LAPPEENRANTA-LAHTI UNIVERSITY OF TECHNOLOGY LUT
School of Engineering Science
Degree Programme in Chemical Engineering

Sanna Lindström

ATP-BASED MONITORING SYSTEM FOR CIP RINSE WATERS IN ENZYME INDUSTRY

Examiners: Prof. Mari Kallioinen
Dr. Tech. Liisa Puro

ABSTRACT

Lappeenranta-Lahti University of Technology LUT
School of Engineering Science
Degree Programme in Chemical Engineering

Sanna Lindström

ATP-Based Monitoring System for CIP Rinse Waters in Enzyme Industry

Master's Thesis
2020
85 pages, 11 figures, 18 tables and 1 appendix
Examiners: Prof. Mari Kallioinen and Dr. Tech. Liisa Puro

Keywords: ATP monitoring, cleaning in place, CIP, bioluminescence

This thesis was made for Genencor International Oy enzyme plant in Jämsänkoski, Finland. The product packing lines are cleaned with cleaning in place (CIP) method and the cleaning verification is performed with a traditional culture method, total viable count (TVC). The TVC method is labor-intensive and slow, the results being available only after 2–3 days. The aim of this thesis was to examine if an ATP-based monitoring method could be used for the cleaning verification to gain the result more quickly.

The objective was to test one pen-based rapid ATP monitoring system and find out if it could be used reliably for CIP cleaning verification. The ATP method was used side by side with the traditional TVC method to analyze CIP rinse water samples and to see if the results aligned. In addition, the effect of various sample conditions, environmental factors, and measurement details to the ATP results were tested. Additionally, the changes in the annual costs and analysis times caused by the possible method change were calculated.

According to the results obtained in the experimental part, the ATP method seems to work well with the CIP rinse water samples. The ATP results aligned well with the TVC results and a good correlation between the two methods was obtained. Based on the results, optimal measurement conditions and protocols were established. Based on the analysis time and cost calculations, the method change to ATP method would save a significant amount of time annually and the ATP method would probably be more cost-effective than the TVC method.

The results suggest that this ATP monitoring method is suitable for reliably assessing the cleaning result. However, further tests are needed. A future aim is to transfer the cleaning verification from laboratory to production personnel, so it is important to see if the method works in the field as well and if the results obtained by multiple operators align. In the future, it would also be beneficial to examine if the method could be used for other applications as well.

TIIVISTELMÄ

Lappeenrannan-Lahden teknillinen yliopisto LUT
School of Engineering Science
Kemiantekniikan koulutusohjelma

Sanna Lindström

ATP-mittaukseen perustuva seurantajärjestelmä entsyymiteollisuuden CIP-huuhteluvesille

Diplomityö
2020
85 sivua, 11 kuvaa, 18 taulukkoa ja 1 liite
Tarkastajat: Prof. Mari Kallioinen ja TkT Liisa Puro

Avainsanat: ATP-monitorointi, kiertopesu, CIP, bioluminesenssi

Tämä opinnäytetyö tehtiin Genencor International Oy:n Jämsänkosken entsyymituotantolaitokselle. Tuotteiden pakkauslinjat pestään CIP-pesulla (cleaning in place, kiertopesu) ja pesutulos varmistetaan perinteisellä maljaviljelymenetelmällä, TVC:llä (total viable count, kokonaispesäkeluku). TVC-menetelmä on työläs ja hidas menetelmä ja sen tulosten saamisessa kestää kahdesta kolmeen päivää. Tämän diplomityön tavoitteena oli selvittää, voisiko ATP-mittaukseen perustuvaa menetelmää käyttää pakkauslinjojen pesutuloksen nopeampaan varmistamiseen.

Työn tarkoituksena oli testata yhtä testitikkuihin perustuvaa ATP-menetelmää ja selvittää, voisiko menetelmällä saada luotettavia tuloksia pesujen onnistumisesta. Työn kokeellisessa osassa ATP-menetelmää käytettiin rinnakkain TVC-menetelmän kanssa CIP-huuhteluvesien analysointiin. Lisäksi tutkittiin erilaisten näyteolosuhteiden, ympäristötekijöiden ja mittauksen yksityiskohtien vaikutusta ATP-tuloksiin. Lisäksi laskettiin mahdollisen menetelmän vaihdoksen aiheuttamat muutokset analysointiajoissa ja -kustannuksissa.

Kokeellisessa osuudessa saatujen tulosten perusteella ATP-menetelmä näyttäisi toimivan CIP-huuhteluvesien analysoinnissa. Saadut ATP-tulokset korreloivat hyvin TVC-tulosten kanssa. Tulosten perusteella ATP-menetelmälle laadittiin optimaaliset mittausolosuhteet ja menettelytavat. Aika- ja kustannuslaskelmien perusteella ATP-menetelmään siirtyminen säästäisi merkittävästi analysointiaikaa vuosittain ja ATP-menetelmä olisi luultavasti myös kustannustehokkaampi kuin TVC-menetelmä.

Tulosten perusteella ATP-menetelmä soveltuu pesutuloksen luotettavaan arviointiin, mutta jatkotutkimuksia tarvitaan siitä huolimatta. Tulevaisuudessa pesutuloksen varmistaminen on tarkoitus siirtää laboratoriosta tuotantotyöntekijöille, joten on tärkeää tarkastaa menetelmän toimivuus tuotanto-olosuhteissa. On myös tärkeää varmistaa, että useiden henkilöiden saamat tulokset vastaavat toisiaan. Lisäksi tulevaisuudessa olisi hyödyllistä selvittää, voiko menetelmää käyttää myös muihin käyttötarkoituksiin tehtaalla.

TABLE OF CONTENTS

1 INTRODUCTION 7
2 PRODUCT SAFETY IN ENZYME PROCESSING PLANT 10
2.1 HACCP 10
2.2 Product soiling and biofilms 11
2.3 CIP process 13
2.4 Bacteria enumeration methods in food diagnostics 15
2.4.1 Cultural methods 15
2.4.2 Other methods 17
3 BIOLUMINESCENCE 19
3.1 Firefly bioluminescence 19
3.2 Bioluminescence kinetics 21
3.3 Luciferin and luciferase 22
4 ATP MONITORING WITH BIOLUMINESCENCE 23
4.1 Adenosine triphosphate 23
4.2 Amount of ATP in microbes 24
4.2.1 Effect of species 25
4.2.2 Effect of biomass 26
4.2.3 Effect of environment 27
4.3 ATP monitoring methods 28
4.3.1 ATP extraction 29
4.3.2 Separation of free ATP 30
4.4 Factors affecting ATP monitoring 31
4.4.1 pH 32
4.4.2 Temperature 32
4.4.3 Interference 33
4.5 Lower and upper ATP limits 34
4.6 ATP versus cell counts 35
5 MATERIALS AND METHODS 37
5.1 Samples 37
5.2 ATP method 38
5.2.1 EnSURE luminometer 38
5.2.2 AquaSnap test devices 39
5.2.3 SuperSnap test devices 41
5.3 TVC method 42

5.4 Test protocol 43
6 RESULTS AND INTERPRETATION 51
6.1 TVC versus ATP results 51
6.2 ATP content of microorganisms 54
6.3 Additional tests 55
6.4 Comparison to earlier results with another luminometer 66
6.5 Analysis time and costs 68
6.6 Environmental impact, safety, and ergonomics 69
6.7 Measurement error 70
7 CONCLUSIONS 72
8 SUGGESTIONS FOR FUTURE RESEARCH 75
REFERENCES 76
APPENDICES 84
Appendix 1. CIP rinse water results 84

ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
AODC	acridine orange direct count
ATP	adenosine triphosphate
CCP	critical control point
CFU	colony forming units
CIP	cleaning in place
CoA	coenzyme A
FCM	flow cytometry
FSMS	food safety management system
GHP	good hygiene practices
GMP	good manufacturing practices
HACCP	hazard analysis and critical control points
LoD	limit of detection
oPRP	operational prerequisite program
PCA	plate count agar
PCR	polymerase chain reaction
PI	propidium iodide
RLU	relative light unit
SFCA	surfactant-free cellulose acetate
TDC	total direct count
TVC	total viable count
VBNC	viable but nonculturable

1 INTRODUCTION

Cleaning in place (CIP) is widely used in the food industry to clean the food processing systems without dismantling them (Tetra Pak 2015, 3). Traditionally, the cleaning verification of the most crucial points, for example packing lines, is performed with traditional culture-based methods, such as total viable count (TVC).

In Jämsänkoski enzyme plant, a variety of different types of enzymes are produced, the most important of which are enzymes for food and feed industries. CIP cleaning is used for example for product transfer and packing lines. CIP cleaning verification is performed in the plant laboratory with a traditional TVC method. However, the results are available only after 2–3 days. This prevents any immediate actions if problems in the cleaning occur. Additionally, TVC methods are rather labor-intensive and require a variety of equipment and resources, as well as expertise.

One of the most promising methods to replace TVC methods in especially CIP verification are ATP methods. All microorganisms contain ATP which is why ATP monitoring can be used to assess the microbial quality of a sample in different applications (Hammes et al. 2010, 3915–3916; Karl 1980, 763). ATP methods do not give an exact number of microorganisms present in the sample but offer an estimate adequate for many applications (Hammes et al. 2010). In CIP verification it is important to reliably get the result whether the cleaning was successful or not instead of the exact number of microorganisms.

Adenosine triphosphate (ATP) monitoring is already a rather widespread technique in the food industry (Shama & Malik 2013, 116). However, there are no well-established practices or standardized methods for ATP monitoring for most of the applications. This is mostly because the ATP monitoring systems are very diverse, each of them providing different results that are not comparable against each other. The results also depend on the sample type as well as environmental conditions and other factors during the measurement (Hammes et al. 2010; Shama & Malik 2013). This makes it difficult to create a universal protocol for different circumstances and applications.

In addition, the bioluminescent systems are still under study and the mechanisms are not yet fully understood. Nevertheless, ATP monitoring offers promising methods to use in multiple different applications in the food industry and many other industries as well. However, there is only limited amount of data available on ATP monitoring in this specific application and environment.

These circumstances lead to a situation where ATP monitoring methods are often desirable, but the method needs rather comprehensive testing and validation before use. Sometimes multiple different ATP monitoring systems need to be tested to find the system suitable for a certain application. Additionally, optimal measurement circumstances and protocols should be carefully tested and considered.

The aim of this thesis was to test if an ATP monitoring method could be used for CIP cleaning verification in the plant. Replacing the TVC method with a rapid ATP method would allow immediate corrective actions if problems occur with the cleaning. Immediate actions would prevent the possible contamination of the product caused by insufficiently cleaned distribution or packing lines.

If the ATP method proves to be suitable for this application, a future aim would be to transfer the CIP verification from the laboratory to be performed by the production personnel in the field. Therefore, a robust, simple, and portable method is needed. The method should also provide reproducible methods between multiple operators.

One pen-based ATP method was tested a year earlier with unsatisfactory results. Therefore, another pen-based ATP method was chosen to be tested in this thesis and the results were also compared to the results obtained from the first ATP method tested earlier. Unfortunately, it was not possible to test the two ATP methods side by side with the same samples.

In the experimental part of this thesis, the rapid ATP monitoring system was tested for CIP rinse water samples. In addition, cooling water and additional water samples were used to gain more results. The currently used TVC culture method was used side by side with the ATP method to gain information about the correlation between the two methods.

Some additional tests were also performed with the ATP method to find out the optimal conditions and operational procedures. This included sample conditions such as pH and temperature as well as operational details and variables. The ATP method was also tested for enzyme products to see if the method could be later used in more versatile applications. Lastly, the analysis time and costs of the ATP and TVC methods were compared, as well as the environmental impact, safety and working ergonomics of the methods.

2 PRODUCT SAFETY IN ENZYME PROCESSING PLANT

The product safety in an enzyme processing plant consists of multiple different aspects. In this thesis, the focus is on the food and feed safety aspect. Even though an enzyme processing plant is not a straightforward food processing plant, many of the products are manufactured for the food and feed industry. Therefore, the regulations concerning food and feed safety must be followed. A systematic approach to follow the regulations and ensure the safety of the products is called a food safety management system (FSMS).

FSMS consists of multiple elements that can be roughly divided into two parts. The basis consists of prerequisite programs, such as good hygiene practices (GHP) and good manufacturing practices (GMP), as well as regulations and standards, such as ISO 22000, FSSC 22000 and FAMI-QS. In addition, own-check systems such as HACCP are important in ensuring food and feed safety. (European Commission 2016, 2.)

2.1 HACCP

Hazard analysis and critical control points (HACCP) is a systematic approach for identifying and managing risks related to food safety. The HACCP system is an important part of the self-monitoring systems of the food industry. The HACCP system covers the whole production chain and focuses on avoiding the hazards rather than only analyzing the finished products. (European Commission 2016, 2, 10.)

Hazards are biological, chemical, or physical agents or a condition of food or feed that can cause adverse health effects. The hazard analysis includes hazard identification, qualitative and quantitative evaluation of each hazard and setting control measures to prevent, reduce or eliminate the effect of each hazard. (European Commission 2016, 10–12.)

Identifying critical control points (CCP) is an essential part of the HACCP system. Critical control points are the critical steps of the process at which control can be applied to prevent, reduce, or eliminate hazards. (European Commission 2016, 10–12.) Other important points regarding food safety that cannot be counted as CCPs can be defined as

operational prerequisite programs (oPRP) that are measures essential in reducing the likelihood of a specific hazard occurring. (European Commission 2016, 13; SFS-EN ISO 22000:2018, 14.)

It is also necessary to set critical limits for measures associated with each CCPs and oPRPs, although oPRPs can typically have targets rather than critical limits. In addition, it is essential to have monitoring procedures and to plan corrective actions for each CCP and oPRP. (European Commission 2016, 13–14.)

There are multiple possible CCPs and oPRPs in the food and feed industry. One important control point is the cleaning verification of product packing lines because the final product is often inspected prior to packing and contaminated packing lines could therefore contaminate the product as well.

2.2 Product soiling and biofilms

Products that are processed in the production system may cause soiling. Product constituents are adsorbed on the pipe and equipment surfaces and can be difficult to remove. There are two types of soils: water-soluble and those that are insoluble in water. Water-soluble soils are easier to remove and they typically contain sugars and some minerals. Insoluble soils in turn are more difficult to remove and they consist of fats, oils, proteins, carbohydrates, minerals, and salts. (Tetra Pak 2015, 5–6.)

Soiling is a contamination risk and soils also act as nutrients promoting the growth of microorganisms. Biofilms are formed by microorganisms that attach to each other and to a surface. In addition to microbes, biofilms consist of water and extracellular matrix, such as polysaccharides and proteins. (Costerton 2007, 7, 20–21; Kanematsu & Barry 2015, 4–5.)

The biofilm protects bacteria from effect of environmental conditions, such as chemicals and flow. In biofilms, bacteria can also catch more nutrients and share nutrients with each other. Therefore, microorganisms prefer growth as a biofilm especially when there is a

shortage of available nutrients or when the environmental conditions are not favorable. The ability and tendency of microorganisms to attach to a surface depends on the structural properties of the organism, surface properties as well as environmental conditions. The accumulation of a biofilm assists the attachment of other microbes. (Kanematsu & Barry 2015, 5–6.) Biofilms can also cause or advance material corrosion and increase energy consumption by blocking pipes and filters (Costerton 2007, 7).

Soil and biofilm removal

Cleaning of production systems and equipment should be effective enough to remove the microorganisms but also all product residues and biofilms that support the growth of microorganisms. However, biofilms and the microorganisms that are attached to it can be very difficult to remove. The biofilm protects microorganisms from various external factors, such as flow and detergents. (Kanematsu & Barry 2015, 6; Shi & Zhu 2009, 407.)

Soil and biofilm removal are based on three main mechanisms: mechanical force, thermal force, and chemical force. In addition to these forces, the time used for each step of the cleaning is also an important parameter in cleaning. (Tetra Pak 2015, 9, 16; Walton 2008, 8–9.)

Mechanical force is based on the forces created by the fluid flow of water and cleaning agents. The flow must be turbulent and the velocity at least 1.5 m/s. Inadequate flow is often one of the main reasons for cleaning problems. (Tetra Pak 2015, 10–12.)

Thermal force is created with the temperature of water and detergents. The temperature used for cleaning depends on the type of the soil and the detergents that are used. The effectiveness of a detergent is typically increased when the temperature increases. However, if the temperature is too high, protein crosslinking and denaturation may happen making the soil harder to remove. (Tetra Pak 2015, 15–16; Walker 2008, 68–69.)

Chemical force is created with detergents. The detergent type and its concentration are chosen based on the type of the soil. A combination of acidic and alkaline detergents is often used. Additional components, such as surfactants, complex-forming agents, and oxidation agents, can also be used. (Tetra Pak 2015, 13, 27; Walton 2008, 6–7.)

2.3 CIP process

Cleaning in place (CIP) is a widely used cleaning method for production systems and equipment in the food industry. CIP cleaning takes place without disassembling the system. CIP systems are usually automated. (Walton 2008, 1–2.) In addition to the CIP distribution lines, the CIP system can either consist of one centralized CIP station or multiple decentralized CIP stations (Moerman et al. 2014, 330–332).

CIP processes can be roughly divided into two types: single-use CIP or recovery CIP. Single-use systems utilize the same cleaning solutions and water only once, disposing of them immediately after use. In recovery CIP some of the solutions and water are recycled. Single-use CIP systems are usually very expensive to operate but have a smaller chance of cross-contamination. Recovery CIP systems in turn are more economical to operate due to decreased water, detergent and energy requirements but are more prone to contamination. (Walton 2008, 2.)

There are many variations in CIP processes, depending on the application and the type of soils and other impurities that need to be removed. A typical CIP process consists of seven main stages (Walton 2008), which are shown in figure 1.

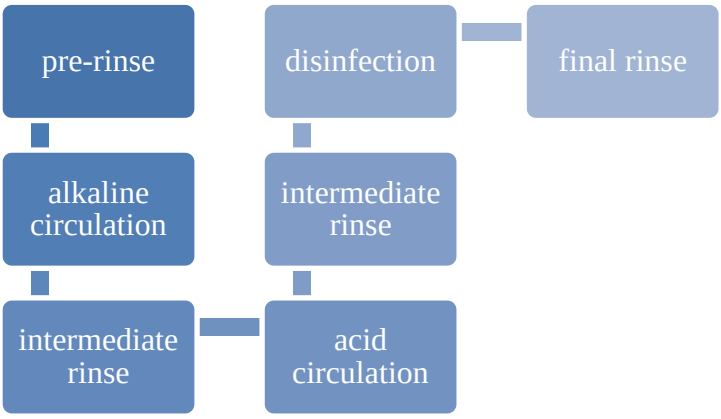

FIGURE 1. Main stages of CIP process

Pre-rinse cycles serve the purpose of removing most of the water-soluble soils and product residues from the system. Pre-rinse in recovery CIP usually utilizes recovered water

from intermediate rinse and final rinse cycles. (Moerman et al. 2014, 318; Walton 2008, 3.)

