (CEH Lancaster)
Staphylococcus aureus	*NCTC 13143*	Negative control test strain	G. Rhodes (CEH Lancaster)

Table 3: Bacterial strains used for this chapter.

4.2.1.2. Equipment

The MicroSnap devices as well as the Ensure luminometer and the dry block incubator were provided by Hygiena International Ltd, Guilford. The MicroSnap (MS) devices are stored in a cold storage room and allowed to acclimatise to room temperature before each test.
The buffer used throughout all the methods as a diluent and for the controls was 0.1 Tris HCl, sterilised by filtration through a 0.22µm filter.
Throughout all methods used in this research project, Table 4 was used to estimate the number of bacterial cells in a sample, in order to compare with the CFU/ml generated by the Microsnap system.

Table 4 Estimated concentration of bacteria

Estimated Cells/ml	1 in 10 Dilution series
10^9	Neat/10^0 (1 loop full into 1ml of buffer)
10^8	10^{-1}
10^7	10^{-2}
10^6	10^{-3}
10^5	10^{-4}
10^4	10^{-5}
10^3	10^{-6}
10^2	10^{-7}
10^1	10^{-8}
10^0	10^{-9}

Table 4 Estimated number of bacterial Cells/ml, when 1 sterile loop full of cells were taken from an overnight culture and suspended into 1ml of TRIS buffer.

4.2.2 Methods

4.2.2.1 Introductory training on the Microsnap kit at Hygiena International Ltd
To gain experience on the correct usage and an understanding of the current applications of the MicroSnap system, practical training was conducted at Hygiena's

laboratory Guilford, over a one-week time period. It began by using the Total, EB, E. coli and the Coliform MicroSnap systems, to swab multiple surfaces around the Hygiena building, which was a demonstration of one of the devices current applications. The devices were also used to detect microbial contamination of a food sample and compared against the spread plate method. A sample of minced beef was ground up and suspended in a buffer solution, and then serial diluted by factors of 10 until $10^{-4.}$ 1ml samples of dilutions 10^{-1} – 10^{-4} was pipetted into each of the MicroSnap Enrichment tubes (Total, EB, Coliform/*E. coli*). MS Total was incubated for 7 hours at 30ºC, EB for 7 hours at 37ºC and *E. coli*/Coliform enrichment tubes for 6 hours at 37ºC. After the allotted time, 100ul aliquot of each tube was pipetted into the corresponding detection tubes, and the *E. coli* and Coliform detection tubes were incubated for a further 10 minutes. Each tube was then read using the Ensure luminometer. The same dilution samples were plated on to three different selective media, TSA (Total bacterial count), VRBGA (selective for Enterobacteriaceae) and Brilliance Agar (selective for *E. coli* and coliform bacteria). The cultures were incubated at 37ºC and the colonies were counted after 24 hours and compared to the RLU values from Microsnap.

The results delivered by MS Ensure luminometer are given as Relative light units (RLU), with a maximum 4-digit display of 9999, and are displayed in the result tables below. The RLUs are then converted to colony forming units (CFU), See appendix 4-6 for RLU to CFU conversion tables given by Hygiena. It has been advised by Hygiena that an RLU of 1-2 however could be background or error, and therefore consider a true value when given an RLU above 2.

4.2.2.2 Initial analysis of the MicroSnap system on a range of bacterial strains

The first stage of testing involved using a variety of strains on all four MicroSnap systems, this experimental method was conducted to analyse the performance of each kit in detecting the target bacteria. See appendix 1-3 for the detailed step by step Microsnap protocol.

One loop of each of the five strains shown in Table 3 was suspended into 1ml of Tris buffer, and serial diluted to 10^{-3}. 1ml of each 10^{-3} bacterial dilutions were transferred

into Total, EB and Coliform/*E. coli* enrichment tubes, and incubated according to Hygiena's instructions. After the initial incubation a 100ul aliquot of samples from the Coliform/*E. coli* enrichment tubes were dispensed into the corresponding detection tubes, activated with the snap valve and placed back into the dry block for the second incubation step. On completion of the incubation stage, samples from MS Total and EB were transferred to detection tubes, each tube was then placed in to the luminometer and read.

4.2.2.3 Repeated test and culturing

Repeated tests were conducted to analyse the MicroSnap systems on a variety of strains, due the results explained in 4.3.1. Three strains were chosen for the repeat; *Pseudomonas syringae subsp. Phaseocola, E. coli HB101* and *Citrobacter freundii. C. freundii* produced the highest RLU's and therefore was chosen to compare it's given RLU's from MS to CFU grown on plates. Dilutions from *C. freundii* were plated out pre and post the enrichment stage, to identify the level of growth occurring over the 6-7 hours incubatory period within the proprietary growth media.

One loop of each strain was suspended into 1ml of buffer and serial diluted to 10^{-3}, 1ml of each strain's 10^{-3} dilution was transferred to one of the four MS enrichment tubes, in triplicate. The tubes were activated and incubated for the allotted time. The 10^{-3} dilution of *C. freundii* was diluted further to 10^{-4} , 100ul of dilutions 10^{-2} – 10^{-4} were plated out in triplicate and incubated at 37ºC for 24 hours. After the MS incubation times 100ul samples were transferred to the correct detection tubes, MS *E. coli* and Coliform were returned to the dry block for the secondary incubation time. MS detection tubes were each placed into the Ensure luminometer and RLU's were read, and then converted to CFU's using Hygiena International Ltd.'s conversion tables (Appendix 4-6).

Samples were taken from one Ms Total enrichment tube containing *C. freundii,* and serial diluted to 10^{-2}, 100ul of the neat sample and each dilution were plated out in triplicate. Ms Total was chosen as it contains no other selective agent other than Apyrase (see literature review section... for details), and therefore will not interfere with the growth of the bacteria.

4.2.2.4 Testing alternative E. coli strains

E. coli J53, was used to repeat method 4.2.2.2 in triplicate on all four MS systems. This was repeated due to the consistently low RLU readings given by MS *E. coli* explained in 4.3.2.

A new strain of *E. coli* was ordered after results explained in 4.3.3. *E. coli 12241* (NCTC) had tested positive at both 6 and 8 hours in Hygiena's analysis, as such this strain was certain to produce β-glucuronidase, the enzyme required for MS *E. coli* detection. The established method 4.2.2.2 was repeated, but only using MicroSnap *E. coli* and performed in triplicate.

4.2.2.5 Analysis on an environmental water sample

Testing the Microsnap systems ability to detect microbial contamination in water involved collecting a 500ml pond water sample from a pond located on the Lancaster University Campus. 100ml of the water was filtered in a 0.22µm filter, the filter paper was then washed and resuspended by 2ml of Tris buffer, thus becoming 50 times more concentrated, this was repeated in triplicate and transferred into MicroSnap Total and *E. coli*. 100ml of water was used as WHO guidelines state that one *E. coli* cell per 100ml of water is a positive result, and therefore current standard methods test 100ml samples. Three 1ml samples of neat pond water were also transferred to the MS enrichment tubes. The standard Microsnap protocol was then followed with all tubes.

4.3 Results

4.3.1 Results from initial bacterial testing

Bacterial strains:	**MS Total RLU**	**MS EB RLU**	**MS Coliform RLU**	**MS *E. coli* RLU**
C. freundii	8628	9999	9794	0
***E. coli* HB 101**	2231	9350	3096	13
Staphylococcus aureus	5130	19	0	0
S. rubidaea	3250	1525	0	0
Pseudomonas syringae	0	0	2	1

Table 5 RLU results of five bacterial strains, using MicroSnap Total, EB, Coliform and E. coli

	MS Total CFU	**MS EB CFU**	**MS Coliform CFU**	**MS *E. coli* CFU**
C. freundii	>5,000--TNTC	>1,000-TNTC	>10,000-TNTC	0
***E. coli* HB 101**	>1000 - TNTC	>1,000-TNTC	>10,000-TNTC	<200
Staphylococcus aureus	>5,000 TNTC	<12	0	0
S. rubidaea	>5,000 - TNTC	>1000 - TNTC	0	0
Pseudomonas syringae	0	0	<10	<10

Table 6 The conversion of RLU's given in Table 5, to CFU/ml, TNTC= Too numerable to count.

All the MicroSnap systems detected their target bacterial strains, MS *E. coli* only detected results for *E. coli HB,* MS Coliform detected both *C.freundii* and *E. coli HB*, MS EB detected all strains minus *Pseudomonas syringae,* as did MS total, which

both gave an RLU of 0, shown in table 5. However poor detection of the *Pseudomonas syringae* species by MS Total was unexpected, and therefore a repeat was needed to deduce whether this result was due to practical error, or the system itself. MS Coliform gave a 0 RLU reading for *Staphylococcus aureus* as expected, which eludes to the existence of some specificity within the system, MS EB however did detect 12 or less CFUs for *Staphylococcus aureus,* which should not be the case, as it is not an Enterobacter.

Using Table 4 (bacterial number estimation table) it was determined that each 10^{-3} sample used contained around 10^6 bacterial cells per ml. This was indicated by MS Total, EB and Coliform with the first three strains displayed in Table 6, and with MS total and EB for *S. rubidaea.* The sensitivity of the MicroSnap systems, Total, EB, and Coliform appeared high as the results were relative to the number of bacterial cells per ml in the samples, however this could not be fully determined as the system has a 4-digit RLU output display limit. Due to MS *E. coli* detecting less than 200 CFU's when analysing a sample containing around 1 x 10^7 cells/ml *E. coli* HB 101 (Table 6), a repeat was needed to determine whether this result was an anomaly or an issue with the MS system.

4.3.2 Detecting RLU's of three bacterial strains and comparisons to plate counts

Table 7 RLU's from three bacterial strains

Strains	**Test**	**MS Total RLU**	**MS EB RLU**	**MS Coliform RLU**	**MS *E. coli* RLU**
C. freundii	*1*	9561	8039	8839	0
	2	8399	9791	7848	1
	3	7767	3422	9237	0
E. coli HB	*1*	9439	9949	7606	14
	2	9377	9437	7440	15
	3	9097	9558	6632	13
Pseudomonas syringae subsp. Phaseocola	*1*	407	13	4	0
	2	21	12	4	0
	3	285	17	4	0

Table 7 RLU generated from three bacterial strains, using MicroSnap Total, EB, Coliform and E. coli devices.

Table 8 RLU's converted to CFU

Strains	**Mean Total RLU**	**CFU/ml**	**Mean EB RLU**	**CFU/ml**	**Mean Coliform RLU**	**CFU/ml**	**Mean *E. coli* RLU**	**CFU/ml**
C. freundii	8576	>5000-TNTC	7084	>1000-TNTC	8641	>10,000	0.33	<10
E. coli HB	9303	>5000-TNTC	9648	>1000-TNTC	7226	>10,000	14	<200
Pseudomonas syringae	237.7	<1000	14	>1000-TNTC	4	<20	0	0

Table 8 The mean RLU calculated from data in Table 7, and the corresponding CFU/ml

Pre-enrichment of *C. freundii:*

Dilutions	**1**	**2**	**3**
10^{-2}	TNTC	TNTC	TNTC
10^{-3}	TNTC	TNTC	TNTC
10^{-4}	TNTC	TNTC	TNTC

Table 9 Plate counts from the Pre-enriched C.freundii, plated in triplicate for three dilutions, all samples were too numerable to count (TNTC)

Post-enrichment of *C. freundii:*

Dilutions	**1**	**2**	**3**
10^{0}	TNTC	TNTC	TNTC
10^{-1}	TNTC	TNTC	TNTC
10^{-2}	TNTC	TNTC	TNTC

Table 10 C.freundii plate counts, plated after the 6 hour incubatory enrichment period, plated in triplicate for three dilutions, all samples were too numerable to count (TNTC)

MicroSnap Total, EB and Coliform detected all three strains tested (table 7), although *Pseudomonas syringae* gave lower than expected results, which appeared to be due to a possible low production of ATP by the bacteria. MicroSnap E. *coli*

results continued to be in contrast with the actual cells/ml in the sample (10^6), repeatedly producing low RLU's, however it was again selective only for *E. coli HB.* All the plates showed growth too numerable to count, as shown in tables 9 and 10 and therefore a numerical figure could not be quantified and compared to the MS system. Due to the lawn of growth on the plates, it was decided that for further analysis using plate counts, the plates were to be incubated at 30^{o}C, in preference to 37 ^{o}C, as a lower temperature may decrease the bacterial growth, and higher dilutions would be used, allowing for the counting of colonies.

