Identification and quantification of microbiological risks in board production

A study of ATP bioluminescence and redox potential

Identifiering och kvantifiering av risker kopplade till mikrobiologi vid kartongproduktion

En studie om ATP bioluminescens och redox potential

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2022-06-13

Abstract

Stora Enso is a well-established provider of renewable solution packaging and board materials. Board products for liquid packaging and folding cartons for food are particularly sensitive to the microbiological contaminants. In the manufacturing process, microbiological growth occurs due to environmental favourable conditions. Most of the microorganisms eventually die in the board machine. The microflora in the process consists mainly of bacteria from genera *Bacillus* and *Paenibacillus,* and these species have a unique characteristic of forming endospores. The spores are non-vegetative capsules transformed from the dying bacteria, with its purpose of protecting the bacterial genome from the unfriendly environment as it passes through in the board machine. The levels of spores found in the board products are usually low, but sudden peaks of spores in the board products are unwanted for food and health safety. Bacterial and spore cultivation is a standard method to investigate microbiological activity. The method is inaccessible due to 48-hour incubation time for obtaining test results and does not provide any overview in a short time frame of where in the process that might triggered sporulation. In the long run, it can cause production losses that affect the prospects of the board production.

The purpose of this study is to investigate a new analysis method to facilitate identification and quantification of microbiological activity with ATP bioluminescence (Adenosine Triphosphate) measurement and redox potential, and as well investigate the main process site that possibly causes the issue. ATP bioluminescence measures the concentration of ATP in relation to the number of bacterial cells. The study was limited to the broke system due to the closed system and accumulation of microorganisms. The broke system manages the reuse of downgraded new paper board. For the laboratory work, 15 process positions were selected in the broke system. Pulp were sampled from each process position for each laboratory workday, and measurements of ATP bioluminescence, redox potential, pH, temperature, TOC (total organic carbon), retention time, and bacterial and spore cultivation were completed as well. The collected data were studied in a multivariate analysis and correlograms were produced for each process position. In addition, a parallel study of a lab scale broke tower simulation was performed to further investigate if the broke tower could be the source of the microbiological activity.

The obtained results showed low correlations between the process parameters and the total bacterial and spore concentration. Similar results were obtained for ATP bioluminescence and redox potential, resulted in a low correlation to the total bacterial and spore concentration as well. The simulation gave additional insight in the function of ATP bioluminescence and redox potential.

The conclusion is that ATP bioluminescence is an accessible method due to high repeatability, however the reliability is lacking. The analysis is not reliable because of low correlation between the total bacterial and spore concentration. Redox potential is both a reliable and accessible method to identify and quantify the microbiological growth in the system, because it signifies the environmental conditions for the bacterial growth. It has been proven in the broke simulation. Further research is needed to understand the representation in relation to the bacterial growth behind each analysis in order to fully consider the analysing measurement applicable. Due to low correlations between the variables in each process position, any assumptions cannot be considered in any specific process position that might be main cause of raised spore values in board products.

Sammanfattning

Stora Enso är en väletablerad leverantör av förnybara lösningar för förpackningar och kartongmaterial. Kartongprodukter för vätskeförpackningar och vikkartong för livsmedel är särskilt känslig för mikrobiologisk aktivitet. I tillverkningsprocessen sker mikrobiologisk tillväxt på grund av gynnsamma miljöförhållanden. De flesta av de vegetativa cellerna dör så småningom i kartongmaskinen, men en stor del av mikrofloran består av bakterier från släktena *Bacillus* och *Paenibacillus*. Dessa arter har en unik egenskap att bilda endosporer. Sporerna är icke-vegetativa kapslar som transformerats från de döende bakterierna, med syftet att skydda bakteriegenomet från den ogynnsamma miljön som passeras i genom maskinen. Nivåerna av sporer som finns i kartongprodukterna är låga, men plötsliga förhöjda värden av sporer i kartongprodukterna är oönskade för livsmedels- och hälsosäkerheten. Bakterie- och sporodling är en standardmetod att undersöka den mikrobiologiska aktiviteten. Metoden är otillgänglig vars resultat visas efter 48 timmar inkubation. Metoden ger ingen översikt var i processen som kan ha framkallats sporulering, och detta bildar en diffus överblick var i processen inom kort tidsram som kan ha bidragit till sporuleringen. På lång sikt kan det orsakas produktionsförluster som påverkar kartongproduktionens framtidsutsikter.

Syftet med denna studie är att undersöka nya analysmetoder för att underlätta identifiering och kvantifiering av mikrobiologisk aktivitet med en ATP bioluminescens (Adenosin Trifosfat) mätning och redoxpotential och även undersöka den huvudsakliga processposition som orsakar det huvudsakliga problemet. ATP bioluminescens mäter koncentrationen av ATP i förhållande till antalet vegetativa celler. Studien begränsades till utskottssystemet på grund av slutet system och ansamling av mikroorganismer. För laborationsarbetet valdes 15 processpunkter ut i utskottssystemet. Utskottsmassan togs från varje processpunkt för varje laborationsdag, och mätningar av ATP bioluminescens, redoxpotential, pH, temperatur, TOC (totalt organiskt kol), retentionstid och bakterie- och sporodling genomfördes också. De insamlade data studerades i en multivariat analys och korrelogram togs fram för varje processpunkt. En parallell undersökning genomfördes med en labbsaklig simulering av utskottstornet för att ytterligare undersöka om tornet är möjligtvis källan till den mikrobiologiska aktiviteten.

De erhållna resultaten visade låg korrelation mellan processparametrarna och den totala bakterie- och sporkoncentrationerna i samtliga processpositioner. ATP bioluminescens och redox potential erhöll låg korrelation mellan den totala bakterie- och sporkoncentrationen, men simuleringen gav tydligare förståelse i de båda mätningarnas funktion.

Slutsatsen är att ATP är en tillgänglig metod eftersom det är repeterbar. Däremot har det visats att den är opålitlig, på grund av den låga korrelationen mellan den totala bakteriella och sporhalten. Redox potential är pålitlig att använda, vilket har visats i simuleringen. Den förklarar de miljömässiga förhållandena i systemet och på så sätt stärks förståelsen om den bakteriella tillväxten. Vidare forskning behövs för att förstå betydelsen i samband med den bakteriella tillväxten bakom varje mätning för att analysmetoden ska vara applicerbar. På grund av låga korrelationer mellan variablerna i varje processposition kan några antaganden inte beaktas i någon specifik processposition som kan vara huvudorsaken till förhöjda sporvärden i kartongprodukter.

Key terms and abbreviations

ATP bioluminescence measurement: measuring the cellular material relating to "energy carrier molecule" Adenosine triphosphate (ATP)

Redox potential: measure electric charge in a sample, the electron transfer between the molecules in a sample

Extracellular ATP: free ATP released from dying or as stress response. The term is equivalent to free ATP

Intracellular ATP: cellular ATP inside active bacteria, generating energy for metabolism

Broke system: recirculation system of fibres

White water system: recirculation system of process water

Pulper: first stage of entering broke system. Rotor inside breaking fibre flocculation

16s rRNA: a ribosomal gene is commonly used for identification and classification of microbiological culture from the 16s rRNA phylogenetic tree

Bacillus: bacterial genera found in board production. Species with sporulation ability

Paenibacillus: bacterial genera found in board production. Species with sporulation ability

Spores: non-reproductive capsules produced from sporulation

Sporulation: defence mechanism gram-positive bacterium possesses to protect its genome from unconditional environment. The term is equivalent to endospore formation

CFU: colony forming units of bacteria on petrifilm

Growth cycle: the life cycle of an organisms, including growth, stagnation and decline phases

Total organic carbon: total organic carbon (TOC). A measurement of the total organic carbon in white water

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1. Introduction

Stora Enso is a well-established global provider of renewable solution packaging materials, wooden construction and board. The primary goal of the manufactured products is to be renewable, recyclable and fossil-free, for providing a reduction of the carbon footprints. The large variety of businesses give Stora Enso an important role in the global bioeconomy (Stora Enso 2022a).

Liquid packing in the leading investments for the company's profits and sustainable goals. The investments have never before been significant due to corresponding global demand. Environmental and climate issues are evident issues that have come to affect people worldwide. Some of the solutions to stagnate these issues, is to eliminate plastics utilization and reduce carbon dioxide emissions. However, the awareness of these issues has not been noticeable for a long time. During the past 15 years companies have started to consider environmental issues more seriously and have transformed their goals towards sustainable values. The environmental and climate changes that occur today are the driving force of using natural resources with efficient strategies. Investors and institutions consider today companies to integrate eco-consciousness into their economical strategies, as a long-term profitable vision for the company and the environment. Whereas the regulations and policies have an impact in the adaptation to climate and environmental changes. Companies are as well observant about peoples' purchase behaviour, which in recent years has shifted to consume more sustainable options (Stora Enso 2022a).

For Stora Enso to match the consumers requirements and maintain a high quality, the challenges are deeply infused in the closed systems in the board manufacturing. Board making is a comprehensive and complex process. The board production is primarily extensive due to the essential water system. The principle of manufacturing is to maintain a low fibre and water consumption, as the water consumption per tonne board product in 2020 was estimated at $2{,}3m^3$ (Stora Enso 2022a). The water level in the production is required to maintain constant without affecting the quality and rate of the production. The water maintenance includes multiple purification steps as water plays an important role in manufacturing (Paulapuro et al. 2000).

The circular systems contribute to higher process temperatures and increased suspended solid particles (Hamm & Schabel 2007). This brings an accumulation of microorganisms in the systems. The board quality reduces drastically simultaneously the process quality and the operation of the production aggravates in the long term (Blanco et al. 1996). Moreover, this increases the risks of limiting the shelf life despite the coating films integrated in liquid food packages, as the microorganisms can migrate into the food products. Therefore, it is important to limit the amount avoiding the risks (Suominen et al. 1996).

The production provides a recurring control of microbiological growth in board products to ensure its quality. The identification of microorganisms in the board products gives a history of the process situation. It requires a control of microbiological growth in the systems as well, because of the favourable environment in the system allowing them to thrive. The microbiological growth is inevitable in any site of the process. A control early in the process requires to avoid affected board products. However, it is crucial to identify any plausible sites in the system that extraneously contribute to excessive growth (Väisänen et al. 1998).

Responsible consumption and production (SDG 12), Climate action (SDG 13) and Life on land (SDG 14) are the three striving fundamental UN's sustainable goals for Stora Enso (Stora Enso

n.d.). Material efficiency is an essential work from extracting wood from forest to the final board product. However, this study covers the board production material efficiency, where the quality of the board is the crucial parameter in the production. The production rate and the economic issues are closely dependent. Quality problems lowers the production rate, as the board needs to be discarded and recirculated back into the system and it relies on the customers' requirements. The significant goal for the production is to utilize freshly produced pulp for board manufacturing in order to maintain a high standard, but the broke system is a remarkable innovation in pulp and board production which provides different aspects of the situation (Paulapuro et al. 2000).

1.2 Aim

The essential aspect of this study is to identify which point in the complex process can lead to excessive microbiological growth. Conventional bacterial and spore cultivation is the common method performed in root-cause analyses for mapping the system and identifying possible sources of microbiological disturbances. The problem with this method, it requires 48 hours to obtain a test result, meaning that these causes cannot be identified within a short time frame. This leads to the microbiological contamination transferring throughout the process and reaching into the final board product before the cause can be identified. The aim in this study is to examine new analysing methods to facilitate identification and quantification of increased microbiological activity in the board process.

1.3 Research questions

Is ATP bioluminescence measurement a reliable and accessible method to identify and quantify microbiological growth?

Is redox potential measurement a reliable and accessible method to identify and quantify microbiological growth?

Which process position is the main cause of microbiological growth in board machine?

2. Board production

In the board machine, the board is manufactured by the fibre furnish pumps through a head box onto a wire. Before the fibres enter in the board machine, a fibre concentration of 0,1% is obtained with water and additional chemicals, for avoidance of fibres to flocculate. The function of the head box is to distribute and orientate the fibres. Due to multiple layers in board products, there is a set of three headboxes. On the wire the board sheet is formed during the forming process, as the board sheet dewaters alongside gravity and vacuum boxes (Norman 2008a). After the sheet forming the board sheet transfers further to the press section, where it presses through cylinders, as shoe press is the common variation. The water from the dewatering collects in wire pits and further transfers to the short and long circulation beneath. In the drying section, the dry content reached 95% (Norman 2008b). The pressed board sheet enters the drying section, where the sheet transfers on wires through heated cylinders (Stenström 2008). Lastly, the board reaches coating and calandering to obtain the optical and physical qualities.

2.1 Problem areas in board production

The board machine is a partly closed system, whereas water and fibres are the main inlet and outlet in the production. The water inside the production is recirculated multiple times before it is purified for the outlet. Because the water is kept constant, fresh water is constantly provided in the system. The fibre flow follows a similar principle throughout the production. The

production includes numerous purification steps connected to each other, with the task of recovering water and fibres lost during the process stages. The fundamental purpose is board and paper production to reduce water consumption and material losses (Norman 2008b). Stock preparation, stability control, white water systems, fibre recycling system and broke system support essential parts of the system enclosure of the board machine (Paulapuro et al. 2000). Behind system closure, there are extensive economic and environmental benefits, mainly regarding the reducing water and fibre consumption (Norman 2008b)

In stock preparation, the stock is refined to avoid fibre aggregations. The stock quality is prepared in the stock preparation varies depending on the product, the concentration of which is regulated. Any addition of broke fibres is added in this step. The white water system includes short and long circulation. From the inlet box, the stock is sprayed on a wire. The fibre concentration on the wire increases at the same rate as the water is removed. During forming, an excess of fibres and water are constantly captured in wire pits. The excess fibres are then separated from the water and added to the stock preparation. The circulation is called the short circulation. This is involved in the long circulation, as the recover fibres and water preparation implicate the contributing part of the long circulation. The recovered water contains additives and chemicals that have been earlier added into the stock (Paulapuro et al. 2000). Before the recovered fibres are entered the stock preparation the fibre concentration is prepared to 1%. The dilution is accomplished with the white water from the long circulation (Norman 2008b).

The long circulation is further complex and includes purification steps and concentration controls, such as disc filters, see figure 1. From the broke tower, the broke mass is pumped to the disc filter. The pulp is separated from the white water by the disc filter. The white water is then converted depending on its purity from fibres to clear filtrate. The white water in the long circulation regulates the concentration of the broke (Hamm & Schabel 2007).

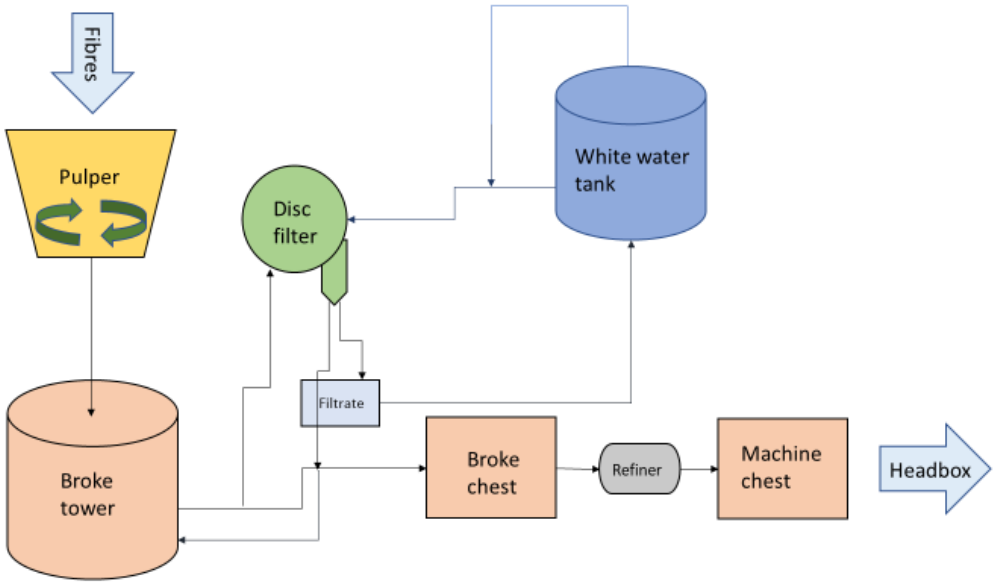

Figure 1. Simplified chart of broke system.

The process conditions in the board machine must be kept constant in order to achieve the established product qualities and high runnability. The underlying factor to this is variations in the chemical stability of the white water system and the wet portion of the board machine. On the other hand, parameters can acquire different roles and meanings in the process, and this needs to be considered when optimizing and reviewing the process. Temperature, pH, flow rate, contamination of undesirable substances and flow conditions are examples of parameters that can cause disturbances in the production (Paulapuro et al. 2000).

The broke system is a part of the white water system, whose task is to process fibres captured from different sections of the board machine. Fibres that reach the broke system originate from different sections of the board machine, mainly due to web breaks and trimming the board. Depending on the location of web breaks these fibres are trapped in pits (Norman 2008b). The main principles of the broke system are to transport the broke mass, convert the broke mass into stock, store and regulate the fibre concentration in the process (Paulapuro et al. 2000).

The flow in the broke system is not constant, the outcome is due to disturbances and stoppages causing prominent variation of incoming fibres. The variation of fibre concentration affects the current flow in the broke system. However, the level and purity of white water is dependent on the concentration of broke fibres entering its system, meaning there are several circumferences involved. The surplus of fibres is captured in pulpers, located under the board machine. The fibres can come in wet and dry batches (Norman 2008b). Its functions may vary depending on its position. The fibre concentration and dry matter content give each individual pulper a distinctive function to separate the fibres. Pulpers consist of rotors whose direction is opposite to the incoming mass, and breaks fibre webs and flocculation. Pulpers remain stationary during high production rates. In case of web breakage, the pulpers are induced, and the white water enters the system for breakage of fibre flocculations (Paulapuro et al. 2000).