The first detergent circulation is usually alkaline circulation, which is used to remove especially the organic soils from the surfaces and keep them suspended or dissolved in the solution (Walton 2008, 3). Pure sodium hydroxide is often used at 0.5–2.0 % concentration. Too high concentrations of alkaline detergent may cause protein crosslinking making the proteins harder to remove. The second detergent circulation is usually performed with an acidic detergent to remove inorganic soils. (Tetra Pak 2015, 13.)

Intermediate and final rinses are used to remove residual dirt and detergents from the system. In addition to these steps, a chemical or heat-based disinfection step may also be added before the final wash if needed. (Moerman et al. 2014, 318–320; Walton 2008, 4–5.)

CIP process in Jämsänkoski

In Jämsänkoski plant there are multiple CIP stations and distribution lines. Each line has a filter that prevents particles and soils from returning to the system and causing possible clogs. A typical CIP process in the plant consists of three stages: pre-rinse, alkali circulation and final rinse. Pre-rinse and final rinse are performed using domestic water which is filtered before use. For some CIP processes, the final rinse water can be reused for pre-rinse for upcoming cleaning cycles. The water coming from pre-rinse is always disposed of.

The dirt built up in the plant consists mostly of organic substances, including biomass, proteins, carbohydrates, and sugars. Therefore, an alkaline cleaning solution has been chosen for use. The cleaning solution is 1–2 % sodium hydroxide with the minimum temperature of 60 °C. The sodium hydroxide concentration of the solution is monitored with conductivity measurements and titration.

The cleaning results of the most critical points, for example packing lines, are monitored with a total viable count (TVC, chapter 2.4.1) analysis. A CIP rinse water sample is taken from the final rinse water during the last minute of the cleaning cycle and is analyzed in

the laboratory as soon as possible with the TVC method. In addition, a sample of plant cooling water is analyzed on a weekly basis with the TVC method.

2.4 Bacteria enumeration methods in food diagnostics

The enumeration of bacteria in the food industry has traditionally been done by cultural methods, such as total viable count, TVC. However, cultural methods are time- and labor-consuming and rather sensitive to environmental circumstances. Therefore, new advanced methods have been developed to meet the demand of quicker and more reliable results.

2.4.1 Cultural methods

Cultural methods are the most traditional enumeration methods in food diagnostics. Cultural methods include conventional colony count methods such as TVC and modified cultural methods such as 3M™ Petrifilm™ (Jasson et al. 2010, 711–714).

TVC

There are multiple different terms for methods that are used to enumerate the number of bacterial colonies that are produced on an agar plate under defined medium and incubation conditions. Examples of used terms are ‘standard plate count’, ‘heterotrophic plate count’, ‘total bacterial count’, ‘total viable count’ and ‘colony count’. All these terms basically mean the same type of method but may be used in different countries or different industries. (Payment et al. 2003, 41.) In this thesis, the term ‘total viable count’ and shortened version ‘TVC’ are used in all contexts, even though the original source of information has used another term.

TVC is the most traditional enumeration method for microorganisms. TVC is used for estimating the number of viable and culturable heterotrophic microorganisms. The term of heterotrophs includes all microorganisms that use organic nutrients for growth. (WHO 2003, 2.) TVC is in many cases used for estimating the concentration of bacteria present

in the sample but also yeasts and molds can be grown on the agar and identified by their appearance. TVC has been the main method to assess microbial quality of drinking water for decades and is recommended in many drinking water guidelines (Sartory et al. 2004).

There is no universal TVC method but a wide variety of test conditions that give different kinds of results. The main variables in TVC methods are temperature (typically between 20 °C and 40 °C), incubation time (from a few hours to 1–2 weeks) and nutrient conditions. The agar is nonselective so that a wide range of microorganisms can grow on it. (WHO 2003, 2.) However, TVC method is in fact a selective method because the conditions determine which microorganisms can grow.

Even though many types of microorganisms can grow on TVC plates, only a small portion of all microorganisms can be grown and detected with TVC methods (WHO 2003, 2). Multiple studies show that only 1 % or less of the microbial community can be grown on TVC plates.

For example, Siebel et al. (2008) tested 200 drinking water samples and found out that only an average of 1.6% of the total cell count could be cultivated. Hammes et al. (2008) reported that 0.001–6.5 % of cells from samples taken from different drinking water treatment steps were culturable. Amann et al. (1995) collected information from multiple studies showing that only around 0.1 % of the bacteria in different types of water samples are culturable. Therefore, TVC method is not a reliable representation of the total microbial load in the sample.

It is important to remember that TVC methods do not necessarily give an exact number of microorganisms present in the sample but a colony forming units count. Some cells exist aggregated in groups and sometimes cells cannot be separated by mixing and therefore one colony can be formed either by one cell or a group of cells (Costerton 2007, 3).

TVC methods are typically time-consuming and labor-intensive methods. In addition, the results are often available only after 2–3 days, which prevents immediate actions if the results are alarming. Due to the nature of TVC methods, the accuracy of them is rather poor and the results are highly dependent on the conditions during analysis.

2.4.2 Other methods

In addition to traditional culture-based methods, there are multiple other enumeration methods available. The downsides of these methods are often high costs and the need of laboratory-scale equipment and expertise. Some of the alternative methods are presented below.

Microscopic methods

Microscopic methods for enumeration of microbes include for example epifluorescence microscopy and laser-scanning microscopy (Rinta-Kanto et al. 2004; Siebel et al. 2008). Despite method development, microscopic techniques remain time- and labor-consuming methods (Hammes et al. 2008, 270).

There are multiple different stains that can be used with fluorescence microscopy, such as DAPI (4',6-diamidino-2-phenylindole) (Vang et al. 2014) or SYBR Green (Rinta-Kanto et al. 2004). Acridine orange direct count (AODC) utilizes acridine orange staining and epifluorescence microscopy (Deininger & Lee 2001; Eydal & Pedersen 2007).

Total direct count (TDC) is a rapid microscopic method to enumerate bacteria typically with the help of fluorescent dyes. Unlike TVC methods, TDC can detect nonculturable microorganisms. (Tominaga et al. 2018, 118.) However, TDC is limited to high cell concentrations (greater than 10^7 cells/ml) and does not distinguish active cells from inactive cells (Postgate 1969).

Flow cytometry

Flow cytometry (FCM) is a potential method for enumerating the total cell concentration and has often been used to compare results from ATP methods with cell counts. In FCM, cells are directed one by one through a laser beam and different parameters are measured using photomultiplier tubes. Traditional FCM detects both viable and dead cells but it can be combined with different staining methods, such as propidium iodide (PI) staining (Hammes et al. 2010; Nescerecka et al. 2016a) or SYBR Green (Hammes et al. 2008; Nescerecka et al. 2016a; Siebel et al. 2008) to distinguish viable cells. Different staining methods can also be used to target specific cells (Jasson et al. 2010, 719).

The main advantage of FCM over TVC is that it detects all bacteria regardless of their culturability. Other advantages of FCM are that it is fast (around 20 minutes), sensitive and accurate. (Hammes et al. 2008; Siebel et al. 2008.) The standard deviation for FCM method is low, below 5 percent (Siebel et al. 2008). However, FCM requires specific equipment and expertise.

Fulford et al. (2004) compared the cell counts of dental water unit systems obtained from TVC and FCM methods and reported that FCM generally gave 50 to 500 times higher results than TVC. Siebel et al. (2008) compared the results obtained from FCM and TVC methods for the same samples and reported that the FCM method gave results two orders of magnitude higher than the TVC method.

Polymerase chain reaction methods

Polymerase chain reaction (PCR) or real-time polymerase chain reaction (qPCR) methods do not normally distinguish between dead and viable bacteria. However, they can be coupled with for example viability discrimination step in which only the DNA from intact cells is detected or with an enrichment step following the PCR. (Barbau-Piednoir et al. 2014, 131.)

According to Bottari et al. (2015, 46), qPCR is a more sensitive method than ATP methods. However, higher costs, environmental inhibitors and overestimation of cells are possible downsides of the PCR methods.

Adenosine triphosphate methods

All living organisms contain adenosine triphosphate, ATP. Therefore, ATP methods can be used for the enumeration of microorganisms in various applications. The most used ATP monitoring methods are based on firefly bioluminescence. Bioluminescence and ATP monitoring are discussed in more detail in the following chapters.

3 BIOLUMINESCENCE

Bioluminescence is the production and emission of light by a living organism through chemical reactions. Bioluminescence has been studied for multiple centuries, but the reaction mechanisms have been deciphered and understood only during the past century. There are some parts of the bioluminescent reactions that are still not fully understood. Therefore, the research still goes on, but the main principles and mechanisms are understood adequately to be able to utilize bioluminescence in for example ATP monitoring.

Bioluminescence has been reported in a wide variety of organisms and only a minority of them has been properly studied. Bioluminescence is most common in marine organisms, but there are also bioluminescent species on land. (Kahlke & Umbers 2016, R313.) Bioluminescence systems are very diverse from species to species.

The most studied bioluminescent organisms are fireflies and especially the North American firefly, *Photinus pyralis*. Fireflies belong to the order Coleoptera and family Lampyridae. (Marques & Esteves da Silva 2009, 62.)

3.1 Firefly bioluminescence

The bioluminescent light is produced through a reaction of a substrate luciferin and an enzyme luciferase in the presence of certain co-substrates and cofactors, the most important of which are ATP and Mg^{2+} (Kahlke & Umbers 2016, R313). The ATP requirement of bioluminescence was first discovered by McElroy (1947). In 1949, McElroy and Strehler discovered that Mg^{2+} is also required in the reaction.

The overall reaction of firefly bioluminescence can be divided into two main reactions (equations 1 and 2) presented below (DeLuca & McElroy 1978, 3; Fraga 2008, 147; Fraga et al. 2006, 929).

$$E + LH_2 + ATP \cdot Mg^{2+} \leftrightarrow E \times LH_2 \cdot AMP + PP \cdot Mg^{2+} \quad (1)$$

$$E \times LH_2 \cdot AMP + O_2 \rightarrow E + AMP + CO_2 + \text{oxyluciferin} + \text{light} \quad (2)$$

In which E is luciferase, LH_2 is D-luciferin, PP is pyrophosphate, AMP is adenosine monophosphate and $LH_2 \cdot AMP$ is luciferyl adenylate.

The reactions contain multiple steps. In the first reaction (equation 1), the substrate luciferin and ATP form a complex with the enzyme luciferase. An enzyme-bound intermediate luciferyl adenylate ($E \times LH_2 \cdot AMP$) is then formed in the presence of Mg^{2+}. Pyrophosphate is released in this reaction. (Day et al. 2004, 10–11; DeLuca & McElroy 1978, 3–4.)

In the second reaction (equation 2), luciferyl adenylate ($E \times LH_2 \cdot AMP$) is oxidized into intermediate dioxetanone which is then decarboxylated leading to the formation of excited oxyluciferin. When oxyluciferin returns to its ground state, light is emitted. (Day et al. 2004, 10–11; DeLuca & McElroy 1987, 3.)

In addition to the light-producing reaction, there are also lateral reactions, the most important of which is one where the luciferyl adenylate ($E \times L \cdot AMP$) complex follows the path towards the synthesis of dehydroluciferyl adenylate ($E \times L \cdot AMP$) complex and dehydroluciferin (L). The reactions are presented below (equations 3 and 4). (Fontes et al. 1997, 445; Fraga et al. 2005, 5206-5207; Fraga et al. 2006, 929; Rhodes & McElroy 1958, 1534; White et al. 1980, 3199, 3203).

$$E \times LH_2 \cdot AMP + O_2 \leftrightarrow E \times L \cdot AMP + H_2O_2 \quad (3)$$

$$E \times L \cdot AMP + PP \cdot Mg^{2+} \leftrightarrow E + ATP \cdot Mg^{2+} + L \quad (4)$$

In which O_2 is oxygen, $L \cdot AMP$ is dehydroluciferyl adenylate, H_2O_2 is hydrogen peroxide and L is dehydroluciferin. The reactions of the firefly bioluminescent system discussed in this chapter are presented in figure 2. The reaction pathway concerning coenzyme A (CoA) mentioned in the figure is discussed in chapter 3.2.

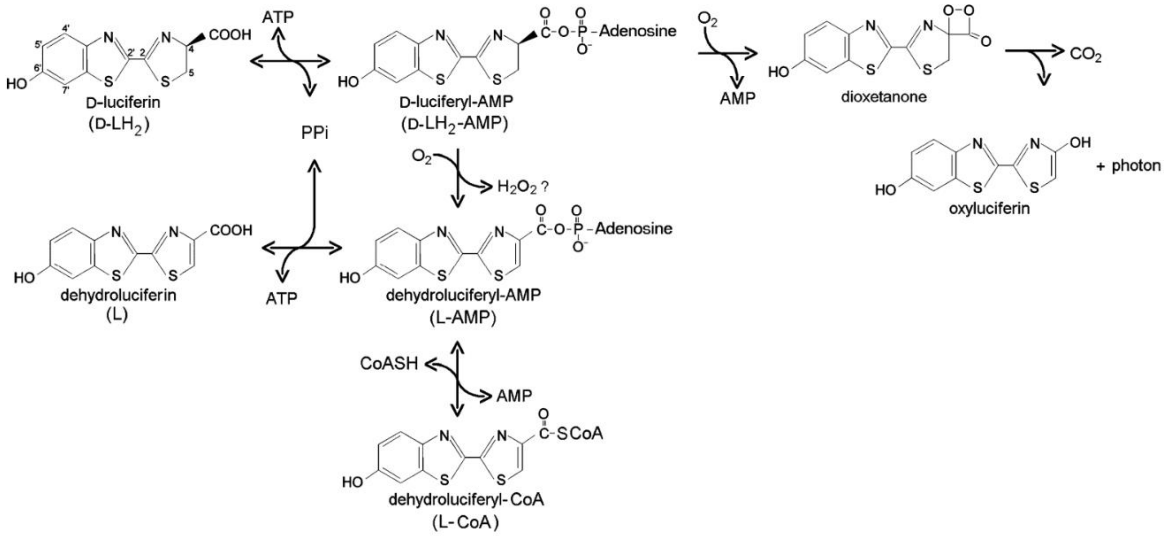

FIGURE 2. Firefly bioluminescence reactions (Fraga et al. 2005, 5207, modified)

According to Fontes et al. (1998, 192) E × L·AMP accounts for 16 % of total LH_2 consumption. However, the reaction pathways and their share of the LH_2 consumption are still not fully confirmed.

3.2 Bioluminescence kinetics

It has been known for decades that in firefly luciferase catalyzed reactions the light is first emitted as a flash which then decays rapidly even if there are excessive amounts of luciferin and ATP left (Airth et al. 1958, 520; McElroy & Seliger, 1963). It is evident that there are some inhibitory factors affecting the light production during bioluminescent reactions. However, the reaction kinetics and the inhibitory effects of multiple different factors have been and are still under constant debate.

The most common theory has been that one of the products, dehydroluciferin, is the main inhibitory factor. Fontes et al. (1997) demonstrated that dehydroluciferin can have an inhibitory effect on the light emission as L·AMP tightly binds the luciferase enzyme. The inhibition by dehydroluciferin was already discussed by Airth et al. (1958) but at the time the compound was called oxyluciferin.

The dehydroluciferin-AMP complex can also react with coenzyme A (CoA) yielding L·CoA (equation 5). The luciferase is then liberated to be used in the light producing reaction, leading to increased light intensity. (Airth et al. 1958, 522–523; Fontes et al. 1997, 193.)

$$E \times L{\cdot}AMP + CoA \leftrightarrow E + L{\cdot}CoA + AMP \quad (5)$$

3.3 Luciferin and luciferase

As stated in chapter 3.1, bioluminescent reaction is an enzymatic reaction between luciferin, luciferase, and multiple cofactors. Luciferin is a common name for substrates in bioluminescent reactions. However, there are multiple types of luciferin which have notable differences in their structure. All Lampyridae members as well as click beetles share a common luciferin typically called firefly luciferin or beetle luciferin. (Day et al. 2004, 8.) The structure for firefly luciferin is presented in figure 3.

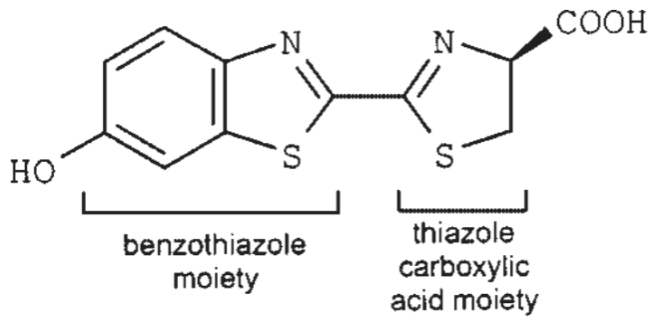

FIGURE 3. Firefly luciferin (Day et al. 2004, 10)

Luciferase in turn is a generic term for enzymes that can catalyze light emission. The luciferases of the members of Lampyridae family are all closely homologous but have minor differences. (Day et al. 2004, 8.) All luciferases utilize an oxidation reaction even though the bioluminescent systems differ from each other. The firefly luciferase molecule is folded into two distinct domains, a large N-terminal domain and a small C-terminal domain, separated with a wide cleft (Conti et al. 1996, 289–290).

4 ATP MONITORING WITH BIOLUMINESCENCE

ATP-based hygiene monitoring is used for the enumeration of microorganisms in a variety of food processing industries including for example dairy industry (Lindell et al. 2018; Renaud et al. 2017) and brewing (Carrick et al. 2001). In addition to the food industry, ATP monitoring has gained interest in other applications as well, such as water quality assessment (Amy et al. 2011; Eydal & Pedersen 2007; Nescerecka 2016b) and healthcare (Alfa et al. 2015; Pierce et al. 2019).