4.3.3 Testing of E. coli J53

Table 11 RLU results from *E. coli J53*

	MS Total	**MS EB**	**MS Coliform**	**MS *E. coli***
Test 1 RLU's	7821	9580	5613	12
Test 2 RLU's	8513	9975	5881	29
Test 3 RLU's	8052	9754	6733	0
Mean RLU	8128	9769	6075	14
CFU/ml	>5,000 - TNTC	>1,000- TNTC	>10,000	<200

Table 11 RLU results of E. coli J53 given by the four MicroSnap systems in triplicate, the mean RLU, and the converted CFU/ml

The MS *E. coli* system delivered low RLU's for *E. coli J53*, which was similar to that of *E. coli HB*. Possible reasons for this could be that *E. coli J53* and *E. coli HB* were lab strains, and therefore may not be expressing the genes for β-glucuronidase enzyme; the target for MS *E. coli*.

4.3.4 E. coli NCTC 12241

Table 12 RLU and CFU/ml for *E. coli NCTC 12241*

Test	RLU	CFU/ml
1	75	<5,000
2	49	<1,000
3	1	<10
Mean	41	<1,000

Table 12 RLU and corresponding CFU/ml results of E. coli 12241

The RLU detected by MS *E. coli* for *E. coli 12241* only marginally increased from previous *E. coli* strains (sections 4.3.1- 4.3.3), when tested with a 10^6 cells/ml sample. The results began to highlight a clear issue involving the MicroSnap *E. coli* system, which was consistently detecting lower than expected levels of bacteria. Previous thoughts described in 4.3.3 that the low RLU counts were due to the use of lab strains of *E. coli* became unlikely, as low RLUs were also observed using non-lab strains (*E. coli* 12241). Therefore, it was decided that that there may be another underlying issue to the system itself, which needed to be tested moving forward.

4.3.5 Pond water analysis

Table 13 shows results from the testing of untreated pond water, extracted from a local pond and tested immediately. Table 14 displays results after the pond water was filtered and resuspended in buffer to become 50x more concentrated than the neat untreated water sample (see methods 4.2.2.5).

The results from MicroSnap Total showed an approximate increase of x50 RLU and CFU/ml counts, from the neat water sample to the concentrated version, which was as anticipated. However, MS *E. coli* only slightly increased, from less than 100 to less than 200 CFU/ml, from the neat sample to the 50x concentrated sample, this indicated that there may be an upper limit to what levels of *E. coli* contamination the

MS system could detect. These results combined with previous *E. coli* tests eluded to a potential limitation of the MS *E. coli* system.

Neat water:

	1	***2***	***3***	**Mean RLU**	**CFU/ml**
MS Total	7	8	4	6	<10
MS *E. coli*	10	4	12	9	<100

Table 13 Testing of neat pond water with MS Total and E. coli, given as RLU and converted CFU/ml

Concentrated:

	1	**2**	**3**	**Mean RLU**	**CFU/ml**
MS Total	316	148	163	209	<1,000
MS *E. coli*	16	13	14	14	<200

Table 14 The RLU results converted to CFU/ml of 50x concentrated pond water sample, using MS Total and E. coli

4.4 Discussion

Although there is increasing interest and acceptance for ATP based bacterial detection systems for multiple disciplines, there is still discrepancies among the scientific community regarding the applicability of these systems in routine testing. The uncertainty concerning the reliability and sensitivity of these methods, is due to several reason, free non-bacterial ATP present in a sample, difference's in ATP content among varying species, substances which may inhibit the luciferase, luciferin reaction and the sensitivity to different strains (Sciortino and Giles, 2012, Omidbakhsh, Ahmadpour and Kenny, 2014). The first aim of this chapter was to determine the MicroSnap systems sensitivity and specificity. This was first analysed by using pure cultures of bacteria to test each MS system, the strains used were chosen in accordance to the MS targets (4.2.1.1 and figure 6). After a series of scientific investigation, using positive and negative control strains, it was decided that the MS systems Total, EB and Coliform all appear to detect their target strains as results show in tables 5 and 6 all three systems produce high Relative Light Units (RLU). Apart from some anomalies including the poor detection of *Pseudomonas*

syringae subsp. Phaseocola by MS Total. Ms *E. coli* also displayed specificity as it produced a negative result (0 RLU) for the negative control strains, and a positive result (> 0 RLU) for the positive *E. coli* control strains (4.3).

However, one major concern about the findings was that MicroSnap *E. coli* consistently produced lower RLU than should be expected when presented with a 10^6 cells/ml load. The initial hypothesis concerning this issue was that the strains used (*E. coli HB* and *E. coli J53*) were Lab strains, and thus may not have produced the enzyme β-glucuronidase. However, after testing *E. coli 12241,* which had been previously verified as producing a positive result by Hygiena, MS *E. coli* still produced low RLU (4.3.4), it was apparent that more investigation was needed. Other studies have inferred challenges with ATP based detection methods relating to the amount of variation of ATP load among different bacterial strains and species (Conn, Charache and Chappelle, 1975). Therefore, because of these results, it was not possible at this point of the project to explore the second part of aim 1, which was to determine whether the systems were sensitive enough to detect low numbers of bacteria in a sample, (1 CFU/ 100 ml) in accordance to WHO guidelines (WHO, 2019).

The second aim presented in this chapter was to determine if the MS systems delivered reproducible RLU's, therefore multiple repeats were conducted throughout each experimental procedure. Although positive correlations have been observed in multiple studies between RLU's and results from quantitative microbiology such as plate counts, this correlation becomes weaker with low bacterial sample concentrations, which means that it remains difficult to conclude on the reliability of ATP based methods (Gibbs et al., 2014). The RLUs generated in the investigation were converted into CFU/ml in accordance with the conversion tables provided by Hygiena, the CFU's were then compared to the actual number of bacteria in that sample, found by using table 4 in 4.2.1. The MS Total, EB and Coliform systems consistently produced RLUs which when converted to estimate CFU's, correlated to the number of bacterial cells in a sample. However, at this point MS *E. coli* is not producing reliable and reproducible results.

Aim 3 of this chapter was to assess whether the MS system could be used to test environmental water samples which may contain different sediment loads, and a complex mix of bacterial load. In order to proceed with this aim, the MS system must first be able to demonstrate reliable and reproducible results using pure culture control strains. However as explained (4.3.4), MS *E. coli* did not meet these requirements and therefore the planned water spiking and environmental tests could not be initiated. Although in parallel an experimental method was conducted to compare the results of a pure environmental vs a filtered concentrated sample (4.3.5). Upon the environmental water test, another problem was identified, MS *E. coli* appeared to have an upper limit to its detection capabilities. MS Total displayed a times 50 increase in microbial load, in a 50 times concentrated sample, from the original neat water sample, however this did not occur for the MS *E. coli* system, as it was expected to. As of yet however, it remains unclear to what extent this upper limit may exist.

At this stage of understanding, two hypotheses can be inferred regarding the data generated throughout the investigations. The first hypothesis is that the low CFUs/ml detected my MS *E. coli* are due to the batch of MS enrichment and detection tubes used in previous methodology. It is possible that a fault may have passed through the production process for this batch, and therefore the next stages of this investigation will involve comparing MS results from the original batch used in previous methods to a new batch of devices.
The second is that the low RLUs could also be a result of mechanical issues, such as the Ensure luminometer being defective and not detecting the light produced by MS *E. coli* detection devices. Alternatively, the dry block incubator may not be reaching or remaining at the set temperature. Although the dry block incubator was checked at different temperature settings with a thermometer, therefore this was not the cause of the issues. Mechanical issue with these pieces of equipment seem unlikely because they appear to work correctly with all other MicroSnap systems (Total, EB, Coliform).
It is difficult to arrive at any conclusion as to the nature of issues surrounding MS *E. coli* without more quantifiable results, therefore further research should examine strategically by running each test in triplicate alongside plate counts to get a quantitative comparison.

4.5 Conclusion

The primary aim of this chapter was to use scientific means to investigate whether all four MicroSnap systems were sensitive to their targets, the answer to that question concerning MS *E. coli*, was yet to be determined. After initial analysis, it remained unclear as to whether the system can be applied to water testing. As such, the plans to use the MicroSnap system on water samples spiked with known concentrations of bacteria were halted. As well as plans to compare MS against current standard water testing techniques. Proceeding with the investigation will mainly focus on deducing the problems raised by MS *E. coli*.

Due to the satisfactory results generated by MicroSnap Total and EB throughout initial testing, combined with the knowledge that current water testing techniques mainly involve the detection of indicator bacteria such as *E. coli* and other coliforms (Ramírez-Castillo et al., 2015), it was decided that MicroSnap *E. coli* and Coliform would be the main focus moving forward.

Exploring the hypotheses raised after the production of low RLU and CFU by MicroSnap *E. coli*

5.1 Introduction and Aims

Unsafe drinking water is a vehicle for diseases with communicable diseases (including water borne diseases) being one of the leading causes of child mortality in developing countries (Washdata.org, 2019). To ensure potable water the biological parameters of water quality should be regularly monitored by a standardized detection method (WHO, 2018). The cruciality of regular monitoring is clear (see section 3.2), and the appropriate detection technique is fundamental to any monitoring scheme (Ramírez-Castillo et al., 2015).

There is an increasing need for water quality tests to not only be rapid and cheap, but that are just as accurate and reliable as current standardized methods (Rajapaksha et al., 2019). Chapter 1 focused on the analysis of the MicroSnap (MS) systems as a potential candidate for a rapid water testing technique. The results from those investigations highlighted that the MS *E. coli* system displays false negatives, or an underestimation of bacterial load. The initial hypothesis concerning these issues was that the *E. coli* strains used lacked the ability to produce the enzyme β-glucuronidase, which is what MS *E. coli* detects. However, this theory was halted when E. coli 12241 was tested and also produced an underestimation.
The limitations of the MS *E. coli* system to detect *E. coli* strains led to the following 6 hypotheses:

1. The Ensure luminometer is at fault, whereby it does not efficiently detect the signal produced by MS *E. coli.*
2. Strains of *E. coli* used do not produce enough β-glucuronidase for detection.
3. The induction of β-glucuronidase in the enrichment stage is poor.
4. Poor binding of ATP to luciferin and therefore failure to produce light for detection.
5. The nutrient broth in the enrichment step is not sufficient for growth of *E. coli* strains.

6. The extractant/lytic agent may not be lysing the *E. coli* cells enough for detection thus producing low relative light units.

This chapter aims to address each of the hypotheses above, in order to determine the principle issue(s) which lead to the MS *E. coli* system underestimating, or not detecting the *E. coli* cells. It also aims to deduce whether these issues arose due to the batch of kit used throughout the study, or whether they occur in all MS *E. coli* systems produced.

5.2 Materials and Methods

5.2.1 Bacteria and growth conditions

Bacteria used in this chapter are described in Table 15. Prior to use in conjunction with MS kits, all *E. coli* strains were cultured on nutrient agar for up to two days at 30-37^{o}C or in nutrient broth with shaking at 37^{o}C overnight.