A recurring problem area in the board machine is the broke tower. Due to the large volume, there is a lack of mixture in the broke mass, and due to the ingesting excess fibres, the retention time is high. This gives rise to high bacterial growth. Of which in the event of a web break, the situation is further aggravated in connection with increased mass in the tower and the flow remains stagnant for several hours. This causes difficulties in maintaining control in the broke tower because web breaks are unpredictable. One possible strategy to maintain control in the broke tower is to maintain a constant low tank level in the tower (Kiuru et al. 2010).

The white water system includes careful control of contamination. The white water is reused several times in the manufacturing process before switching to healthier water, and frequently fresh water is added to the system to maintain adequate levels. The runnability of the manufacturing process and the product quality are largely due to the accumulation of substances originating from the stock preparation and the broke. In white water may exhibit high microbiological activity (Nordkvist 2005).

2.2 Biocides

Biocides are bactericidal chemicals added at various bacterial and spore-affected process positions in the machine. However, the consumption of biocides has decreased in recent years due to the contributing effect to chemical variations in the manufacturing process, as well as web breaks and lack of runnability are caused. Knowledge about biocides is limited regarding the impact on humans and the compatibility with the constituent chemicals in the manufacturing process (Blanco et al. 1996), (Kiuru 2011). In recent years, alternative biocides have been

developed and have shown to be more cautious against biofilms. Aggressive biocides cause bacterial resistance, leading to difficulties to maintain control in the process. Instead of completely killing the bacterial culture, the alternative biocides can maintain bacterial control to a certain extent, by providing the bacterial culture with limited access to nutrients. Bacterial control is maintained through complex formation of biomolecules that plays essential parts in metabolism of the bacteria. The effect of the alternative biocides contributes to reduced changes in the vegetative cell membranes, therefore the resistance to biocides is avoided. However, it is rather uncertain the effectiveness of alternative biocides (Blanco et al. 1996).

3. Bacterial flora in board production

It is shown that the board machine has a unique and diverse bacterial flora, whereas the abundant genera are *Bacillus* and *Paenibacillus*.

Väisänen et al (1998) isolated and identified 390 bacterial strains in two printing board machines. The identification was performed partly with 16s rRNA sequencing, whereas the main contaminants were found in the wet end. *Bacillus coagulans* and other *Bacillus* species, *Burkholderia cepacia*, *Ralstonia pickettii* were the main bacterial groups identified in its part of the board process. Kolari et al (2001) identified *Bacillus* species and *Paenibacillus* from three different mills. The bacterial flora is specific to individual mills.

3.1 Bacilli

142 of *Bacillus* species are included in the phylogenetic tree of 16s rRNA gene. The species have its own characteristics in shape and form, the morphology can distinguish them from each other. *Bacillus cereus* are described as square shaped. However, there is still an ongoing debate of division between the species. Within a *Bacillus* group, the bacterial species can be additionally divided into further groups based on the core genes. Identification of new bacteria gives a rather complex mapping in the phylogenetic tree, and the scientists are concerned about the impact of it (Logan & Vos 2015).

Microorganisms belonging to the genera *Bacillus* are the largest contaminants in food packaging (Suominen et al. 1997). This aerobic bacterial species is very extensive and has unique characteristics that distinguish them from many other bacterial species. *Bacillus* species possess the ability of forming endospores, a contributing property of survival in adverse environments (Madigan & Martinko 2006). *Bacillus cereus* is a species that is found in various types of food. It originates from soil and water, but through different transfers the cells reach into food.The board products are the common cause of food contamination (Andersson et al. 1995).

Paenibacillus is included in the phylogenetic tree of 16s rRNA, however it was earlier included in genera *Bacillus*, from increased knowledge of mapping species this genus has become a separate despite many common characteristics. It is an endospore forming and aerobic bacterium (Logan & Vos 2015).

3.2 Nutrients

The bacterial cell consists of 95% hydrogen, carbon, nitrogen, sulphur, calcium, magnesium, phosphorus and iron. These are the fundamental building blocks for the structure of lipids and proteins, nucleic acids and carbohydrates, essential for the survival and growth of bacteria and are interdependent.

Manganese, cobalt, molybdenum, nickel and zinc are elements that play an essential part of the enzymatic activity in the vegetative cells. The turnover of the metals depends on the surrounding environment the bacterium lives in, as the conditions for growth can vary for the bacterium. However, bacteria are not affected to the same extent in case of deficiency as the macromolecules (Prescott et al. 2002).

Glucose is the primary carbon source for the bacterium. Glucose has a high redox potential which interferes with high energy sources. Bacteria convert glucose into highly valuable energy through reducing reactions. Microorganisms that possess this function are called heterotrophs. Researchers have shown that bacteria are adaptable, i.e., its capacity of utilizing extensive variation of carbon sources (Prescott et al. 2002).

3.3 Growth cycle

Each bacterial species has its own growth cycle, undergoing four growth stages, see figure 2. The *lag phase* is when the culture is incubated in a fresh growth medium. The conditions in the environment have a considerate impact on the life of microorganisms. The initiation of growth is slowed at this stage. During favourable conditions, the bacterial cells grow and are divided in binary fission, which is when the microorganisms enter *log phase*. The cell division is vegetative, the septum breaks up the cell in two and forms two bacterial cells (Logan & Vos 2015). The exponential growth decelerates until its stagnation as limits of nutrients and waste products accumulation in the medium becomes too stressful. The stagnation phase is called the *stationary phase*. Finally, the microorganisms gradually die (Madigan & Martinko 2006).

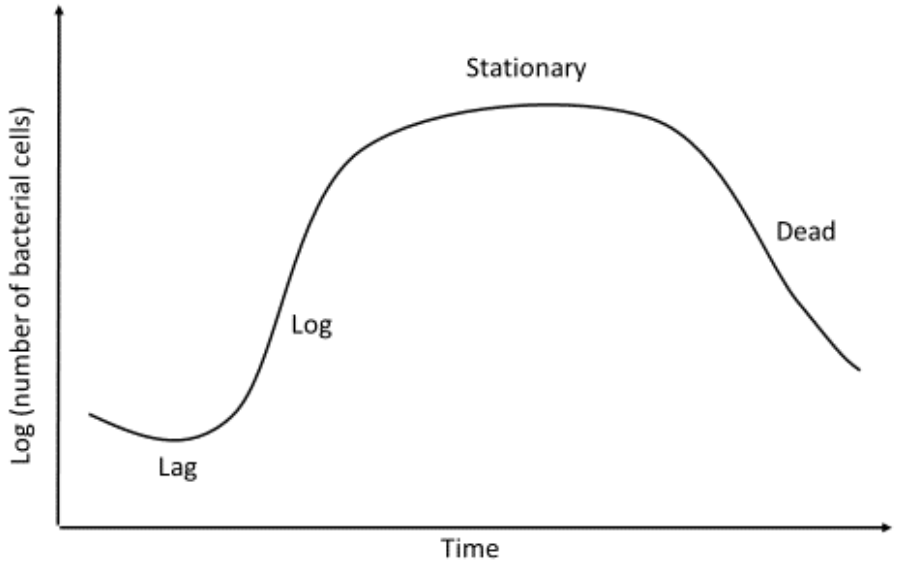

Figure 2. Illustrated growth cycle of bacteria. The y-axis represents the number of bacterial cells and the x-axis represents the duration time. Inspired by Wang et al. (2015).

4. Favourable environment in board machine

The board machine operates in slightly alkaline conditions (Thomassin et al. 2006), and the optimum temperature for stock preparation is 46 to 54°C (Paulapuro et al. 2000). The pH range for *Bacillus cereus* have shown to be between 5,0-9,0, while acid conditions is being an inconsiderate environment for the species as it has the ability of entering the survival mode, sporulation. The awareness of pH range provides thriving conditions for the bacterial culture in

the medium, by which the sporulation is avoided. pH variations are needed to be evaded as well (Thomassin et al. 2006). Various *Bacillus* species have its optimum growth temperature in between 20 and 40°C (Logan & Vos 2015). The environment is conditional for a great variety of bacterial populations. Higher temperature exceeding 60°C inhibits microbiological growth (Paulapuro et al. 2000).

The extensive situation of a board process is to pinpoint the exact site where the considerable conditions contribute to bacterial growth. A part of the bacterial culture can be found in additives. Starch, kaolin, CMC (carboxymethyl cellulose), and bentonite are additives in board and paper production. Clays and minerals originate from natural mines and the storage in tanks could be a possible source of microbiological activity in paper and board systems (Väisänen et al. 1998).

The white water has a considered culture medium for bacterial growth, as the carbon content is high with a ratio to nitrogen and phosphorous (C/N 40:1 and C/P 90:1). The conditions provide the production of polysaccharides, the main composition of biofilms (Väisänen et al. 1994). Starch is often used for surface sizing. It is a natural carbohydrate, making it a natural primary carbon source for the bacterial population (Väisänen et al. 1998). Biofilm formation is one of significant survival strategies bacteria bring out (O'Toole et al., 2004). A colonial population of microorganisms forms the biofilm. The formation of biofilms is initiated by planktonic microorganisms attach onto a surface, for example on steel (Öqvist 2008). The formation induces as the environmental conditions trigger the formation. High levels of nutrients activate the planktonic bacteria to transform into colonies. After the attachment the colonies produce its protective layer of extracellular polysaccharide, attracting other vegetative cells. The matrix provides protection from potential fluctuation of hostile environments and assurance of nutrient access. Moreover pH, oxygen, temperature and iron influence the formation triggers (O'Toole et al 2004). The composition of the extracellular matrix mainly consists of glucose, mannose, galactose, rhamnose, fucose, arabinose and other types of sugars (Väisänen et al., 1993). Biofilms have protective properties depending on the environment outside, such as from UV-light. As well the biofilm provides the bacterial culture genetic exchange and secondary metabolite production (O'Toole et al., 2004). Bacterial culture that is found in the board machines generally consist of gram-positive bacteria (Väisänen et al., 1998). *Bacillus* species are often associated in biofilms. The extracellular polysaccharide formation is frequently called "slime". Detachment of biofilms occurs during starvation (O'Toole et al., 2004).

5. Endospore

Endospore formation is a survival strategy possessed by the *Bacillus* bacterium. Endospores are non-vegetative cells and have a function as a storage capsule for the bacterium's biological genome in dormant phase. During its dormant stage, the ATP consumption is absent, there is no metabolic activity (Logan & Vos 2015). The environment inside the endospore is optimal for conserving the genome during harsh conditions outside (Madigan & Martinko 2006). Temperature, pH, aeration, presence of nutrients and minerals have been known to affect the endospore formation (Logan & Vos 2015). The endospores can withstand extreme temperatures and are resistant to chemicals and radiation, such as strong acids and bases and UV radiation. This makes them resistant to dryness and limited nutritional values that vegetative cells usually require. Highly heat-resistant endospores can survive up to 150 °C (Madigan & Martinko 2006).

5.1 Structure and its properties

Endospores have a complex structure. The diameter of a spore is about 1µm in comparison to vegetative cells being 10 times larger. The layers are unique compared to the vegetative cells and are formed during the sporulation. The outer layer is a thin protein-containing layer, called *exosporium.* Under the thin layer there are several spore layers consisting of specific proteins intended for spores. The *cortex* is a thick layer of peptidoglycans that encloses the core of the endospore. It occupies more than half of the endospore volume.

The nucleus contains essential molecules for its survival. Knowledge about the survival of endospore is limited, though there is a strong indication that dipicolinic acid (DPA) plays an important role in the core of the endospore. It is shown that dipicolinic acid promotes resistance to heat and adverse chemicals. Water gives both drying and dehydrating effects to the core for prevention of chemical and physical stress. In the endospore core the dehydrating effect is emphasized (Prescott et al. 2002). Maturation of the dormant cells has a strong influence on the water content in relation to the environmental stress. The content is as low as 10-30%. A vegetative cell has a higher water content due to enzymatic activities and is more prone to heat, making the water have a considerable effect on molecules inside (Logan & Vos 2015). Calcium dipicolinic acid maintains the stability of the nucleic acids contained, which has a contributing effect in protecting the backbone of DNA from heat. Through osmotic mechanisms, once the water is removed from the core of the endospore, the protection is stabilized. The endospore consists of 10–15% dipicolinic acid. The nucleus has a high concentration of *small acid-soluble proteins* (SASPs). The proteins are synthesized during sporulation. During the dormant life of the endospore, these proteins bind to the core of the DNA and its crucial role is to protect the DNA from chemicals, radiation, heat and dehydration (Prescott et al. 2002), (Madigan & Martinko 2006).

5.2 Endospore formation

The endospore formation initiates during the end of the log phase, and it plays an important role in the vegetative cycle which can be observed in figure 3. The cell division in endospore formation is asymmetrical, as a stress response of the inconsiderate environment, which is to produce endospore in abundance for increasing the changes of genome's survival. The sporulation cycle can be classified into seven morphological stages. Stage I is the initiation process of DNA replication. In stage II a septum forms in the end of the bacteria where the crucial DNA is collected, and the membrane of the protoplast forms around the capsule. The spore becomes detached from the mother cell, and the division is asymmetrical. The spore is enclosed by the cytoplasm of the mother cell in stage III. Afterwards the cortex forms, in stage IV, the most rigid layer of the spore. The cortex provides the resistance of the environmental stresses. The cortex becomes covered by a thinner layer, exosporium, formed in stage V. During stage VI the spore matures, and the mother cells are lysed. Then the spore enters the dormant life until it germinates to a vegetative cell, seen in stage VII. In laboratory conditions the sporulation duration is 8 hours (Logan & Vos 2015).

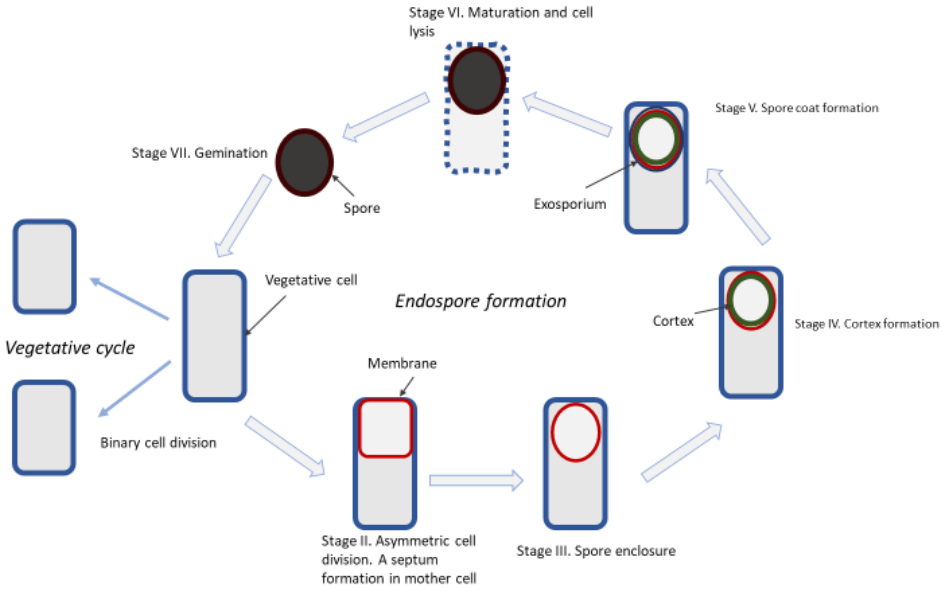

Figure 3. Sporulation and germination cycle, as well as vegetative cycle of a Bacillus species bacteria. Inspired by Errington (2003).

During the conversion from a dormant spore to a vegetative cell, including three important steps; activation, germination, and outgrowth. The activation steps involve the encountering environmental conditions that trigger the germination procedure. From the activation the spores begin to swell and SASPs hydrolyse and the forming nucleus fills with water and all biological syntheses are established. The vegetative cell grows out from the spore coat. A minor shift in the environment can activate the germination, corresponding to a monitor ability of environmental conditions (Logan & Vos 2015).

5.3 Factors of sporulation

5.3.1 Malnutrition

Malnutrition and cell density are the crucial triggers for the bacterial cells. These are two main factors that determine the activation of the endospore formation. During formation, the essential molecule, Spo0A~*P*, is activated by phosphorylation. The molecule is a regulator of sporulation, and its purpose is to initiate the formation by controlling the 200 genes included in the formation. The gene activation requires energy, and the remaining available nutrients in the environment consume during the activation process. Initiation is controlled when the branched chain amino acids (BCAAs) and guanosine triphosphate (GTP) is restricted in the bacterial cells, of which CodY regulates the signals and binds BCAAs with GTP (Sonenshein 2005). The bacterial cells are dependent on natural carbon sources, mainly the starvation of the bacteria is regulated reliant on its primary source of nutrition. For *Bacillus* species glucose is the primary and vital carbon source. Yet the survival mechanism allows the bacteria to consume another accessible organic compound in the existing medium. In contrast, optimal glucose intake enhances cell growth (Mamzira et al. 2012). Kemppaninen (2013) demonstrated that nutrient depletion is the most effective method to inhibit sporulation. σ^B is a protein, its activation induces during the environmental stresses, principally during starvation. The protein regulates activation of numerous survival mechanisms for searching for new resources, motility and eliminating its neighbouring bacteria. The most essential function the protein possesses is its regulation ability of sporulation. However, sporulation is the least survival strategy and it is

activated if the primary mechanisms cannot resist from the environmental stresses (Reder et al. 2012).

Calcium dipicolinic acid is the essential acid form in the core of the endospores. The concentration of calcium plays a crucial part in endospores resistance mechanisms, especially against temperature. The natural source of calcium in the external environment, the amount found in the board systems influence the anticipation of sporulation (Nordkvist 2005). The sporulation time is five times longer in absence of calcium ions than the presence of it (Nguyen Thi Minh et al. 2011).