4.1 Adenosine triphosphate

Adenosine triphosphate (ATP) is present in all living organisms. Living cells convert the energy obtained from sunlight or nutrients into energy-rich ATP. When ATP is hydrolyzed to adenosine diphosphate (ADP), energy is made available to the cell for their activities, such as protein synthesis. Chemical energy in cells is not stored as ATP but rather as fats and carbohydrates which are then metabolized when needed. In addition to providing energy for cells to use, ATP is also involved in chemical signaling between cells. (Campbell & Farrell 2011, 438–441.)

Adenosine triphosphate (figure 4) is a nucleotide consisting of three main parts: a nitrogenous base (adenine), a sugar (ribose) and three phosphate groups bound to ribose (Encyclopaedia Britannica 2020). Energy is released when the bonds between the phosphate groups are hydrolyzed. ADP and free phosphates are then recycled back into ATP. (Campbell & Farrell 2011, 438–439).

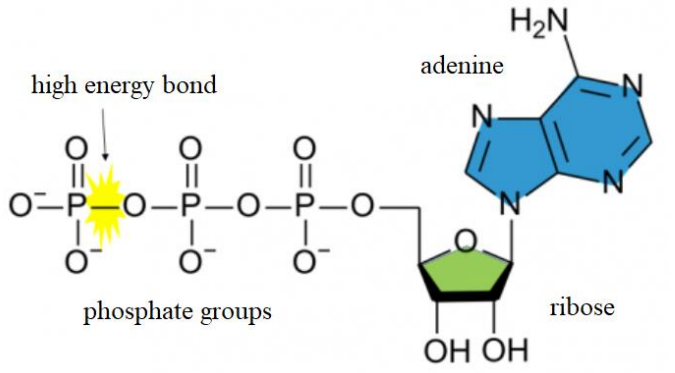

FIGURE 4. ATP molecule (Donaher 2016, modified)

A typical ATP pool of an aqueous sample consists of two types of ATP: microbial ATP inside the cells and free ATP in the sample. Free ATP has traditionally been thought to originate from dead or stressed cells (Abushaban et al. 2019, 2; Hammes et al. 2008, 269, 276; Venkateswaran et al. 2003, 375). However, studies also show that cells release ATP into extracellular matrix and ATP acts as a signaling agent among cells (Choi et al. 2014; Feng et al. 2015). When using the ATP method to evaluate the microbial burden of the sample, it is important to differentiate the non-microbial free ATP from the microbial intracellular ATP.

4.2 Amount of ATP in microbes

The amount of intracellular ATP is not constant between different cells which is one of the reasons why ATP and cell count results cannot be compared directly to each other. However, average ATP contents per cell have been proposed in multiple studies. In this chapter, some examples of ATP contents per cell obtained from different studies are presented. Some of the ATP contents per cell presented were originally reported as moles per cell (mol/cell). In these cases, the value was converted into grams per cell (g/cell) using the molecular mass of an ATP molecule (507.18 g/mol).

The most typical ATP per cell values reported in different studies vary approximately between 1×10^{-18} and 1×10^{-15} grams per cell. A value of 1×10^{-15} grams per cell (Stanley 1989) has commonly been used in calculations and similar values have been obtained in earlier studies, for example by Hamilton and Holm-Hansen (1967). Smaller values between 1×10^{-18} and 1×10^{-17} g/cell have typically been obtained in more recent studies. Examples of values for drinking water and seawater microorganisms are presented in table 1.

TABLE 1. ATP per cell values from literature for drinking water and seawater samples

g/cell	cell count method	sample type	reference
8.6×10^{-16}	FCM	seawater	Abushaban et al. 2019
8.9×10^{-17}	FCM + PI	drinking water	Hammes et al. 2010
7.61×10^{-17}–2.43×10^{-16}	FCM + SYBR Green	drinking water	Hammes et al. 2008
6.87×10^{-17}	FCM + SYBR Green	drinking water	Siebel et al. 2008
3.5–5.6×10^{-17}	TDC	drinking water	Vang et al. 2014

However, it should be noted that in more recent studies with lower ATP per cell values the focus has been on analyzing microorganisms from natural environments whereas many older studies with higher values were performed with laboratory-grown microorganisms. It should also be noted that various methods to measure the ATP per cell concentrations as well as cell counts were used. Therefore, the values from different studies are not fully comparable.

In addition to differences in the methods and sample types, also multiple intracellular and extracellular factors affect the ATP content of cells. Most important of these factors are presented in the following chapters 4.2.1–4.2.3.

4.2.1 Effect of species

Species of the microorganism is one of the main factors affecting the ATP content of the cell. The ATP content varies from species to species and in general, the ATP content of yeast and fungal spores are found to be higher than that of bacteria (Shama and Malik 2013).

Shama and Malik (2013) have collected information about ATP contents of different microbial cells from multiple studies. In this comparison, the ATP concentrations for bacteria were between 1.72×10^{-18} and 1.27×10^{-12} g/cell (3.4×10^{-21} and 2.5×10^{-15} mol/cell), most of the values being around the middle of the range. The values for yeast and fungal spores were higher than for bacteria in average, around 1×10^{-14} and 1×10^{-13} grams per cell.

Kodaka et al. (1996) reported that the average ATP content of Gram-positive bacteria is approximately 12 times higher than that of Gram-negative bacteria. Also, Venkateswaran et al. (2003) reported higher ATP content per cell for Gram-positive bacteria. However, the difference could be partly explained by issues in detecting Gram negative bacteria, probably due to incomplete cell lysis caused by the cell wall structure (Turner et al. 2010, 193–194).

4.2.2 Effect of biomass

Another factor affecting the intracellular ATP content is the biomass or biovolume of the cell. Larger cells typically have more ATP than smaller cells (Eydal & Pedersen 2007, 370; Hammes et al. 2010, 3921–3922; Siebel et al. 2010, 3). Therefore, an average ATP per cell approach underestimates the number of small cells and overestimates the number of larger cells.

Hamilton & Holm-Hansen (1967) reported that in the five genera of marine bacteria they studied, the ATP content was from 0.3 to 1.1 % of the cell carbon content. In another study, Holm-Hansen (1970) showed that the ATP content of multiple algal species also correlated well with the cell carbon content. Karl (1980) proposed the following correlation between biomass and ATP content: biomass (g C) = 250 × ATP (g ATP).

It has been proposed that the ATP per biomass or biovolume value would be much more constant than ATP per cell, even between different species when the growth conditions and growth phases are similar. This could partly explain the differences in ATP per cell values typically obtained in studies. (Eydal & Pedersen 2007; Wang et al. 2009.) However, the ATP per biomass approach is also only a rough estimate and the values vary between studies (Karl 1980, 763–765).

4.2.3 Effect of environment

Another important group of factors affecting the ATP content of cells are environmental conditions, such as the availability of nutrients. Cells cultured in the laboratory seem to generally have a higher ATP-content than those isolated from natural environments (Hammes et al. 2010, 3921; Shama & Malik 2013, 118). It has been reported that the values of ATP per cell are on average two orders of magnitude higher in laboratory cultures than in natural systems (Wilson et al. 1981).

Wilson et al. (1981) also stated that the ATP content of cells cultivated in nutrient rich media is higher than the content of cells cultivated under oligotrophic conditions. Pridmore et al. (1984) reported that the amount of ATP per cell decreased in lake water under nitrogen or phosphorus deficiency. Hamilton and Holm-Hansen (1967) reported that the ATP content of aged and starved cells was about 20 % of the concentration found in exponentially growing cells. According to Gengenbacher et al. (2010, 86), it seems that ATP level downregulation might be an important survival strategy for bacteria during nutrient starvation.

Some bacteria produce bacterial endospores when they are exposed to environmental stress. These spores can survive more extreme environmental conditions than vegetative cells. Spores can maintain viability on surfaces for a rather long period of time. In general, the ATP content of spores is lower than that of vegetative bacterial cells. (Fajardo-Cavazos et al. 2008; Shama & Malik 2013.)

Many bacteria, especially many pathogens, can also enter the ‘viable but nonculturable’ state (VBNC) under chemical or environmental factors. In this state, bacteria maintain their viability but are unable to grow on artificial laboratory media. The bacteria become culturable again upon resuscitation. (Oliver 2000, 277; Shama & Malik 2013, 119.) For example, *Escherichia coli* and many *Salmonella* spp. can enter VBNC state (Oliver 2010, 415).

According to Oliver (2010, 417), the cell size is typically reduced but the ATP level may remain relatively high in VBNC state. Oliver also noted that it seems that pathogens are

not able to initiate disease in VBNC state, but their virulence is retained after resuscitation. It was reported by del Mar Lleo et al. (2007) that some highly active antibiotics did not act on bacteria in VBNC state.

4.3 ATP monitoring methods

Bioluminescence-based ATP methods can be roughly divided into two different types: cuvette-based and pen-based methods. The general principle remains the same, but the measurement protocol varies between different methods. Typically, surface samples are taken with swabs and liquid samples with swabs, sample dippers or by pipetting. The light emitted in the reaction is measured with a luminometer.

In cuvette-based methods the sample is transferred into a cuvette and necessary reagents are added. If the sample is taken with a swab, the ATP is typically released from the swab with a diluent. After mixing the sample and the reagents, the cuvette is measured with a luminometer. (Hawronskyj & Holah 1997, 81.) Cuvette-based methods typically require more laboratory facilities and expertise than pen-based methods.

In pen-based methods the sample is transferred into a pen-type device with a swab, a sample dipper or by pipetting. The pen contains all necessary reagents but the system for releasing the reagents to react with the sample vary between different devices. After the activation of the reaction, the pen is put into the luminometer for measuring. The luminometers used in pen-based methods are typically lightweight and handheld. Therefore, the advantages of pen-based methods over cuvette-based methods are their portability and ease of use.

Luminometers from different manufacturers share the same basic operational principles, even though they may differ in construction, use, and interface. Traditionally luminometers have been equipped with more sensitive photomultiplier tubes as light sensors but advanced photodiodes have become increasingly popular. Photodiodes are lighter and require lower voltage than photomultiplier tubes. (Hygiena n.d.b.) Luminometers equipped

with a photodiode are also more stable and robust thus requiring less maintenance (Meighan 2011, 7).

Luminometers typically express the results as relative light units, RLU. The RLU is not a standardized unit but each manufacturer defines the value for one RLU for their own system instead. Therefore, the RLU values from different ATP monitoring systems should not be directly compared to each other. (Hygiena n.d.c.) In addition to differences in methods, luminometers and RLU values, different commercial ATP tests have different kinds of reagents from different sources. These factors together may cause very significant variations in the results between systems.

ATP methods have typically been reported to have a good precision and repeatability when replicate samples are analyzed with the same system (Fulford et al. 2004; Hammes et al. 2008; Siebel et al. 2008). However, there are two steps during the measurement during which even small failures can affect the results significantly. These two steps are ATP extraction and separation of free ATP and they are presented in more detail in the following chapters 4.3.1 and 4.3.2.

4.3.1 ATP extraction

The most crucial step in ATP monitoring is the extraction of intracellular ATP from the cells which can be performed with various different methods including for example chemical extraction (Luo et al. 2009), microwave (Tsai 1986), continuous DC voltage (Lee & Cho 2007) and ultrasound (Law et al. 2003). Poor extraction can lead to low results and false negative readings as some of the intracellular ATP remains inside the cells and does not react during the measurement.

In some applications, it is also important to differentiate between ATP from somatic cells and bacterial ATP. Somatic ATP can originate from food or other product residues. Somatic ATP can be separated enzymatically or physically. (Bottari et al. 2015, 37; Dostálek & Brányik 2005, 86; Shinozaki et al. 2013, 4.) In CIP rinse water monitoring, it is not

necessary to differentiate ATP sources since product residues and bacterial contamination are both unwanted and should be removed as properly as possible.

4.3.2 Separation of free ATP

ATP methods cannot themselves distinguish intracellular ATP that directly indicates the presence of microorganisms from extracellular free ATP. Therefore, another important step of ATP monitoring is the separation of free ATP. The presence of free ATP during the measurement can increase the results significantly and therefore lead to false conclusions. However, in some applications such as cleaning verification, all ATP can sometimes be measured as one since any ATP residues indicate that the cleaning was not fully successful. Therefore, the importance of the separation of free ATP depends on the application.

The amount of free ATP in samples may vary greatly depending on for example sample type, sample handling and environmental conditions. Hammes et al. (2010) tested 102 water samples from different sources including bottled water, tap water and freshwater. They reported that the amount of free ATP in the samples varied between 0 and 97 % of the total ATP regardless of the sample type. Hammes et al. (2008) reported free ATP percentages between 12 and 100 % in drinking water samples taken from different treatment steps. Siebel et al. (2008) used a filtration (0.1 µm) step to segregate free ATP from drinking water samples and reported free ATP concentrations below 5 %. These results show that the amount of free ATP varies significantly and cannot be estimated in advance.

The extraction of free ATP can be performed mechanically using filtration (Hammes et al. 2010; Siebel et al. 2008) or enzymatically (Shinozaki et al. 2013; Venkateswaran et al. 2003). However, it should be noted that filtration may damage cell walls and lead to the release of intracellular ATP outside the cells too early, which in turn lowers the results for intracellular ATP content (Eydal & Pedersen 2007, 371).

Some of the pen-based methods have been developed so that it is possible to separate the amount of free ATP from the total ATP content of the sample and thereby get the amount

of microbial ATP. This often requires two different pens: one for measuring free ATP without cell lysis and one for measuring the total ATP with cell lysis.

There is rather contradictory information about the stability of ATP molecules outside of cells (Fajardo-Cavazos et al. 2008, 5160; Shama & Malik 2013, 120). Dostálek and Brányik (2005, 86) stated that ATP is quickly broken down when cells die. Alfa et al. (2015) tested the stability of ATP dried on healthcare environmental surfaces as well as in liquid suspension. They reported that high levels or ATP were still detected after 14 and 29 days even though no viable organisms could be detected. Schuerger et al. (2008) in turn concluded based on their studies that ATP could survive years or even decades under martian conditions.

Based on the variation in the amount of free ATP and the uncertainty about the stability of the ATP molecules outside of cells, it can be concluded that in most applications it is crucial to separate the free ATP from the sample before measurement. However, in some applications all ATP can be measured as one. It should also be noted that if the sample types, microorganisms present in the samples and environmental conditions stay nearly the same, it is not probably necessary to separate free ATP every time the measurements are performed. In these situations, the amount of free ATP can be estimated based on earlier results and taken into account in calculations.

4.4 Factors affecting ATP monitoring

There are multiple factors that can affect the bioluminescent reaction, for example by changing the wavelength of the light emission or interfering with the reaction. These factors should be considered when planning ATP monitoring and their effect should be tested before establishing an ATP monitoring protocol. The most important of these factors are presented below.

4.4.1 pH

Bioluminescence is based on enzymatic reactions which is why the pH can have a strong effect on the reaction and thereby on the light emission. Enzymes have a pH optimum where the activity of the enzyme is the highest (Yoo et al. 2017, 46–47). According to Hawronskyj & Holah (1997, 81), the optimal pH for the reaction is 7.75. Steghens et al. (1998) discovered that the maximum emission of light in the ATP measurement occurred at pH 7.8 when the pH was tested between pH 6.8 and 8.8 and was adjusted with steps of 0.5.

The pH can also affect the peak wavelength of the light emission. The light emission in the case of firefly bioluminescence is yellow-green and it peaks in 562 nm (Seliger et al. 1964). It has been studied by Seliger & McElroy (1964) that in acidic conditions (pH < 6.5) the intensity of the yellow-green emission decreases and the emission is shifted to red, peaking at 616 nm. Similar remarks have been made in other studies, such as Morton et al. (1969).

Many commercial reagents for ATP monitoring include buffering agents so that the effect of the acidic or alkaline pH should remain rather minimal. Many manufacturers also claim that the pH of the sample should have no effect on the measurements. However, it is important to know the pH of the samples and test the effects of very low or high pH values. It is often recommended to adjust the pH of the sample before measurement if the original pH is very low or high.

4.4.2 Temperature

Luciferase enzyme functions best in the temperature of 20–22 °C (Hawronskyj & Holah 1997, 81, Hygiena n.d.c.). In colder temperatures the reaction is slower, and the results may be low. Thereby also false negatives are possible. The best performance is therefore typically gained when the temperature of the reagents and samples as well as the ambient temperature are all near 20–22 °C.

However, Hygiena claims that the ambient temperature does not affect the operation of the luminometer provided that the instrument has time to equilibrate to the temperature conditions. The instrument will sense changes in the environmental temperature and will automatically re-calibrate itself. (Hygiena n.d.c.)

4.4.3 Interference

Multiple studies have been performed to examine if cleaning chemicals and sanitizers affect ATP measurements. Velazquez & Feirtag (1997) defined substances that reduce the light signal as quenchers and substances that increase the light signal as enhancers. They studied the effect of multiple cleansers and sanitizers and reported that many of them acted as an enhancer at low concentrations but had a quenching effect at high concentrations.

There are multiple possible reasons for the enhancing and quenching effects of cleaning and disinfecting agents. Lappalainen et al. (2000) stated that cleaning agents can act as ATP releasing agents disrupting the cell walls of microorganisms and also inactivate both the ATPase enzymes and ATP converting enzymes, depending on the type and concentration of the agent. In addition, cleaning agents can also denature the luciferase enzyme leading to poor ATP measurement performance. Different combinations of these effects can lead to very varying results.

Turner et al. (2010) tested the effect of multiple common disinfecting agents and reported decreased ATP results in presence of disinfectants. They also reported that residual bleach has the potential to quench the light emission so significantly that a false negative reading is provided.

The whitepaper by LuminUltra (2016) mentioned that other than chemicals, possible interference can be caused by multiple other sources including suspended solids, dissolved solids, heavy metals, biocides, and organics. They also mentioned that sample color can cause inhibition.

Amy et al. (2011) reported that high salt concentrations in water samples affect the ATP measurements by significantly reducing the light emission. A salt concentration of 4 g/l reduced the amount of light by 50 % compared to a 0 g/l concentration. With a salt concentration of 10 g/l, only 20 % of the full light emission is achieved. They also pointed out that the effects of interfering factors can be reduced or removed by filtration.

Many commercial bioluminescence reagents have been designed so that the interference caused by for example cleaning chemicals or salts is reduced to a minimal level. Nevertheless, the possible interference of these factors should still be considered and minimized as well as possible.