Table 1: Bacterial strains used in this chapter

Species	**Strain**	**Description**	**Source**
Escherichia coli	NCTC 12241	Recommended positive control	Culture collections England
Escherichia coli	NCTC 12241	Recommended positive control	Hygiena international Ltd, Guilford
Escherichia coli	NCTC 12923	Positive control	Hygiena international Ltd, Guilford
Escherichia coli	NCTC 9001	Positive control	Hygiena international Ltd, Guilford

Table 15 Positive control strains used in this section of the project

5.2.1.2 Equipment

The TSA (Tryptic soy agar) and brilliance agar, the CHDG extractant, Maximum recovery diluent buffer (MRD) and the second (L2) luminometer were provided by Hygiena International Ltd, Guilford.

5.2.2 Methods

5.2.2.1 Testing the Ensure Luminometer

The first hypothesis was that the Ensure luminometer used, led to the low RLU's generated throughout testing in chapter 1. This method was conducted to explore this. The enrichment step of the MS system was skipped, as it was the luminometers ability to detect a signal being tested, not the MS ability to grow the bacteria.

MS *E. coli* detection devices were used in duplicate to swab overnight cultures of two *E. coli* strains, NCTCC 12241 and 12923. The detection devices were then incubated for 37ºC for 10 minutes as standard. The devices were placed in to the first luminometer (L1), which had been demonstrated in all the MS experiments described in chapter 1 and then a second luminometer currently being using in Hygienas Guilford laboratory (L2). Hygienas cal check calibration system was also used on both L1 and L2 after this procedure, the system is a stick like device which emits a specific light intensity, for the luminometer to detect.

5.2.2.2 MicroSnap batch testing and E. coli strain comparisons

This method was conducted to investigate whether the results in chapter 1 arose due to any of the hypotheses (2-6) occurring in the specific batch of MS *E. coli* tubes being used throughout this study, or whether these problems are across all the MS *E. coli* tubes manufactured. To do this, the batch of MS *E. coli* tubes used throughout this project (B1) was compared against a new batch of tubes provided by Hygiena (B2).

The four *E. coli* strains listed in Table 15 were 1/10 serially diluted in maximum recovery diluent (MRD) buffer. From these suspensions, 1ml of the 10^{-5} dilution was pipetted into MS *E. coli* enrichment tubes from B1 and B2 in triplicate. The tubes were then incubated under standard recommended conditions. After incubation 100µl of 10^{-5} and 10^{-6} dilution of each strain was spread plated on to TSA, and brilliance agar plates, and incubated overnight at 37ºC. The conversion table (Appendix 4) was then used to convert the RLU output to CFU/ml. Brilliance chromogenic plates were used as they detect the same targets as the MS *E. coli* and coliform systems do. Purple colonies are produced on the plates in the presence of β-glucuronidase, which indicates *E. coli*, and pink colonies are indicators of β-galactosidase in other coliforms, therefore the number of purple colonies should match up with the number of CFU/ml detected by the *E. coli* Microsnap.

Statistical analysis was performed on the results, to generate a p value comparing the difference from the B1 and B2 data sets for each *E. coli* strain.

5.2.2.3 Extractant efficiency test

The extractant or lytic agent is used in the MS *E. coli* tubes to lyse the bacterial cell and release the enzymes to allow for detection. This method was performed to test the efficiency of this (hypothesis 6).
Two serial dilutions were performed using four overnight cultures of *E. coli* strains. The first comprised of one loop full of bacterial cells in 1ml of MRD buffer, then 1 in 10 serially diluted. The other was the same, with the addition of 150µl of extractant added at 10^{-1} dilution and then serially diluted. The volume of extractant was chosen because there is 150µl of extractant in the MS detection tubes. The 10^{-5} and 10^{-6} of the MRD dilutions, and the 10^{-4} and 10^{-5} of the extractant dilutions were plated out on TSA and Brilliance plates and incubated overnight at 37ºC, to compare the number of cells grown.
The test was repeated due to TNTC plates (see section 5.3.3), using 1ml of extractant instead of 150µl as before, and further serially diluted to 10^{-6} and 10^{-7}. The MRD series was also further diluted to 10^{-7} and 10^{-8}, both series were spread plated out on TSA and Brilliance and incubated overnight at 37ºC.

5.2.2.4 Extraction comparisons

Continuing investigations into hypothesis 6, this method was conducted to analyse how the original extractant in the MS detection devices compares to a different extractant (CHDG) used by Hygiena and against distilled water. Both *E. coli* and coliform detection devices were investigated as they contain the same extractant and therefore should produce the same percentage of cell lysis.

E. coli 12241 and 12923 were serially diluted to 10^{-4}, 1ml of the 10^{-4} dilution of both strains was pipetted into the corresponding MS *E. coli* and coliform enrichment devices and incubated according to the manufactures instructions. The tubes from several *E. coli* and Coliform detection snap valve devices were removed and the bulbs washed of original extractant, clean unused plastic tubes then were put in place of the old tubes. Distilled H_2O (150µl) was placed in to the four clean *E. coli*

tubes and the four clean coliform tubes. CHDG (150µl) was pipetted into another four clean *E. coli* tubes and four clean coliform tubes.
After the enrichment stage 100µl of 12241 was taken and was pipetted into two standard E.coli detection devices with original extractant, two containing H_2O and two containing the CHDG extractant, this was repeated for strain 12923, and also on the coliform detection devices (figure 1).

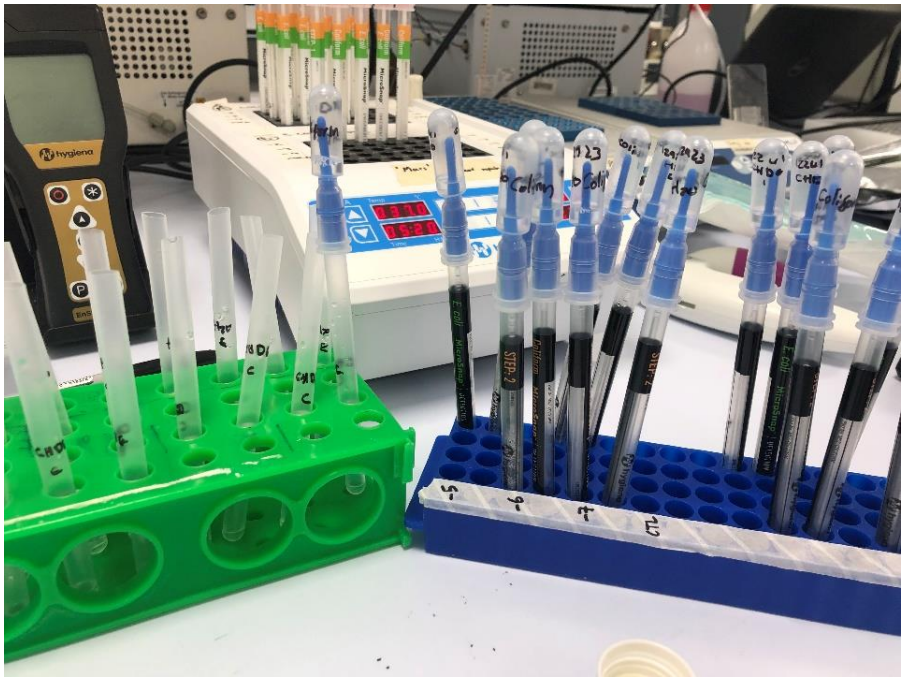

Figure 7 Image showing E. coli and coliform detection devices with clean tubes containing the CHDG extractant and distilled water, as well as the enrichment devices incubating in the background of the image.

5.2.2.5 Analysis of E. coli cell supernatant

This method was carried out to explore the hypothesis explained in 5.3.4, by analysing the supernatant of cells suspended in buffer and spun down. Extracellular products will be contained within the supernatant, while the pellet will contain the bacteria cells.

E. coli 12241 and 12923 was suspended in 1ml of MRD buffer and transferred into Eppendorf tubes. The tubes were spun at 6000rpm for 6 minutes, the supernatant was transferred to fresh Eppendorf's and re-spun, the supernatant was then transferred to *E. coli* and Coliform enrichment tubes and then the corresponding

detection tubes as standard. The samples were also spread plated on brilliance agar and incubated overnight at 37ºC.

5.3 Results

5.3.1 Testing the luminometer detection devices (Hypothesis 1)

Luminometer 1 and 2 were compared using two positive control strains (Table 16). Although the RLU outputs for each test repeat, across the two luminometers appear varied from one another, table 16 shows that only the RLU's in test 1 *E. coli* 12241 lead to variation in the converted CFU's/ml. L1's detected RLU converts into <1,000 CFU and L2 detects <500 CFU, all other test repeats using L1 and L2 generate the same CFU/ml as each other. L1 and L2 also both detected the correct level of light intensity, during the confirmatory test using Hygienas calibration system (see method 5.2.2.1). These results suggest that the low RLUs by MS *E. coli* in previous investigations were not a result of the luminometer being used. These results also highlight the fact that changes in the RLU do not always equate to differences in the CFU/ml.

Table comparing RLU output from two luminometers

	E. coli 12241				*E. coli* 12923			
	L1 RLU's	**CFU/ml**	**L2 RLU's**	**CFU/ml**	**L1 RLU's**	**CFU/ml**	**L2 RLU's**	**CFU/ml**
Test 1	51	<1,000	31	<500	727	>10,000	531	>10,000
Test 2	114	<5,000	83	<5,000	687	>10,000	507	>10,000

Table 16 Comparison of two luminometers used with Hygienas MicroSnap E. coli kits on two positive control strains, with 2 repeats (Tests 1 and 2), and CFU/ml found using Hygienas conversion table (Appendix 4). L1= luminometer used throughout this project, provided by Hygiena, L2= A luminometer used in Hygienas Guilford site.

5.3.2 Comparison of MicroSnap tube batch numbers

Experiment 5.2.3 was conducted to compare the original batch (B1) with a new batch of MS *E. coli* tubes(B2). The results from table 17 show that across the two batches of devices the results generated were similar, for example both B1 and B2 detected <500 CFU/ml for the original *E. coli* 12241strain (see appendix 4 for conversion table). However, B1 overall appeared to have a higher sensitivity than B2, for strain 9001 for example B1 detected <200 CFU/ml, whereas B2 detected 0 CFU/ml. Statistical analysis was performed on these data sets, to generate a p value comparing the difference from B1 and B2 for each *E. coli* strain. Using a 5% confidence level, all P values generated were above 0.05, therefore it cannot be concluded that there is a statistically significant difference (Cumming, Fidler and Vaux, 2007). These results suggested that the results gained through previous study may not be related to the specific batch of MS tubes being used, as no significant difference was observed. Table 18 shows the CFU/ml calculated using the plate count method, the CFU/ml of the 10^{-5} dilution plates were too numerable to count (TNTC).

Table 17 Comparison of two batches of MS devices

		RLU's from B1	**RLU's from B2**	**P value=**
Original Escherichia coli NCTC 12241	Test no. 1	21	5	
	Test no. 2	66	31	
	Test no. 3	15	45	0.743
	Mean:	34	27	
	CFU/ml:	<500	<500	

New Escherichia coli	Test no. 1	20	22	
NCTC 12241	Test no. 2	21	1	
	Test no. 3	22	3	0.140
	Mean:	21	9	
	CFU/ml:	<500	<100	
Escherichia coli	Test no. 1	837	159	
NCTC 12923	Test no. 2	19	74	
	Test no. 3	916	655	0.434
	Mean:	591	296	
	CFU/ml:	>10,000	<10,000	
Escherichia coli	Test no. 1	0	0	
NCTC 9001	Test no. 2	35	0	
	Test no. 3	8	1	0.257
	Mean:	20	0	
	CFU/ml:	<200	0	
Control MRD		0	0	

Table 17 The RLU's generated by the original batch of MS devices (B1) versus the new batch of devices (B2), analysed using a 10^{-5} dilution of four E. coli strains in triplicate (test no.1 – 3), the mean RLU's were calculated and then converted to CFU/ml using Hygiena's conversion tables. A T test was performed on this data to calculate the p values for each E. coli strain.