5.3.2 Cell density

Bacteria have the function of communicating via signals cell to cell, *quorum sensing*. Quorum sensing is a mechanism whose function is to regulate the behaviour of bacteria in relation to their environment. The conditions and strategies change based on the environmental conditions. During accumulation of high bacterial densities, the bacterial cells start to communicate in terms of the nutrient depletion becoming more evident for the entire population (Bischofs et al. 2008). As the cell density increases, the extracellular secreted peptide begins to accumulate among the cells. The cells communicate through transmitted secretions (Faqua & Greenberg, 1998). The secretion is produced until the bacterial cells' maximum concentration is reached. The increased concentration of secretion responds to increased values of CSF (Competence and Sporulation Factor) in the bacterial cells. CSF activates the response regulator Spo0 (Sonenshein 2000). Endospore formation also occurs in biofilms when the growth takes place to the stage of competition and location (Vlamakis et al. 2008).

5.3.3 pH

The influence of pH on sporulation is unclear, to this extent in which pH range affects the sporulation. Despite optimum growth environment for bacteria exists in alkaline conditions, Nguyen Thi Minh et al. (2011) obtained a spore culture in pH 8,0. In fact, the influence of sporulation does not depend on the pH itself, the environment relies on multiple factors such as aeration, heat, pressure, and water activity. The environmental factors during growth of bacterial cells, has later a deep influence of the sporulation. The conditions are delicate for the bacterial cells. Any minor disturbance activates the sporulation process. Moreover, it has been observed that lowering the pH in the microbiological environment could be an optional strategy to decelerate the sporulation. The spore yield is maximal when aeration, heat, pressure and water activity are in optimal range. In another study by Nguyen Thi Minh et al. (2008) it is explained that the alkaline conditions enhance the mineralization of spores. This contributes to higher heat resistance. Thomassin et al. (2006) examined bacterial culture grown in different pH levels, from 5,5 to 9,0 with a growth rate variation. The bacterial culture was set in acid shock at pH 4,0. The intracellular pH is sensitive to the variation of the environmental pH, though *Bacillus cereus* showed considerable results as to be adaptable in acid conditions. The bacterial cells have the ability to maintain the differential pH value, between the environmental and intracellular pH. Wilks et al. (2009) explains the bacterium has adaptable mechanisms to alkaline shocks. Depending on the environmental shocks the bacterial cells are constrained to the catabolic related genes activation. Alkaline mediums induce a higher abundance of genes than in an acid environment.

Paenibacillus is an acid tolerant species. The sporulation can occur at low pH. Casadei et al. (2000) showed that acid medium reduces the spores' heat resistance. Thus, multiple variables, such as organic acid, low pH and the environmental medium contribute to heat sensitivity.

5.3.4 Temperature

The sporulation is more efficient at 37°C. At lower temperatures, such as 20°C the sporulation activity declines, and this results in lower spore concentration. Through temperature variations spores evolve their resistance to endure the harsh environmental stresses (Reder et al. 2012). Species from genera *Bacillus* are psychrophilic and mesophilic (Logan & Vos 2015).

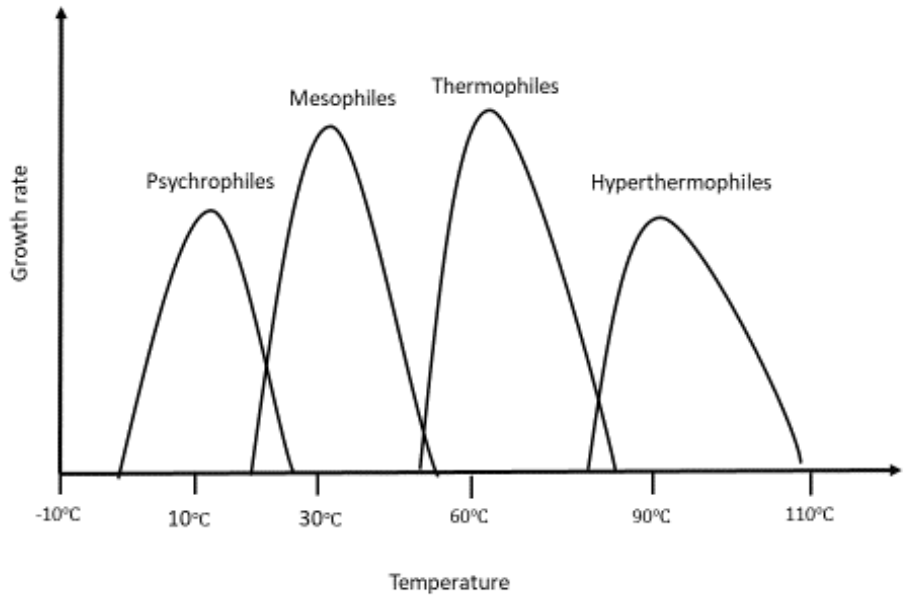

Figure 4. Growth curve of microorganisms living in different thermal conditions. Inspired by Thomas et al. (2008).

5.3.5 Aeration

Microorganisms of genus *Bacillus* are aerobic. To operate metabolic synthesis for generating energy for growth, abundant oxygen in the extracellular environment is the most essential and necessary for the vegetative cells to survive. Aerobic microorganisms cannot operate their metabolic mechanisms in the absence of oxygen, because of oxygen's role of functioning as a terminal electron acceptor. During metabolic synthesis oxygen reduction products release in the intracellular medium. In fact, oxygen is toxic to aerobic microorganisms, but survival ability in the presence of defence mechanisms. Essential enzymes are the key of the defence reactions, able to convert the toxic oxygen products into feasible molecules.

A superoxide anion (O_2^-) converts by reduction reaction during the first step as the oxygen (O_2) induces into the system by interacting with cellular building blocks. The reaction is damageable to the vegetative cell, and the secondary products increase the oxygen toxicity by further reactions. The superoxide anion reacts with hydrogen peroxide within the cell, and from the reaction it releases hydroxyl radicals ($OH^{\cdot}$). The radical attacks important organic compounds in the cell and produces singlet oxygen (O_2^*), a highly reactive molecule. To be able to survive the presence of oxygen, the superoxide anion must diminish. Enzyme, Superoxide Dismutase,

converts the superoxide anion to ground state oxygen and hydrogen peroxide. Abundance of aerobic microorganisms contain the enzymes Catalase or Peroxidase in abundance to eliminate hydrogen peroxide. Catalase initiates the reaction to ground state oxygen and water by utilizing hydrogen peroxide as an oxidant and reductant. Higher concentration of Catalase and Peroxidase in the vegetative cells intend to inhibit the formation of superoxide anion by effectively reducing hydrogen peroxide to form hydrogen oxide radicals (Hentges 1996).

The aeration effect has shown that saturated oxygen in water medium inhibits the sporulation. Oxygen has a toxic effect in microbiological metabolism, due to the accumulation of hydrogen peroxide which disintegrates with the enzymes Catalase and NADH peroxidase (Sarrafzadeh et al. 2013).

Toxification of oxygen in the bacterial medium as well as intracellular, links to the various forms of oxygen molecules. Hydrogen peroxide is strongly related to bacterial growth and sporulation mechanisms. It is a highly reactive molecule, aggressively attacks organic compounds in the bacterial cells. A high concentration of hydrogen peroxide inhibits the involved enzymes to fully decompose. It consistently depends on which species of *Bacillus* is involved in the culture. The sensitivity relies on the adequate levels of NADH peroxidase and Catalase. Once the sporulation is triggered, there is no matter of regaining oxygen supply to the medium. This explains the importance of the time the microorganisms have been exposed to oxygen depletion. The optimal oxygen condition is dependent on complex metabolism, whereas the culture medium gives an indicator of the intracellular reactions, such as pH (Sarrafzadeh et al. 2013).

6. ATP bioluminescence measurement

ATP bioluminescence measurement is a method using light reaction for detecting microbiological contamination at the cellular level. Compared to examination of conventional bacterial cultures, this method is adaptable and faster. The test results from an ATP bioluminescence measurement is received within a few minutes, while bacterial and spore cultivation results are obtained after 48-hour incubation.

Adenosine triphosphate (ATP) is a vital molecule for utilizing and storing energy. The vegetative cells are dependent on the ATP synthesis as the consumed energy from the ATP molecules maintain continuous proper function. Its structure consists of three phosphate groups bonded to adenine, which is the base of a molecule. The molecule contains high energy because of electron negative charges between the phosphate groups. When ATP hydrolysis to adenosine diphosphate (ADP) and further to adenosine monophosphate (AMP) high amounts of free energy releases (Dunn & Grider 2022).

Mainly the ATP synthesis occurs in the cellular respiratory cycle in the matrix and plays major parts in other synthesis in the vegetative cells. The involvement of ATP is crucial for DNA and RNA synthesis (Dunn & Grider 2022).

The heterotrophic bacteria gain energy from organic compounds with high oxidative capacity, such as carbohydrates, lipids and proteins. In order to attain the energy, the compounds degrade through oxidation, which is involved in multiple steps in the cellular respiration. In bacterial cellular respiration there are essential processes involved first Embden-Meyerhof-Parnas pathway, secondly Krebs cycle, and thirdly membrane-bound electron transport oxidation with oxidative phosphorylation. During the process ATP molecules are synthesised, providing

energy for other important cellular activities and processes, crucial for the growth of the cell (Jurtshuk 1996).

In addition to intracellular ATP, involved in the bacterial metabolism, ATP can be found in extracellular sites. The release of ATP varies during the growth phases, infers the extracellular ATP concentration. During cell death, a rapid release of ATP occurs, besides it has also been found that vegetative cells have the ability of releasing ATP during cell growth as a survival strategy of providing energy and nutrients for adjacent bacterial communities. The release of extracellular ATP during the log phase represented 3-5% of the total ATP concentration. The peak of leaking ATP was recorded during the late log phase and the start of the stationary phase (Mempin et al. 2013). For Ihssen et al. (2021) obtained 3-5% of extracellular ATP of total ATP during early growth phase and 11-17% during death phase. It is possible that the leakage of ATP is a response of stress and damage.

Despite similar trends between the studies of Mempin et al. (2013) and Ihssen et al. (2021), the temporal patterns and peak values vary. The assumption of the observations determines the interaction of cell growth, cell lysis, active ATP secretion or uptake. During which phase extracellular ATP produces the most differences between the species and growth environment. In the stationary phase *Bacillus cereus* both the intracellular and extracellular ATP obtained a considerable drop, but for *E. coli* the extracellular ATP remained fluctuate which can be assumed of a gradual conversion to fermentation (Ihseen et al. 2021).

Kiuru (2011) reports that ATP measurement is an adequate method to determine microbiological activity. The level of free ATP, representing extracellular ATP, is relatively low. The low value of free ATP signifies the total ATP, meaning the ATP originates from the vegetative cells. Moreover, free ATP is an indication of dead cells during lysis.

Measuring ATP involves a two-step bioluminescent luciferin and luciferase reaction, the light is the measuring product of the reaction see eq. (1). Luciferase is an enzyme with a function of a catalysator, particularly in presence of magnesium ions due to implication of the conversion of D-luciferin. The enzyme generates light as a by-product in the oxidation of D-luciferin. The substrate is originally derived from North American firefly *Photinus pyralis* (Morciano et al 2017).

$$ATP + luciferin + O_2 \rightarrow oxyluciferin + AMP + PP_i + CO_2 + light \quad (1)$$

The produced quantified light is directly proportional to the ATP amount in the sample (Ivančić et al. 2008).

7. Redox potential measurement

The redox potential measurement is a method to illustrate the oxidation and reduction reactions in microbiological culture. Microorganisms utilize the environment through oxidation reactions to gain its energy. This results in a reduction in the environment, due to oxygen deficiency and production of reducing compounds. This means if a molecule has a higher electronegativity than hydrogen, the molecule has a positive redox potential. The molecule is capable of accomplishing an oxidation (Reichart et al. 2007). The redox potential and its electric charge are expressed in Nernst equation, see eq (2) and (3) (Adams & Moss 1995).

$$[Oxidant] + [H^+] + ne^- \rightarrow [Reductant] \quad (2)$$

$$Redox\ potential = E_0 + \frac{R \times T}{n \times F} \times ln \frac{[Oxidant] \times [H^+]}{[Reductant]} \quad (3)$$

E_0 is the normal redox potential in the system, *R* is the gas constant, *T* is temperature, *n* is the number of electrons, *F* is Faraday's constant.

Nernst equation expresses that the concentration of hydrogen ions affects the redox potential. The association between pH and redox potential can be explained, for every unit of pH increases the redox potential decreases. A positive redox potential reflects a low pH (Adams & Moss 1995).

Although the redox potential is a complex indicator of microbiological contamination, it is a highly useful tool for qualitative and quantitative determination. The redox potential decreases throughout the bacterial growth and the shape of the curve are specific to the speciesde of microorganism. Redox potential profiles can identify the metabolic changes in the microbiological culture and its life phases (Reichart et al. 2007). Higareda et al. (1997) investigated the influence of glucose and glutamine depletion through redox potential profiles. The results demonstrated that glucose and glutamine depletion is directly related to the oxygen uptake rate by the microbiological culture as it resulted in considerable decrease. (Adams and Moss 1995) explains that oxygen is a powerful oxidizing agent, which results in a positive redox potential. The concentration itself is a parameter that affects the redox potential. In the initiation of a metabolism, the redox potential is high, see figure 5. This trend could be observed in redox potential rate and measurements of dissolved oxygen. The oxygen uptake can be further determined through distinction of glucose and glutamine depletion. This method can be used to further determine the growth phases. A sudden mark of depletion can be marked as the beginning of the death phase (Higareda et al 1997).

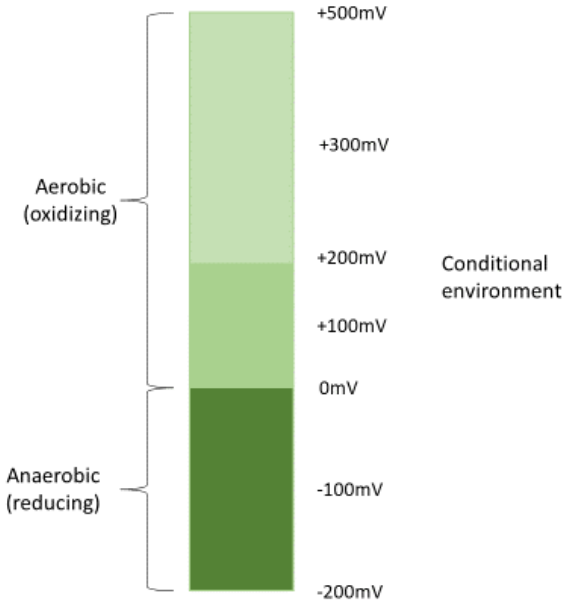

Figure 5. Redox potential intervals of aerobic and anaerobic bacteria based on information by Adams and Moss (1995).

During log phase, the substrate consumption and redox potential is opposite correlated to the oxygen uptake. Lee et al. (1998) reported that the redox potential behaviour correlates with metabolic changes, which is thoroughly associated with microbiological growth. The bacterial growth requires favourable environmental conditions to promote degradation of nutrients. The oxygen supplement inhibits the degradation; however, the oxygen was found to enhance the growth instead as the degradation was completely related to the bacterial concentration (Shrout & Parkin 2006).

8. Multivariate analysis

In this master thesis it requires a statistical and analytical tool to handle all extensive data and target the most valuable, because forming simple diagrams for each process sample for all the process and quality parameters takes far too long a time to analyse. Therefore, there are more sophisticated tools that have the ability of simplifying work, and to understand certain trends and conjunctions between the chosen variables through correlations. In this case assemble a correlogram is optional to illustrate the extent of correlation between multiple variables (Renberg 2001).

9. Material and method

9.1 Methodology

Laboratory work was performed at Stora Enso. The laboratory work consisted of sample testing and was performed for 8 weeks.

The broke system has constantly been a question regarding being the source of high microbiological activity and spore values. The long circulation is tightly closed which causes accumulation of nutritious substances and conditions the system operates in favour of the microorganisms and its growth. The study has been limited to 15 process positions in the broke system to observe any early microbiological development throughout the system. In table I, a short motivation for each process position is presented in order to gain understanding of the situation this study revolves around.

Table I. A short motivation of the chosen process positions in broke system.

Process position	**Motivation**
Pulper 1	Mostly stationary → oxygen depletion, malnutrition, biofilm→ spores
Pulper 2	Mostly stationary → oxygen depletion, malnutrition, biofilm→ spores
Pulper 3	Mostly stationary → oxygen depletion, malnutrition, biofilm→ spores
Pulper 4	Mostly stationary → oxygen depletion, malnutrition, biofilm→ spores
Pulper 5	Mostly stationary → oxygen depletion, malnutrition, biofilm→ spores
Pulper 6	Mostly stationary → oxygen depletion, malnutrition →biofilm→ spores
Broke tower	Large volume tank → favourable environment, biofilm → microbiological activity

Broke chest 1	Continuous line from pulpers and broke tower→ favourable environment, biofilm→ microbiological activity
Broke chest 2	Continuous line from pulpers and broke tower → favourable environment, biofilm → microbiological activity
Disc filter	Continuous line from pulpers and broke tower →favourable environment → microbiological activity
Broke refiner	Continuous line from pulpers and broke tower →favourable environment → microbiological activity
Centre layer chest 1	Continuous line from pulpers and broke tower →favourable environment → microbiological activity
Centre layer chest 2	Continuous line from pulpers and broke tower →favourable environment → microbiological activity
White water tower 1	Nutritious water, favourable environment → microbiological activity
White water tower 2	Nutritious water, favourable environment → microbiological activity

The initial part of the laboratory work was to hold interviews with the staff to gain additional insight in production, daily routines and work, and situation of the board machine. The motivation of each selected process sample conveys from the gathered knowledge for long and short circulation implicated with the information of microbiology. Each process sample has its unique function in the process resulting in specific mediums for the microbiological growth.

9.2 Laboratory structure

All process positions were collected in sterile bottles, and all following process positions were tested according to the table II below.

Table II. Each process position is marked with an "X" for each examined parameter.