4.5 Lower and upper ATP limits

In many ATP monitoring applications, it is important to set thresholds for the ATP results. ATP monitoring systems typically come with default limits, but the limits should be established according to the sample type, object of cleaning and other unique needs. There are also examples of these limits in literature, but they are established for a certain ATP monitoring system and sample type and therefore cannot be used universally.

In CIP cleaning verification, two limit values are typically set, and they divide the cleaning result into ‘pass’, ‘caution’, and ‘fail’. There are multiple protocols used to determine the limits. The lower ‘pass’ limit is typically determined by cleaning the location properly and taking an average of the results obtained after cleaning. All results below this lower limit are ‘pass’. (Hawronskyj & Holah 1997, 82; Hygiena 2013a, 20.)

The upper limit in turn can be defined in multiple different ways. Hawronskyj & Holah (1997, 82) suggested that the upper limit is determined by the results before cleaning. Hygiena (2013a, 21) in turn suggests to either multiply the lower limit by three or calculate a standard deviation of the results for the cleaned surface, multiplying it by three and adding this to the lower limit. The results above the upper limit are ‘fail’ which means that the location should be recleaned and retested immediately.

The results between the lower and upper limits are defined as ‘caution’ which means that the location should be either recleaned or monitored more carefully, depending on the situation. However, it is also possible to use only one threshold to divide the results into ‘pass’ or ‘fail’. One limit is often used because it is simple and clear regardless of the situation. It should be noted that the different locations may require different limits.

4.6 ATP versus cell counts

When comparing ATP results to cell counts, it should be remembered that both results are used to estimate the number of bacteria in a sample, but the methods determine different things. It is important to remember that some cell count methods detect only culturable cells, some only viable cells and some detect both viable and dead cells. ATP methods in turn detect only viable cells if free ATP is separated. Therefore, the methods and the results should not be confused with each other or used as synonyms to each other.

In literature, obtained correlations between cell count and ATP methods vary greatly. Typically, the correlations between TVC and ATP results are rather poor or even nonexistent, but the correlations between FCM and ATP results are significantly better. This is probably mostly due to the non-culturability of a significant portion of the cells with TVC. Some of the correlations from different studies are presented in table 2.

TABLE 2. Correlations between cell count and ATP results

R^2	sample type	cell count method	reference
0.004	different water samples	TVC	Hammes et al. 2010
0.31	drinking water	TVC	Siebel et al. 2008
0.69	drinking water	FCM + SYBR	Siebel et al. 2008
0.72	seawater	FCM + SYBR	Abushaban et al. 2019
0.80	different water samples	FCM + SYBR	Hammes et al. 2010
0.81	colostrum feeding equipment	ACC Petrifilm	Renaud et al. 2017
0.84	drinking water	TVC	Deininger & Lee 2001
0.9993	*E. coli*	TVC	Turner et al. 2010
0.9995	*S. aurelius*	TVC	Turner et al. 2010
0.999999	*Bacillus* sp.	TVC	Shinozaki et al 2013

As can be seen from the table, also the correlations between TVC and ATP results vary greatly between studies. The correlation can be very good when pure bacteria are analyzed. The correlations for samples containing a wider variety of bacteria are in turn significantly poorer. Problems with the mixtures of multiple different species rise from the possible presence of nonculturable microorganisms and the variation of the ATP content of different species. These are probably the main reasons to decrease the correlation between TVC and ATP results. In addition, sample properties may cause problems in either or both methods decreasing the correlation.

It should be noted that as discussed in chapter 2.4.1, TVC is not a single method that is performed similarly in different studies, but rather a group of cultivation methods where the test conditions may vary. In addition to differences in the cell count methods, it should also be noted that the methods to measure the ATP content of the samples differ between the studies.

5 MATERIALS AND METHODS

In this chapter, the materials and methods used in this thesis are presented. At first, CIP rinse water and cooling water samples are discussed in more detail. Next, ATP and TVC methods and the equipment are presented. Lastly, the testing protocol for the ATP method is explained.

5.1 Samples

In this thesis, 50 CIP rinse water samples and 9 cooling water samples were analyzed with both TVC and ATP methods. The pH values of most of the samples were also analyzed. In addition, four water samples were collected from less regularly cleaned parts of the plant in order to get samples more highly contaminated by bacteria and other residues. The samples were analyzed as soon as possible after their arrival at the laboratory. If the sample could not be analyzed in a couple of hours, it was stored in a refrigerator.

The CIP rinse water samples are typically slightly alkaline, the pH value being between 8 and 9. The samples usually contain only very small amounts of product residues. They may contain very small amounts of microbes, proteins, salts, and formulation chemicals such as sugars and polyols. In addition, small concentrations of sodium hydroxide from the CIP cycle may be present, as the slightly elevated pH value indicates. However, the conductivity of the last rinse water during the CIP cycle is monitored and therefore the pH should not be much higher than 9 and the sodium hydroxide concentration should remain very low.

The plant laboratory analyzes hundreds of CIP rinse water samples annually. The TVC results for the CIP rinse water samples are typically low, more than half of the annual results being <1 CFU/ml. Less than 10 % of the annual results are above 100 CFU/ml and only around 1–2 % are above 1000 CFU/ml.

The cooling water samples in turn are closer to neutral, their pH values being typically between 7 and 8. Cooling water samples should not contain other possibly interfering

agents either. However, their TVC results are usually somewhat higher than CIP rinse water results. Typical results are a couple of hundreds CFU/ml, some results exceeding 1000 CFU/ml annually.

5.2 ATP method

ATP measurements were carried out with a pen-based ATP method from Hygiena. The equipment included Hygiena EnSURE™ luminometer, AquaSnap™ Total water test pens and SuperSnap™ surface test pens. In this thesis, the pens are called test devices.

5.2.1 EnSURE luminometer

The luminometer used in this thesis was Hygiena EnSURE luminometer (figure 5). Hygiena also has an updated version of the luminometer, EnSURE Touch, with a touch screen and a shorter measurement time. EnSURE and EnSURE Touch are similar in sensitivity and overall performance. Nonetheless, the older model was used in this thesis.

FIGURE 5. Hygiena EnSURE luminometer (Hygiena 2013a, 6)

The luminometer is handheld, lightweight, fully portable and battery powered. The luminometer is equipped with an advanced photodiode sensor that is not damaged by drops or

shakes. The sensitivity of the EnSURE luminometer is reported to be 0.1 femtomoles of ATP. (Hygiena 2016.)

There are multiple different test devices available to be used with EnSURE luminometers ranging from liquid and surface ATP tests to tests that detect specific bacteria, such as *E. coli*, Coliform, *Listeria* spp. and Enterobacteriaceae. Some of the test devices can also be used with other luminometers by Hygiena, but the EnSURE luminometer is the most sensitive of them. (Hygiena 2016.)

Test plans, test locations, user IDs and results can be saved to the memory of the EnSURE luminometer. The luminometer can be connected to a computer and Hygiena's SureTrend data analysis software via USB. The software offers tools for example for result analysis and reporting as well as data storage. (Hygiena 2016.)

5.2.2 AquaSnap test devices

The water samples were analyzed using Hygiena AquaSnap Total devices which contain all the needed reagents and utilize advanced liquid-stable chemistry. Traditionally the reagents, especially enzymes, have been stabilized by lyophilization prior to use. Lyophilized reagents need to be reconstituted with a liquid. Hygiena in turn uses a newer and more advanced stabilization method called liquid-stable chemistry which is cheaper to manufacture and provides better accuracy and reproducibility. (Hygiena n.d.b.) In addition, the test devices consist of fewer components and less plastic than other ATP test devices and are also fully recyclable (Hygiena n.d.d.).

Hygiena ATP test devices have a unique Snap-Valve™ technology in which the reagents are released to contact with the sample by snapping a valve on top of the device. Many other ATP test devices use foil seals or similar to separate different compartments inside the device and require breaking the foils with the sample collection tip to combine the reagents with the sample. This may lead to leaving sample behind on the foil and therefore lower or variable results. (Hygiena n.d.d.)

AquaSnap Total test devices measure both free and microbial ATP from the sample. Hygiena also offers AquaSnap Free test devices that measure only the amount of free ATP in the sample, but these tests were not available for use in this thesis. AquaSnap Total tests can detect down to 0.1 femtomoles of ATP (Hygiena 2014).

The sample collection tip of AquaSnap devices is a honeycomb dipper which collects exactly 100 ul of liquid sample when used correctly. The Snap-Valve design allows the release of reagents to the sample right before the test device is measured, meaning that the samples can be collected into the devices earlier and the reaction can be started just before measurement to avoid any delay during the measurement.

ATP measurement with AquaSnap

The ATP measurements with the AquaSnap devices were carried out according to the instructions given by the manufacturer. Only some minor additions or changes were made. The method execution is explained below (Hygiena 2013a, 10–13) and also presented in figure 6.

1. The test device is allowed to come to the room temperature
2. The test device is shaken so that the ATP extraction reagent comes to the bottom of the tube
3. The sample collection device is removed from the outer tube and the dipper tip is dipped into the sample for two seconds
4. The sample collection device is placed back into the tube
5. The test device is shaken to mix the sample and the extraction reagent inside the tube
6. The bioluminescent reaction is activated by bending the bulb on the top of the sampling device so that the valve breaks
7. The bulb is squeezed 2–3 times to release all liquid into the bottom of the tube
8. The test device is shaken gently in a side-to-side motion for 5 seconds
9. The device is inserted into the luminometer and measured without any delay

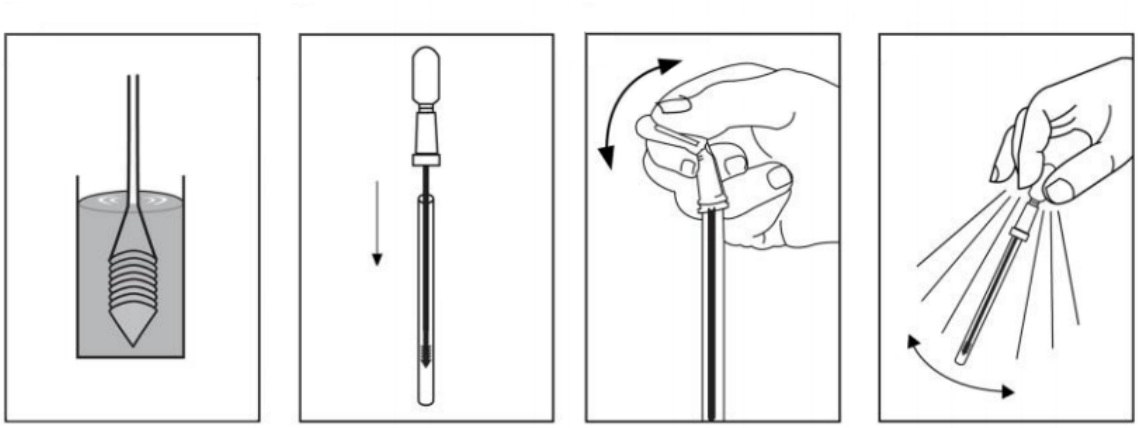

FIGURE 6. Analyzing liquid samples with AquaSnap (Hygiena 2014, modified)

It is important to perform the measurement in the same way every time. Especially any unnecessary delay between reaction activation (step 6) and measurement (step 9) causes too low and variable results since the amount of light will begin to decay rapidly (Hygiena n.d.c.). The luminometer should always be kept in an upright position during the measurement.

5.2.3 SuperSnap test devices

In addition to liquid test devices, also surface test devices were tested in this thesis. The surface test devices chosen for the tests were Hygiena SuperSnap devices. SuperSnap tests are the most sensitive surface ATP tests of Hygiena, being 4 times more sensitive than Hygiena's UltraSnap surface ATP tests. According to Hygiena, SuperSnap tests are resistant to the effects of sanitizers at working concentration (Hygiena 2013a, 24). These swabs work in a similar way than AquaSnap devices, but the sample collection tip is a cotton swab that is pre-moistened with a mild extraction solution (Hygiena 2013a, 10).

When used for surface ATP monitoring, the surface is swiped with the cotton tip in a zig-zag fashion and the swab is rotated at the same time (figure 7). A typical sampling area is 10 times 10 centimeters. The swab is then placed into the tube, the valve is snapped as with AquaSnap devices and the content of the bulb is squeezed into the bottom of the tube. After 5 seconds of shaking, the device is measured with the luminometer without any delay. (Hygiena 2013a, 10–13.)

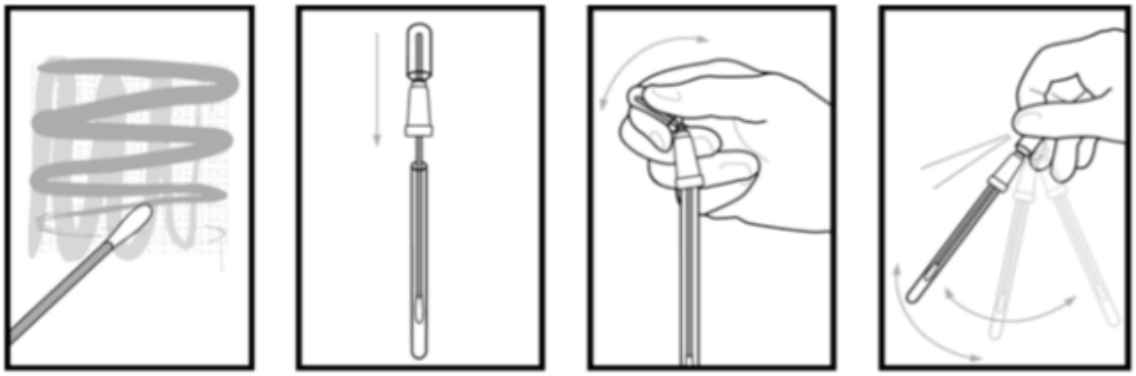

FIGURE 7. Analyzing surface samples with SuperSnap devices (Hygiena n.d.e.)

SuperSnap devices are primarily designed for surface ATP testing but they can be used for water samples as well. However, it is not possible to collect an exact amount of liquid sample with the cotton swab. Therefore, the results are only vague and do not show the real ATP level of the sample. Although, this can be avoided by pipetting an exact sample amount directly into the tube without using the sample collection tip.

The problem with surface swabs like SuperSnap is that it is not possible to distinguish microbial ATP from free ATP. Even when the surface is cleaned from microbes, there might be product residues causing a relatively high ATP result. Therefore, the result interpretation is rather difficult.

5.3 TVC method

The TVC method used in Jämsänkoski plant is based on SFS-EN ISO 4833-1:2013 standard. The culture medium is Plate Count Agar (PCA) which consists of tryptone, yeast extract and glucose. The PCA agar is melted and cooled to 45 °C before use.

The CIP rinse water samples are typically diluted in serial 10-fold dilutions until 10^{-2} dilution. Cooling water samples are diluted until 10^{-3} or 10^{-4} dilutions. One milliliter of each dilution is pipetted onto separate plates. Approximately 15 ml of 45 °C PCA is then poured onto each plate and the plates are rotated gently in a circular motion to mix the sample with the agar. The plates are then allowed to solidify and placed into incubators

in an inverted position. The plates are incubated at 30 °C for 72 hours or at 37 °C for 48 hours.

After incubation, all colonies from plates with 30 to 300 colonies are counted using a microscope. The result is expressed in colony forming units per milliliter, CFU/ml, and is calculated as below (equation 6). If there are no colonies on a plate, the result is expressed as <1 CFU/ml.

$$\frac{\text{CFU}}{\text{ml}} = \frac{X_1 + X_2 + \ldots + X_n}{Y_1 + Y_2 + \ldots + Y_n} \tag{6}$$

In which X is the number of colonies counted from the plate and Y is the actual volume of sample plated.

In this thesis, some plates with more than 300 colonies were divided into 2 or 4 equal sectors and the colony count of one sector was counted and multiplied with the number of the sectors to get the colony count of the whole plate. Some plates with less than 30 colonies were also included to be able to compare the results with the ATP results.

5.4 Test protocol

The basic testing protocol included analyzing the CIP rinse water and cooling water samples side by side with ATP and TVC methods. This was done to see if the ATP method worked properly and if there was any correlation between the results of the two methods. In addition, four dirtier water samples were prepared and analyzed with both methods. Also, some additional tests were done to see how the ATP method performed and how different conditions affected the ATP measurement. The additional tests are presented in table 3 and in more detail below the table.

TABLE 3. Test protocol

number	test
1	linearity
2	precision
3	background noise and limit of detection
4	test with pure bacteria
5	pH test
6	temperature test
7	measurement delay test
8	sample stability in AquaSnap
9	AquaSnap shelf life at room temperature
10	filtration test
11	water samples with surface test devices
12	test with product samples

Linearity

Omidbakhsh et al. (2014) compared the linearity of four different luminometers and swabs, one of them being Hygiena EnSURE luminometer and SuperSnap surface swabs. They reported very good linearity between ATP results and the real ATP concentration for EnSURE luminometer. However, the measuring range of EnSURE (from 0 to 9999 RLU) is narrower than that of the other luminometers that were tested so a full comparison between the linearity of the systems cannot be made.

For the linearity test in this thesis, an ATP standard was used. The standard had been sent to the laboratory diluted and divided into small aliquots. The temperature during the shipping was not appropriate which probably has had an effect to the ATP concentration of the solution. Therefore, the exact ATP concentration of the standard was unknown, so it was not possible to see the correlation between the RLU result and the actual ATP concentration. Hygiena reports that with EnSURE luminometer and AquaSnap devices 1 RLU corresponds to 0.1 femtomoles of ATP (Hygiena 2013b).

The linearity test was done by melting the frozen ATP standard aliquot in room temperature and immediately diluting it. The dilutions were 10-fold dilutions between 10^{-2} and 10^{-5}. The RLU result for every dilution was measured.

Precision

Precision is the closeness of multiple measurements to each other. Precision can be further divided into repeatability and reproducibility, which are often confused with each other. In this thesis, the term repeatability is used when the same operator repeats the measurement in the same experimental conditions. Repeatability is also used when two luminometers are tested side by side, but the operator remains the same. Reproducibility is in turn used when the operator changes, but the experimental conditions remain the same.

Plate count methods such as TVC are rather sensitive to small factors during the analysis because they are based on the growth of living organisms. In this thesis, the TVC method was mostly carried out without using replicates due to limited resources. Therefore, there is no data about the repeatability or reproducibility of the method. However, it is known that the precision of the method is only moderate. For example, Siebel et al. (2008) reported a relative standard deviation (RSD) of 14 % for the TVC method. RSD is calculated as the ratio of the standard deviation to the mean of the replicate results.