Table showing CFU/ml of four strains of *E. coli* found using spread plate method

Bacterial strain	Dilutions	Brilliance Agar plate counts	**CFU/ml**	TSA plate counts	**CFU/ml**
Original *Escherichia coli* NCTC 12241	10^{-6} 10^{-5}	668 TNTC	**6680** **TNTC**	688 1352	**6880** **13520**
New *Escherichia coli* NCTC 12241	10^{-6} 10^{-5}	940 TNTC	**9400** **TNTC**	628 TNTC	**6280** **TNTC**

Escherichia coli NCTC 12923	10^{-6} 10^{-5}	952 TNTC	**9520** **TNTC**	1088 TNTC	**10880** **TNTC**
Escherichia coli NCTC 9001	10^{-6} 10^{-5}	900 TNTC	**9000** **TNTC**	905 TNTC	**9050** **TNTC**
Control MRD		0	**0**	0	**0**

Table 18 Plate counts from four E. coli strains on brilliance and TSA agar plates, and the conversion of the plate counts to CFU/ml of that dilution sample, most of the 10^{-5} dilutions was too numerable to count (TNTC).

5.3.3 Extractant efficiency: testing hypothesis 6

All plates containing the MRD dilution series, and the 150µl extractant series were TNTC. When repeated with 1ml of extractant, only one plate was countable, a TSA plate containing *E. coli* 12241 at 10^{-7} dilution. The plate count was 960 colonies, therefore 9600 CFU/ml, all other plates showed growth TNTC. There did appear to be some evidence of lysis of the *E. coli* cells in the 1ml extractant series as plates visually appeared to have less growth than the MRD plates which had complete bacterial lawn. However, this could not be confirmed as counting the colonies proved difficult.

5.3.4 Comparisons of two extractants and water

MS coliform produced higher RLU's across both strains of *E. coli*, and across all three variables than MS *E. coli* (Table 19). Although interestingly MS coliform also detected RLU's for both strains in water, which was unexpected as there should be little to no extraction of the enzymes. When comparing the converted CFU values from MS *E. coli* to MS coliform; in some instances, there is a large difference in RLU and no change in CFU. An example of this is Test number 1 of original extractant, strain 12923; MS E. coli detected 433 RLU which converted to >10,000 CFU, and MS coliform detected 6030 RLUs and still >10,000 CFU's. Overall, however MS coliform appeared more sensitive in detecting *E. coli* strains, such as test 1 and 2 for the original extractant with strain 12241, MS *E. coli* detected less that 5,000 CFU, and MS coliform detected more than 10,000.

A hypothesis was formed that that this may be due to the distribution of β-galactosidase and β-glucuronidase in the *E. coli* cells. β-galactosidase may be

expressed or excreted extracellularly and therefore more easily detectable by the MS coliform system without much lysis of the cells. The *E. coli* system however detects β-glucuronidase which may be present more intracellularly and therefore due to poor extraction, MS *E. coli* produces a low signal output. The original extractant which is used in the MS detection tubes appeared to cause more cell lysis, than both CHDG and water overall.

Table 19: Comparison of MS *E. coli* and coliform on under three conditions

		MS *E. coli* RLU's		MS Coliform RLU's	
		Strain 12241	Strain 12923	Strain 12241	Strain 12923
Original extractant	Test 1	77	433	304	6030
	Test 2	85	1110	4934	6882
	CFU/ml	**<5,000**	**>10,000**	**>10,000**	**>10,000**
		<5,000	**>10,000**	**>10,000**	**>10,000**
H_2O	Test 1	0	7	163	272
	Test 2	0	3	152	114
	CFU/ml	**0**	**<50**	**<5,000**	**<10,000**
		0	**<20**	**<5,000**	**<5,000**
CHDG	Test 1	3	188	3375	1905
	Test 2	4	127	6745	2375
	CFU/ml	**<20**	**<5,000**	**>10,000**	**>10,000**
		<20	**<5,000**	**>10,000**	**>10,000**

Table 19 The RLU results of two E. coli strains generated by MS E. coli and coliform detection devices, with the original extractant, CHDG (different extractant) and distilled water, tests 1 and 2 are the repeats of each variable. CFU's converted using table 4 in appendix.

5.3.5 Analysis of suspended cell supernatant

MS coliform which detects β-galactosidase, generated higher RLU and CFU outputs for both strains compared to MS *E. coli* (table 20). This suggested that the supernatant contained high levels of extracellular β-galactosidase. MS *E. coli* detects β-glucuronidase, and due to the low CFU/ml detected for both strains, it eludes to β-

glucuronidase being more intracellular and secreted less. However, it is possible that either there were still *E. coli* cells in the supernatant, or that some β-glucuronidase enzyme was extracellular as MS E*. coli* device detected CFU's.
Strain 12923 gave higher RLU's in both tests, more so in the coliform test, which suggest that strain 12923 produces more β-galactosidase extracellularly. This was confirmed using Brilliance agar plates (Figure 8), the plates with strain 12241 produced majority purple colonies, which indicated β-glucuronidase, and the plates with 12923 were almost all pink colonies, which represents the β-galactosidase enzyme.

Table 20 RLUS and CFU of cell supernatant

	MS Coliform RLU		MS *E. coli* RLU	
	E. coli 12241	*E. coli* 12923	*E. coli* 12241	*E. coli* 12923
Test 1	171	871	6	107
CFU/ml	<5,000	>10,000	<50	<5,000
Test 2	311	398	3	35
CFU/ml	>10,000	>10,000	<20	<500

Table 20 RLU's generated by MS E. coli and coliform from the analysis of cell supernatant of two strains of E. coli, tests 1 and 2 are the repeats of each variable.

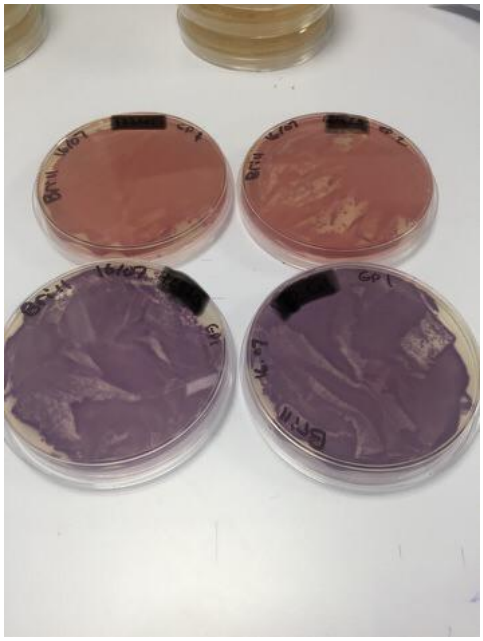

Figure 8 Photograph taken of four brilliance agar plates, two containing strain 12241 and two with 12923.

5.4 Discussion

Previous investigations showed that MS *E. coli* consistently underestimated the bacterial load in samples, further investigations described here addressed those findings and analysed the potential causes of the low Relative light units and CFU's/ml. The first investigation considered whether these issues were batch specific, in that there was a fault which possibly occurred during manufacturing process, either with the luminometer or MS *E. coli.* This hypothesis meant that the issues observed would stop when systems were replaced by a new batch of devices or a new luminometer.

Methods 5.2.2.1 and 5.2.2.2 tested this hypothesis, results comparing the original batch (B1) to a new batch (B2) of MS *E. coli* tubes, and the comparison of two luminometers, were not dissimilar from each other. Thus, the results gained in previous study were not related to the specific batch of kit being used.
Another hypothesis to be investigated was the efficiency of the extractant fluid in MS *E. coli* detection tubes. Bacteria may not export the β-galactosidase and β-glucuronidase enzymes into the media, and therefore need an extractant/lytic agent to release the enzymes more efficiently. Once released the enzymes can bind to the pro-luciferin molecule and cleave the pro group, which is a manufactured substrate of β-glucuronidase (MS *E. coli)* or β-galactosidase (MS coliform). **T**he luciferin then binds to ATP in the presence of luciferase to produce light for detection. Without that first lysis step none of this reaction can occur, and no RLU's would be produced. Strains of *E. coli* were serial diluted; one with buffer alone, the other with added extractant, and then plated out to compare the amount of growth. Apart from one plate (5.3.3), there was no observable differences between the extractant plates and the buffer plates, which indicated poor cell lysis by the extractant. The enzyme β-glucuronidase is one of the most common biomarkers to test for the presence of *E. coli* cells, as a large majority of other coliforms lack this. β-glucuronidase detection can also identify VBNC cells, which traditional methods cannot (Satoh et al., 2020). However, difficulties in detection present because different *E. coli* cells produce varying amounts of enzyme and at different times, which becomes even more varied when multiple strains are considered. Due to this it makes it even more imperative for an efficient lytic agent to be used (Omidbakhsh, Ahmadpour and Kenny, 2014).

MS *E. coli* was compared with the MS coliform detection devices using two *E. coli* strains, the original extractant was also compared to an alternative from Hygiena (CHDG), and distilled water. On average the original extractant caused more cell lysis than the alternative and water, as more RLU's were detected. MS coliform detected higher CFU's for every sample compared to MS *E. coli* (table 19), this result was interesting as both detection devices contain the same extractant fluid, which in theory should produce the same level of cell lysis and thus the same detection. Although, MS coliform detection device works by detecting the enzyme β-galactosidase, not and β-glucuronidase as MS *E. coli.* Suggesting that there is either a much higher production of β-galactosidase by *E. coli* strains, or that β-glucuronidase is more concentrated intracellularly and thus due to poor extraction is not being released for detection.

5.5 Conclusion

The overall conclusion relating to the low RLU values for MS *E. coli* is likely to be due to a combination of low β-glucuronidase production in some *E. coli* strains with poor lysis of cells by the extractant. Further investigation will focus on quantifying the level of cell lysis by the extractant. Through discussions with Hygiena it was decided that they would attempt to alter the extractant and send samples for testing and comparisons to the original extractant.

One of the original aims of this project was to compare the accuracy and reliability of MicroSnap to current standardized methods of water analysis. Moving forward it was decided that there was no reason to investigate this in this project, as MS *E. coli* currently produces underestimations of contamination and thus not comparable to current methods.

Analysis into the extent of bacterial cell lysis caused by the extractant in the MicroSnap *E. coli* detection tubes and comparisons with a newly developed extractant.

6.1 Introduction and Aims

Works in chapters 1 and 2 suggested that the low RLU detection by MS *E. coli* appears to be due to a combination of poor cell lysis and low levels of β-glucuronidase by some *E. coli* cells. No growth differences were observed between *E. coli* plates with only buffer and plates with added extractant (see 5.3.3). This confirmed that the extractant was insufficient in causing adequate cell lysis. If the extractant in MicroSnap *E. coli* cannot lyse the bacterial cells and release the enzymes, then the enzymes cannot trigger the luciferin/luciferase reaction and produce relative light units to measure. It was also theorised that *E. coli* cells may not produce or export β-glucuronidase efficiently enough for detection, due to results from MS coliform. MS coliform consistently gave higher RLU's for every sample compared to MS *E. coli,* suggesting that there is either a much higher production or exportation of β-galactosidase by *E. coli* strains.
Therefore, the aim of this chapter was to further investigate the extractant/lytic agent in the MicroSnap *E. coli* and coliform detection tubes and determine the extent of cell lysis.