Process positions/Tests	**Redox potential**	**ATP measurement**	**Temperature**	**pH**	**TOC**	**Tank level**	**Storage experiment**	**Bacterial and spore cultivation**
Pulper 1	x	x	x	x				x
Pulper 2	x	x	x	x				x
Pulper 3	x	x	x	x				x
Pulper 4	x	x	x	x				x
Pulper 5	x	x	x	x				x
Pulper 6	x	x	x	x				x
Broke tower	x	x	x	x		x	x	x
Broke chest 1	x	x	x	x		x	x	x
Broke chest 2	x	x	x	x		x	x	x
Disc filter	x	x	x	x				x
Broke refiner	x	x	x	x				x
Centre layer chest 1	x	x	x	x				x
Centre layer chest 2	x	x	x	x				x
White water tower 1	x	x	x	x	x			x
White water tower 2	x	x	x	x	x			x

9.3 ATP bioluminescence measurement

Testing the ATP concentration was performed with a Clean-Trace™ Luminometer LM1 3M, see figure 6, and with a reagent kit Clean-Trace™ Biomass kit 3M. A-diluent was mixed with B-reagent for establishing the reconstructed Enzyme. 100µL of a sample was pipetted into a cuvette, and 100 µL of Extractant XM. The mixture was gently mixed for 2 seconds and was set at a minimum 60 seconds. 100 µL of reconstructed Enzyme was pipetted into the mixture and was swirled for 2 seconds. The cuvette was inserted into the chamber of the luminometer for measuring the total ATP amount. The analysed values were measured in RLU (Relative light unit).

Figure 6. ATP bioluminescence instrument.

The kit was stored in a refrigerator between the usage. It is important that the kit is at room temperature during the procedure.

9.4 Redox potential measurement

The redox potential was measured on all process positions with METTLER pH 12 213, see figure 7. The electrode was placed in each process sample in 60 seconds. The unit for this analysis is mV.

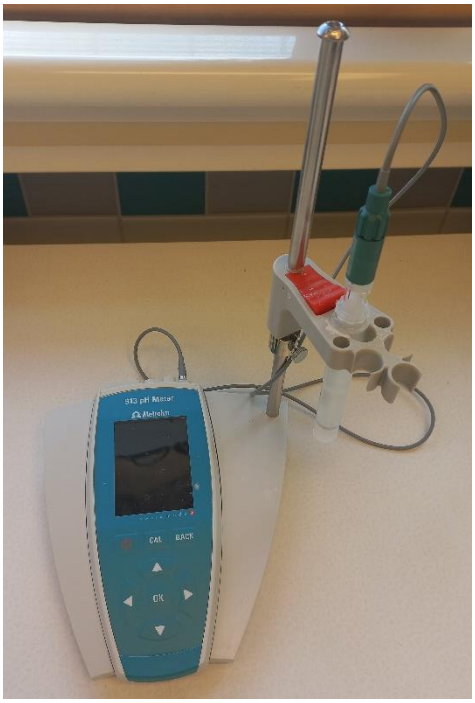

Figure 7. Instrument for redox potential measurements.

9.4.1 Reproducibility of ATP measurement and Redox potential measurement

ATP measurement is a material demanding method, because of the high number of investigated process positions the statistical analysis was limited. Each laboratory session was time demanding, including collecting process positions, measurement of redox potential, total ATP, temperature, pH, bacterial and spore cultivation and counting total bacterial and spore colonies. Therefore, statistical analysis was performed separately to incorporate a statistical foundation in this study. The redox potential measurement was involved as well. This investigation was performed lastly after circa 8 weeks of data sampling. Three process positions for this experiment were selected based on which obtained highest and lowest ATP values. Disc filter obtained overall the highest total ATP amount, see figure 10, in contrast of white water tower 1 with lowest total ATP amount. The third component was broke chest 2 with a stable variation. 10 total ATP and redox potential measurements were performed on each sample and coefficient of variation was calculated, see eq. (4).

$$CV(\%) = \frac{standard\ deviation}{average} \tag{4}$$

9.5 Temperature

The temperature was measured on all process positions with a VWR calibrated electronic thermometer with stainless steel stem.

9.6 pH measurement

For all process positions the pH level was measured with a METTLER FiveGo F2 Toledo pH-meter. The pH-meter was calibrated with calibration solutions of pH 4 and 7 with 102% linearity.

9.7 Retention time

For calculation of retention time, see eq. (5) below, a limitation of the system was taken in consideration due to the complexity of the long circulation system. The broke tower incorporates a fundamental element of the hypothesis, consequently the analysis was narrowed to broke tower, broke chest 1 and 2. The data of fibre concentration, tank level, total volume and fibre flow for each process sample was collected from the internal program system.

$$Retention\ time = \frac{total\ volume[m^3] \cdot \frac{fibre\ conc.[\%]}{100} \cdot \frac{tank\ level[\%]}{100}}{fibre\ flow\ [\frac{tonne}{hour}]} \tag{5}$$

9.8 Total organic carbon

TOC is a measure of total organic carbon samples, possibly giving an indication of possible nutrient is starch and other additives in the white water system is nutrient access to the microbiological activity.

Total organic carbon (TOC) was measured in white water tower 1 and 2, according to SS-EN 1484:1997, with the instrumentation seen in figure 8. The total concentration of organic carbon was presented in mg/L.

Figure 8. Instrument of TOC

9.9 Board sample cultivation, cultivation temperature 37°C

This method was achieved according to SCAN-C 60:02. The number of spores were presented in CFU/g.

9.10 Bacterial and spore cultivation

All glass material was sterilized by autoclaving in 121°C with a pressure of 1,2bar. The spore cultivation was set up in a serial dilution with a factor of 0; 0,5 and 10^{-1}. The bacterial cultivation was set up in a serial dilution with a factor of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}. All process positions were diluted with Ringers solution with the specific amount to achieve the dilution factor of circa 15%. The Ringers was prepared with 1 tablet of Ringers in 500mL deionized water.

Process positions from broke chest 1 and 2, disc filter, pulper 2, 3, 5 and 6, centre layer chest 1 and 2, broker tower, and broke grinder acquire higher fibre concentration. These process positions required additional dilution of a diluent, to facilitate separation between fibres and microbiological consistency. For preparation of diluent, 1 drop of Tween80 (Monooleate polyoxyetylenesorbitan) in 500 mL flask of Ringers solution. The dilution factor was obtained between 8 to 20. For each microbiological solution and dilution, 1 mL was pipetted onto aerobic count plate, petrifilm. Afterwards, the petrifilms were incubated for 48 hours in 32°C. This method was performed according to SCAN-C 60:02. The total bacterial and spore concentration was measured in CFU/mL.

9.11 Broke tower simulation

Broke tower level and retention time is a hypothesis to be a possible issue of high bacterial and spore level, and with this method it strengthens the hypothesis. Broke pulp was collected in 14 sterile bottles for each storage day. The bottles were stored in an incubator of 40°C, see table III. The temperature was set due to the observed process temperature of the broke tower.

Table III. Presentation of storage temperature and days for broke tower simulation.

	Temperature (°C)	**Storage days**
Broke tower pulp	40	0 to 8

For each day two sterile bottles were taken out and measurements of ATP, redox potential and pH were performed, as well as bacterial and spore cultivation was carried out. The measurements were repeated 6 times each and a 95% confidence interval was calculated.

9.12 Correlation analysis between microbiological measurement and process parameters

The correlation analysis was separated into 15 calculations for each process position, as the environment for each is unique and a higher number of collected data causes incorrect analysis. Totally 18 samples were analysed. The correlograms were formed in program MATLAB 9.12.0.1884302 (R2022a) with function [R,PValue]=corrplot(tabl). The figure below shows a correlogram of the broke tower. All variables for broke tower were correlated with each variable forming a completion of the assembled results. The correlation coefficient *R* shows in the chart, see figure 9, an example of obtained correlations. The Pearson correlation coefficient, p-value, can be included in the analysis to investigate significance of R. The obtained correlation coefficients in the correlograms are the essential part of analysis. Nonetheless, the coefficient of determination R^2 is a more familiar variable. R^2 measures the prediction of an outcome in a linear regression. The lines seen in each bivariate correlation is the regression line. R^2 is squared R, a higher value of R represents a higher R^2. A negative indication sign in correlogram indicates an increase of the variable (x-axis) results in a decrease in the represented process parameter (y-axis). A decrease of the variable results in an increase of the process parameter gives a positive indication sign.

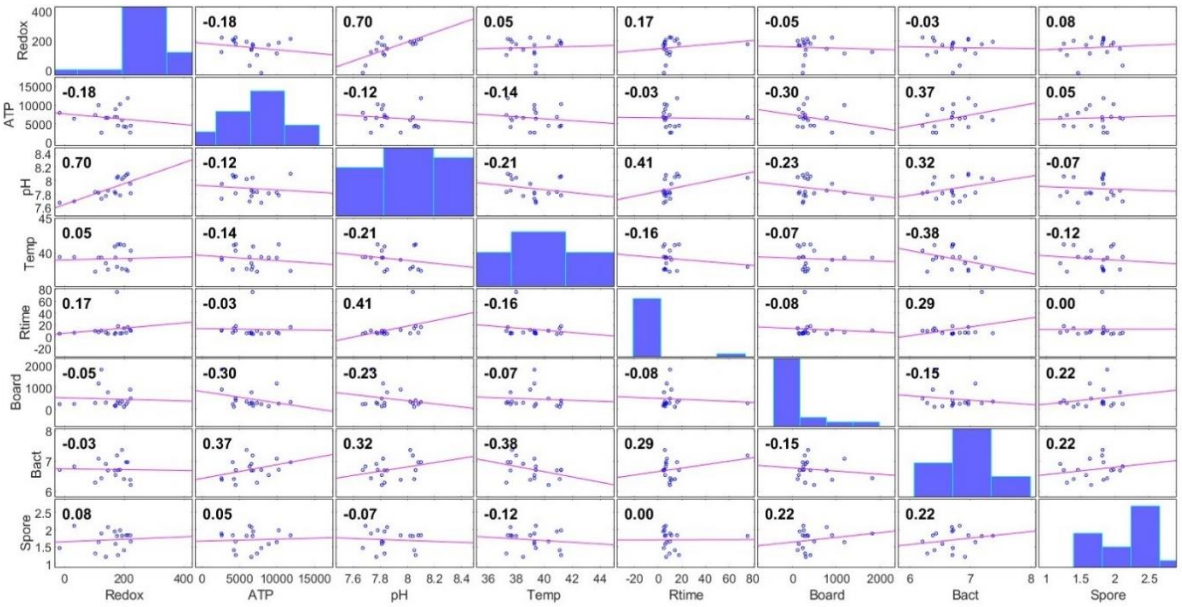

Figure 9. An example of a correlogram of broke tower.

The correlation analysis was achieved for each process position. The focus was the correlation for total ATP, redox, potential, process variables with total bacterial and spore concentration.

10. Results

10.1ATP bioluminescence measurement

For ATP bioluminescence measurement, there is a minimal correlation between board samples, total bacterial and spore concentration, see in table IV.

Most process positions obtained positive correlation in relation to the total bacterial concentration. This indicates that an increase in total ATP correlates with an increase in total bacterial concentration. Despite this, pulper 2, pulper 5, centre layer chest 2 obtained a negative correlation, a contradiction to the principle and significance of ATP measurement. In relation to spore concentration, the negative correlation is obtained in the majority of the process positions. Pulper 5 obtained the highest correlation, however the correlation is positive, indicating an increase in total ATP signifies an increase in spore amount. This contradicts the obtained positive correlation between total ATP and the total bacterial concentration. The correlations give an unclear illustration in which process position the induction of microbiological activity.

In table IV, it shows that ATP values decrease in correlation to board samples in most of the process positions. This signifies that the total ATP values are smaller than the spore amount that is present in board samples.

Table IV. Table presents the correlation coefficient (R) for each process position between bioluminescence ATP measurement with board sample at 37°C, total bacterial concentration, spore concentration.

Position/Correlation coefficient (R)	**Total ATP/Board 37°C**	**Total bacterial conc. /Board 37°C**	**Spore conc./Board 37°C**	**Total ATP/Total bacterial conc.**	**Total ATP/Spore conc.**
Pulper 1	-0,1	-0,2	0,5	0,2	-0,3
Pulper 2	0,0	-0,1	-0,1	-0,4	-0,1
Pulper 3	-0,1	0,0	0,0	0,4	0,1
Pulper 4	0,1	0,2	0,1	0,0	-0,2
Pulper 5	-0,1	-0,5	0,2	-0,5	0,5
Pulper 6	-0,3	-0,2	-0,3	0,0	0,0
Broke tower	-0,3	-0,2	0,2	0,4	0,1
Broke chest 1	0,0	0,4	-0,2	0,2	-0,4
Broke chest 2	-0,2	-0,3	-0,1	0,2	-0,4
Disc filter	-0,1	0,0	0,3	0,0	-0,1
Broke refiner	-0,1	-0,3	0,6	0,2	-0,4
Centre layer chest 1	0,0	-0,4	0,0	0,4	-0,1
Centre layer chest 2	0,0	-0,1	0,2	-0,1	0,1
White water tower 1	-0,1	0,2	0,2	0,3	-0,1
White water tower 2	-0,1	0,1	0,5	0,2	-0,1

The total bacterial concentration correlated with board samples shows similar results, however the obtained results do not strengthen the significance of ATP measurement due to low correlations. In pulper 5, it is shown that the correlation between total ATP and board samples is -0,1, while the correlation between total bacterial concentration and board samples is -0,5. It was expected to obtain an amplification in correlation between total ATP and board samples, and presumably give an indication of the impact the microbiological activity might have in relation to the final product.

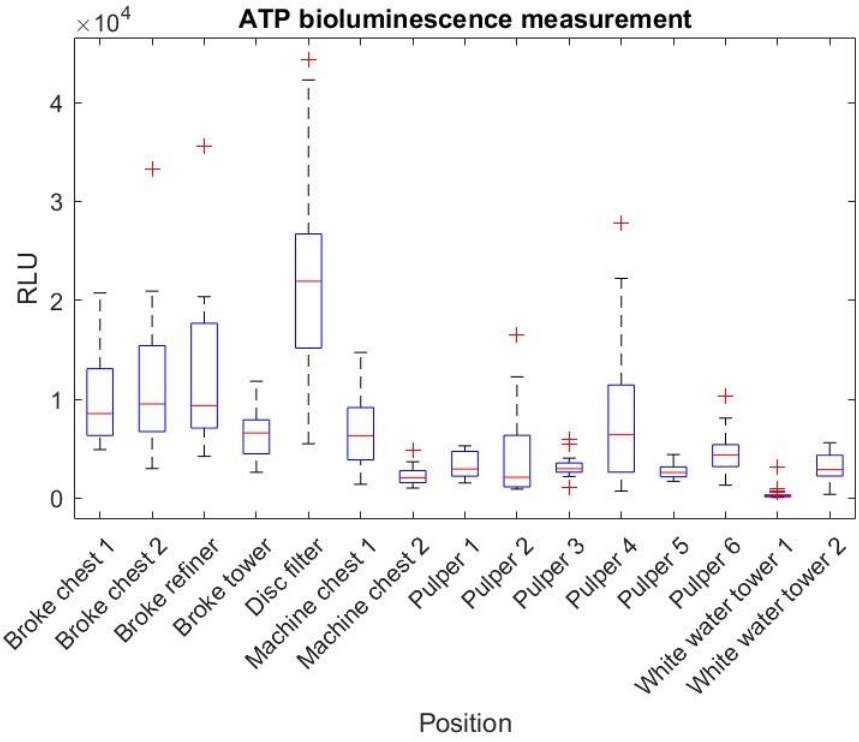

Figure 10. Boxplot of ATP measurement for each process position.

Boxplot for ATP measurements, see figure 10, shows the interval each position obtained. The red crosses represent outliers and are shown in most of the process positions, and the boxes representing the interquartile range. The aim was to obtain variations to observe the influence it might have on the correlations. This is due to process variations possibly having an underlying effect on the data, therefore eliminating outliers is not relevant. The box plot illustrates that the disc filter has the highest variation, its median at 2,3 10^4 RLU. The rest of positions attained a median under 10^4 RLU.

10.2Redox potential measurement

The results in each position obtained low correlations between the total bacterial and spore concentration. Redox potential is used to illustrate the environment for bacterial growth. A high value of redox potential indicates a high oxidative environment for the bacteria, as the metabolism relies on oxidative molecules. The bacterial culture endeavours to transform to a reducing environment through its metabolism in the specific environment. The negative correlation, see table V, indicates a higher total bacterial concentration gives a lower redox potential. Pulper 2 has a noticeable correlation. The correlation between redox potential and spore concentration is mostly positive in abundance of process positions. However, the correlation is not strong in any of the positions which indicates an unclear conclusion regarding the contradiction.

Table V. Table presents the correlation coefficient (R) for each process position between redox potential with total bacterial and spore concentration and total ATP.

Position/Correlation coefficient (R)	**Redox potential/Total bacterial conc.**	**Redox potential/Spore conc.**	**Redox potential/Total ATP**
Pulper 1	-0,2	0,3	-0,3
Pulper 2	-0,4	-0,4	0,1
Pulper 3	-0,1	0,2	-0,2
Pulper 4	-0,2	-0,1	-0,2
Pulper 5	0,2	0,2	0,1
Pulper 6	0,2	0,0	-0,2
Broke tower	0,0	0,1	-0,5
Broke chest 1	-0,2	0,1	-0,5
Broke chest 2	-0,3	0,1	-0,6
Disc filter	-0,1	0,1	-0,3
Broke refiner	-0,3	-0,1	-0,3
Centre layer chest 1	0,2	0,1	-0,1
Centre layer chest 2	0,2	0,5	0,2
White water tower 1	-0,2	0,1	0,2
White water tower 2	-0,3	0,2	-0,3

The negative correlation between redox potential and total ATP shows an increase in total ATP signifies a decrease in redox potential. The highest correlation was obtained in broke chest 1 and 2 and broke tower.