ATP methods are typically more precise than TVC methods. In this thesis, the repeatability of the ATP method was examined in two different parts: repeatability between measurements and repeatability between luminometers. Multiple samples used in this thesis were analyzed in 2–4 replicates. The RSD between these replicate results is considered as the repeatability between measurements. It can also be considered as the repeatability between AquaSnap test devices, because one test device can only be measured once to avoid any measurement delay.

If the ATP method is further utilized in the plant, the idea is to have multiple luminometers in different parts of the plant. Therefore, there should be no difference between the results given by separate luminometers. The repeatability of the method between two luminometers was tested by analyzing 14 samples side by side with two separate Hygiena EnSURE luminometers. Separate AquaSnap devices were used to avoid any measurement delay. Measurements were carried out in a similar manner with both luminometers.

Hygiena states that EnSURE luminometers will stay in calibration for the life of the luminometer and therefore yearly calibrations are not needed. Hygiena luminometers also

self-calibrate at start up. However, a documented calibration is often needed for example for audits and certificates and therefore periodic calibrations are often performed. Hygiena offers calibration kits for testing the luminometer and the ATP test devices. (Hygiena 2013a, 14.) However, it was not possible to use these kits in this thesis. The effect of the calibration was tested together with the repeatability between luminometers, since one of the luminometers was calibrated over a year ago and the other a week ago.

The second part of the precision, reproducibility, could not be examined during this thesis, because the measurements were all carried out by a single operator. In the future, the reproducibility should be examined with multiple operators analyzing the same samples.

Background noise and limit of detection

Background noise of a system can be caused by the instrument itself or by chemical interference (Hygiena n.d.d.; Meighan 2011, 7). It is important to know the background noise level of the system to be able to assess the sensitivity of the system and thereby the lowest levels of ATP that can be detected (limit of detection, LoD). If the issue of the background noise level is not addressed, it may cause misinterpretations of the results. The background noise of the system was examined by measuring the test device without sampling. In total, 5 measurements were made during different days.

The limit of detection may vary greatly between different systems. In multiple studies average values of 1×10^2 to 1×10^4 CFU/ml have been reported for multiple commercial luminometers (Alfa et al. 2013; Sciortino & Giles 2012; Turner et al. 2010). It was also demonstrated in these studies that the limit of detection for Gram positive bacteria is generally lower than for Gram negative bacteria. As discussed in chapter 4.2.1, incomplete cell lysis seems to be a problem when analyzing the ATP content of Gram negative bacteria.

Hygiena states that the EnSURE luminometer and AquaSnap test devices can detect down to 1000 CFU/ml or less (Hygiena 2015) or 0.1 femtomoles of ATP or less (Hygiena 2013b). As discussed in chapter 5.4.1, it was not possible to use the ATP standard in this thesis to test the limit of detection as an ATP concentration. Due to the variability of

intracellular ATP content of cells, it is also rather vague to determine the limit of detection as a cell count.

Test with pure bacteria

The ability of the ATP assay to detect pure bacteria was examined by using a suspension of *Bacillus subtilis*. Serial 10-fold dilutions from 10^{-2} to 10^{-7} were made in duplicate using dilution water containing peptone and sodium chloride and CIP rinse water sample with the TVC result of <1 CFU/ml and the ATP result of 0 RLU. This was done to mimic the situation where the CIP rinse water sample would contain a significant number of bacteria. The dilutions were then analyzed with the ATP method as well as the TVC method.

pH test

The effect of sample pH was tested by adjusting the pH value of one water sample with a dilute base solution and two different dilute acids. The original pH of the sample was 8.35. The sample was separated into three different sterile bottles. One sample fraction was adjusted with 0.1 M sodium hydroxide, one with 0.1 M hydrochloric acid and the last one with 0.1 M acetic acid. The pH values of the samples were adjusted in the steps of around 0.6–1.2 units. The ATP measurement was performed after every adjustment step.

Temperature test

Hygiena states that the EnSURE luminometer re-calibrates itself when the environmental temperature changes. Therefore, the ambient temperature should have no effect on the measurement in reasonable temperature range. However, as discussed in chapter 4.4.2, the test devices should be in room temperature when used, as well as the sample.

The AquaSnap test devices should be stored long-term in the refrigerator but can be stored up to 4 weeks at room temperature. (Hygiena n.d.c.) The effect of cold AquaSnap test devices and cold samples were tested by using devices and samples directly from the refrigerator (+5 °C).

Measurement delay test

As discussed in chapter 5.2.2, it is important to measure the test device as soon as possible after the reaction activation. The effect of time delay between the reaction activation and

the measurement was examined by reading the same test device multiple times after different delays. The first reading (0 seconds) was made immediately after test activation and normal 5 seconds of shaking. The following readings were made in 30 second intervals. The samples used were three water samples intentionally contaminated with bacteria.

Sample stability in AquaSnap

The sample stability in AquaSnap test device means the time the sample can be kept in the AquaSnap test device before activating the reaction and measuring the device. Hygiena recommends activating and measuring the test devices no longer than 30 minutes after sample collection to avoid reductions in RLU results (Hygiena n.d.c.).

The effect of the delay between collecting the sample with the AquaSnap device and measurement was studied by collecting the same sample with five different AquaSnap devices at the same time. Each device was then activated after a different delay. The devices were measured immediately after activation.

AquaSnap shelf life at room temperature

In many situations, the AquaSnap devices are needed in the field where refrigerators are not necessarily available. Therefore, it is important that the test devices can be stored at room temperature at least for a while.

According to Hygiena, the shelf life for AquaSnap devices in room temperature is 4 weeks if the devices are kept out of light (Hygiena n.d.c.). The shelf life of AquaSnap devices in room temperature was examined by measuring the same sample at the same time with test devices that had been stored in room temperature for 2 days, 2 weeks and 4 weeks.

Filtration test

A filtration method was also tested to assess the amount of free ATP in the samples and to remove the effect of pH or any cleaning agent or other residues possibly present in the samples. Disposable bottle filters with 0.45 µm SFCA (surfactant-free cellulose acetate) membranes were chosen for the test so the bacteria could be retained on the membrane efficiently. A smaller pore size was not chosen so that the sample could pass through the

membrane with as little vacuum pressure as possible and the stress caused to the bacteria would remain minimal.

The filtered sample volume was 50 ml. After the filtration, the bacteria were let to recover on the membranes for 30 minutes. The bottle filter system was covered with a lid and placed in a laminar flow cabinet to protect it from contamination. After 30 minutes the membrane was swiped thoroughly with Hygiena SuperSnap swabs to collect all the microorganisms retained by the membrane. The test swabs were activated and measured without delay.

The amount of free ATP was measured by pouring the filtrate into a sterile cup and using an AquaSnap device to analyze it. In addition, the filtrate was also analyzed with the TVC method to ensure that no microorganisms passed through the membrane. The filtration test was also performed with a wastewater sample to see if the method worked as planned.

Water samples with surface test devices

The analysis of water samples was also tested with surface test devices (SuperSnap). As discussed in chapter 5.2.3, it is not recommended to take water samples with the cotton tip of the SuperSnap device, because the sampling volume may vary a lot leading to variable results.

SuperSnap devices were tested with two water samples both by taking the sample with the cotton tip of the device and by pipetting 100 µl of the sample directly into the tube. The samples were also analyzed with AquaSnap devices to see if there is any difference between the results given by SuperSnap and AquaSnap devices.

Test with product samples

The ATP method was also tested with enzyme product samples to see if it would be possible to use the method for a quick estimate of the microbial load of the products or process samples. Sometimes this kind of quick enumeration is enough instead of the TVC result that takes 2–3 days.

Most of the product and process samples are dark brown and some of them are very turbid. They contain multiple different chemicals, such as formulation chemicals that may cause interference in addition to the color and turbidity. Additionally, it is possible that the enzymes interfere with the method.

6 RESULTS AND INTERPRETATION

In this chapter, the results obtained in this thesis are presented and interpreted. The exact information about the samples were left out due to confidentiality reasons and therefore the samples are sorted only with sequential numbering. It should be noted that each table has its own sample numbers starting from 1 and the numbers do not correlate between tables, if not otherwise mentioned.

6.1 TVC versus ATP results

In this chapter, the ATP and TVC results for the CIP rinse water, cooling water and additional water samples are presented and the correlation between the ATP and TVC results is discussed. It should be noted that the TVC results are expressed as CFU/ml, but the ATP results do not include 'per volume' in the RLU unit. Therefore, the RLU result is always essentially per sampled volume, which is 100 µl in the case of AquaSnap devices. For clarity however, the results are compared using the units CFU/ml and RLU as it is given by the instrument.

CIP rinse water samples

Most of the samples analyzed side by side with TVC and ATP methods were CIP rinse water samples (appendix 1). Based on both TVC and ATP results, the microbial loads of most of the CIP rinse waters were very low. Only nine of the samples gave a TVC result above <1 CFU/ml and only five of them were higher than 1 CFU/ml. These five samples with the highest microbial load and their results are presented in table 4. The two methods aligned very well with low microbial loads, both giving consistently low results for the same samples. Therefore, it could be rather safely concluded that if the sample contains very little or no microorganisms, the ATP method works as intended. It also seems that there is no free ATP in these samples that would affect the results, but this conclusion should be confirmed with samples containing a higher microbial load.

TABLE 4. The results of the five CIP rinse water samples with the highest microbial load

sample	ATP (RLU)	mean ATP (RLU)	RSD (ATP) (%)	TVC (CFU/ml)	TVC/ATP ratio
11	4	4	-	31	8
34	28 41	35	27	4	0
48	4	4	-	31	8
49	586 572 614 626	600	4	6025	10
50	148 146 145	146	1	1214	8

The methods also align well with higher results. With four of the samples, the TVC result (CFU/ml) is approximately 10 times (8–10) the ATP result. Sample 34 is an exception, the ATP result being 35 RLU and the TVC result 4 CFU/ml. This may be due to multiple different factors, such as the presence of nonculturable microorganisms or free ATP.

Cooling water samples

Nine cooling water samples were analyzed for this thesis. The results are presented in table 5 below. The results for the cooling water samples show the same relation between the TVC and ATP results as the CIP rinse water samples: the TVC result is roughly 10 times (8–17) the ATP result. The pH values were all between 7 and 8.

TABLE 5. Cooling water sample results

sample	pH	ATP (RLU)	ATP (RLU)	mean ATP (RLU)	RSD (ATP) (%)	TVC (CFU/ml)	TVC/ATP ratio
1	7.78	20	21	21	3	245	12
2	7.66	25	24	25	3	310	12
3	7.56	45	-	-	-	420	9
4	7.61	51	-	-	-	587	12
5	7.54	49	51	50	3	458	9
6	7.68	51	55	53	5	670	13
7	7.52	28	24	26	11	200	8
8	7.85	99	97	98	1	1150	12
9	7.68	41	45	43	7	715	17

Additional water samples

Because of the relatively low microbial content of CIP rinse water and cooling water samples, four additional water samples with intentionally higher microbial levels were also prepared for analysis. The results of these samples are presented in table 6.

TABLE 6. The results of additional water samples collected from less regularly cleaned parts of the plant

sample	pH	ATP (RLU)	ATP (RLU)	mean ATP (RLU)	RSD (ATP) (%)	TVC (CFU/ml)	TVC/ATP ratio
1	7.78	380	390	385	2	4700	12
2	7.66	786	791	789	0	8012	10
3	7.61	1009	1026	1018	1	11450	11
4	7.54	1294	1289	1292	0	14055	11

The ratio between TVC and ATP results is roughly 10 (10–12) as it was with CIP rinse water and cooling water samples. This suggests that the good correlation between TVC and ATP results continues from the lower microbial levels to higher levels as well. It should, however, be noted that these water samples do not fully represent CIP rinse water samples in sample composition and the type of microorganisms present. Unfortunately, CIP rinse water samples with higher microbial load were not available during this thesis. Moreover, only two samples exceeded 1000 RLU and therefore no clear conclusions about the correlation in higher microbial levels should be made according to these results.

Correlation between TVC and ATP results

In figure 8, the results for all samples that were analyzed side by side with TVC and ATP methods are presented. This includes the CIP rinse water, cooling water and additional water samples presented above as well as some samples analyzed during the additional tests. The lowest results (below 3 RLU) are removed as well as one outlier.

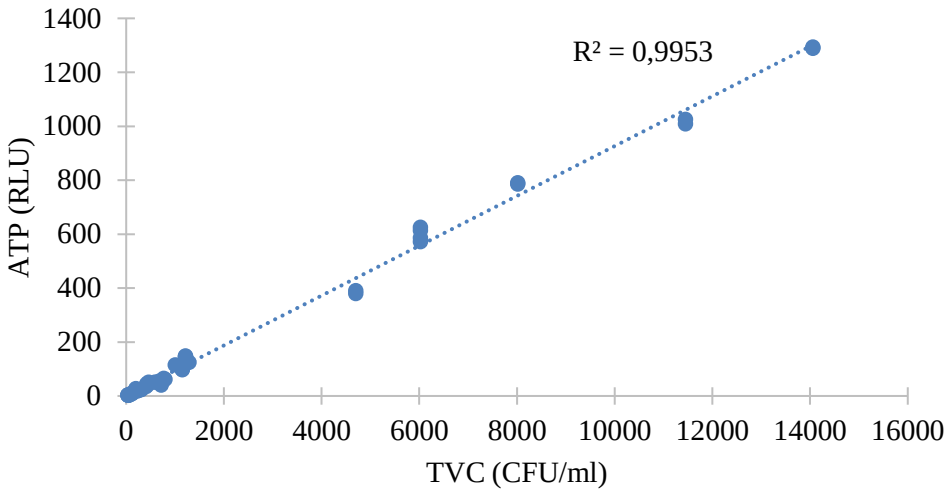

FIGURE 8. The ATP result as a function of the TVC result (n = 47)

As can be seen from the graph, the TVC and ATP results correlate very well. A majority of the results are below 200 RLU and 2000 CFU/ml and if only these results are plotted in the graph, the correlation is somewhat lower, the R^2 being 0.92. However, these correlations are very good compared to most of the correlations found in literature. As discussed in chapter 4.6, the correlation between TVC and ATP results for different water samples containing different bacterial species ranged between 0.004 and 0.84. The high correlations obtained in this thesis are probably due to rather constant microbial species present in the CIP rinse water and cooling water samples as well as stable sample and environmental conditions. When all of the 47 results presented in figure 8 are considered, the average ratio between the ATP and TVC results is 11 and can be rounded to 10 to get an estimate of the microbial load that is accurate enough for the application.

6.2 ATP content of microorganisms

Hygiena (2013b) states that with the combination of EnSURE luminometer and AquaSnap Total devices 0.1 femtomoles (0.1×10^{-15} moles) of ATP equals to 1 RLU. Unfortunately, it was not possible to confirm this relation with an ATP standard during this thesis. However, the relation given by Hygiena was used to calculate the relationship between ATP and TVC results.

According to the results for CIP rinse water samples, 1 RLU corresponds to approximately 10 CFU/ml. With the relation for ATP concentration and RLU given by Hygiena, the correlation between TVC results and ATP concentration would be as follows: 1 CFU/ml equals to 1×10^{-17} moles of ATP, which is 5.07×10^{-15} grams of ATP. According to these calculations, one colony forming unit detected by TVC method would contain 5.07×10^{-15} grams of ATP. This is rather similar to ATP contents of cells reported in multiple other studies (chapter 4.2). However, it should be noted that the TVC method does not detect all microorganisms and does not count individual cells either (chapter 2.4.1). In addition, this result is based on multiple assumptions and should be used with care.

6.3 Additional tests

Linearity

The linearity test was performed with four ATP standard dilutions. The results are presented in table 7.

TABLE 7. Linearity test results for ATP standard

dilution	ATP (RLU)
10^{-2}	1010
10^{-3}	109
10^{-4}	11
10^{-5}	1

As mentioned in chapter 5.4, the measuring range of the EnSURE luminometer is from 0 to 9999 RLU and therefore only four ten-fold dilutions could be included in the standard dilution series. With these four points the results show a high degree of linearity, the correlation coefficient *r* calculated with Excel's regression tool being 0.9999. This result suggests that there are no issues with the linearity of the ATP measurement. However, a more comprehensive test series and a proper ATP standard with a known ATP concentration would be needed to make reliable conclusions.

Precision

The precision of the ATP method was examined as the repeatability of the method in two different parts: repeatability between measurements and repeatability between luminometers. The repeatability between measurements can be seen from the RSD values of multiple replicate results. The RSD values are presented in the result tables in appendix 1 and chapter 6.1. It should be noted that some of the results are so small that the RSD is high even though the difference between the results is only a few RLU units. All in all, the RSDs for replicate measurements are small, mostly below 5 %, meaning that the ATP method is highly repeatable.

In addition, the repeatability between luminometers was examined by analyzing 14 samples side by side with two separate ENSURE luminometers. These results are presented in table 8. Luminometer 1 was used for all the measurements included in this project and luminometer 2 was used only for this repeatability test.

TABLE 8. Repeatability between luminometers

sample	ATP (RLU)		RSD
	luminometer 1	luminometer 2	(%)
1	586	572	2
2	614	626	1
3	51	55	5
4	28	24	11
5	99	97	1
6	148	146	1
7	41	45	7
8	0	0	-
9	1	2	47
10	380	390	2
11	786	791	0
12	0	0	-
13	0	0	-
14	20	20	0

The results show that there is no significant difference between the results provided by two different EnSURE luminometers. The RSD values are mostly <5 % as they were with the repeatability between measurements using the same luminometer. When the results

are >100 RLU, the RSDs are below 2 %. There is only small irregular variation that is probably due to other factors than the luminometer itself.

As discussed in chapter 5.4, the effect of the luminometer calibration was also tested at the same time with the repeatability between luminometers. Luminometer 1 was calibrated over a year ago and luminometer 2 one week ago. The results confirm that the calibration does not affect the results, at least if the calibration was performed approximately one year ago.

Background noise

The background noise level was tested by analyzing multiple AquaSnap devices without sampling. The measurement was done during 5 different days and every time the result was 0 RLU.

The results suggest that there is no background noise caused by the instrument or the equipment in existing conditions. This conclusion is in line with the information given by Hygiena (n.d.a.). Changes in the environmental conditions may cause a small level of background noise. It should also be noted that some samples may cause chemical interference resulting in increased background noise. This could be tested with an ATP standard solution. However, because the system itself does not cause background noise, the possible noise caused by the sample should remain rather minimal.