6.2 Materials and Methods

6.2.1 Bacteria and growth conditions

E. coli NCTC 12923 was used throughout this chapter, because in previous testing it appeared to produce higher RLU when used with MS *E. coli,* compared to other strains (Chapter 2). It was provided by Hygiena international Ltd, Guilford and prior to usage was cultured on nutrient agar for up to two days at 30-37^{o}C or in nutrient broth with shaking at 37^{o}C overnight.

6.2.1.2 Equipment

All MicroSnap equipment was provided by Hygiena International Ltd, Guilford, as well as the new altered extractant fluid, named extractant 2 (E2).

6.2.2 Methods

6.2.2.1 Quantifying the detection level of MS E. coli

The bacterial load at which the MS *E. coli* system is unable to detect in a sample was tested. A 1 in 10 serial dilution of an overnight grown fresh culture of *E. coli* NCTC 12923 was performed, down to 10^{-9} with sterile pond water. Pond water was used in place of distilled water in an attempt not to stress or kill the bacteria, the pond water was sterilized by filtration, twice through a 0.22µm filter.
Dilutions 10^{-5} to 10^{-9} were testing in triplicate using the *MS E. coli* system (see appendix 1-3 for instructions), and 100µl of each was spread plated, including a control of the unfiltered and filtered water.

6.2.2.2 Comparing the effects of time on the extraction efficiency

According to the manufacturer's instructions the incubation time of the detection step is 10 minutes, in order to investigate whether increasing the incubation time allowed for more cell lysis by the extractant, this incubation time was increased.
E. coli NCTC 12923 was serially diluted to 10^{-6}, samples of 10^{-5} and 10^{-6} were incubated according to the manufactures instructions. After the enrichment stage, an

aliquot of each dilution sample was immediately placed into the MS *E. coli* detection devices, in triplicate, and tested with the luminometer (0 mins). This was repeated in triplicate every 10 minutes for 1 hour.

The results are presented in graphical form (figure 10), and standard deviation error bars were inferred to provide statistical information on the data set.

6.2.2.3 Comparison of the level of cell lysis under different extractants

This method compared the extent of cell lysis occurring, with the original extractant against an extractant that has been altered by Hygiena International Ltd and sent for trial testing, to determine whether it should replace the original currently in the detection tubes.

Three serial dilutions were performed using *E. coli* NCTC 12923, the first contained no extractant, only buffer and *E. coli,* the second contained the original extractant (E1), and the third contained the new extractant (E2). Dilutions 10^{-5} to 10^{-9} of each of the three dilution series were spread plated out in triplicate, and incubated overnight at 30^{o}C.

6.2.2.4 Analysis of E2 in MS E. coli detection tubes

This method was designed to give an indication of what future results may show If E1 (original extractant) was replaced by E2 (new extractant) in MS *E. coli.*
E. coli NCTC 12923 was 1 in 10 serially diluted to 10^{-6} in buffer, dilutions 10^{-4} to 10^{-6} were incubated according to the manufactures instructions. After the enrichment step, the dilutions were transferred to the detection tubes in triplicate and 100µl of E1 or E2 was added to the corresponding MS *E. coli* detection tubes. The detection tubes were incubated for 10 mins, and then detected using the luminometer.

6.3 Results

6.3.1 The minimum bacterial load which MS detects

A dilution series was analysed by MS *E. coli*, for dilutions 10^{-5} and 10^{-6} MS *E. coli* gave RLUs which equated to CFUs/ml above 10,000 which are acceptable bacterial numbers for those samples. However, from dilutions 10^{-7} onwards MS fails to detect numbers of bacteria. When compared to plate counts (table 21 and figure 9), the number of colonies observed for dilutions 10^{-5} and 10^{-6} match the CFUs given by MS. Although plates 10^{-7} to 10^{-9} showed growth (in the thousands for plate 10^{-7}) which was not detected by MS *E. coli*. These results suggest that MS *E. coli* has a lower limit of detection around 100-1000 CFUs/ml, which does not adhere to the current acceptable parameters of drinking water; of 0-1 CFU/ml (WHO, 2019).

Table showing RLU's and CFU/ml of *E. coli 12923* found using a 1 in 10 dilution series

Dilution	Repeat 1 (RLUs)	Repeat 2 (RLUs)	Repeat 3 (RLUs)	Mean CFUs/ml
10^{-5}	3209	4147	3068	>10,000
10^{-6}	652	51	1022	>10,000
10^{-7}	0	2	3	<10
10^{-8}	0	0	0	0
10^{-9}	1	1	0	<10
Control (Filtered water)	1			
Control (Unfiltered water)	2			

Table 21 RLU results from dilutions of 10^{-5} - 10^{-9} of E. coli 12923, and the average CFU found using the conversion table provided by Hygiena International Ltd (Appendix 4)

CFUs/ml of *E. coli 12923* found using spread plate counts

Dilution	Plate counts	CFUs/ml
10^{-5}	TNTC	NA
10^{-6}	896	8960
10^{-7}	197	1970
10^{-8}	32	320
10^{-9}	4	40
Control (Filtered water)	0	0
Control (Unfiltered water)	73	730

Table 22 Plate counts from dilutions of 10^{-5} - 10^{-9} E. coli 12923 and two control plates using nutrient agar.

Figure showing a serial dilution of E. coli 12923 grown on nutrient agar

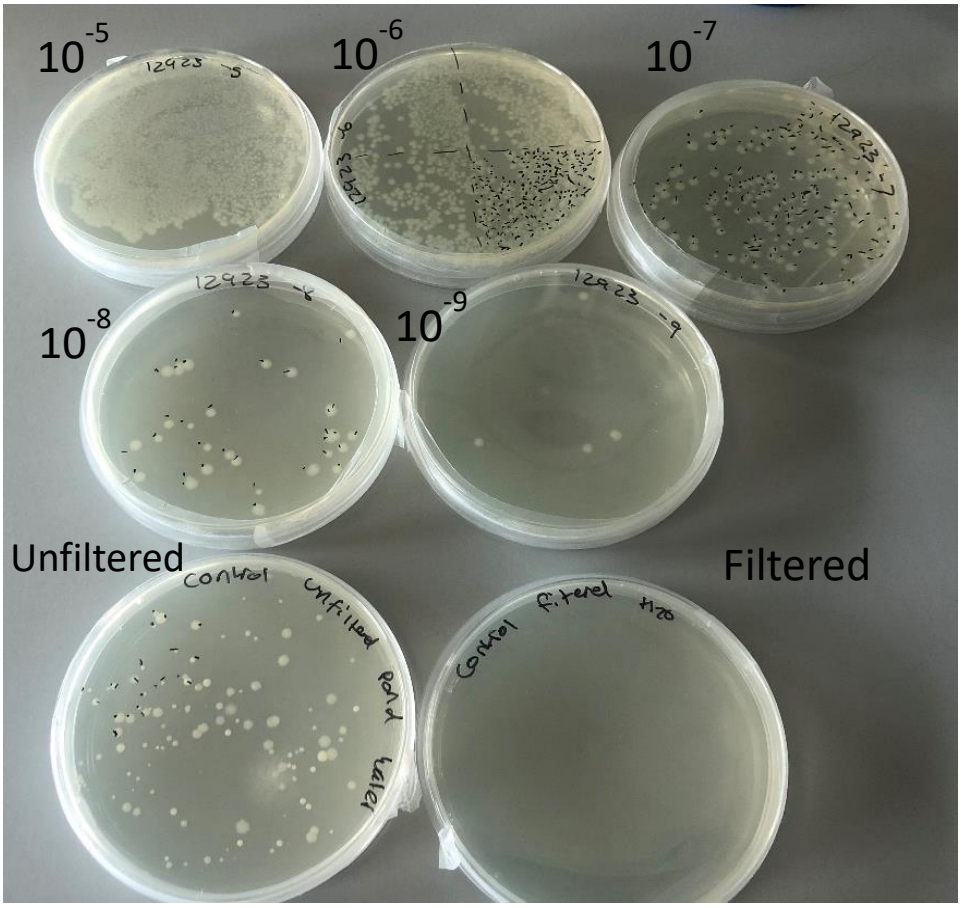

Figure 9 Photograph taken of seven nutrient agar plates containing a dilution series of E. coli 12923, starting with 10^{-5} in the top left-hand corner to 10^{-9} , and two control plates of filtered and unfiltered water, used to compare against RLU's generated by the MS E. coli system.

6.3.2 Extractant efficiency vs. incubation time

Bacterial lysis is observed after the recommended 10-minute incubatory period; as the results show in table 23 and figure 10 that RLU values were generated after this time. This was expected as 10 minutes is the amount of time suggested by Hygiena International Ltd. However, as can be observed graphically by figure 10 the largest amount of lysis for the 10^{-5} dilution occurred between 10 and 20 minutes, and between 30 and 40 minutes for the 10^{-6} dilution. These findings suggested that the RLU results generated by MS *E. coli* may be improved when the readings are taken at a period longer than currently set in the manufacturer's instructions, for example at 20 minutes. These improved results could be due to the bacterial sample being allowed to incubate with the extractant for an extended period, which increases the percentage cell lysis occurring, and therefore a higher proportion of the β-glucuronidase biomarker is released for detection. Standard deviation error bars

were calculated in figure 10 to provide statistical information. The length of the error bars for dilution 10^{-5} are larger, and thus the standard deviation of that data set is larger (Cumming, Fidler and Vaux, 2007). A graphical representation of CFU/ml was not completed because at present, the conversion tables only reach <300 RLU to convert in to CFU; as the majority of the RLU's (table 23) are above this, the graph did not show and increase over time after the 10 minute interval.

The mean RLU's taken for two dilution series over time.

Time (mins)	10^{-5} dilution (RLU)	**Mean RLU**	10^{-6} dilution (RLU)	**Mean RLU**
0	**1**. 7 **2**. 5 **3.** 2	**5**	**1.** 0 **2.** 0 **3**. 0	**0**
10	**1.** 1136 **2**. 1171 **3.** 1401	**1236**	**1.** 0 **2.** 3 **3.** 1	**1**
20	**1.** 7313 **2.** 2437 **3.** 4139	**4628**	**1.** 29 **2.** 209 **3.** 279	**172**
30	**1.** 2021 **2.** 5896 **3.** 3474	**3797**	**1.** 190 **2.** 386 **3.** 118	**231**
40	**1.** 3005 **2**. 7594 **3.** 2974	**4524**	**1.** 950 **2.** 527 **3.** 600	**692**
50	**1.** 4589 **2.** 4155 **3.** 4802	**4515**	**1.** 485 **2.** 716 **3.** 1085	**765**
60	**1**. 6819 **2**. 5476 **3**. 4556	**5617**	**1.** 582 **2.** 990 **3.** 806	**792**

Table 23 Actual and average RLUs from two dilutions (10^{-5} and 10^{-6}) of E. coli 12923 using MS E. coli detection tubes, when incubated on Hygiena International Ltd dry block incubator for increasing time intervals. The dilutions were tested in triplicate for each time period, shown as 1. 2. 3. in the table.