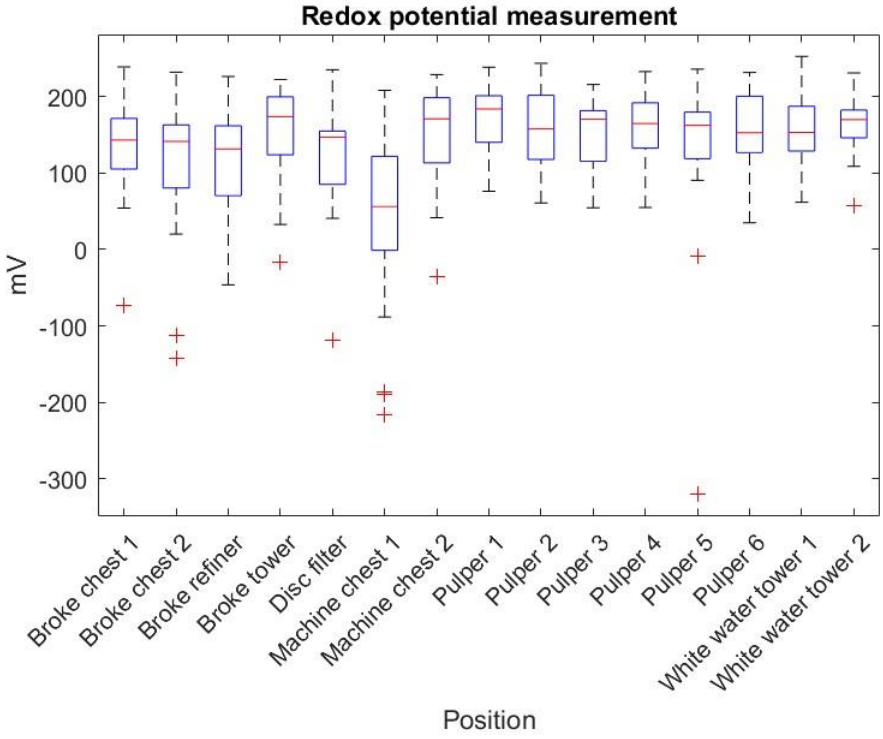

Figure 11. Box plot of redox potential measurement for each process position.

All the outliers shown in the box plot, see figure 11, are negative in redox potential, could reveal an inconsiderate environment for the microbiological growth. Possibly the outliers give prognose of spore formation in the process position. Overall, the redox potential is attained in an interval of 200 to 50mV.

10.3Reproducibility of ATP and redox potential measurement

Coefficient of variation (CV) is a comparable measurement between separate methods. The reproducibility of ATP measurement obtained with average of 10,8%, while redox potential measurement obtained of 17,2%, see table VI.

Table VI. Reproducibility of ATP bioluminescence and redox potential in three chosen process positions.

CV (%)/Process positions	**Broke chest 2**	**Disc filter**	**White water tower 1**	**Average**
Redox potential	32,6	10,4	8,52	17,2
ATP measurement	11,5	5,76	15,0	10,8

10.4Source of affecting microbiological activity

Finding the essence of microbiological activity in the board process is rather complex due to all variables, including a suitable environment that inhibits the spore concentration to grow throughout the process. TOC, retention time, pH and temperature were examined in this study. The obtained results indicate there is a low correlation between the examined variables and the total bacterial and spore concentration.

The correlation between total bacterial concentration and pH, see table VII, in most of the process positions giving a positive correlation. This indicates that a higher pH features an increase in the total bacterial amount. The highest correlation is obtained in pulper 2, and the lowest is in pulper 4. For spore concentrations, the correlation is negative, meaning that a higher pH lowers the risks of sporulation. Pulper 4 indicates to attain lowest risk of spores while pulper 5 to have the highest.

An interesting aspect is the correlation between board samples and pH, in relation to the correlation between spore amount and pH. The correlation between board samples and pH is mostly negative in most positions, meaning an increase in pH and the drying section in the board machine are considerable effects of eliminating vegetative cells in the product.

Table VII. Table presents the correlation coefficient (R) between pH with the total bacterial and spore concentration, as well as correlation between board samples.

Position/Correlation coefficient (R)	**Total bacterial conc./pH**	**Spore conc./pH**	**Board sample 37°C/pH**
Pulper 1	0,5	-0,2	-0,7
Pulper 2	0,6	-0,1	-0,2
Pulper 3	0,4	0,3	-0,1
Pulper 4	0,0	-0,4	-0,6
Pulper 5	0,0	0,3	-0,6
Pulper 6	-0,3	-0,1	0,3
Broke tower	0,3	-0,1	-0,2
Broke chest 1	0,1	0,1	-0,4
Broke chest 2	0,0	-0,2	-0,3
Disc filter	-0,4	-0,3	-0,3
Broke refiner	0,2	-0,1	-0,3
Centre layer chest 1	0,4	-0,2	-0,6
Centre layer chest 2	0,4	0,2	-0,4
White water tower 1	-0,2	-0,5	0,1
White water tower 2	-0,2	-0,2	-0,4

The boxplot for pH, in figure 12, shows that the environment throughout the broke system is alkaline. Outliers are obtained in disc filter, machine chest 1, pulper 1, pulper 2, pulper 4, pulper 5; meaning that the rest of positions acquire stable conditions.

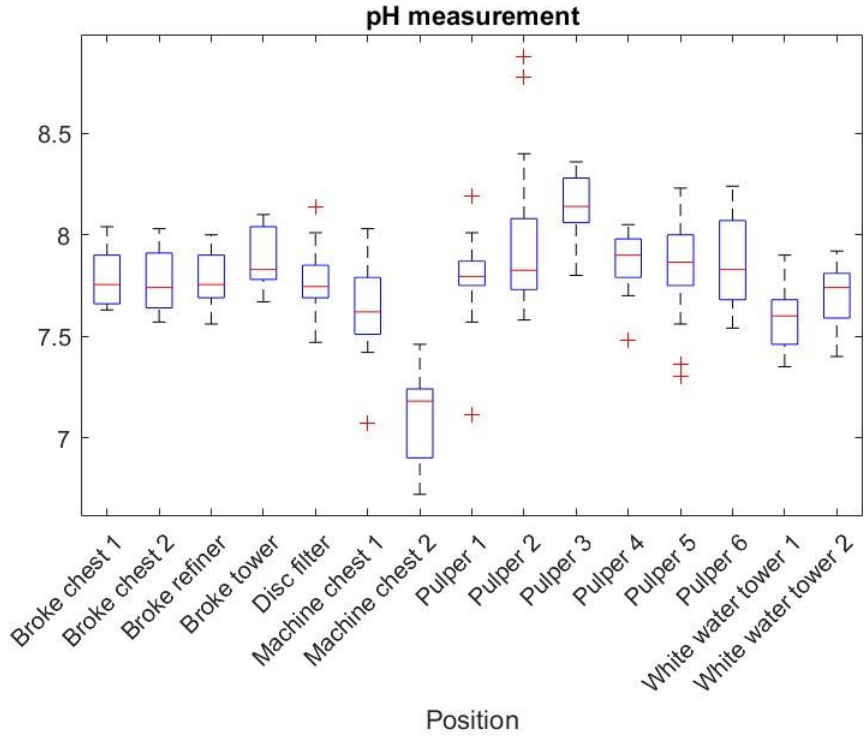

Figure 12. Box plot of pH measurement for each process position.

A higher temperature lowers the total bacterial concentration, which the negative correlation between the total bacterial concentration and temperature indicate, see table VIII. Pulper 1 obtained the highest negative correlation. While pulper 2 and white water tower 1 obtained the highest positive correlation, which signifies an increase in temperature intensifies the risk of microbiological activity. Most positions obtained a negative correlation between spore concentration and temperature. This indicates that the risks of sporulation decrease by increase of temperature.

Table VIII. Table presents correlation coefficient (R) between temperature and total bacterial and spore concentration in each position.

Position/Correlation coefficient (R)	**Total bacterial conc./Temperature**	**Spore conc./Temperature**
Pulper 1	-0,5	-0,4
Pulper 2	0,5	0,1
Pulper 3	0,1	-0,3
Pulper 4	0,9	0,6
Pulper 5	0,1	0,0
Pulper 6	-0,2	-0,2
Broke tower	-0,4	-0,1
Broke chest 1	-0,1	-0,1
Broke chest 2	-0,2	-0,2
Disc filter	0,3	0,4
Broke refiner	-0,3	-0,5
Centre layer chest 1	-0,2	0,3
Centre layer chest 2	-0,3	-0,3
White water tower 1	0,5	0,2
White water tower 2	0,3	-0,1

The temperature measurement seen in figure 13 below, shows the temperature ranges and outliers for all positions. The highest temperatures were obtained in broke refiner and machine chest 1, while the lowest in pulper 6 and white water tower 1.

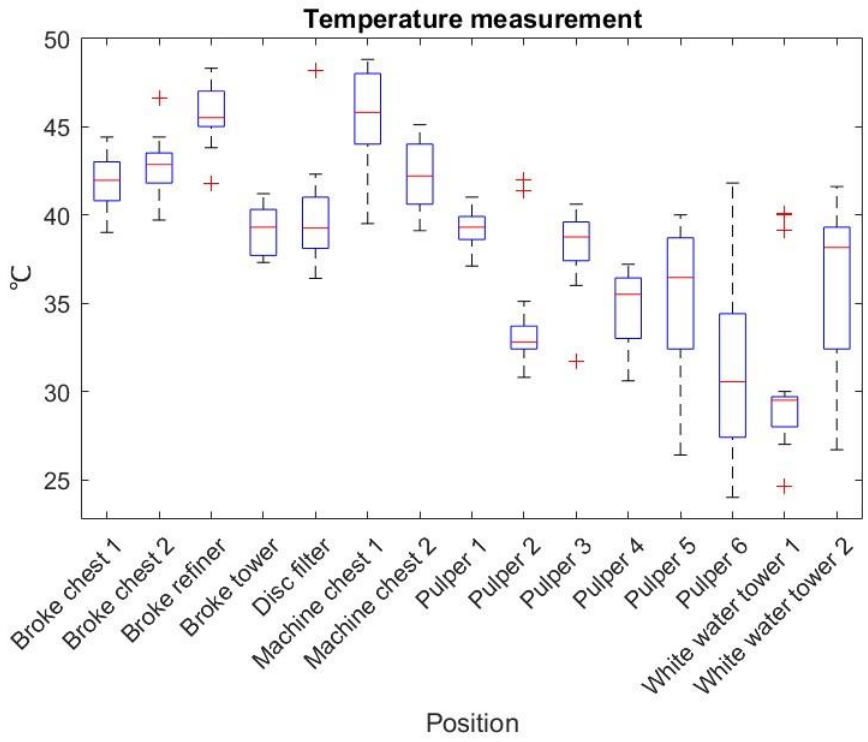

Figure 13. Box plot of temperature measurement for each position.

The microbiological activity increases the longer the culture rests in the towers, see table IX, more interestingly in the broke tower. Presumably the large volume has an impact on this parameter. The retention time does not affect the spore concentration in broke tower. In machine chest 2 intends to attain the increasing influence, though the influence on the spore concentration is similar.

Table IX. Table presents the correlation coefficient (R) between retention time with total bacterial and spore concentration in each position.

Position/Correlation coefficient (R)	**Total bacterial conc. /Retention time**	**Spore conc./Retention time**
Broke tower	0,3	0,0
Machine chest 1	0,0	0,0
Machine chest 2	0,2	0,2

The variation of retention time shown in the box plot, see figure 14. The retention time in broke chest 1 and 2 are low in comparison to the broke tower. The retention time in broke tower is median 8 hours, while in the broke chests less than 1 hour.

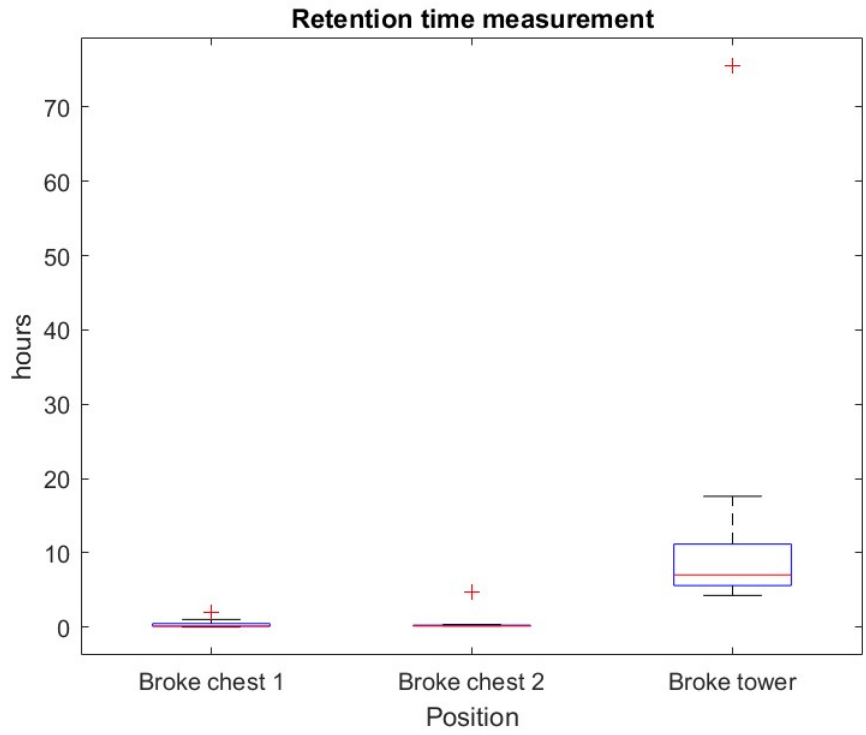

Figure 14. Box plot for retention time for broke chest 1 and 2 and broke tower.

The correlations between spore concentration and TOC, see table X are negative in both white water towers, meaning the risks of sporulation lowers by the increasing amount of organic carbon in the medium. White water tower 1 obtained the highest correlation between the spore concentration, in contrast to whiter water tower 2 obtained highest correlation between the total bacterial concentration. This means that the microbiological culture favours a nutritious environment.

Table X. Table with presented correlation coefficients between TOC with total bacterial and spore concentration.

Position/Correlation coefficient (R)	**Total bacterial conc./TOC**	**Spore conc./TOC**
White water tower 1	0,0	-0,4
White water tower 2	0,2	-0,2

The box plot below, in figure 15, presents the data of TOC in white water tower 1 and 2. Outliers are shown in both positions despite the differences in the concentration of organic carbon in the white water.

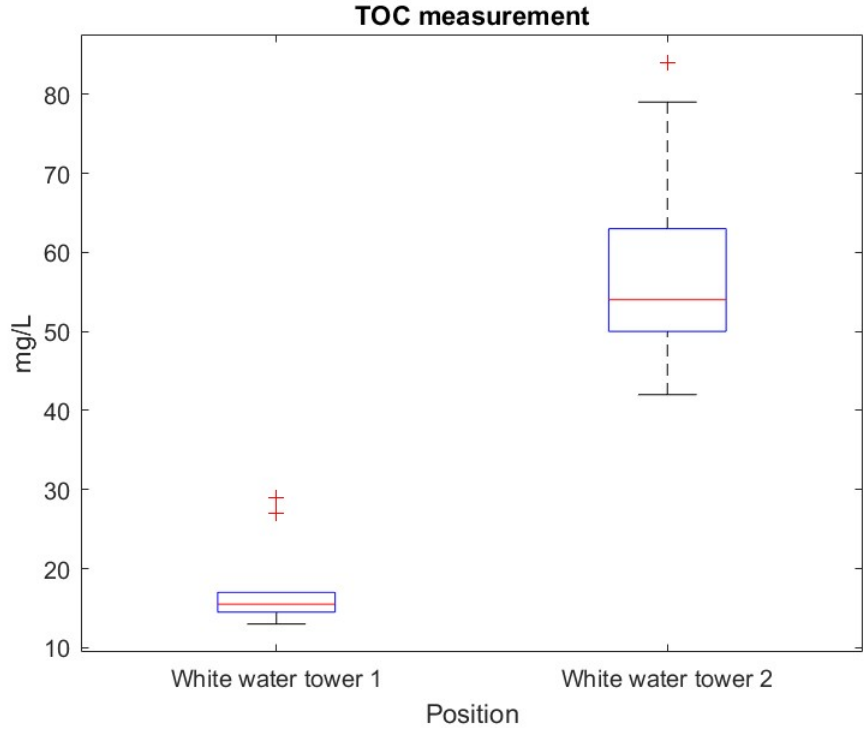

Figure 15. Box plot of TOC measurement for each process position.

Broke chest 1 and 2, broke refiner, broke tower, disc filter and machine chest 1 obtained in a higher median of the total bacterial concentration, see figure 16, indicating of a higher microbiological activity, while pulpers obtained in a lower range.

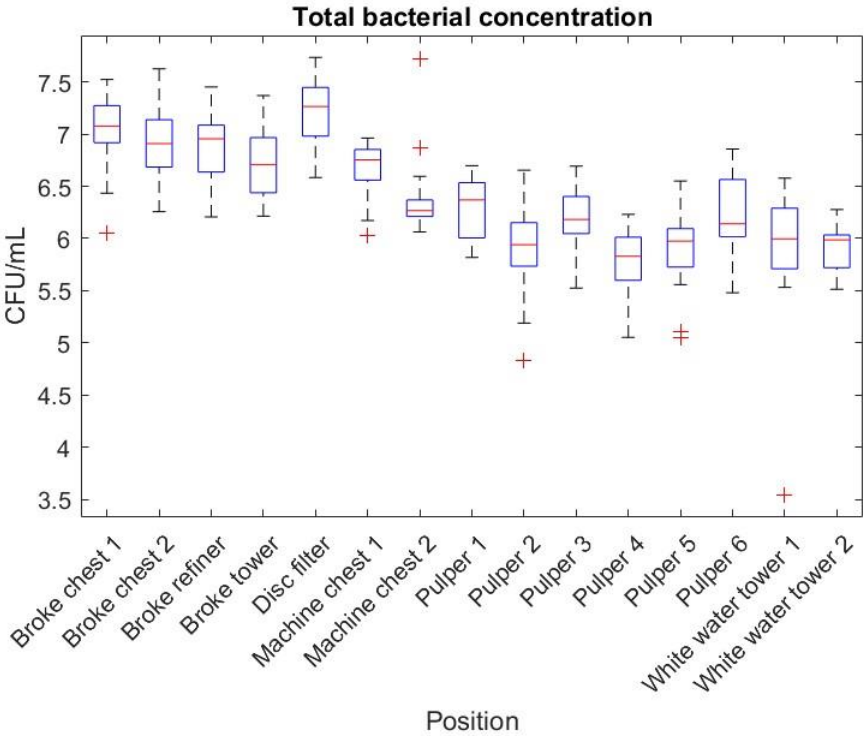

Figure 16. Box plot of log10 total bacterial concentration for each process position.