Test with pure bacteria

The TVC and ATP results for pure *Bacillus subtilis* bacteria are presented in table 9. The bacterial suspension was diluted with dilution water as well as CIP rinse water sample with the result of <1 CFU/ml and 0 RLU. Ten-fold dilutions were used from 10^{-2} to 10^{-7} in both dilution series. As discussed in chapter 5.4, the measuring range of the EnSURE luminometer is rather limited. In addition, the TVC method also has its limitations concerning the number of colonies that can be counted from one plate (chapter 5.3). Therefore, additional two-fold dilutions (marked with '-> 1/2' in the table 9) were made of some of the ten-fold dilutions to get more data points.

TABLE 9. ATP test with pure bacteria

dilution	CIP rinse water			dilution water		
	ATP (RLU)	TVC (CFU/ml)	TVC/ATP ratio	ATP (RLU)	TVC (CFU/ml)	TVC/ATP ratio
10^{-2}	5566	-	-	5512	-	-
10^{-3}	589	-	-	586	-	-
-> 1/2	236	-	-	245	-	-
-> 1/2	125	1285	10	115	1005	9
10^{-4}	62	796	13	65	768	12
-> 1/2	39	426	11	35	402	11
-> 1/2	21	224	11	19	228	12
-> 1/2	11	119	11	10	128	13
10^{-5}	6	84	14	6	88	15
-> 1/2	3	38	13	3	46	15
10^{-6}	1	8	-	1	7	-
10^{-7}	0	1	-	0	2	-

Because of the limitations of the result range of the methods, only the results between the dashed lines in the table 9 were used for the comparison of the results. From these results, it can be seen that the TVC result is roughly 10 times (11–15) the ATP result, as with previous results of this thesis. The results are also plotted in figure 9. There is a good correlation between the TVC and ATP results, the R^2 being 0.9883 and 0.9992.

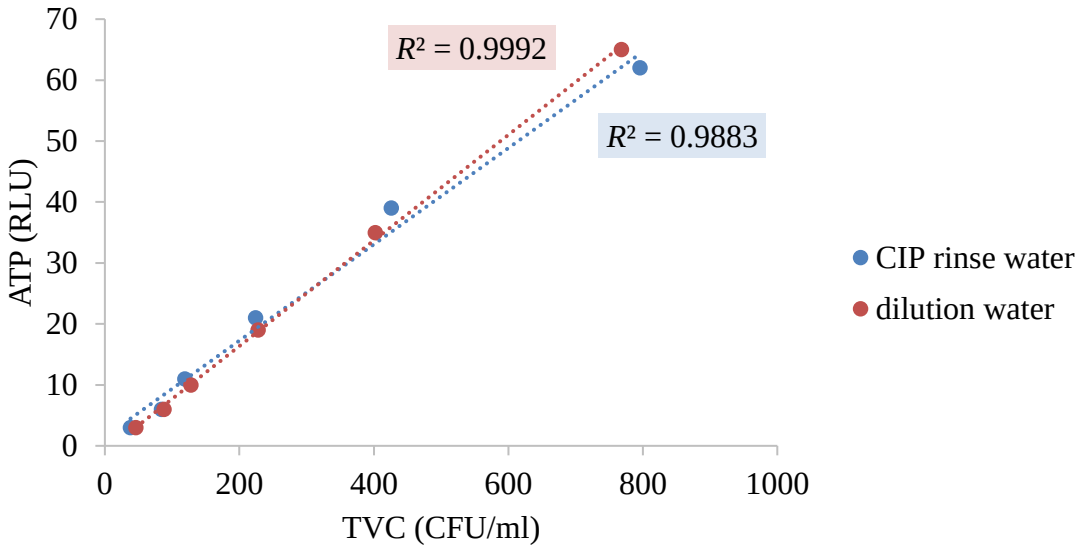

FIGURE 9. The ATP result as a function of the TVC result for *Bacillus subtilis*

The pH of the dilution water was 7.00 and the samples diluted with it should not contain any other interfering agents either. The pH of the CIP rinse water sample used for the dilutions was 8.56. However, the results of both dilution series are very similar, suggesting that the pH or any other factors, such as cleaning chemical or product residues possibly present in the CIP rinse water samples do not have a significant interference to the measurement.

In addition to the correlation between the methods, this dilution series can be used to examine the linearity of the ATP method with bacteria. The ATP results for dilutions from 10^{-2} to the two-fold dilution of 10^{-5} were used. The linearity of the results is very good, the correlation coefficient *r* being above 0.99 in both dilution series. The correlation coefficient was calculated with Excel's regression tool.

Bacillus subtilis is not the most ideal microorganism to use for the comparison between TVC and ATP results. *Bacillus subtilis* forms spores and as discussed in chapter 4.2.3, the ATP content of spores is generally lower than that of vegetative bacterial cells (Fajardo-Cavazos et al. 2008; Shama & Malik 2013). However, as sporulation is tightly related to starvation, the handling times were kept as short as possible and it was assumed that not much sporulation had happened before the analysis.

pH test

The effect of the sample pH was tested by adjusting the sample pH with two acids and a base. The results of the test are presented in table 10.

TABLE 10. Results of the pH test

hydrochloric acid		**acetic acid**		**sodium hydroxide**	
ATP (RLU)	**pH**	**ATP (RLU)**	**pH**	**ATP (RLU)**	**pH**
69	8.35	69	8.35	69	8.35
74	7.45	71	7.65	70	8.99
73	6.52	72	6.66	65	9.65
66	5.43	58	5.54	54	10.25
54	4.29	51	4.35	39	11.02
47	3.69	45	3.75	-	-

When the same results are plotted (figure 10), it can be easily seen that the results are in line with the optimal pH (approximately 7.8) for the ATP assay found in literature (chapter 4.4.1; Hawronskyj & Holah 1997, 81; Steghens et al. 1998).

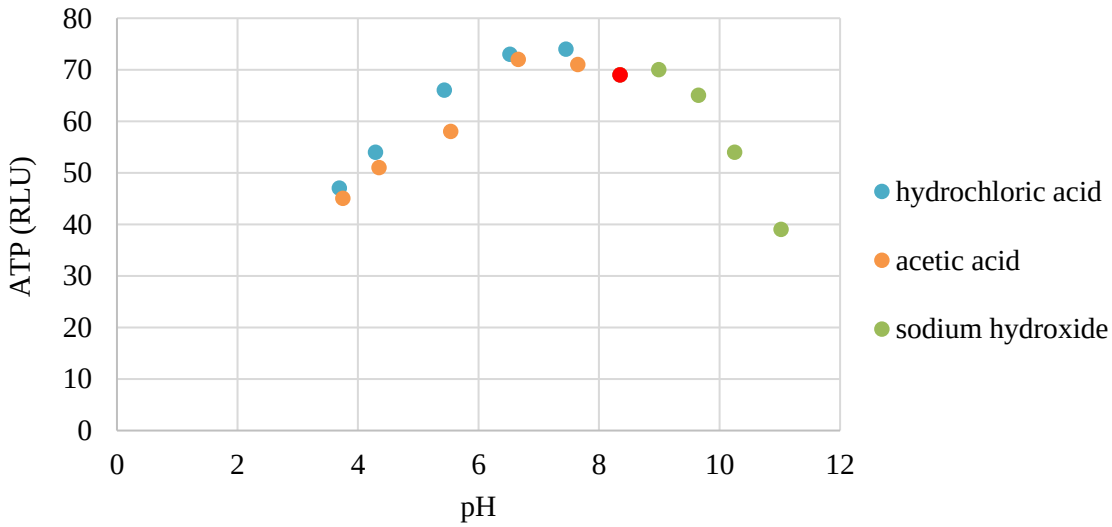

FIGURE 10. The ATP result as a function of pH

According to these results, the reaction works best approximately between pH 6 and 10. When the pH value decreases or increases more, the RLU results drop. Most of the CIP rinse water samples are between pH 8 and 9 and the method seems to be working well inside this range. As discussed in chapter 5.1, the pH of CIP rinse water samples should not often rise above 9. During this thesis, only 1 CIP rinse water sample with a pH value slightly above 9 was encountered.

Of course, these results are just directional and a more comprehensive test series with greater RLU values should be prepared to determine a more exact optimal pH range for the assay. However, it seems that it is rather safe to conclude that the pH values of the samples are usually in the optimal range and the pH should not therefore cause any problems.

Temperature test

The results for the test in which cold AquaSnap test devices and cold samples were used are presented in table 11. The test devices and samples were used straight from the refrigerator meaning that the temperature was approximately +5 °C.

TABLE 11. Results of the temperature test

sample	both +20 °C ATP (RLU)	sample +5 °C ATP (RLU)	AquaSnap +5 °C ATP (RLU)	both +5 °C ATP (RLU)
1	51	28	20	11
2	586	345	299	204
3	99	59	44	19

The results show that the temperature of the test devices as well as the temperature of the sample both have a significant effect on the results. In both cases, the results are lower when the devices and the samples are not in room temperature. Cold test devices seem to have a greater effect than cold samples. These results support the instructions given by Hygiena (Hygiena n.d.c.) to always allow the test devices and samples to come to room temperature before measurement.

Measurement delay test

The effect of the measurement delay was tested by activating the test devices and measuring them after different delays in 30 second intervals. The point 0 includes 5 seconds of shaking. The results are presented in table 12.

TABLE 12. Results of the measurement delay test

delay (s)	sample 1 ATP (RLU)	sample 2 ATP (RLU)	sample 3 ATP (RLU)
0	3809	3766	6300
30	2003	2395	4250
60	1177	1347	2750
90	686	658	2111
120	332	472	1258
150	185	235	811
180	94	82	411

The results of the time delay test are also presented as a graph in figure 11. The results show that the intensity of the light decreases rapidly after reaction activation, as discussed in chapter 5.2.2. (Hygiena n.d.c.).

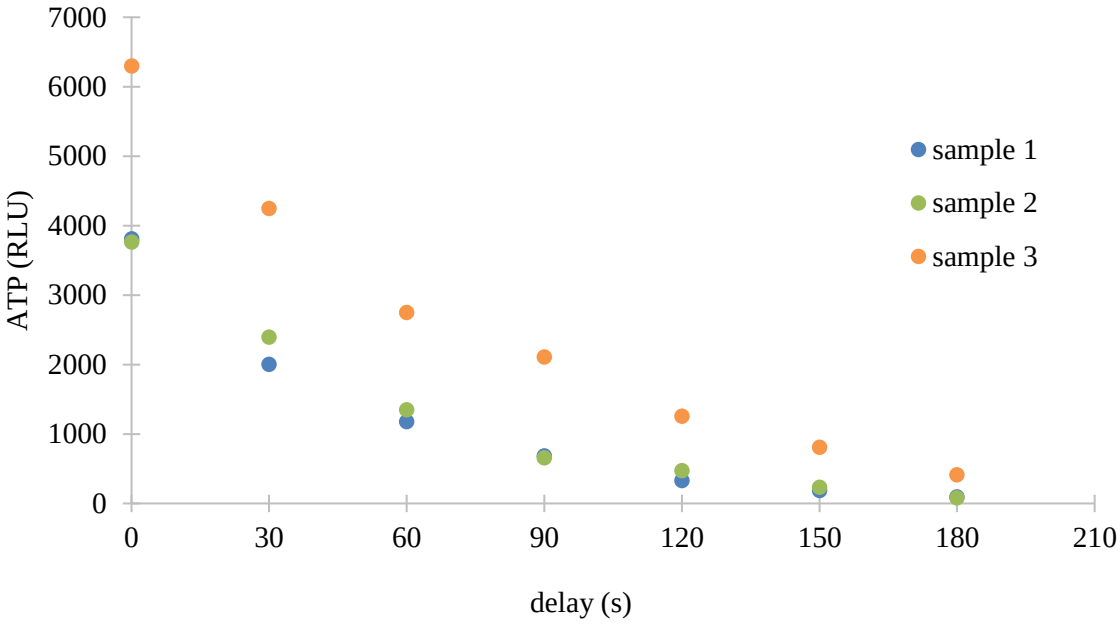

FIGURE 11. The ATP result as a function of measurement delay

The decay rate of the light depends on the system, but according to these results, it is undeniable that with this equipment any delay between test activation and actual measurement can cause lower results or even false negative or false ‘pass’ results. Therefore, the test device should be read as soon as possible after the activation and 5 seconds of shaking.

Sample stability in AquaSnap

Sample stability in AquaSnap devices was tested by taking a sample into multiple separate devices and analyzing them after different delays. The results are presented in table 13.

TABLE 13. Sample stability in AquaSnap

sample	delay (min)	ATP (RLU)	%
1	0	380	100
	30	371	98
	60	344	91
	90	256	67
	120	191	50
2	0	786	100
	30	769	98
	60	705	90
	120	414	53
	180	332	42
	240	198	25

The results show that 30 minutes after the sample collection, the ATP result is still 98 % of the result obtained directly after sample collection. After one hour, the result has dropped to around 90 % and after two hours to around 50 %. Therefore, it is important to measure the sample with the AquaSnap devices preferably within 30 minutes of sample collection. This conclusion is in line with the instructions given by Hygiena (Hygiena n.d.c.).

AquaSnap shelf life at room temperature

The shelf life of AquaSnap test devices at room temperature was tested. The results are presented in table 14.

TABLE 14. Shelf life results of AquaSnap devices in room temperature

AquaSnap stored in +20 °C	ATP (RLU)
10 minutes	99
2 days	105
2 weeks	102
4 weeks	98

The results show that the devices can be stored in room temperature for 4 weeks without it affecting the results. This is in line with Hygiena's instructions (Hygiena n.d.c.).

Filtration test

The filtration test was performed to see if the method could be coupled with a filtration step to minimize the effect of any interfering factors present in the sample. The amount of free ATP in the samples was also examined with the filtration test by measuring the filtrate with AquaSnap devices. The results of the filtration tests are presented in table 15. AquaSnap devices are marked with 'AS' and SuperSnap devices with 'SS'. In the ATP results, 'max' means that the result given by the luminometer was 9999 RLU exceeding the measuring range.

TABLE 15. Filtration test results

sample	before filtration			filter		filtrate	
	TVC (CFU/ml)	AS (RLU) 100 µl	SS (RLU)	SS (RLU) 50 ml	SS (RLU) 100 µl	AS (RLU) 100 µl	TVC (CFU/ml)
1	587	51	5	2549	5	0	0
2	6025	600	41	6889	14	0	0
3	$5x10^6$	max	-	max	-	8476	2

First of all, the test with the wastewater sample (sample 3) shows that the filtration is working correctly: the filtrate gives a TVC result of 2 CFU/ml meaning that almost all of the microorganisms were retained by the filter. However, the filtrate gives a high ATP result of 8476 RLU meaning that the sample probably included a significant amount of free ATP. According to these results, the microorganisms are withheld by the membrane, but free ATP is passed through.

The results with cooling water sample (sample 1) and CIP rinse water sample (sample 2) in turn show that these samples contained only a little or no free ATP. This is necessarily not the case with all CIP rinse water and cooling water samples, but it can be assumed that free ATP would not cause significant interference in this kind of samples.

Even though the filtration part seems to work well, the problem is the swabbing with the SuperSnap devices. It is difficult to gather all the microorganisms with the swab. This can be seen as very low results when the membrane is swabbed with the SuperSnap devices and the result is expressed as RLU/100 µl to be comparable with the AquaSnap result.

When the same membrane (sample 2) was swabbed again with a new SuperSnap device, the result was 2588 RLU and for the third device 1812 RLU. This shows that a significant portion of ATP is left behind on the membrane even after multiple swabs.

The results show that it is not possible to gather all the microorganisms from the membrane with one swab. The filter material is porous, and the surface is uneven, which are probably the main reasons for swabbing problems. Some of the microorganisms may pass partly through the membrane remaining inside the membrane depth and are therefore not detected in filter surface ATP test or filtrate ATP test. Therefore, this is not a reliable method to measure the ATP content of the samples.

The microorganisms are also affected by the stress caused by the filtration, which probably affects the ATP content of the cells by breaking down the cell walls and releasing microbial ATP changing it into free ATP. Nevertheless, this would increase the amount of free ATP in the filtrate. In the cases where the result for ATP in the filtrate is 0 RLU, it is rather safe to conclude that not a significant portion of the microbial ATP has become free ATP during filtration.

The filtration method could be further developed if needed to make it applicable. However, if the cleaning verification is transferred from the laboratory to production personnel, it is not possible to use a filtration method. Therefore, it is not important to test and develop the filtration method any further for this currently planned application of the ATP method. If the method however is later used for any other application in the laboratory, the development of the filtration method could be considered.

Water samples with surface test devices

Two water samples were also analyzed with SuperSnap test devices to see if the surface devices could be used for water samples as well. The results are presented in table 16.

TABLE 16. Water sample results with surface test devices

sample	AS	SS dipped	SS dipped	SS pipetted
1	36	15	3	37
2	80	11	35	75

The results show that SuperSnap devices cannot be used for water samples by dipping the cotton tip into the sample because the cotton tip is not able to take a precise amount of sample. Therefore, the results are varying and too low. However, the SuperSnap devices seem to work well if 100 µl of the sample is pipetted directly into the tube of the device. The results given by SuperSnap devices do not differ significantly from the results given by AquaSnap devices.

Test with product samples

The results obtained with the ATP method for the product samples are presented in table 17. Exact information about the samples were left out due to confidentiality reasons, but the samples included two final products and one concentrate sample. The samples were rather dark brown and slightly turbid.

TABLE 17. Test with product samples

sample	TVC (CFU/ml)	ATP (RLU)
1	$1.8x10^5$	59
2	980	25
3	498	0

The results show, as expected, that the method cannot be used for the product samples. The sample color and the turbidity seem to cause so much interference that the ATP results remain very low compared to the TVC results. It is also possible that other factors, such as chemicals or enzymes, present in the sample cause interference. Further testing would be needed to find the cause and to be able to see if the ATP method could be used with product samples with some changes or additions to the method.

6.4 Comparison to earlier results with another luminometer

Another luminometer (called ‘luminometer B’) and water test devices from a different supplier were tested prior to this thesis in 2019. The test devices functioned in a rather similar way and the measurements were performed similarly than with Hygiena EnSURE

and AquaSnap. There were, however, some differences in the reaction activation mechanism.

The exact components of the test device reagents or the precise details of the luminometers are not known and therefore the systems cannot be directly compared to each other. It is known, however, that the luminometer B has a photomultiplier as the light sensor whereas EnSURE has a photodiode. Each system also has different sensitivities and their own RLU scale which means that the RLU values can vary significantly from one system to another.