Average MS E. coli RLU's vs Time

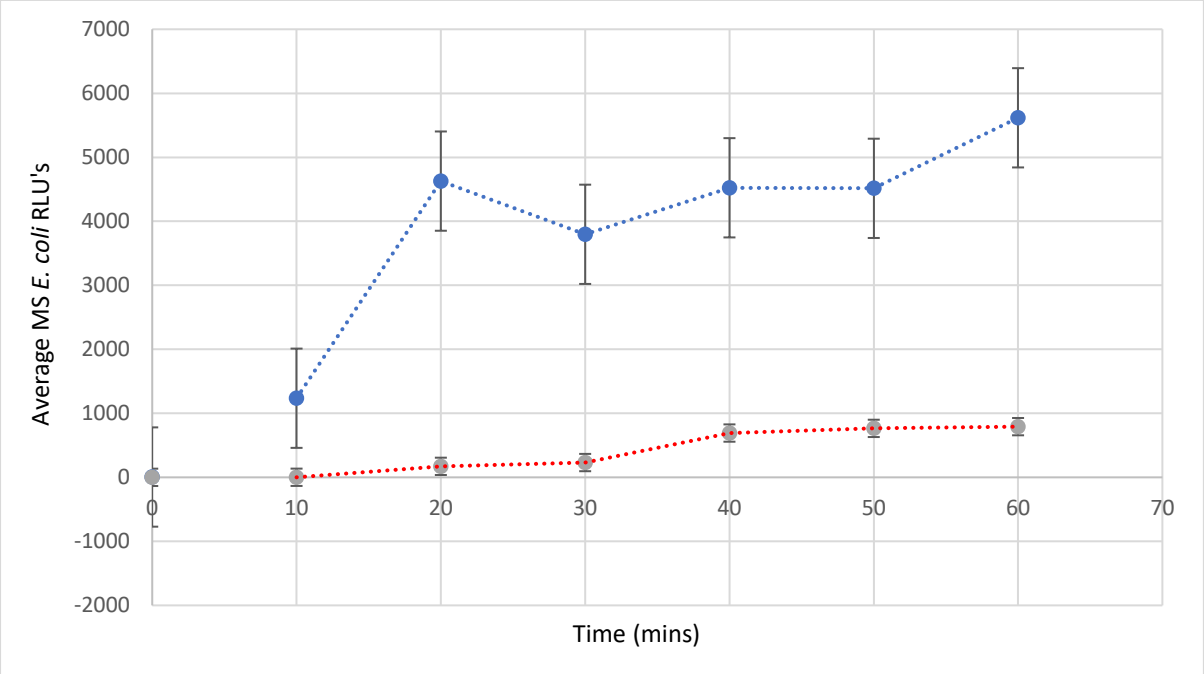

Figure 10 Mean RLUs from dilutions of E. coli 12923, when incubated at 37°C in the dry block incubator during the detection stage of MS E. coli for different time intervals. Red line: 10^{-6} dilution, Blue line: 10^{-5} dilution. Standard deviation error bars were used for statistical analysis.

6.3.3 Comparison of extractants on cell lysis

The plate counts shown in table 24 and figure 11 clearly display the growth differences between the three dilution series. The highest amount of growth was observed on the plates with no extractant added, which contained a lawn of bacteria. Growth was reduced in the original extractant plates (E1), but almost no growth was observed on the E2 plates containing the newly developed extractant. These results suggest that the new extractant (E2) is more efficient at causing cell lysis of *E. coli*, than the extractant currently used in the MS systems.

Spread plate counts for *E. coli 12923*, comparing two extractants

		Colony no. per dilution				
Extractant:	Repeat number:	**10^{-5}**	**10^{-6}**	**10^{-7}**	**10^{-8}**	**10^{-9}**
No extractant	**1.** **2.** **3.**	TNTC	TNTC	TNTC	TNTC	TNTC
E1	**1.** **2.** **3.**	117 105 81	73 66 80	77 62 64	100 50 60	52 74 43
E2	**1.** **2.** **3.**	3 1 2	1 0 1	1 0 0	0 0 0	0 0 0

Table 24 Plate counts from dilutions 10^{-5} to 10^{-9} of E. coli 12923, with either no extractant, the original extractant (E1), or the new extractant (E2). (TNTC: Too numerable to count)

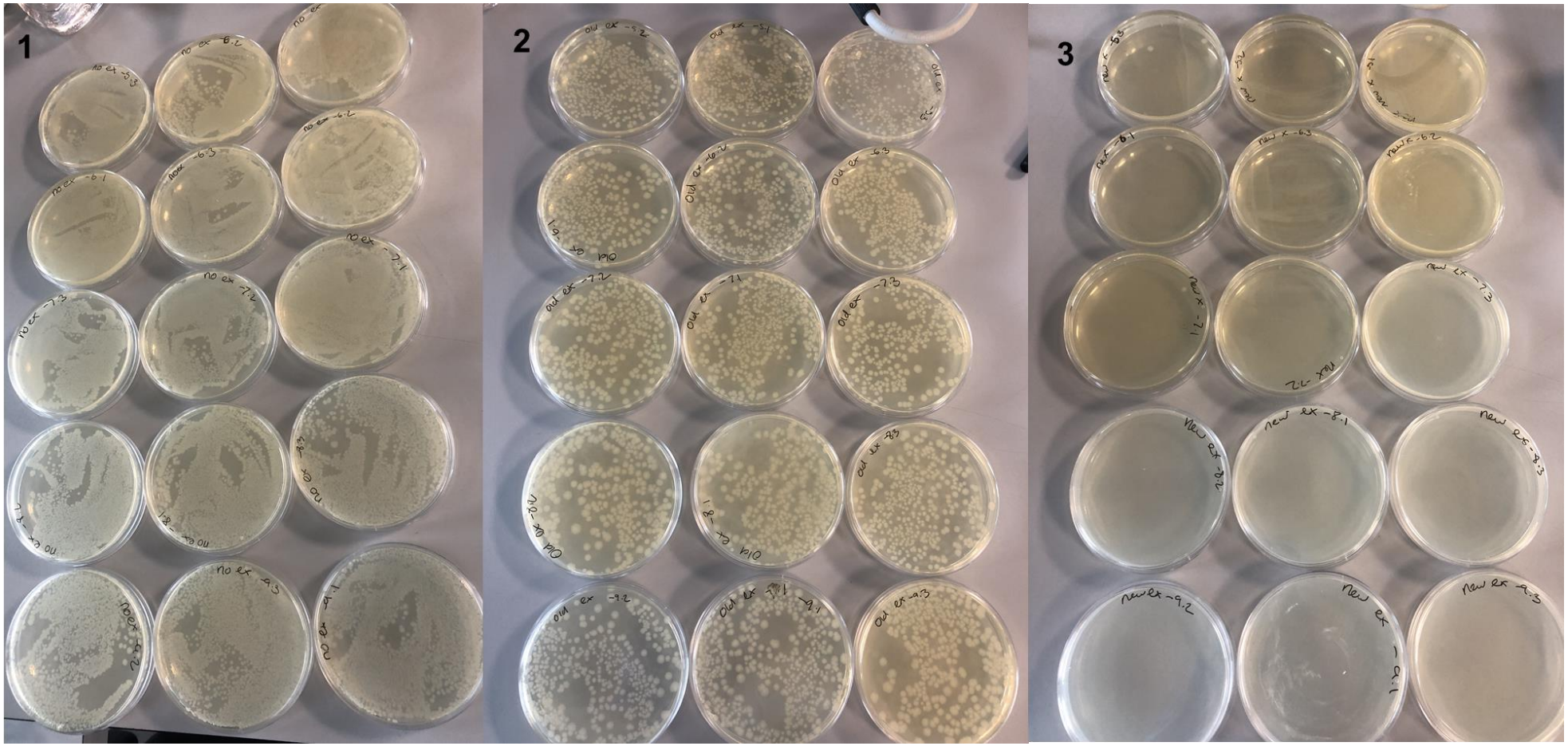

Figure 11 Photograph taken of three 1 in 10 dilution series of E. coli 12923 *on nutrient agar in triplicate. Image 1: E. coli* 12923 dilution *series with no extractant added, Image 2: E1, E. coli containing the original extractant used in the MS system. Image 3: E2, a dilution series of E. coli with added extractant, newly developed by Hygiena International Ltd.*

6.3.4 The new extractants effects on RLUs in MS E. coli detection tubes

When comparing the CFU's/ml of each of the dilutions across E1 and E2 samples, there is almost a 10-fold increase for every sample. This indicates that the new extractant (E2) delivers 10 times the amount of cell lysis than the original extractant for the same duration of time.

Table comparing RLUs derived from testing two extractions

E1 RLUs			
Test repeat	Dilutions 10^{-4}	Dilutions 10^{-5}	Dilutions 10^{-6}
1	12	7	0
2	52	10	1
3	111	3	1
CFU's/ml	**<1,000**	**<50**	**0-10**
E2 RLUs			
1	540	67	4
2	602	81	15
3	730	129	11
Mean	**>10,000**	**<5,000**	**<100**

Table 25 RLUs by MS E. coli of three dilutions tested in triplicate the first with only the original extractant (E1), and the second with the new extractant added (E2). The CFU's were generated using the mean value RLU of the test repeats for each dilution, and then converted using appendix table 4

6.4 Discussion

MicroSnap *E. coli* and MicroSnap coliform detection tubes contain the same extractant/lytic agent, therefore in theory when presented with the same sample (same bacterial load), both systems should detect the same number of bacteria and produce the same relative light units. However, this has not been the case; MS *E. coli* consistently underestimates the bacterial load and producing lower RLUs than MS coliform for the same sample. This led to the hypothesis that the results were due to a combination of both poor production/exportation of β-glucuronidase, and poor cell lysis by the extractant.

This chapter aimed to investigate the extent of cell lysis by the extractant. MS *E. coli* was compared against plate counts over a dilution series of *E. coli* cells (6.2.2.1). MS produced RLUs which appeared to correlate to that of plate counts for the lower dilution samples (up to 10^{-6}), however for the higher dilutions (10^{-7} - 10^{-9}) MS detected little to no bacteria, but growth was still present on plates. These results indicated that there was a lower limit of bacteria at to which the can system detect. It was estimated to be between 100-1000 CFU/ml, at this number of cells, there is not enough lysis occurring and enzymes present for adequate detection by the system. This evidence is comparable to results found in a study which compared four leading ATP detection systems for surface swabs. The study found that there was significant difference in the lower detection limits for all four systems, with one brand having a lower detection limit of 6.17×10^{5} CFU, before It reported an RLU value above 0 (Omidbakhsh, Ahmadpour and Kenny, 2014).

The efficiency of the extractant appeared to improve the longer the detection tubes were incubated on the dry block incubator (6.3.2). However, it was decided to compare the original extractant (E1) against a new extractant (E2) which Hygiena International Ltd had developed. Figure 11 showed very clearly the effect that the extractants had on bacterial growth, with almost no growth appearing on the E2 plates compared to E1, which indicated much higher cell lysis. The E2 extractant was then placed into MS *E. coli* tubes and compared against the original MS tubes.

Results from this method (6.3.4) showed almost a 10-fold increase in detection capabilities with tubes containing E2.

These are promising results for Hygiena International Ltd to take on board, by either increasing the incubation time in the detection stage, or replacing the original extractant with the newly developed extractant, the systems detection capabilities can be improved.

7. Discussion

7.1 Background

Globally many people only have access to unimproved water sources such as wells and springs and over 144 million people are still dependent on surface water, which includes rivers, lakes, and streams. Often, these types of water sources become contaminated, leading to water-borne diseases (Washdata.org, 2019, WHO, 2019). An adequate monitoring system seems to be crucial in reducing the incidence of disease (Rajapaksha et al., 2019). However, monitoring a complex water system from catchment to user is already a difficult task and proves more challenging in low income, less established communities. Due to the lack of resources and infrastructure, sufficient surveillance in these areas remains a challenge, and thus the detection of water pollution is slow (Ramírez-Castillo et al., 2015). Detection technologies are the forefront of any monitoring scheme, however even with advancements in these technologies the disease burden caused by waterborne pathogens persists (UNESCO, 2019). Therefore, this project focused on the potential deployment of a new rapid water monitoring system in developing countries.

Current detection technologies are mainly culture-based and therefore time consuming and often require the transportation of the water sample to a central laboratory for bacterial enumeration (Rajapaksha et al., 2019, Rompré et al., 2002). More rapid technologies have become standardized for water testing, such as PCR and ELISA's. However, these methods are expensive and often require complex equipment and trained personnel to perform, and therefore they are not suitable for field deployment in low resourced communities (Gunda, et al 2016, Rompré et al., 2002).