A similar trend as in the total bacterial concentration is shown in spore concentration as well, shown in box plot below, see figure 17. Pulper 1 obtained the highest number of outliers. The interquartile illustrates there is a small variation in obtained spore amount.

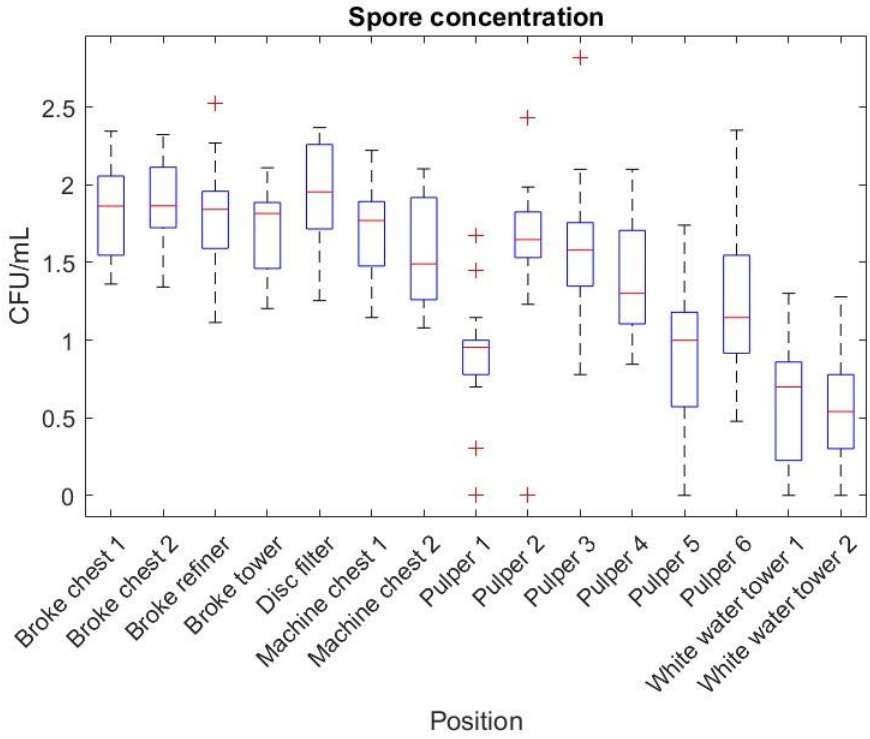

Figure 17. Box plot of log 10 spore concentration for each process position.

10.5 Broke tower simulation

Broke tower simulation was a separate study to strengthen the hypothesis about the influence of broke tower potentially have on microbiological activity. Bacterial and spore cultivation, ATP measurement, redox potential and pH were examined to prove the hypothesis. The broke pulp was stored in full sterile bottles in 8 days, a long period of time compared to the actual retention time and tank level. The total ATP followed approximately the growth phase of bacterial concentration, see figure 18. In figure 19, the most interesting feature was day 7 and 8, illustrating the drop in total ATP value as the spore concentration increased.

The bacterial concentration and total ATP are considered to approximately follow the growth cycle. The issue that the curves do not follow accordingly can be dependent on different causes.

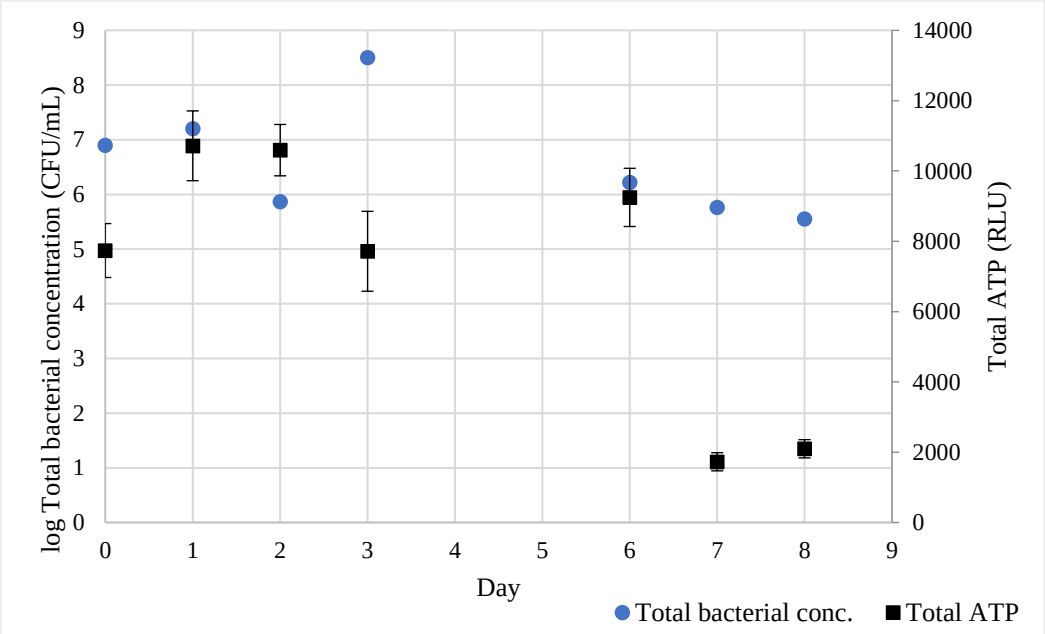

Figure 18. Total bacterial and total ATP concentration in broke tower simulation, 95% confidence interval. Blue and circular plot: total bacterial concentration, Black and square plot: total ATP.

The growth phases are not clear as expected. For the bacterial concentration, day 0 to 2 is intended to signify the lag phase. However, an unusual deviation is observed on day 2. The bacterial concentration should not decrease in this case. Day 3 to 5 indicates the stationary phase, meaning the maximum cell density and nutrient accessibility is achieved in the broke mass. During day 4 and 5, was during a weekend which hindered to performing any measurements, this could have given enhanced illustration of the evolvement on the measurements.

The values between these days should not shift significantly as it does between day 2 and 3. Day 6 to 8, the observations are clear of a death phase. For total ATP, the curve does have a clearer form than the bacterial concentration, despite day 3 showing a deviation. The curve is intended to increase, as day 3 to 5 designate the stationary phase. Day 7 and 8 shows that a death phase occured in the sterile bottle.

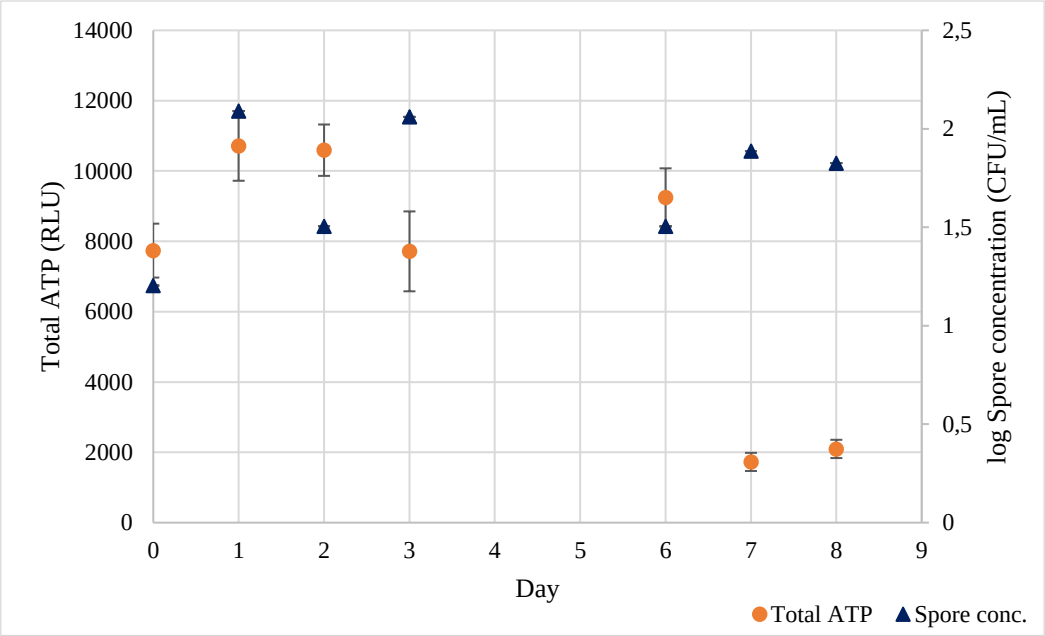

Figure 19. Total ATP and spore concentration in broke tower simulation, 95% confidence interval. Blue and triangular plot: spore concentration, Orange and circular plot: total ATP.

Figure 20 showcases a clear development of redox potential in the storage simulation. The redox potential drastically decreased for each storage day. The development indicates oxidizing rate the bacterial culture contributes, as the redox potential represents the amount of nutrients that is available. During the storage day the nutrients are consumed, indicating that a bacterial growth occurred. During day 6 to 8 indicates a death phase of the bacterial culture which assumes the redox potential rate due to the stagnation illustrated in the figure.

The confidence intervals in day 2 and 3 do not show any significant difference, the uncertainty is considerable. Though day 6, 7 and 8 shows a significant difference, which indicates that the redox potential is rather approved according to the hypothesis.

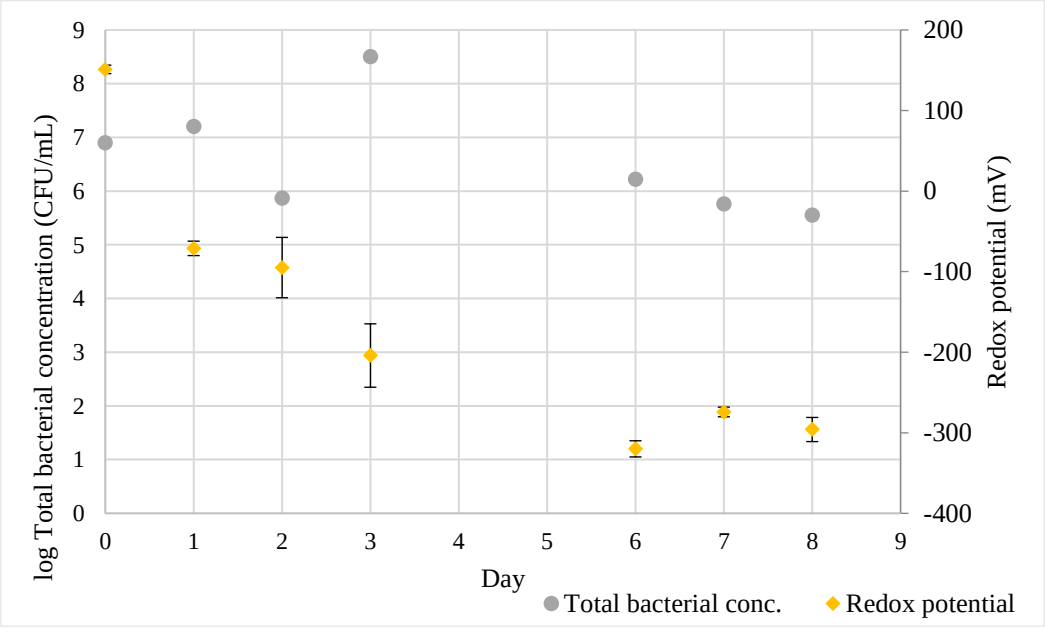

Figure 20. Total bacterial concentration and redox potential rate in broke tower simulation, 95% confidence interval. Grey and circular plot: total bacterial concentration, Yellow and squared plot: redox potential

The spore concentration for each day, see figure 21, is rather uncertain due to the high variation between day 1, 2 and 3 showing a decrease and then an increase in spores. The same trend is shown in day 6, 7 and 8. As the redox potential decreases for each day, as the nutrients become limited, the sporulation becomes for significant, following the accordance of the bacterial concentration.

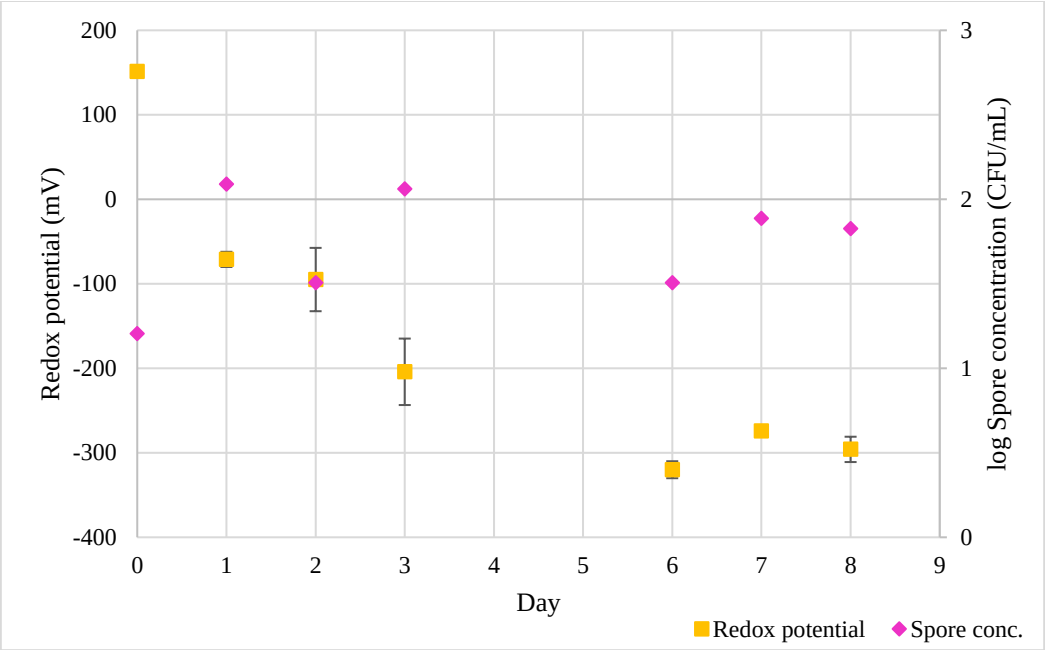

Figure 21. Spore concentration and redox potential rate in broke tower simulation, 95% confidence interval. Yellow and squared plot: redox potential, Pink and rhombus plot: spore concentration.

The pH rate is obtained in figure 22 and 23 for respectively bacterial and spore concentration. The pH rate has not evolved as considerably as redox potential. The major pH drop, from pH 8 to 6,6, occurred in between day 0 and 2. The pH level was stabilized during the days after. The bacterial amount increased simultaneously as the pH level was reduced, similar trend was exhibited by the spore concentration.

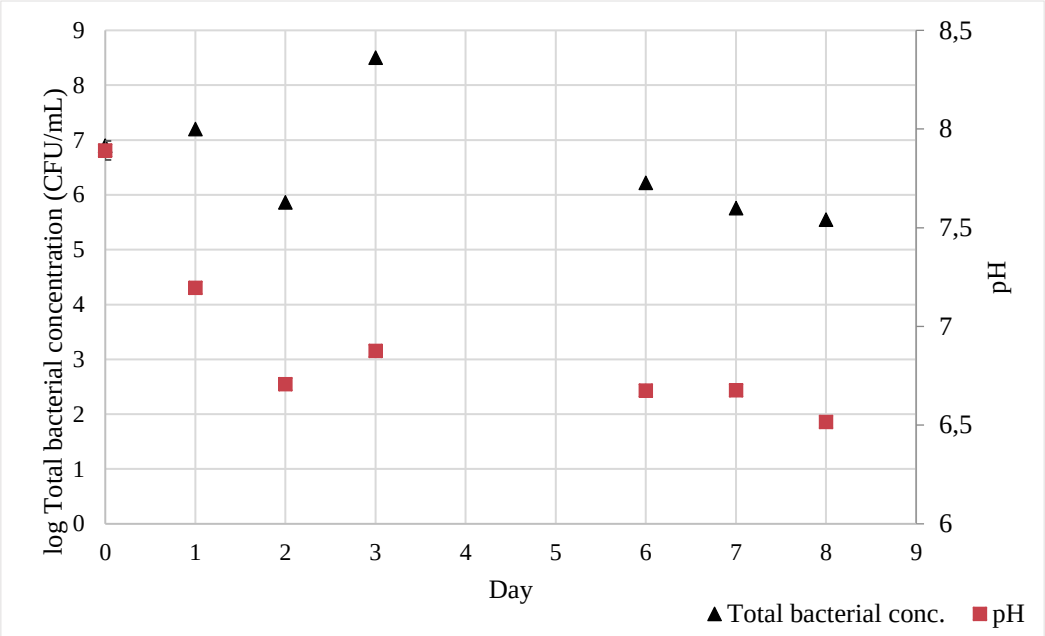

Figure 22. Total bacterial concentration and pH rate in broke tower simulation, 95% confidence interval. Black and triangular plot: total bacterial concentration, Red and squared plot: pH

The sporulation was triggered from day 1, see figure 23. Though the certainty is questionable due to contradicted variation between day 2 and 3, as well as between day 3 and 6.

No variations in the confidence intervals of 95% occurred in pH measurements, meaning the medium is stable to this variable. The results for each day obtained a significant difference.