The two luminometers and the test devices cannot be compared also because the samples that were tested were different for both systems. Therefore, it was not possible to find the reasons for the result variation between the luminometers. Nevertheless, the systems can be compared according to for example their background noise and repeatability.

The results obtained with luminometer B are not presented in this thesis, but a short summary of the results is presented here. When TVC results were <1 CFU/ml, the ATP results given by luminometer B ranged from 5 to 245 RLU. The test devices without sampling gave background noises ranging from 5 to 20 RLU. There was no clear correlation between TVC and ATP results: the ratio between the TVC and ATP results ranged between approximately 1 and 100. In most cases, the TVC result was higher than the ATP result but there were multiple exceptions as well. Replicate samples also gave variable results with high RSDs. All in all, the results did not seem logical and some factor was very probably interfering with the measurement.

Possible reasons for very variable results obtained from luminometer B are numerous. One of the most probable reasons is sample interference caused by pH, cleaning chemical residues or product residues. In addition, the differences in the reaction activation mechanism and light sensors probably cause variance in the results between the luminometers. Hygiena has also performed well in the comparisons of different luminometer systems. Hygiena systems have shown very good linearity, repeatability, sensitivity, as well as high accuracy. (Hygiena n.d.a., Hygiena n.d.d., Omidbakhsh et al. 2014; Kupski et al.

2010.) The good performance of Hygiena luminometer compared to luminometer B is one reason that probably affects the results.

6.5 Analysis time and costs

One goal of this thesis was to calculate if a possible method change from TVC to ATP method for CIP rinse water and cooling water samples would affect the analysis time and costs. The times and costs used in the calculations are not exact but estimated with the available information.

As mentioned earlier in this thesis, the time from sampling to results with the TVC method is 2–3 days (48–72 hours). However, most of this time is used for the incubation. The active time needed for the method is around 30 minutes per CIP rinse water or cooling water sample. This includes melting of the agar, sample dilution and plating, as well as colony counting and calculations. The time estimate does not include for example agar preparation or dishes. In addition, the results have to be transferred into computer manually.

The costs of the TVC method are approximately 0.60 € per CIP rinse water sample and between 0.80 € and 1.00 € per cooling water sample depending on the dilution and rounded to closest 5 cents. The cost estimates include the equipment needed such as petri dishes, pipettes and agar but does not include water or electricity needed for agar preparation, dishes, autoclave, or incubator. Therefore, the total time and cost for the TVC method are higher than the estimates presented here.

The time needed for the ATP method from sampling to results is approximately 3 minutes including switching on the luminometer, sampling with the test device, reaction activation and measuring the device. The luminometer gives the result after 15 seconds. The annual costs of the ATP method consist practically only of the test devices that cost 3.39 € each. In addition, possible annual calibrations cost around 100 € per year, but they are not obligatory for the function of the luminometer. The luminometer itself costs approximately 2500 € but it is only a one-time cost.

The active analysis times and costs per one CIP rinse water sample are presented in table 18. In addition, the times and costs per 100, 500 and 1000 samples are calculated.

TABLE 18. Active analysis time and costs of CIP rinse water samples

number of samples	TVC (CIP rinse water)		ATP	
	time (h)	costs (€)	time (h)	costs (€)
1	0.5	0.6	0.05	3.39
100	50	60	5	339
500	250	300	25	1695
1000	500	600	50	3390

Based on the calculations, the method change from TVC to ATP would save 225 hours of work every year if 500 CIP rinse water samples are analyzed annually. If the number of samples is 1000, 450 hours of work would be saved every year. The cooling water samples are analyzed weekly, so the annual saving of active work would be more than 20 hours there as well.

Even though the annual saving in the analysis time would be significant with the ATP method, the consumables for the ATP method are more expensive than for the TVC method. However, as discussed above, the cost estimate for the TVC method does not include for example electricity or water needed for the analysis. In addition, savings in analysis times can be considered as savings in costs as well since considerably less work is needed to obtain the result. All in all, the method change from TVC to ATP method would save both active analysis times and costs.

6.6 Environmental impact, safety, and ergonomics

In addition to changes in the analysis time and costs, it is important to consider the aspects of environmental impact, safety and ergonomics of the ATP method. The test devices used in the ATP method are made of plastic and are fully recyclable. They contain no harmful chemicals. Only one device is needed for one sample whereas the TVC method

requires multiple petri dishes and pipette tips per one sample. The ATP method also requires only an insignificant amount of water and electricity compared to the TVC method and therefore the environmental impact is lower than the impact of the TVC method.

The ATP method improves the working safety compared to the TVC method because it removes for example the need to handle hot agar or autoclave. The ATP method also offers improvements to working ergonomics because the measurement is quick and can be performed while standing. The TVC method in turn includes multiple steps which require sitting or where the working position is not otherwise ideal.

6.7 Measurement error

The possible measurement error of TVC and ATP methods were considered to see if the ATP method would offer more reliable results than the TVC method. The measurement error can be divided into consistent systematic error and unpredictable random error. The error can be caused by multiple different factors, such as instruments, environment, sample conditions, and human error.

The TVC method itself is rather prone to error because the method is based on artificial cultivation of microorganisms and unsuitable growth conditions, such as temperature and nutrients, can affect the results significantly. The equipment, such as pipettes, thermometers or incubation cabinets can also cause error. In addition, the TVC method contains multiple steps where human error can affect the results considerably. Most important of these steps are preparation of agar and diluent, pipetting, diluting, counting the colonies, calculating, and inserting the results into computer.

The ATP method can be considered to have less sources for error. The equipment, including test devices and the luminometer, can cause errors when not functioning, stored, or used properly. The sample pH or cleaning agent interference can also cause error. In addition, the human-based error, such as measurement delay, wrong sample temperature or test device temperature, or neglect of instructions, can cause significant error. However, there is no need for counting, calculation or inserting the results, because the results given

by the luminometer are stored in the memory of the luminometer and can also be easily transferred into the computer. The fewer method stages and automatization decrease the possibility of human error occurring.

7 CONCLUSIONS

The currently used traditional cultural method for the CIP cleaning verification of product packing lines is slow as well as labor- and time-consuming. In order to minimize the product release time and workload and still ensure product quality and safety, a quicker method is required for the application.

In this thesis, a pen-based ATP method from Hygiena was tested side by side with the traditional TVC method for CIP rinse water and plant cooling water samples. The results of these two methods aligned very well with all water samples that were tested, the TVC results being in average 10 times the ATP results. It should, however, be noted that the correlation obtained between the TVC and ATP results is only valid for the sample types analyzed in this thesis. The ATP method should be carefully tested prior to using it with any other sample type or application.

The ATP method gives the result in approximately 3 minutes including all steps whereas the TVC method takes 2–3 days. In addition to being remarkably quicker than the TVC method, the ATP method can also be considered as a more reliable method. The ATP method has a better repeatability and detects all viable microorganisms regardless of their culturability. The ATP method also contains less sources for errors due to for example fewer measurement steps and high degree of automatization.

Even though the ATP method seems to perform well in stable conditions, it is known that the sample pH, temperature and cleaning agent or product residues may affect the results. Therefore, different sample conditions were also tested during the experimental part. According to the results it can be concluded that the optimal sample pH for the ATP measurement is between 6 and 10 and the temperature of the sample as well as the test devices should be close to +20 °C. In addition, it was found out that the method does not work for product samples that are typically dark and somewhat turbid. Therefore, until the issue has been examined further, the method can only be used for samples that are colorless and clear.

A filtration method was also tested to minimize the effect of sample pH and cleaning agent residues, but it was proved unsuitable for the application. The filtration tests however confirmed that there was no significant amount of free ATP in the CIP rinse water or cooling water samples. In addition, some operational details, such as measurement delay, sample stability in AquaSnap test device and AquaSnap shelf life at room temperature, were examined and tested.

Lastly, the effects of the possible method change were discussed. The ATP method would decrease the workload and shorten analysis times significantly. The consumables needed for the TVC analysis are cheaper than the ATP test devices, but the TVC method also requires electricity and water that are not taken into account in the calculations. The lesser requirements in terms of consumables, water and electricity also make the environmental impact of the ATP method considerably lower than that of the TVC method. Additionally, the ATP method would improve work safety and ergonomics. All in all, it can be concluded that the ATP method would be a more cost-effective, environmentally friendly, and safer method for the application.

No lower or upper limits for the ATP result were suggested in this thesis because more results for CIP rinse water samples with a higher microbial load are needed to reliably calculate the limits. Inadequate consideration of the limits can cause unnecessary repetitions of cleaning causing additional costs and delays. However, the worst consequences would be undetected contaminations of the product because of false ‘pass’ results.

According to the results obtained in this thesis, it can be rather safely concluded that the method is suitable for reliably assessing the cleaning result of the CIP cycle. In this application, it is not important to get the exact number of microorganisms present in the sample, but it is important to reliably get the result whether the cleaning was successful. The method seems to be suitable for reliably assessing the microbial load of cooling water samples as well. The currently used TVC method offers only a broad estimate and considering the non-culturability issues discussed earlier, the ATP method could be a more accurate method for all microorganism types.

To conclude, a simple and quick system that produces reliable and reproducible results is needed to assess the microbial load of the CIP rinse water samples from the product packing lines in order to release products as quickly as possible without compromising the product safety or quality. The ATP method by Hygiena tested in this thesis proves to be a promising method and would significantly decrease the analysis times and costs as well as offer improvements to work safety and ergonomics.

8 SUGGESTIONS FOR FUTURE RESEARCH

The next step in this project would be purchasing a couple of these luminometers and continue testing them side by side with the TVC method to obtain more data. One luminometer could be used by the production personnel and one in the laboratory to see if there are any differences between the results and to find out if the luminometer could be used reliably in the production environment. In addition, it would be important to gain results from multiple different operators to see if there are any differences between them.

An ATP standard could be purchased to test for example the linearity of the method and the interference caused by cleaning and formulation chemicals. In addition, the correlation between the ATP concentration and the RLU result could be further examined with the ATP standard and thereby also the amount of ATP in the microorganisms could be studied further.

Even though the SuperSnap devices can only be used for water samples by pipetting the sample directly into the device, they could be further applied for applications that require surface sampling. One possible application would be verifying if surfaces have been cleaned properly. Even though ATP tests do not detect viruses directly, SuperSnap devices could be used for evaluating the effectiveness of hygiene procedures, for example in the current COVID-19 situation. Hygiena also offers different types of other test devices compatible with the EnSURE luminometer that could be tested and applied for example for pathogen testing in the laboratory.

REFERENCES

Abushaban, A., Salinas-Rodriguez, S. G., Mangal, M. N., Mondal, S., Goueli, S. A., Knezev, A., Vrouwenvelder, J. S., Schippers, J. C. & Kennedy, M. D. 2019. ATP Measurement in Seawater Reverse Osmosis Systems: Eliminating Seawater Matrix Effects Using a Filtration-Based Method. Desalination, 453. 1–9.

Airth, R. L., Rhodes, W. C. & McElroy, W. D. 1958. The Function of Coenzyme A in Luminescence. Biochimica et Biophysica Acta, 27. 519–532.

Alfa, M. J., Fatima, I. & Olson, N. 2013. Validation of Adenosine Triphosphate to Audit Manual Cleaning of Flexible Endoscope Channels. American Journal of Infection Control, 41: 3. 245–248.

Alfa, M. J., Olson, N. & Murray, B.-L. 2015. Adenosine Tri-phosphate (ATP)-Based Cleaning Monitoring in Health Care: How Rapidly Does Environmental ATP Deteriorate? Journal of Hospital Infection, 90: 1. 59–65.

Amann, R. I., Ludwig, W. & Schleifer, K. H. 1995. Phylogenetic Identification and in Situ Detection of Individual Microbial Cells Without Cultivation. Microbiological Reviews, 59: 1. 143–169.

Amy, G. L., Salinas Rodriguez, S. G., Kennedy, M. D., Schippers, J. C., Rapenne, S., Remize, P., Barbe, C., de O. Manes, C., West, N. J., Lebaron, P., van der Kooij, D., Veenendaal, H., Schaule, G., Petrowski, K., Huber, S., Sim, L. N., Ye, Y.,Chen, V. & Fane, A. G. 2011. Water Quality Assessment Tools. In: Drioli, E., Criscuoli, A. & Macedonio, F. (ed.) Membrane-Based Desalination: An Integrated Approach (MEDINA), 1st edition. London: IWA Publishing. 3–32. ISBN 9781843393214.

Barbau-Piednoir, E., Mahillon, J., Pillyser, J., Coucke, W., Roosens, N. H. & Botteldoorn, N. 2014. Evaluation of Viability-qPCR Detection System on Viable and Dead Salmonella Serovar Enteritidis. Journal of Microbiological Methods, 103. 131–137.

Bottari, B., Santarelli, M. & Neviani, E. 2015. Determination of Microbial Load for Different Beverages and Foodstuff by Assessment of Intracellular ATP. Trends in Food Science & Technology, 44: 1. 36–48.

Campbell, M. K. & Farrell, S. O. 2011. Biochemistry, 7th edition. Pacific Grove: Brooks/Cole. ISBN 9780840068583.

Carrick, K., Barney, M., Navarro, A. & Ryder, D. 2001. The Comparison of Four Bioluminometers and Their Swab Kits for Instant Hygiene Monitoring and Detection of Microorganisms in the Brewery. Journal of the Institute of Brewing, 107: 1. 31–37.

Choi, J., Tanaka, K., Liang, Y., Cao, Y., Lee, S. Y. & Stacey, G. 2014. Extracellular ATP, a Danger Signal, is Recognized by DORN1 in Arabidopsis. Biochemical Journal, 463: 3. 429–437.

Conti, E., Franks, N. P. & Brick, P. 1996. Crystal Structure of Firefly Luciferase Throws Light on a Superfamily of Adenylate-Forming Enzymes. Structure, 4: 3. 287–298.

Costerton, J. W. 2007. The Biofilm Primer, 1st edition. Berlin, Heidelberg: Springer. ISBN 9783540680215.

Day, J. C., Tisi, L. C. & Bailey, M. J. 2004. Evolution of Beetle Bioluminescence: The Origin of Beetle Luciferin. Luminescence, 19: 1. 8–20.

Deininger, R. A. & Lee, J. 2001. Rapid Determination of Bacteria in Drinking Water Using an ATP Assay. Field Analytical Chemistry & Technology, 5: 4. 185–189.

del Mar Lleò, M., Benedetti, D., Tafi, M. C., Signoretto, C. & Canepari, P. 2007. Inhibition of the Resuscitation from the Viable but Non-Culturable State in Enterococcus faecalis. Environmental Microbiology Reports, 9: 9. 2313–2320.

DeLuca, M. & McElroy, W. D. 1978. Purification and Properties of Firefly Luciferase. Methods in Enzymology, 57. 3–15.

Donaher, A. 2016. What Is ATP and What Does It Do? [Online]. [Accessed 1 June 2020]. Available at https://www.luminultra.com/what-is-atp-and-what-does-it-do/

Dostálek, P. & Brányik, T. 2005. Prospects for Rapid Bioluminescent Detection Methods in the Food Industry - A Review. Czech Journal of Food Sciences, 23: 3. 85–92.

Encyclopaedia Britannica. 2020. Adenosine Triphosphate. [Online]. [Accessed 15 April 2020]. Available at https://www.britannica.com/science/adenosine-triphosphate

European Commission. 2016. Commission Notice on the Implementation of Food Safety Management Systems Covering Prerequisite Programs (PRPs) and Procedures Based on the HACCP Principles, Including theFacilitation/Flexibility of the Implementation in Certain Food Businesses. Official Journal of the European Union, 59: C278. 1–32.

Eydal, H. S. C. & Pedersen, K. 2007. Use of an ATP Assay to Determine Viable Microbial Biomass in Fennoscandian Shield Groundwater from Depths of 3–1000 m. Journal of Microbiological Methods, 70: 2. 363–373.

Fajardo-Cavazos, P., Schuerger, A. C. & Nicholson, W. L. 2008. Persistence of Biomarker ATP and ATP-generating Capability in Bacterial Cells and Spores Contaminating Spacecraft Materials Under Earth Conditions and in a Simulated Martian Environment. Applied and Environmental Microbiology, 74: 16. 5159–5167.

Feng, H., Guan, D., Bai, J., Sun, K. & Jia, L. 2015. Extracellular ATP: A Potential Regulator of Plant Cell Death. Molecular Plant Pathology, 16: 6. 633–639.

Fontes, R., Dukhovich, A. Sillero, A. & Sillero, M. A. 1997. Synthesis of Dehydroluciferin by Firefly Luciferase: Effect of Dehydroluciferin, Coenzyme A and Nucleoside Triphosphates on the Luminescent Reaction. Biochemical and Biophysical Research Communications, 237: 2. 445–450.

Fontes, R., Ortiz, B., de Diego, A., Sillero, A., Günther Sillero, M. A. 1998. Dehydroluciferyl-AMP Is the Main Intermediate in the Luciferin Dependent Synthesis of Ap4A Catalyzed by Firefly Luciferase. FEBS Letters, 438: 3. 190–194.

Fraga, H., Fernandes, D., Fontes, R., Esteves da Silva, J. C. G. 2005. Coenzyme A Affects Firefly Luciferase Luminescence Because it Acts as a Substrate and Not as an Allosteric Effector. The FEBS Journal, 272: 20. 5206–5216.

Fraga, H., Fernandes, D., Novotny, J., Fontes, R., Esteves da Silva, J. C. G. 2006. Firefly Luciferase Produces Hydrogen Peroxide as a Coproduct in Dehydroluciferyl Adenylate Formation. ChemBioChem, 7: 6. 929–935.

Fraga, H. 2008. Firefly luminescence: A Historical Perspective and Recent Developments. Photochemical & Photobiological Sciences, 7: 2. 146–158.

Fulford, M. R., Walker, J. T., Martin, M. V. & Marsh, P. D. 2004. Total Viable Counts, ATP, and Endotoxin Levels as Potential Markers of Microbial Contamination of Dental Unit Water Systems. British Dental Journal, 196: 3. 157–159.

Gengenbacher, M., Rao, S. P. S., Pethe, K. & Dick, T. 2010. Nutrient-Starved, Non-Replicating *Mycobacterium tuberculosis* Requires Respiration, ATP Synthase and Isocitrate Lyase for Maintenance of ATP Homeostasis and Viability. Microbiology, 156: 1. 81–87.