Since it was first described by Chappelle and Levin in in 1968, there has been increasing acceptance for the use of ATP bioluminescence monitoring systems in different industries including the food and beverages industries (Chappelle and Levin, 1968, Carrick et al., 2001). However, there is still a lack of supporting evidence and application in the water sector. Some evidence has suggested that the detection limit of ATP tests is between 10^1 – 10^4 CFU/ per ml of water which means

that they are not currently applicable to test drinking water, where the acceptance level is 0-1 CFU per 100ml (Lee et al., 2017).

After a comprehensive review of the literature, there appears to be only one method which can currently be regarded as a suitable rapid monitoring system, for potable water in low resource communities, the mobile water kit (See 3.6.2). This is claimed to be able to detect down to 2 CFU/100ml of coliforms in up to 1 hour (Gunda et al., 2014). However, this system may prove difficult to perform in the field by persons not scientifically trained. As of yet the system does not distinguish between *E. coli* and other coliforms, and as previously stated (3.3) *E. coli* is considered the most accurate and reliable representative of faecal contamination (Price and Wildeboer, 2017).

The MicroSnap system by Hygiena International Ltd was investigated to determine if it could be a low cost, reliable and rapid water monitoring system that could also be deployed in low resource settings.

7.2 Testing the MicroSnap system

The investigations into the Microsnap system began with initial testing of all four systems (Total count, Enterobacteriaceae (EB), Coliform, and *E. coli*) on pure bacterial cultures, to determine the systems sensitivity, specificity and reproducibility. The main focus was on the MS *E. coli* system as *E. coli* is said to be the most representative indicator for pathogenic contamination (Price and Wildeboer, 2017). The MS E. coli system works in two stages, the first is the enrichment step which contains a proprietary growth media and enzyme inducers for the accumulation of β-glucuronidase within bacteria. The second is the detection stage where an extractant lyses the cells in order to release the enzymes. The free enzymes can then cleave the ‘pro’ group from the luciferin molecules, allowing it to bind to ATP and luciferase, to produce detectable light. Therefore, the system uses β-glucuronidase as a biomarker for the presence of *E. coli*.

Throughout testing the MS Total, EB and Coliform systems produced relative light units (RLU) which correlated to the correct number of CFU’s in a sample. However,

MS *E. coli* was not producing reliable and reproducible results. The RLU's generated by MS *E. coli* were a constant underestimation of bacteria load. Multiple hypotheses were thought of and examined to determine the source of the low RLU's (see Chapter 2). It appeared that the MS system encountered a similar problem to what has been suggested for other ATP systems; poor extraction of biomarkers from bacterial cells (Lee et al., 2017). It was also theorized that this was in combination with low production of β-glucuronidase by *E. coli* cells.

The poor extractant hypothesis was confirmed when a series of *E. coli* on nutrient agar plates showed no growth difference between plates with the extractant and plates without. Due to the insufficient cell lysis, and the low production of β-glucuronidase, there was not enough free enzyme to generate the RLUs.
The extractant went under investigation and was also compared to a new extractant developed and sent by Hygiena International Ltd. The extractants were compared on spread plates of *E. coli* NTCT 12923, along side plates which contained no extractant. Chapter 3, figure 11 shows that plates with the newly developed extractant, almost no growth appears, compared to the original extractant plates.

The extractants were also compared within the MS system, almost a 10-fold increase in RLUs was observed across samples with the new extractant (see 6.3.4)
An investigation was also carried out into the secondary incubation period, during the detection stage. According to the manufacturer's instructions, the secondary incubation is 10 minutes, a method was conducted to see if extending this time frame would allow for more cell lysis by the extractant. It was kept in mind that the MS method was designed to be completed within one working shift, and therefore this extension in time could not exceed this time frame. As such, it was decided that the extension would be maximum 1 hour. The samples from two dilution series were detected with the Ensure luminometer every 10 minutes up to 1 hour. RLUs were detected after 10 minutes for the 10^{-5} dilution, but very little for the 10^{-6} dilution. The largest amount of lysis was observed for the 10^{-5} dilution, between 10 and 20 minutes, and between 30 and 40 minutes for the 10^{-6} dilution. Therefore, it was suggested that an increase in the secondary incubatory time from 10 minutes to 20 minutes should yield results more reflective to actual number of cells, while maintaining the rapid title.

Currently the lower detection limit of MS *E. coli* appears to be around 100-1000 CFUs/ml. As with other ATP based detection methods the MS is not yet sensitive enough to act in accordance with WHO guidelines for drinking water, of 0-1 CFU/1ml (Lee et al., 2017, WHO, 2019). However, promising results occurred when changes to the extractant, or to the incubation time were tested.

7.3 Possible deployment in low income communities

An on-site detection system for water testing could empower people in low income communities to be able to take charge of their own drinking water supplies, and not have to rely on agencies and other bodies, where infrastructure may be lacking. As the literature review explains (see 3.6) there are certain requirements an emerging method should meet in order to become a standardized rapid detection technique, and to be deployed in field testing.

The main requirement is of course the time the method takes, from sample collection and preparation, to the delivery of the results. In order for a method to be considered rapid, it must be able to be completed within one day (Rajapaksha et al., 2019) (Eijkelkamp, et al 2008). MicroSnap *E. coli* and coliform test can be performed within 7 hours, including sample collection. There is also no sample preparation with this method, as a 1ml water sample can be placed directly into the enrichment tubes. With a decreased timeframe, the method must still be as sensitive and specific and as reproducible as current standard methods. They must be able to detect down to 1 CFU per 100ml water sample, in accordance to WHO drinking water legislation (WHO., 2019). As explained the MS system is not currently applicable to drinking water sources as the lower detection limit is too high.

A major requirement, for the deployment of a system in the field, is that it must be easy to use and not require specific scientific training (Rajapaksha et al., 2019). MS has novelties over current methods including the use of the patented snap valve technology, which means that all the reagents are already contained within the tubes, and no need for user to add to or to handle the reagents. The MS tubes also

contains inbuilt pipettes, and printed fill lines, so that the user does not require extra equipment to add the correct amount of sample to the tubes.
For the monitoring system to be deployed in low resource communities, it must be low costing, especially because water monitoring requires consistent and regular testing to be the most effective. As well as the cost per test, the upkeep and maintenance of the equipment should be minimal. (Africa's Water Quality, 2010, Eijkelkamp et al., 2008). The cost per test of MS E. coli/coliform is £2.50, the total cost of the Ensure luminometer and the dry block incubator is £1500. Conventional culturing methods such as agar plates or petrifilm are usually cheaper per test however they require an air incubator to be left on for up to 72 hours for the incubation, with added time for the incubator to reach desired temperature. Changing the incubator type, and reducing the incubation to 7 hours will reduce power usage and thus the cost. There is also additional labour and maintenance cost with these methods as they require specialised equipment such as an autoclave. The ensure luminometer and the dry block incubator require minimal maintenance, the luminometer requires a battery change suggested every few months. The incubator only needs to be turned on around 10 minutes prior to usage and switched off after every test. Compared to current standard rapid tests such as PCR and ELISA, the MS system appears to be a lower cost alternative for water testing, and an easier test than current culturing methods.

On site application is advantageous as it means that there is no requirement to send samples to a central laboratory for testing, especially where testing may be time sensitive (Rajapaksha et al., 2019, Gunda et al., 2016). The MicroSnap system could be performed on site if the dry block incubator can be set up, as it does not require large amounts or constant supply of electricity. Hygiena International Ltd can also provide a standardized protocol to the community, meaning that there is no need for specialised scientific training. However, the difficulties may appear involving the storage of the MS tubes, Hygiena suggest that they should be stored at around 2 – 8^{o}C, which may prove challenging in low resourced settings.

7.4 Outlook for the future

The present version of the MicroSnap system is not applicable for use in the water sector, as the lower detection limit is too high. To increase the sensitivity two changes are proposed. The first change would be increasing the incubation time of MicroSnap *E. coli* tubes in the detection stage from 10 minutes to 20 minutes. The next is replacing the extractant within the detection tubes; either with the accompaniment of the first change, or in keeping to the original protocol. Both should yield results more representative of the actual number of bacteria, as the changes will have allowed for more cell lysis and therefore a higher release of the biomarker β-glucuronidase.

Once the changes are made, the next stages of testing will involve vigorous investigation to determine the systems applicable to water testing. The system should be analysed again with pure cultures of bacteria, as positive and negative control strains, but also with a complex mixture of interference bacteria. These results should be compared to methods which have been widely established for many years as an accurate representation of microbial load, such as the multiple tube method and membrane filtration (Davey, 2011). The results of the MS system should match these methods in terms of its sensitivity and reliability.

It would also be important to test the system on different environmental parameters such as of sediment load or turbidity. Hygiena International Ltd suggests that a number of factors may alter the RLU output including salt content, pH, colour, opacity, turbidity and temperatures. This was not able to be explored due to the inconsistent RLU's generated by MS *E. coli*. However, the deployment of this system will mainly focus on potable drinking water sources, therefore interference by these factors is unlikely

Once the results of bacterial testing become reliable and consistent it may be of interest to create new conversion tables. The RLU to CFU conversion tables given by Hygiena are not specific to water samples. A lengthy dilution series of pure cultures of bacteria in water could be tested and compared to spread plate counts to generate a standard curve and produce water specific conversion tables, to aid in a standardized procedure for drinking water tests. Currently the lowest CFU detection levels given by the conversion tables; specifically, for *E. coli* (figure 12) are <10

CFU/ml, in order to be applied to water testing, the RLU to CFU conversion tables need to be re done to accommodate smaller bacterial load, and display more specific numbers rather than large groupings of CFU. As of yet, there is some disparity between the RLU and CFU, as changes in the RLU output do not always equate to changes in the converted CFU, it would be interesting to see whether a more extensive conversion table would affect this.

Estimated CFU	Equivalent RLU	
	SystemSURE Plus	EnSURE
<10	< 2	< 2
<20	< 3	< 4
<50	< 6	< 7
<100	< 8	< 12
<200	< 12	< 20
<500	< 25	< 35
<1,000	< 50	< 60
<5,000	< 85	< 180
<10,000	< 150	< 300

Figure 12 Conversion table for RLU – CFU for MicroSnap E. coli. Throughout investigations only the Ensure detection method was used, not the systemSure Plus.

8. Conclusion

This project has demonstrated a potential rapid, inexpensive and easy to use quantitative tests for the enumeration of bacteria in drinking water sources. MicroSnap Total, EB and coliform have been successfully tested against known numbers of bacteria. Alterations have been suggested to increase the detection limit of MS *E. coli*, with the enhancements suggested this system could significantly reduce the current time taken by conventional methods to under 8 hours. The system needs to be further validated for its applicability in the water sector, however,

the ease of this detection method makes it a potential candidate for deployment in limited resource communities.

MS as a monitoring scheme could provide an early warning system, allowing communities to isolate a specific water source and allow the polluted water to be treated. The information found has been passed on to Hygiena International Ltd and it has been advised to adjust the method accordingly in order to apply MS to the water sector.