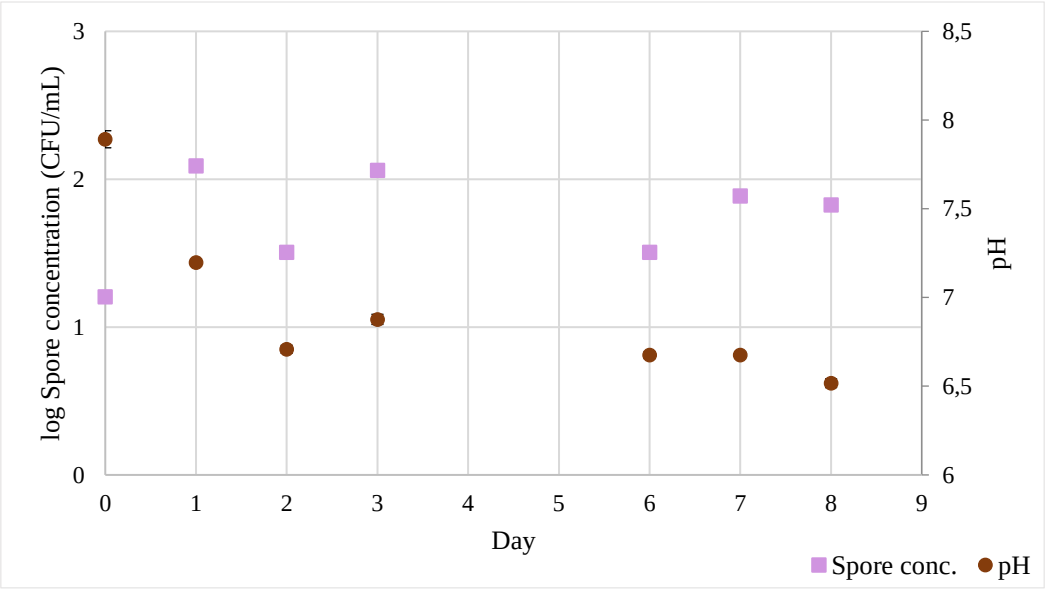

Figure 23. Spore concentration and pH in broke tower simulation, 95% confidence interval. Purple and squared plot: spore concentration, Brown and circular plot: pH

11. Discussion

The expectations were that ATP bioluminescence would correlate with the total bacterial concentration in sample testing, eventually the results in table IV contradicted the hypothesis. The correlations between the total bacterial and spore concentration and the total ATP are low in all selected process positions. This means the ATP bioluminescence is not a reliable

analysing method to quantify the bacterial growth in the system. The negative or positive correlation indicates the conformity to the theories. Most of the positions attained a positive correlation, which demonstrates approximately the principle of ATP measurements. An increase in ATP measurement indicates a higher total bacterial concentration. Some positions obtained positive correlations between the total ATP and spore concentration, which creates an uncertainty around the measurement. The reliability needs to be clarified in order to establish its representation in relation to the growth.

In previous studies from Kiuru (2011) stating that the ATP measurements is a helpful method to detect the microbiological activity in the process. As the obtained amount of extracellular ATP was relatively low, the assumption is made that the total ATP represents the intracellular ATP. Mempin et al. (2013) and Ihssen et al. (2021) presented the level of extracellular gives only a small hint in which growth phase the bacterial culture has entered in. The peak of extracellular ATP is obtained during stationary phase, yet the leakage of ATP initiates during the log phase. The leakage could be a response of stress or harm due to inconsiderate conditions. The highest peak of extracellular ATP reaches during death phase, when all intracellular ATP are released into the medium. In this case, the sporulation in the board process is the fundamental issue, and spore values are the considering indicator of the history in the process. In order to establish ATP measurement for predicting spore values in the board products and in process, the association of ATP to sporulation needs to be clarified. There is uncertainty during which growth phase the sporulation initiated by measuring the activity with ATP bioluminescence. A high value likely gives a “false” value of sporulation, meaning the actual sporulation indicators can be missed. Though the ATP measurement analysed on vegetative cells, the significance of ATP values requires to be in direct relation in order to predict sporulation. During log phase the growth is at its peak, but there is no intention of sporulation. The method would be more applicable if it can be used to indicate the activity during stationary phase for signifying sporulation. During this phase the stress responses are activated, and sporulation is taking place. Another option if the levels of ATP could signify the sporulation as response of death phase. An insightful knowledge about the association could be helpful to predict sporulation in the medium.

Ihssen et al. (2021) presented that an individual species has its own characteristics in behaviour of ATP. During the stationary phase the drop in the amount of extracellular and intracellular attained a noticeable profile for *Bacillus cereus*, while *E. coli* had a gradual decrease in intracellular ATP. Yet, the bacterial culture is not limited to a certain bacterial species, rather to the entire flora. The behaviour of the entire flora, as a singular unit, must be further researched in order to comprehend the level of ATP in perspective of the growth phase.

ATP bioluminescence measurement obtained relatively low variations, with 10,8%, see table VI. White water tower 1 obtained the lowest values of ATP, yet it attained highest variation. In contrast, the disc filter with the highest obtained values has the lowest variation. These results demonstrate that ATP bioluminescence is reproducible, and therefore it is accessible. With ATP measurements it is possible to map the entire system to find the affecting process positions in order to predict any increased microbiological activity. In order to establish the measurement reliability, mapping over a period of time is acquired to understand acceptable levels to minimize risks of raised spore values in board products.

The considerable disadvantage of bacterial and spore cultivation is the 48-hour incubation time, which is a reason of interest in investigating ATP measurement. The method is questionable in measuring the total bacterial concentration. Since the problem does not rely on the bacterial concentration in the process, the method affects the significance of the analysis. It is in fact the spore concentration in the system that provides the comprehensible insight in the history of the board system, as the environmental conditions trigger the sporulation. The total bacterial concentration in each process position obtained limited considerable variations that possibly gave any evident indication of the situation despite the obtained spore peaks in board samples. Spores are small capsules whose cover provides an extreme resistance to harsh chemicals, radiation and heat to protect the bacterial genome. It is an exquisite survival mechanism some bacterial species, such as *Bacillus* and *Paenibacillus* species, possess. The purpose of understanding the microbiological risks in the production system and simultaneously acquire the process and quality conditions for the board production.

Bacterial and spore cultivation does not provide any perception of the situation in the board system, due to the long incubation time and the unexpected peaks of spore levels in board products, although the total bacterial level was maintained moderately constant. The effect of deficient reasons of maintaining bacterial cultivation as a primary method in daily work causing the root cause analysis to be weakened. The infected pulp has been transformed into board products in the immediate moment the process samples have been collected and cultivated. As a matter of fact, the ATP analysis is performed within several minutes, which is an essential advantage of replacing the bacterial and spore cultivation.

In figure 18, the box plot for spore concentration in each process position obtained a higher variation compared to the obtained total bacterial concentration, figure 17. This indicates the spore amount in board samples is based on spore amount in the process rather than the total bacterial amount, as the spores cause the risks of affected products. The results show that spore content in the process should primarily be emphasised in relation to the quantitative analysing method. A suggestion is to eliminate bacterial cultivation from the method and exclusively perform spore cultivation along with ATP measurement. This would give a more comprehensive information to the actual spore levels in the process and the activity, for a direct interpretation of the situation in the process.

Despite the bacterial and spore cultivation being assumed to provide with the exact concentration of the microbiological activity, there were uncertainties in counting bacterial and spore colonies. The spore colonies did not follow the dilution series with factors of 0; 0,5 and 10^{-1} . Higher numbers of colonies were frequently obtained in the dilutions, despite the expected number of colonies being greatest in the undiluted solutions. Explanations to the unpredictable spore results for each process position are rather ambiguous. The spore cells are 10 times smaller than the vegetative cells, lowering the plausibility of pipetting the adequate number of spores. The purpose of Tween80 is separating the bacteria and spores from fibre flocculations, yet the results did not represent its effect. In conclusion the bacterial and spore cultivation is neither accessible nor reliable, despite it signifies the concentrations.

From the broke tower simulation additional insights have gained. Although the ATP values did not result in amplification of the total bacterial concentration, the analysis gives a proposal of future research. The analysis could be applied on every type of pulp and process position, in

order to understand the evolvement of the microbiological activity, because each process position has its own unique medium due to its function.

Redox potential states the environmental status and the nutrient accessibility, even though the obtained results presented low correlations. It gives a fundamental indication of the acid/alkaline conditions, nutrient accessibility, and oxygenation in the medium. Each species in the bacterial flora has its specific conditions in the medium, presumably the medium causes a competitive environment as each species attempts to transform the medium for its specific requirements. Meaning the medium is complex as the variables contribute to the value of redox potential to a certain extent in relation to the bacterial concentration.

Though the boxplot, see figure 12, illustrates the outliers obtained are negative, meaning that the most extreme environment is being reductive, as the pH, oxygen accessibility or metabolism have affected the results and the conditions. The pH implies the conditions for the microbiological activity, whether the environment is oxidative. Providing a stable pH level in the environment inhibits any sporulation, explain Thomassin et al. (2006), in order to maintain the intracellular pH within its range. Metabolism is accomplished through highly oxidative organic compounds, such as glucose is the primary source for the vegetative cells. Abundance of oxidative organic compounds are revealed by a high value of redox potential, as well as an alkaline pH. Depletion of nutrients is designated by a low value in redox potential. The advantage of redox potential is its capacity of signifying the nutrient accessibility and the electric charge in the growth medium. The multiple perspective of redox potential gives a profounder insight of the situation. Higareda et al. (1997) explained determination on the growth phases of a bacterial culture can be examined through similar analysis, by investigating the substrate depletion related by the oxygen intake. The redox potential profile decreases by the low oxygen uptake.

Redox potential measurements obtained relatively low variations, see table V. Meaning the significance of redox potential value is not affected by the 17,2% variation. In figure 12 and 13 profile of redox potential is clearly represented for the medium in the broke tower, despite the rate is during 8 days, as it can be explained that the pH, nutrient and oxygen depletion are not affected during 6 to 10 hours which is the normal retention time in the broke tower. Reichart et al. (2007) and Lee et al. (1998) explained the redox potential development associated with the metabolic changes that represent the bacterial growth. In this simulation, the theory is approved despite the fibrous medium the bacterial culture was growing in. The correlation analysis did not approve the redox potential measurement in process scale; however, broke tower experiment does strengthen the potential to be applicable in process regardless. Higareda et al. (1997) and Lee et al. (1998) explained that redox potential rate is related to the metabolic changes. This means that the performed analysing method of redox potential for each process position was insufficient for this methodology. To monitor the metabolic and environmental changes in the process medium and correlating with the actual retention time in the towers, one suggestion is to apply redox potential on-line in the process. The trend can then be tracked back in time, for gaining insight and understanding of the situation.

The obtained results in table IV gives an illustration of the influence redox potential might have on metabolism in microorganisms, as ATP is one of the most essential molecules in microorganisms' metabolism and fundamental synthesis as Dunn & Grider (2022) explain. The negative indication signs were obtained in majority of process positions, describing the

reductive metabolism the aerobic bacteria possess, as the metabolism reaches its peak the redox potential is then low. The abundant genera in the bacterial flora is *Bacillus* and *Paenibacillus*, which can possibly be confirmed by the obtained results. However, the hypothesis expresses that the correlation between redox potential and total ATP must be positive, because high redox potential indicates high metabolic activities in the oxidative medium. The obtained results contradict this principle. On the contrary, the correlations prove another reality. In metabolic perspective that relates to the aerobic oxidative metabolism, meaning that the redox potential profile decreases in relation to the metabolic behaviour. Therefore, an increase of total ATP implies rather a stagnation in the metabolic rate, because of malnutrition and oxygen depletion (two of main sporulation triggers), which result in sporulation. In this way, the association between the unconditional environment and sporulation can be described and give another significance to the analysis. A high value of ATP can imply a prediction of sporulation. However, this needs to be clarified as the assumptions are based on the obtained weak correlations. Further into this perspective, machine chest 1 and 2 and broke tower attained highest correlation with total ATP in comparison to total bacterial concentration, which can tell that ATP is more likely sensitive to the environment than the vegetative cells itself. It requires further investigation with this in mind.

The purpose of the chosen process positions was to investigate the microbiological development throughout the broke system, from the pulpers to the machine chests. In table I, an overview of the methodology is presented. The information for each process sample was combined with the fundamental knowledge of board production and the microbiology. The choice of method revolved to accomplish as close to the production as possible to identify any correlation with the truth of the production.

The sample tests for each variable and process position were measured once for each laboratory workday, totalling 18 measurements. The method was predominantly defective due to poor resolution of sample testing. Any deviation or considerable trend might have been missed during the 23 hours after that possibly showcase higher correlation with the bacterial and spore amount. The optimal method is to apply the measurements on-line for greater resolution of process data giving perceptively knowledge of the bacterial medium. Because phenomenon can be caused by several underlying factors and giving extensive affect.

Such as temperature, TOC and the other variables could not be determined whether these are the significant factors of microbiological activity, only indicating factors favouring sporulation. Recently discussed applying redox potential and pH measurement on-line. Methods could have been executed laboratory simulations to investigate closely the sporulation triggers and analysing the correlation the microbiological activity. However, extensive laboratory does not take into consideration the process variations.

The box plots in the results show several outliers that usually had been removed, in this case the outliers are most interesting. The box plots give another perspective in the interpretations of outliers and correlations. The boxes illustrate the range of attained data. It is observed that broke tower, broke chest 1 and 2, disc filter and machine chest 1 obtained a higher range in total bacterial and spore amount. However, the positive and negative correlation attain an indication if the process corresponds to the theory.

The sample testing obtained low correlations between the process parameters; therefore, any assumption of which process position is the main cause of sporulation cannot make. Another

factor of this challenge is the complexity of board production. The process includes several circulations, such as broke and white water system. Investigating the white water and the water intake would be another interesting aspect of process position identification. As well as the wire pits, because of low water and fibre flow there is a risk of biofilm formation. Once the vegetative cells have entered one part of the system, all production is then affected. If the process position is identified that is the main cause of the microbiological activity, a profound knowledge is then gained about the bacterial culture and its sporulation triggers.

In each process position there is an interesting trend between the pH and board samples (cultivated at 37°C) are related. The negative trend indicates an increase of spore concentration in board samples is resulted by a decrease in pH. However, the correlations between the total bacterial and spore concentration and pH in each position demonstrated mixed trends. Some positions obtained a positive correlation between the pH and the total bacterial concentration, indicating that it results in transferring bacterial cells into the board products. The correlation between pH and spore concentration showcases the situation, as a decrease in pH results in raised spore concentration. There is an association between this correlation and correlation between pH and board samples, indicating similar trends. The vegetative cells eventually die due to the high temperatures in the machine, and because spores are tough cells they remain in the product.

Comparing broke tower with broke chest 1 and 2, the retention time is notably higher. The retention time in broke tower is normally 6 to 10 hours, while in the broke chests is 20 to 30 minutes. Due to high retention time and a favourable environment in the tower, it increases the risks for biofilm formation on the surfaces inside. Bacterial colonies attach onto the surfaces and produce an extracellular polysaccharide film in which the vegetative cells thrive and communicate until depletion of nutrients and oxygen. The colonies detach from the surface and transfer through the process as O'Toole et al. (2000) describes. Possibly different production variations could precipitate the detachment of biofilms causing sudden spore concentration peaks. The environment inside the biofilms is mostly conditional, when a sudden detachment occurs the colonies appear in broke pulp that eventually is not conditional as in the biofilms. The environmental changes trigger the sporulation. The vegetative cells do not the anticipation of adapting to the new environment in a very short time, resulting in sporulation being the only way out for survival. Thomassin et al. (2006) explains that *Bacillus* are adaptable in extreme pH conditions, but time is presumably a crucial factor for survival.

The purpose of the simulation was to examine the hypothesis of broke tower to be a possible source of microbiological causes in the board system. Kiuru et al. (2010) explained long retention time and high mass level in the tower raises the bacterial growth into the system. The boxplots in figure 16 and 17 show the higher range of total bacteria and spore Kiuru et al. explain.

The broke tower simulation has given a new perspective of ATP bioluminescence measurements, redox potential and pH. Each bacterial culture is unique to its behaviour observing from different analysis; therefore, the simulation gives insight of the bacterial cultures' life cycle in the board process. The total ATP development for 8 days describes the rate of ATP, even though in this experiment the obtained growth curve did not follow the principle of growth phases in figure 2.

In figure 22 and 23, a significant drop in pH occurred in day 2, and in the remaining days the pH rate was stabilized. Presumably the broke pulp showcases a buffer system. The rate of redox potential and pH can be compared to understand the environmental changes and the influence the pH rate has on the redox potential. The results from the simulation approve Thomassin et al. (2006) interpretation of microbiological ability of stabilizing and adapting to the acid conditions. Possibly more resistant bacteria survived and adapted to the acid conditions due to the accumulated waste products, making a competing environment. The spore concentration increases during day 2. In the stationary phase the accumulation of waste products becomes apparent in the drop of redox potential rate, see figure 20 and 21.

11.1 The impact on sustainability

The obtained results do give any high expectations in the microbiological situation. Although, this study can give guidance from the knowledge gaps that are dealt with in the development of identification and quantification of microbiological activity in the board machine. Whereas the contribution to the board machine is suggesting new process positions to be examined or arranging a new method of mapping all process positions. The key to a successful method of applying in daily work must be feasible, fast and reliable, in comparison to the cultivation method. The results of the cultivation method are obtained after 48 hours, meaning the board product during that time has already been affected. The overall situation brings difficulties of repairing the causes before the issue transfers into the board product. Both in short and long term the customers lose interest in investing in the board products, causing logistical delays. During the time of delay, necessary analysis is required to reassure and recover the products, and to avoid the dilemma in later products. Causing a chain reaction by poor quality and delayed transport, and later affecting the customers production of laminated liquid boards and packaging foods. The company attains production losses due to loss of interest as a consequence of customers' production losses. It induces a downfall in economic achievements for all included companies in the production chain for delivering food to people. In the long term this affects the social aspect of this problem because appreciation of the company motivates people to accomplish the required work for maintaining the prospects of sustainability, for workers as well as consumers at the very end of the chain.

12. Conclusion

ATP bioluminescence measurement is an accessible method to quantify the microbiological activity, due to its reproducibility and feasible analysing method, in contrast to bacterial and spore cultivation with 48-hour incubation time. The correlation analysis demonstrated a low correlation between the measurement and the total bacterial and spore concentration, explaining the measurement is not reliable to identify the microbiological growth in board production. The considerable uncertainty is what the ATP bioluminescence values represent in relation to the microbiological growth.