Hamilton, R. D. & Holm-Hansen, O. 1967. Adenosine Triphosphate Content of Marine Bacteria. Limnology and Oceanography, 12: 2. 319–342.

Hammes, F., Berney, M., Wang, Y., Vital, M., Köster, O. & Egli, T. 2008. Flow-Cytometric Total Bacterial Cell Counts as a Descriptive Microbiological Parameter for Drinking Water Treatment Processes. Water Research, 42: 1–2. 269–277.

Hammes, F., Goldschmidt, F., Vital, M., Wang, Y. & Egli, T. 2010. Measurement and Interpretation of Microbial Adenosine Triphosphate (ATP) in aquatic environments. Water Research, 44: 13. 3915–3923.

Hawronskyj, J. & Holah, J. 1997. ATP: A Universal Hygiene Monitor. Trends in Food Science & Technology, 8: 3. 79–84.

Holm-Hansen, O. 1970. ATP Levels in Algal Cells as Influenced by Environmental Conditions. Plant and Cell Physiology, 11: 5. 689–700.

Hygiena. 2013a. A Guide to ATP Hygiene Monitoring. [PDF]. [Accessed 8 April 2020]. Available at https://www.hygiena.com/food-and-beverage-products/ensure-food-and-beverage-processors.html#resources

Hygiena. 2013b. AquaSnap Total Sensitivity and Limit of Detection. [PDF]. [Accessed 17 April 2020]. Available at https://www.hygiena.com/food-and-beverage-products/aquasnap-food-and-beverage.html#resources

Hygiena. 2014. AquaSnap Product Brochure. [PDF]. [Accessed 2 March 2020]. Available at https://www.hygiena.com/other-products/aquasnap-other.html#resources

Hygiena. 2015. Using AquaSnap™ Total & Free for Estimating Bacteria Levels. [PDF]. [Accessed 15 March 2020]. Available at https://www.hygiena.com/aquasnap-difference-food-and-beverage.html

Hygiena. 2016. EnSURE Product Brochure. [PDF]. [Accessed 4 March 2020]. Available at https://www.hygiena.com/food-and-beverage-products/ensure-food-and-beverage-processors.html

Hygiena. n.d.a. A Comparative Study of Commercial ATP Hygiene Monitoring Systems. [PDF]. [Accessed 18 April 2020]. Available at https://www.fooddiagnostics.dk/seekings/uploads/Silliker_Report-A_Comparison_of_ATP_Systems.pdf

Hygiena. n.d.b. ATP system components. [Online]. [Accessed 27 April 2020]. Available at https://www.hygiena.com/other-products/sensor-technology-other.html

Hygiena. n.d.c. Frequently asked questions. [Online]. [Accessed 23 March 2020]. Available at https://www.hygiena.com/frequent-asked-questions-food-and-beverage.html

Hygiena. n.d.d. Hygiena ATP test device design and performance. [Online]. [Accessed 15 April 2020]. Available at https://www.hygiena.com/atp-test-design-performance-other.html

Hygiena. n.d.e. SuperSnap Product Brochure. [PDF]. [Accessed 2 March 2020]. Available at https://www.hygiena.com/supersnap-food-and-beverage.html

Jasson, V., Jacxsens, L., Luning, P., Rajkovic, A. & Uyttendaele, M. 2010. Alternative Microbial Methods: An Overview and Selection Criteria. Food Microbiology, 27: 6. 710–730.

Kahlke, T. & Umbers, K. D. L. 2016. Bioluminescence. Current Biology, 26: 8. R313–R314.

Kanematsu, H. & Barry, D. M. 2015. Introduction. In: Kanematsu, H. & Barry, D. M. (ed.) Biofilm and Materials Science, 1st edition. New York: Springer. 3–8. ISBN 9783319145648.

Karl, D. M. 1980. Cellular Nucleotide Measurements and Applications in Microbial Ecology. Microbiological Reviews, 44: 4. 739–796.

Kodaka, H., Fukuda, K., Mizuochi, S. & Horigome, K. 1996. Adenosine Triphosphate Content of Microorganisms Related with Food Spoilage. Japanese Journal of Food Microbiology, 13: 1. 29–34.

Kupski, B., Ceylan, E. & Stewart, C. 2010. Performance Evaluation of Various ATP Detecting Units. Silliker, Inc., Food Science Center Report. [PDF]. [Accessed 25 April 2020]. Available at https://www.keydiagnostics.com.au/images/PDF/new/systemSURE%20Plus/Rep_Hygiena_ATP%20Instrument%20Evaluation_RPN13922_rev012110.pdf

Lappalainen, J., Loikkanen, S., Havana, M., Karp, M., Sjöberg, A. M. & Wirtanen, G. 2000. Microbial Testing Methods for Detection of Residual Cleaning Agents and Disinfectants - Prevention of ATP Bioluminescence Measurement Errors in the Food Industry. Journal of Food Protection, 63: 2. 210–215.

Law, K. A., Derrick, J. P. & Higson, S. P. J. 2003. Initial Investigations into the Ultrasonic Lysis of Microbial Cells for the Release of Adenosine Triphosphate. Analytical Biochemistry, 317: 2. 266–267.

Lee, D. W. & Cho, Y. 2007. A Continuous Electrical Cell Lysis Device Using a Low Dc Voltage for a Cell Transport and Rupture. Sensors and Actuators B: Chemical, 124: 1. 84–89.

Lindell, I. C., Lundh, Å., Sjaunja, K. S. & Cederholm, M. 2018. Adenosine Triphosphate Bioluminescence for Hygiene Testing of Rubber Liners and Tubes on Dairy Farms. Journal of Dairy Science, 101: 3. 2438–2447.

LuminUltra. 2016. Technical Whitepaper: The Science of 2nd Generation ATP Monitoring. [PDF]. [Accessed 5 May 2020]. Available at https://www.luminultra.com/resource-types/white-papers-slide-decks/

Luo, J., Liu, X., Tian, Q., Yue, W., Zeng, J., Chen, G., Cai, X. 2009. Disposable Bioluminescence-Based Biosensor for Detection of Bacterial Count in Food. Analytical Biochemistry, 394: 1. 1–6.

Marques, S. M. & Esteves da Silva, J. C. G. 2009. Firefly Bioluminescence: A Mechanistic Approach of Luciferase Catalyzed Reactions. IUBMB Life, 61: 1. 6–17.

McElroy, W. D. 1947. The Energy Source for Bioluminescence in an Isolated System. Proceedings of the National Academy of Sciences of the United States of America, 33: 11. 342–345.

McElroy, W. D. & Seliger, H. H. 1963. The Chemistry of Light Emission. Advances in Enzymology and Related Areas of Molecular Biology, 25. 119–166.

McElroy, W. D. & Strehler, B. L. 1949. Factors Influencing the Response of the Bioluminescent Reaction to Adenosine Triphosphate. Archives of Biochemistry, 22: 3. 420–433.

Meighan, P. 2011. Looking at Further Developments in ATP Bioluminescence. International Food Hygiene, 22: 3. 7–9.

Moerman, F., Rizoulières, P. & Majoor, F. A. 2014. Cleaning in Place (CIP) in Food Processing. In: Lelieveld, H. L. M., Holah, J. & Napper, D. (ed.) Hygiene in Food Processing: Principles and Practice, 2nd edition. Cambridge: Woodhead Publishing Limited. 305–383. ISBN 9780857094292.

Morton, R. A., Hopkins, T. A. & Seliger, H. H. 1969. Spectroscopic Properties of Firefly Luciferin and Related Compounds; an Approach to Product Emission. Biochemistry, 8: 4. 1598–1607.

Nescerecka, A., Hammes, F. & Juhna, T. 2016a. A Pipeline for Developing and Testing Staining Protocols for Flow Cytometry, Demonstrated with SYBR Green I and Propidium Iodide Viability Staining. Journal of Microbiological Methods, 131. 172–180.

Nescerecka, A., Juhna, T. & Hammes, F. 2016b. Behavior and Stability of Adenosine Triphosphate (ATP) During Chlorine Disinfection. Water Research, 101. 490–497.

Oliver, J. D. 2000. The Public Health Significance of Viable but Nonculturable Bacteria. In: Colwell, R. R. & Grimes, D. J. (ed.) Nonculturable Microorganisms in the Environment, 1st edition. Washington: ASM Press. 277–300. ISBN 9781475702736.

Oliver, J. D. 2010. Recent Findings on the Viable but Nonculturable State in Pathogenic Bacteria. FEMS Microbiology Reviews, 34: 4. 415–425.

Omidbakhsh, N., Ahmadpour, F. & Kenny, N. 2014. How Reliable Are ATP Bioluminescence Meters in Assessing Decontamination of Environmental Surfaces in Healthcare Settings? PLoS One, 9: 6.

Payment, P., Sartory, D. P. & Reasoner, D. J. 2003. The History and Use of HPC in Drinking-Water Quality Management. In: Bartram, J., Cotruvo, J., Exner, M., Fricker, C. & Glasmacher, A. (ed.) Heterotrophic Plate Counts and Drinking-Water Safety, 1st edition. London: IWA Publishing. ISBN 9781843390251.

Pierce, J., Hiebert, J. B., Mahoney, D., Shen, Q., Peltzer, J., Rahman, F., Johnson, S. & Pierce, J. T. 2019. Development of a Point-of-Contact Technique to Measure Adenosine Triphosphate: A Quality Improvement Study. Annals of Medicine and Surgery, 41. 29–32.

Postgate, J. R. 1969. Viable Counts and Viability. In: Norris, J. R. & Ribbons, D. W. (ed.) Methods in Microbiology, Volume 1, 1st edition. London: Academic Press. 611–628. ISBN 9780125215015.

Pridmore, R. D., Cooper, A. B. & Hewitt, J. E. 1984. ATP as a Biomass Indicator in Eight North Island Lakes, New Zealand. Freshwater Biology, 14: 1. 73–78.

Renaud, D. L., Kelton, D. F., LeBlanc, S. J., Haley, D. B., Jalbert, A. B. & Duffield, T. F. 2017. Validation of Commercial Luminometry Swabs for Total Bacteria and Coliform Counts in Colostrum-Feeding Equipment. Journal of Dairy Science, 100: 11. 9459–9465.

Rhodes, W. C. & McElroy, W. D. 1958. The Synthesis and Function of Luciferyl-Adenylate and Oxyluciferyl-Adenylate. The Journal of Biological Chemistry, 233: 6. 1528–1537.

Rinta-Kanto, J. M., Lehtola, M. J., Vartiainen, T. & Martikainen, P. J. 2004. Rapid Enumeration of Virus-Like Particles in Drinking Water Samples Using SYBR Green I-Staining. Water Research, 38: 10. 2614–2618.

Sartory, D. P. 2004. Heterotrophic Plate Count Monitoring of Treated Drinking Water in the UK: A Useful Operational Tool. International Journal of Food Microbiology, 92: 3. 297–306.

Schuerger, A. C., Fajardo-Cavazos, P., Clausen, C. A., Moores, J. E., Smith, P. H. & Nicholson, W. L. 2008. Slow Degradation of ATP in Simulated Martian Environments Suggests Long Residence Times for the Biosignature Molecule on Spacecraft Surfaces on Mars. Icarus, 194: 1. 86–100.

Sciortino, C. V. & Giles, R. A. 2012. Validation and Comparison of Three Adenosine Triphosphate Luminometers for Monitoring Hospital Surface Sanitization: A Rosetta Stone for Adenosine Triphosphate Testing. American Journal of Infection Control, 40: 8. 233–239.

Seliger, H. H., Buck, J. B., Fastie, W. G. & McElroy, W. D. 1964. The Spectral Distribution of Firefly Light. The Journal of General Physiology, 48: 1. 95–104.

Seliger, H. H. & McElroy, W. D. 1964. The Colors of Firefly Bioluminescence: Enzyme Configurations and Species Specificity. Proceedings of the National Academy of Sciences of the United States of America, 52: 1. 75–81.

SFS-EN ISO 22000:2018. Food Safety Management Systems. Requirements for Any Organization in the Food Chain, 2nd edition. Helsinki: Finnish Standards Association.

SFS-EN ISO 4833-1:2013. Microbiology of the Food Chain. Horizontal Method for the Enumeration of Microorganisms. Part 1: Colony Count at 30 Degrees C by the Pour Plate Technique, 1st edition. Helsinki: Finnish Standards Association.

Shama, G. & Malik, D. J. 2013. The Uses and Abuses of Rapid Bioluminescence-Based ATP Assays. International Journal of Hygiene and Environmental Health, 216: 2. 115–125.

Shi, X. & Zhu, X. 2009. Biofilm Formation and Food Safety in Food Industries. Trends in Food Science & Technology, 20: 9. 407–413.

Shinozaki, Y., Sato, J., Igarashi, T., Suzuki, S., Nishimoto, K. & Harada, Y. 2013. Evaluation of an Improved Bioluminescence Assay for the Detection of Bacteria in Soy Milk. Biocontrol Science, 18: 1. 1–17.

Siebel, E., Wang, Y., Egli, T. & Hammes, F. 2008. Correlations Between Total Cell concentration, Total Adenosine Tri-Phosphate Concentration and Heterotrophic Plate Counts During Microbial Monitoring of Drinking Water. Drinking Water Engineering and Science, 1: 1. 1–6.

Stanley, P. E. 1989. A Review of Bioluminescent ATP Techniques in Rapid Microbiology. Journal of Bioluminescence and Chemiluminescence, 4: 1. 375–380.

Steghens, J., Min, K. & Bernengo, J. 1998. Firefly Luciferase Has Two Nucleotide Binding Sites: Effect of Nucleoside Monophosphate and CoA on the Light-Emission Spectra. Biochemical Journal, 336: 1. 109–113.

Tetra Pak. 2015. Cleaning in place: A Guide to Cleaning Technology in the Food Processing Industry. [PDF].

Tominaga, T., Fujioka, K., Hijikuro, M., Tada, T. & Nishikawa, Y. 2018. Mechanism of Increased Microbial Autofluorescence by Heat Treatment. Letters in Applied Microbiology, 66: 2. 118–123.

Tsai, T. S. 1986. A Microwave Method for the Extraction of Cellular ATP. Journal of Biochemical and Biophysical Methods, 13: 6. 343–346.

Turner, D. E., Daugherity, E. K., Altier, C. & Maurer, K. J. 2010. Efficacy and Limitations of an ATP-Based Monitoring System. Journal of the American Association for Laboratory Animal Science, 49: 2. 190–195.

Vang, Ó. K., Corfitzen, C. B., Smith, C. & Albrechtsen, H. 2014. Evaluation of ATP Measurements to Detect Microbial Ingress by Wastewater and Surface Water in Drinking Water. Water Research, 64. 309–320.

Velazquez, M. & Feirtag, J. M. 1997. Quenching and Enhancement Effects of ATP Extractants, Cleansers, and Sanitizers on the Detection of the ATP Bioluminescence Signal. Journal of Food Protection, 60: 7. 799–803.

Venkateswaran, K., Hattori, N. La Duc, M. T. & Kern, R. 2003. ATP as a Biomarker of Viable Microorganisms in Clean-Room Facilities. Journal of Microbiological Methods, 52: 3. 367–377.

Walker, S. I. 2008. Water Supplies in the Food Industry. In: Tamime, A. Y. (ed.) Cleaning-in-Place: Dairy, Food and Beverage Operations, 3rd edition. Oxford: Blackwell Publishing Ltd. 32–55. ISBN 9781405155038.

Walton, M. 2008. Principles of Cleaning-in-Place (CIP). In: Tamime, A. Y. (ed.) Cleaning-in-Place: Dairy, Food and Beverage Operations, 3rd edition. Oxford: Blackwell Publishing Ltd. 1–9. ISBN 9781405155038.

Wang, Y., Hammes, F., Boon, N., Chami, M. & Egli, T. 2009. Isolation and Characterization of Low Nucleic Acid (LNA)-Content Bacteria. The ISME Journal, 3: 8. 889–902.

White, E. H., Steinmetz, M. G., Miano, J. D., Wildes, P. D. & Morland, R. 1980. Chemi- and Bioluminescence of Firefly Luciferin. Journal of the American Chemical Society, 102: 9. 3199–3208.

WHO. 2003. Expert consensus. Expert Group Meeting Report. In: Bartram, J., Cotruvo, J., Exner, M., Fricker, C. & Glasmacher, A. (ed.) Heterotrophic Plate Counts and Drinking-Water Safety. London: IWA Publishing. ISBN 9781843390251.

Wilson, C. A., Stevenson, L. H. & Chrzanowski, T. H. 1981. The Contribution of Bacteria to the Total Adenosine Triphosphate Extracted from the Microbiota in the Water of a Salt-Marsh Creek. Journal of Experimental Marine Biology and Ecology, 50: 2–3. 183–195.

Yoo, Y. J., Feng, Y., Kim, Y. H. & Yagonia, C. 2017. Fundamentals of Enzyme Engineering, 1st edition. Dordrecht: Springer. ISBN 9789402410242.

APPENDICES

Appendix 1. CIP rinse water results

sample	pH	ATP (RLU)	mean ATP (RLU)	RSD (ATP) (%)	TVC (CFU/ml)
1	8.74	0			<1
2	9.10	0			<1
3	8.84	0			<1
4	8.54	1			<1
5	8.88	1			<1
6	8.44	0			<1
7	8.21	1			<1
8	8.53	1			<1
9	8.48	0			<1
10	8.11	0			<1
11	8.25	4			31
12	8.45	0			<1
13	8.25	0			<1
14	8.11	0			<1
15	8.20	0			<1
16	8.33	0			<1
17	8.41	0			<1
18	8.15	0			<1
19	8.31	1			<1
20	8.54	0			<1
21	8.65	0			<1
22	8.49	0			<1
23	8.11	0			<1
24	8.25	0			<1
25	8.54	0			<1
26	8.33	0			<1
27	8.75	0			<1
28	8.65	0			<1
29	8.44	0			<1
30	8.19	0			<1
31	8.36	0			<1
32	8.88	0			<1
33	8.26	0			<1
34	8.54	28 41	35	27	4
35	7.99	0			<1
36	8.45	0			<1

37	8.22	0			<1
38	8.68	1			1
39	8.81	0			<1
40	8.26	0			<1
41	8.45	0			<1
42	-	0			<1
43	-	0			<1
44	-	1			<1
45	-	0			1
46	-	0			1
47	-	1			1
48	8.11	4			31
49	8.12	586 572 614 626	600	4	6025
50	8.52	148 146 145	146	1	1214