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Appendices

Appendix 1: Instruction manual for MicroSnap E. coli and Coliform from Hygiena International Ltd, adapted for the purpose of formatting this thesis

Step 1: Enrichment

Environmental surfaces and product samples:

1) Collect sample and place in MicroSnap Enrichment Device.
   Samples can be:
   1.1 Surface – swab a 4 x 4 inches (10 x 10 cm) square area or for irregular surfaces swab as much of the surface as possible.
   1.2 Liquid - 1mL beverage or water samples added directly to Enrichment Device.
   1.3 Product - 1mL 10% w/v food homogenate added directly to Enrichment Device. Food homogenate should be prepared using industry recommended diluents and standard microbiological procedures (e.g. 50g in 450ml of diluent as used in AOAC validation studies). Other sample sizes should be validated by user.
2) Re-attach the swab piece back into swab tube. Device should look the same as it did when first pulled from the bag.
3) Activate device by bending bulb back and forth.
4) Separate bulb and swab tube about 1-2 inches from each other, relieving internal pressure, and squeeze bulb to flush all media to bottom of swab tube. Ensure most of enrichment broth is in bottom of swab tube. Place bulb into swab tube firmly to seal device.
5) Shake tube gently to mix sample and enrichment broth.
6) Incubate at 37° ± 0.5°C. For enumeration, incubate for 6 hours. For presence/absence, incubate for 8 hours. ***For large volume filterable liquids:***

1) Collect sample up to 100mL capacity and filter through 0.45 µm filter membrane with diameter 25mm or 47mm.
2) Aseptically remove filter and place into sterile 47mm Petri dish.
3) Aseptically add entire contents of Enrichment Broth (MS1-CECBROTH-2ML) vial to sterile Petri dish.

4) Incubate Petri dish at 37° ± 0.5°C. For enumeration, incubate for 6 hours. For presence/absence, incubate for 8 hours.

Step 2: Detection:

Detection procedure is described below

1) Allow MicroSnap Coliform or *E. coli* Detection Device to equilibrate to room temperature (10 minutes at 22-26° C). Shake test device by either tapping on palm of hand 5 times, or forcefully flicking in a downward motion once. This will bring excess extractant liquid dispersed in tube to bottom of tube. Extractant is necessary to facilitate mixing of enriched sample with solution in tube.
2) Transfer enriched sample to Detection Device.
   2.1 Aseptically remove an aliquot of sample (optimum volume is 0.1mL, or 3 drops) from Enrichment Device and transfer to Detection Device. Enrichment Device can be used as a dropper tip for convenience. Squeeze and release bulb to mix and draw sample into bulb. Remove swab from tube and carefully dispense 3 drops (0.1mL) to fill line marked on bottom of Detection Device. Remaining enriched sample can be returned to Enrichment Device for additional testing.
   2.2 For filtered samples, aseptically pipette 0.1mL of incubated broth from Petri dish to Detection Device.
3) Activate Detection Device by bending bulb to break Snap-Valve. Squeeze bulb 3 times to release reagent.
4) Shake gently for 2 seconds to mix.
5) Incubate Detection Device for 10 minutes (± 0.2 min) at 37°± 0.5°C.
6) After 10 minutes of incubation, insert whole device into luminometer and close lid. Holding unit upright, press “OK” button to initiate measurement. Results will appear after 15 second count down.
7) Result will be displayed in RLU (Relative Light Units). Set thresholds on instrument that correspond to pass/fail levels deemed acceptable.

Appendix 2: Instruction manual for MicroSnap Total from Hygiena International Ltd, adapted for the purpose of formatting this thesis.

Step 1: Enrichment

1. Collect sample and place in MicroSnap Total Enrichment Device (Part # MS1-TOTAL) Samples can be:
   i. Surface - Swab a 4 x 4 inch (10 x 10 cm) square area, or for irregular surfaces, as much of surface as possible to collect a representative sample.
   ii. Liquid - 1mL liquid food, beverage or water samples added directly to Enrichment Device. iii. Product - 1mL of appropriate suspension, e.g. 10% w/v (weight / volume) food homogenate added directly to Enrichment Device. Food homogenate should be prepared by weighing out 10g or 50g of food matrix and adding it to a stomacher bag containing 90mL or 450mL diluent (*Note: Maximum Recovery Diluent was validated in the AOAC PTM study*). For unknown sample contamination, dilutions below 10% should be produced in more diluent by adding 10mL of 10% into 90mL of fresh diluent and repeating for 1% and 0.1%. If replicate samples are required then another 10g or 50g should be removed from the bulk matrix and the dilutions series repeated. Replication can be achieved by drawing multiple 1mL aliquots from either the 10%, 1%, 0.1% dilutions depending on RLUs achieved.
   Note: When performing comparison testing, sample assays must be started within 10 minutes for comparable results between methods. Samples taken can be stored prior to use at 4°C for up to 2 days but must be equilibrated back to ambient before samples are run on MicroSnap and any equivalent methods.
2. Re-attach swab back on to swab tube. Device should look the same as it did when first pulled from bag.
3. Activate Enrichment Device by holding swab tube firmly and using thumb and forefinger to break Snap-Valve by bending bulb forward and backward.
4. Separate bulb and swab tube about 1 – 2 inches from each other, relieving internal pressure, and squeeze bulb to flush all media to bottom of swab tube. Ensure most of enrichment broth is in bottom of swab tube.
5. Re-attach swab back on to swab tube firmly to seal device.
6. Shake tube gently to mix sample with enrichment broth.

7. Incubate at 30 ± 0.5 °C for 7 hours ± 10 minutes.

Step 2: Detection:

Detection procedure is described below. Before beginning Step 2, turn on EnSURE luminometer. If locations have been programmed, select location to be tested.

1. Allow MicroSnap Total Detection Device (Part # MS2-TOTAL) to equilibrate to room temperature (10 minutes at 22 – 26 °C).Shake test device by either tapping on palm of hand 5 times, or forcefully flicking in a downward motion once. This will bring extractant liquid to bottom of tube.
2. Transfer enriched sample from Enrichment Device to Detection Device. Enrichment Device swab can be used as a pipette for convenience.
   i. Squeeze and release Enrichment Device bulb to mix and draw sample into bulb. ii. Remove Enrichment Device swab from tube.
   iii. Open Detection Device by twisting and pulling to remove bulb. Set aside.
      a. Insert Enrichment Device swab tip into top of Detection Device tube (approximately 1 inch or 3 cm) and lightly squeeze Enrichment Device bulb to trickle enriched sample into tube until volume reaches fill line marked on bottom of Detection Device tube. Avoid adding excess sample above fill line, as this can increase variation of test results.
   iv. Remaining enriched sample can be returned to Enrichment Device for additional testing. Reassemble Enrichment Device to original state and return device to incubator. *Note: When testing replicates from same enriched sample, all replicates must be performed within 10 minutes to obtain comparable results.*
   v. Reassemble Detection Device to original state.
3. Activate Detection Device by holding swab tube firmly and using thumb and forefinger to break Snap-Valve by bending bulb forward and backward. Squeeze bulb 3 times to release all liquid to bottom of swab tube.
4. Shake gently to mix.
5. Immediately insert whole device into luminometer; close lid and holding unit upright, press “OK” button to initiate measurement. Results will appear after 15 second count down.

6. Result will be displayed in RLU (Relative Light Units). Set RLU thresholds on instrument to correspond with required CFU limits.

   Refer to “Interpretation of Results” below for correlation.

Appendix 3: Instruction manual for MicroSnap EB from Hygiena International Ltd, adapted for the purpose of formatting this thesis

Step 1: Enrichment

Enrichment procedure is described below

1. Collect sample and place in the MicroSnap EB Enrichment Device (Part # MS1-EB). Samples can be:
   i. Surface - Swab a 4 x 4 inch (10 x 10 cm) square area, or for irregular surfaces, as much of surface as possible to collect a representative sample.
   ii. Liquid - 1mL liquid food, beverage or water samples added directly to Enrichment Device.
   iii. Product – 1mL of appropriate suspension, e.g. 10% w/v (weight/volume) food homogenate added directly to Enrichment Device. Food homogenate should be prepared using standard microbiological procedures. For unknown sample contamination, dilutions below 10% should be made and tested.

2. Re-attach swab back on to swab tube. Device should look the same as it did when first pulled from bag.
3. Activate Enrichment Device by holding swab tube firmly and using thumb and forefinger to break Snap-Valve by bending bulb forward and backward.
4. Separate bulb and swab tube about 1-2 inches from each other, relieving internal pressure, and squeeze bulb to flush all media to bottom of swab tube. Ensure most of enrichment broth is in bottom of swab tube.
5. Re-attach swab back on to swab tube firmly to seal device.
6. Shake tube gently to mix sample with enrichment broth.
7. Incubate at 37 ± 0.5 °C for 6 to 8 hours. (Refer to Tables 2 – 4 for details).

Step 2: Detection:

Detection procedure is described below

Before beginning Step 2, turn on EnSURE luminometer. If locations have been programmed, select location to be tested.

1. Allow the MicroSnap EB Detection Device (Part # MS2-EB) to equilibrate to room temperature (10 minutes at 22 – 26 °C). Shake test device by either tapping on palm of hand 5 times, or forcefully flicking in a downward motion once. This will bring extractant liquid to bottom of tube.
2. Transfer enriched sample from Enrichment Device to Detection Device. Enrichment Swab can be used as a pipette for convenience.
    i. Squeeze and release Enrichment Device bulb to mix and draw sample into bulb.
    ii. Remove Enrichment swab from tube. iii. Open Detection Device by twisting and pulling to remove bulb. Set aside.
        a. Insert Enrichment swab tip into top of Detection Device tube (approximately 1 inch or 3 cm) and lightly squeeze Enrichment Device bulb to trickle enriched sample into tube until volume reaches fill line marked on bottom of Detection Device tube. Avoid adding excess sample above fill line, as this can increase variation of test results.
    iv. Reassemble Detection Device to original state.
3. Activate Detection Device by holding swab tube firmly and using thumb and forefinger to break Snap-Valve by bending bulb forward and backward. Squeeze bulb 3 times to release all liquid to bottom of swab tube.
4. Shake gently to mix.
5. Immediately insert whole device into luminometer; close lid and holding unit upright, press “OK” button to initiate measurement. Results will appear after 15 second count down.
6. Result will be displayed in RLU (Relative Light Units). Set RLU thresholds on instrument to correspond with required CFU limits.

Appendix 4: The RLU to CFU conversion table for MicroSnap E. coli and Coliform taken from Hygiena International Ltd.

For the purpose of this project the Ensure luminometer was used throughout testing, as such the column in appendix 4 which displays Ensure RLU's was used in the conversion of RLUs to CFUs.

Estimated CFU	Equivalent RLU	
	SystemSURE Plus	EnSURE
<10	< 2	< 2
<20	< 3	< 4
<50	< 6	< 7
<100	< 8	< 12
<200	< 12	< 20
<500	< 25	< 35
<1,000	< 50	< 60
<5,000	< 85	< 180
<10,000	< 150	< 300

Appendix 5: The RLU to CFU conversion table for MicroSnap Total taken from Hygiena International Ltd.

The RLUs generated using the MS Total system were converted using this table, as this table shows, the RLUs shown on the Ensure luminometer correlate to that of CFUs in a direct 1ml liquid sample.

RLU (EnSURE)	Equivalent CFU	
	Direct sample e.g. 1mL liquid (or surface swab)	Typical 10% suspension of solid sample
<10	<10	<100/g
<20	<20	<200/g
<30	<30	<300/g
<50	<50	<500/g

<100	<100	<1,000/g
<1000	<1000	<10,000/g
>5,000	TNTC	TNTC

Appendix 6 The RLU to CFU conversion table for MicroSnap EB taken from Hygiena International Ltd.

The RLUs shown on the Ensure luminometer when using MS EB correlate to that of CFUs in a direct 1ml liquid sample.

EnSURE RLU	Equivalent CFU	
	Direct sample e.g., surface swab or 1mL liquid sample	10% suspension of solid sample
<10	<5/mL	<50/g
<25	<12/mL	<100/g
<50	<25/mL	<250/g
<100	<50/mL	<500/g
<250	<120/mL	<1,200/g
<500	<250/mL	<2,500/g
<1,000	<500/mL	<5,000/g
>1,000	TNTC	TNTC