Redox potential measurement is a reliable method to identify growth conditions for microorganisms. This was proven by the broke tower simulation, which showed the environmental development in the broke pulp. However, the method of redox potential was shown to not be accessible due to low correlations between the total bacterial and spore concentration for sample testing, and the simulation demonstrated a different perspective of quantifying the microbiological conditions.

Beneath the board machine revolves several systems in the long circulations. Once the bacteria have entered the system, the whole circulation is then affected, meaning there is not a start and an end of the situation. The broke tower simulation did not confirm that the broke tower is the cause of microbiological activity. The results presented low correlations between the process variables in the 15 process positions; therefore, no assumptions cannot be considered in which position in the system can be the significant cause of high microbiological activity.

13. Future research

Future studies should focus further on the mechanism of ATP bioluminescence in order to understand its significance and be able to apply it in daily routines. The values should signify the quantity of vegetative cells as well as giving an indication in which growth phase the vegetative cells are in, so that the microbiological development throughout the system can then be predicted.

It is important to monitor the environmental conditions directly in the system in order to fully determine the situation. The redox potential analysis is an optional combined with pH measurement, on-line in the board process, because these two analyses can indicate the alkaline and acidic conditions and substrate and oxygen accessibility. From trend data, predictions of microbiological activity can be made.

It would be interesting to examine the white water system, from the water intake to the initiation of broke system to observe any raised bacterial and spore values. As well as investigate biofilms from different positions in the board machine that could be the cause of the microbiological activity, such as wire pits. Further research would be to investigate sporulation triggers through simulations with multiple sporulation triggers regarding environmental changes.

Acknowledgements

I want to thank my supervisor at Karlstad University, Björn Sjörstrand for the support and guidance throughout my project.

I would also like to thank my supervisors at Stora Enso; Kristin Johansson, Karin Lindblom and Lina Barnaba. You have been a great help for me for minor and major inconveniences and guided me in the right direction to accomplish this work.

A special thanks to Daniel Ekbåge for learning and guiding me with multivariate analysis, statistical analysis and reviewing my thesis.

Thank you Våtlab and Mibi for welcoming me into your team and sharing experiences. I have had such a great time with you.

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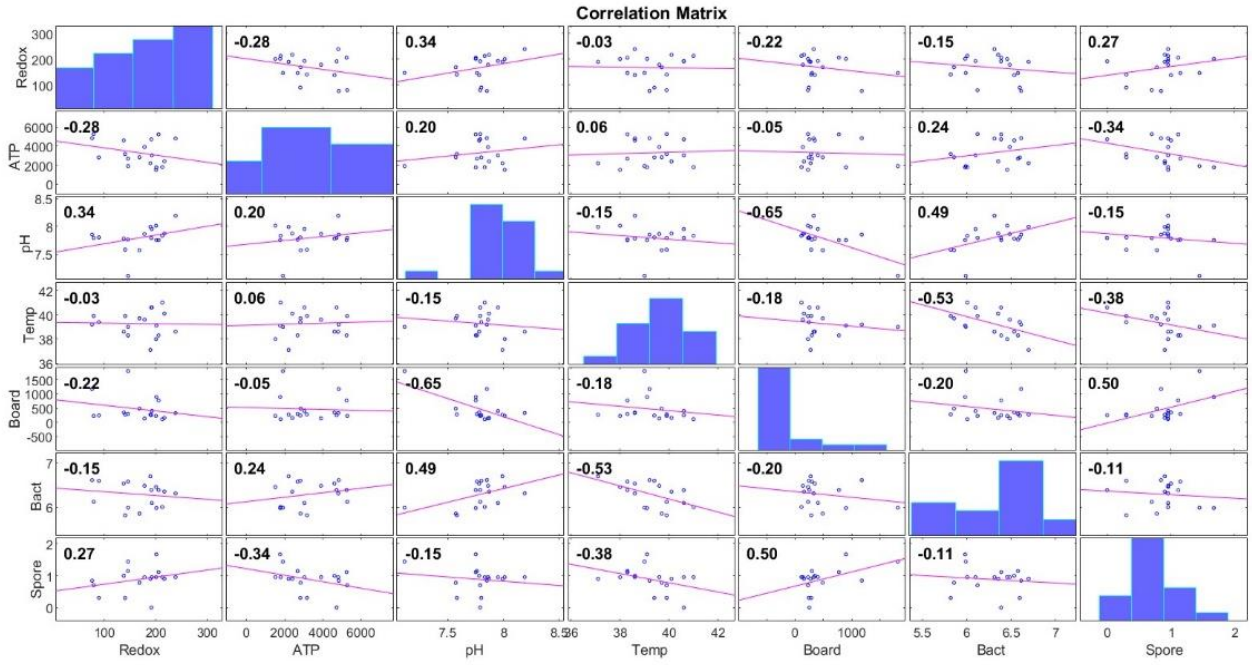
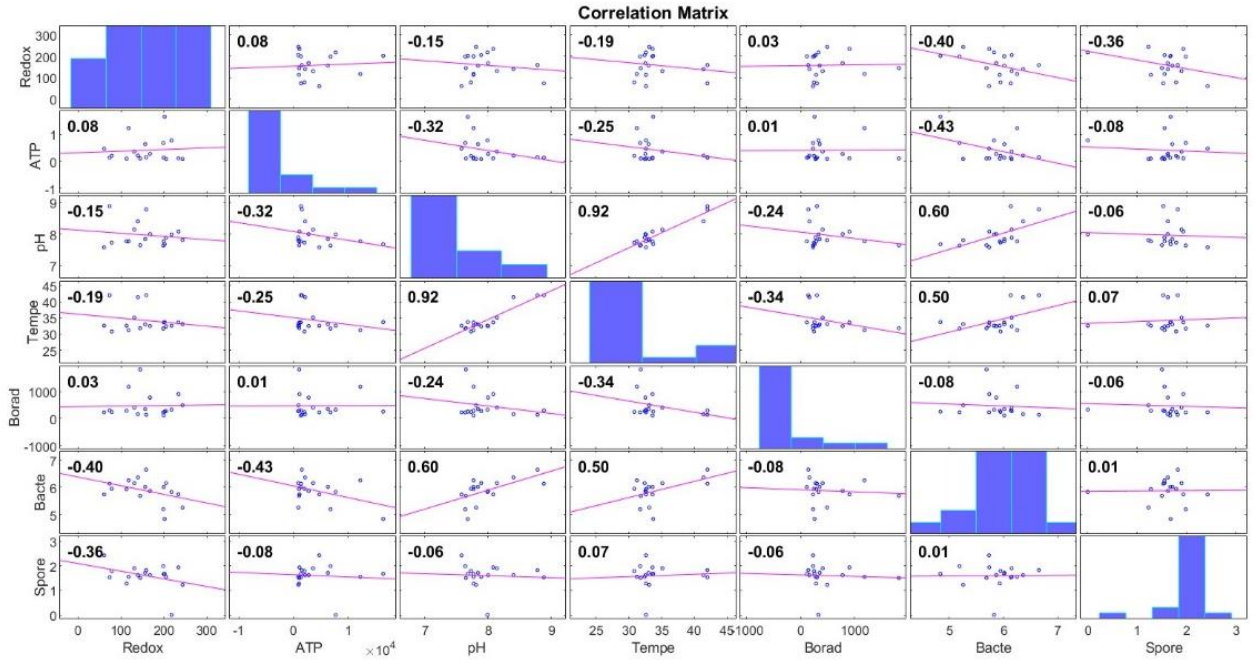
Appendices

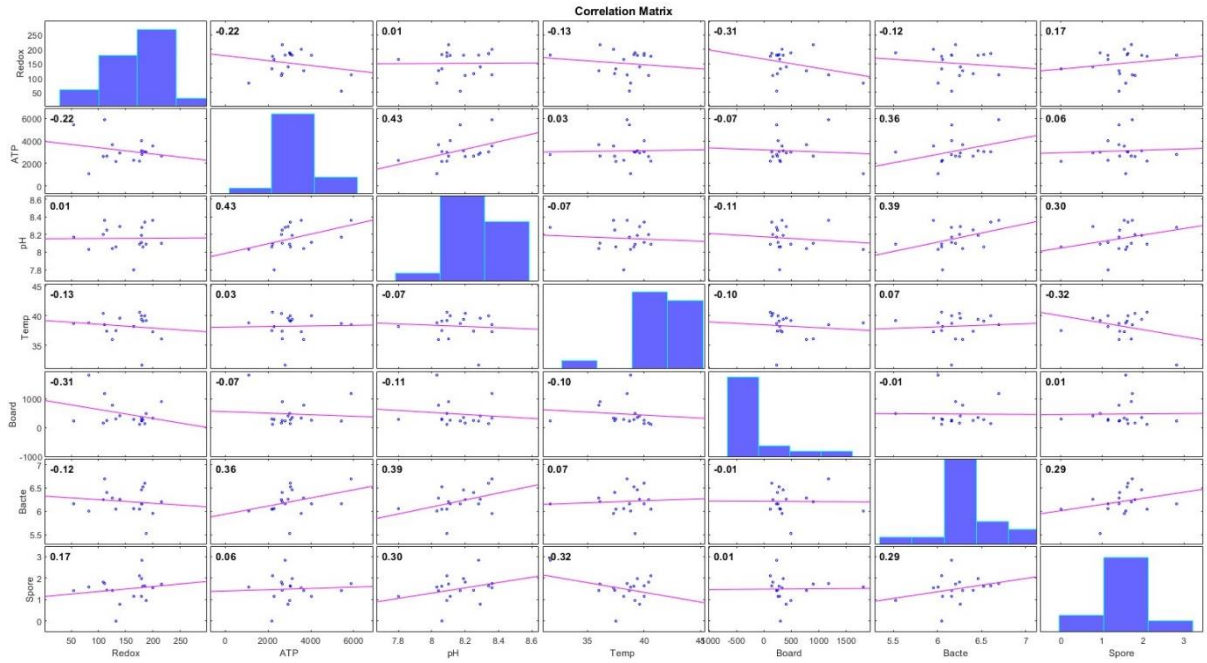
Appendix A. Correlogram of pulper 1.



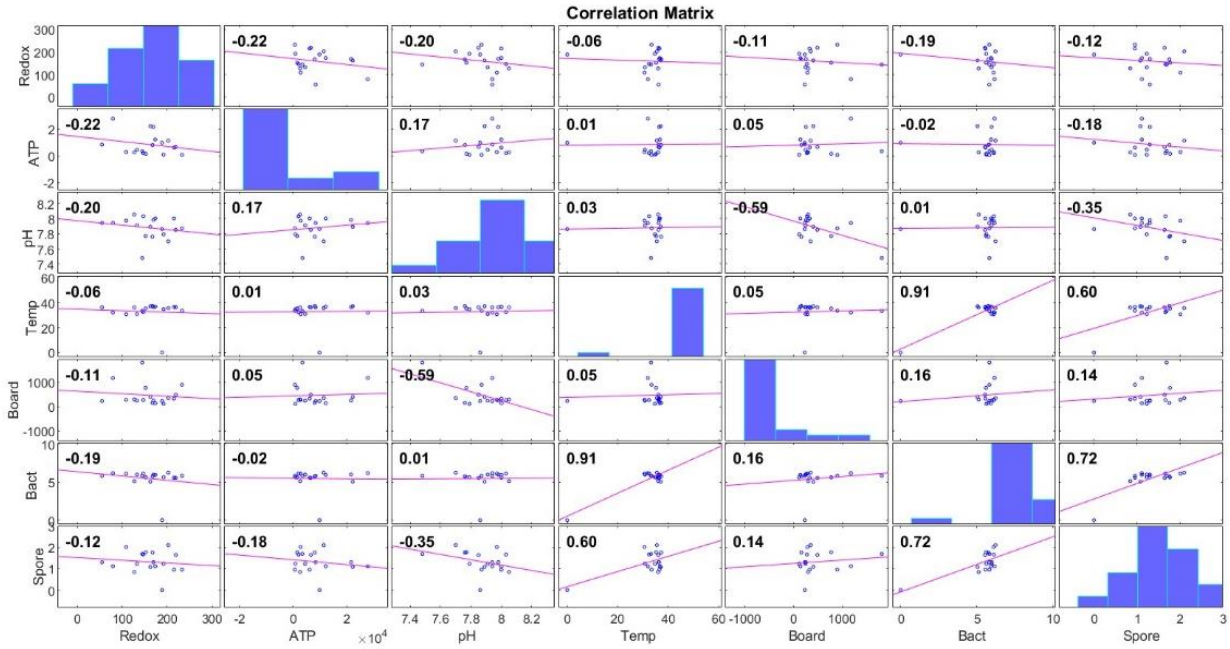
Appendix B. Correlogram of pulper 2.



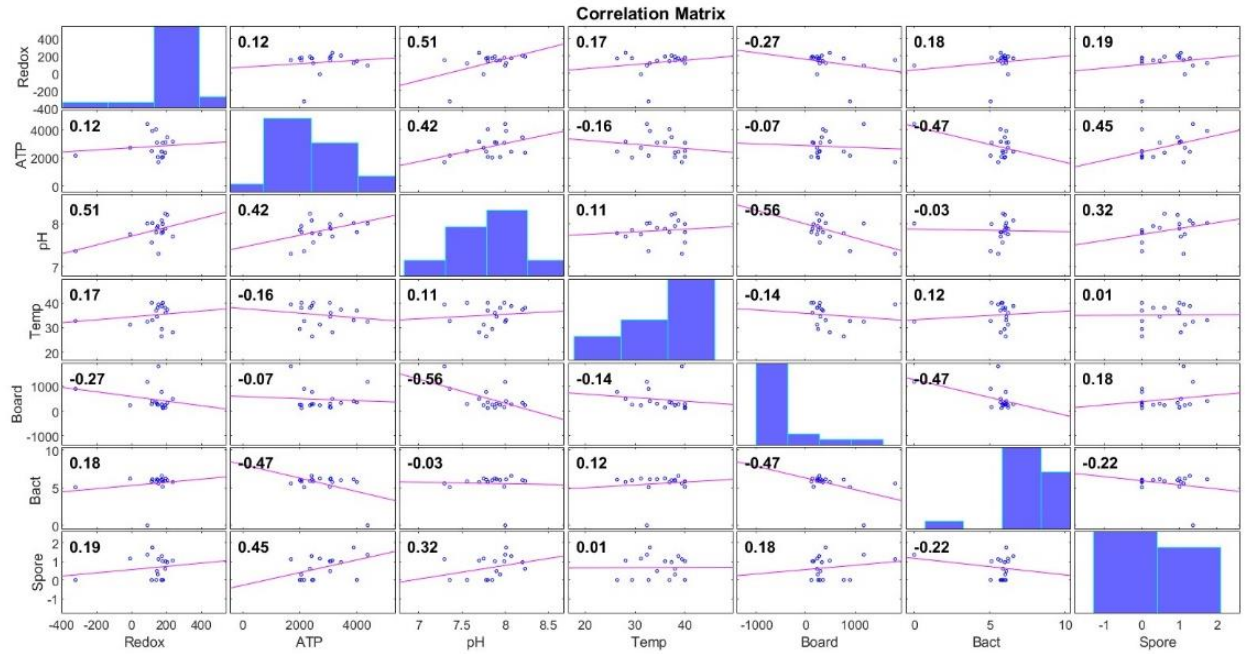
Appendix C. Correlogram of pulper 3.



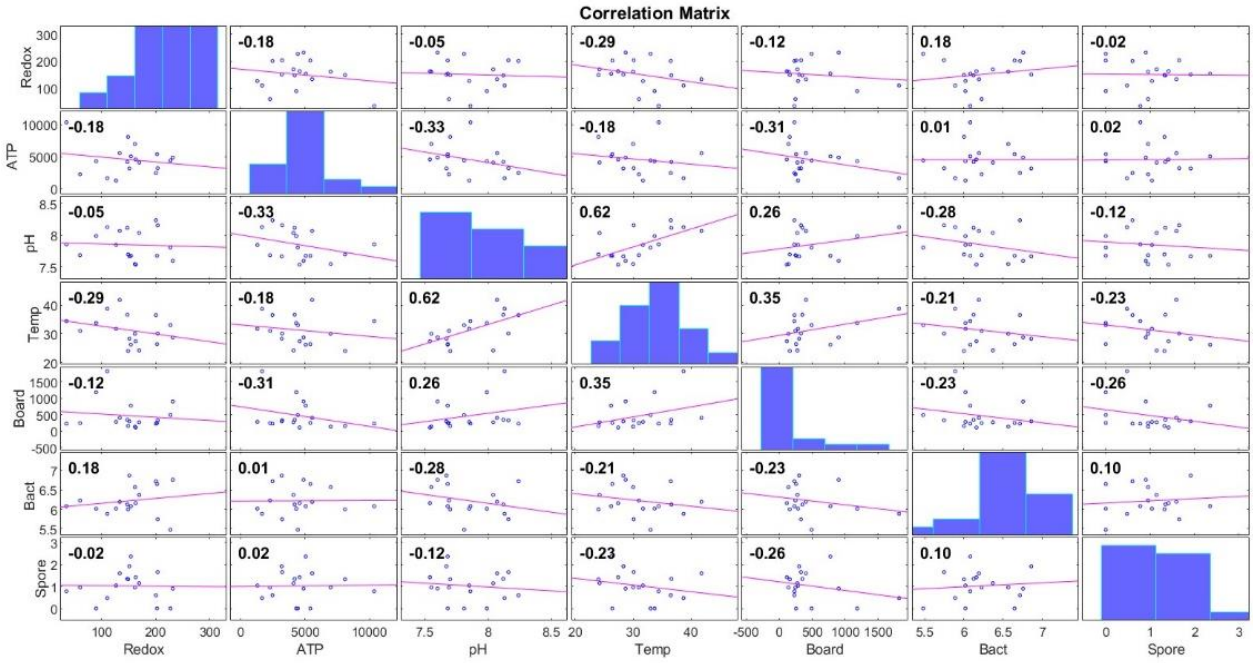
Appendix D. Correlogram of pulper 4.



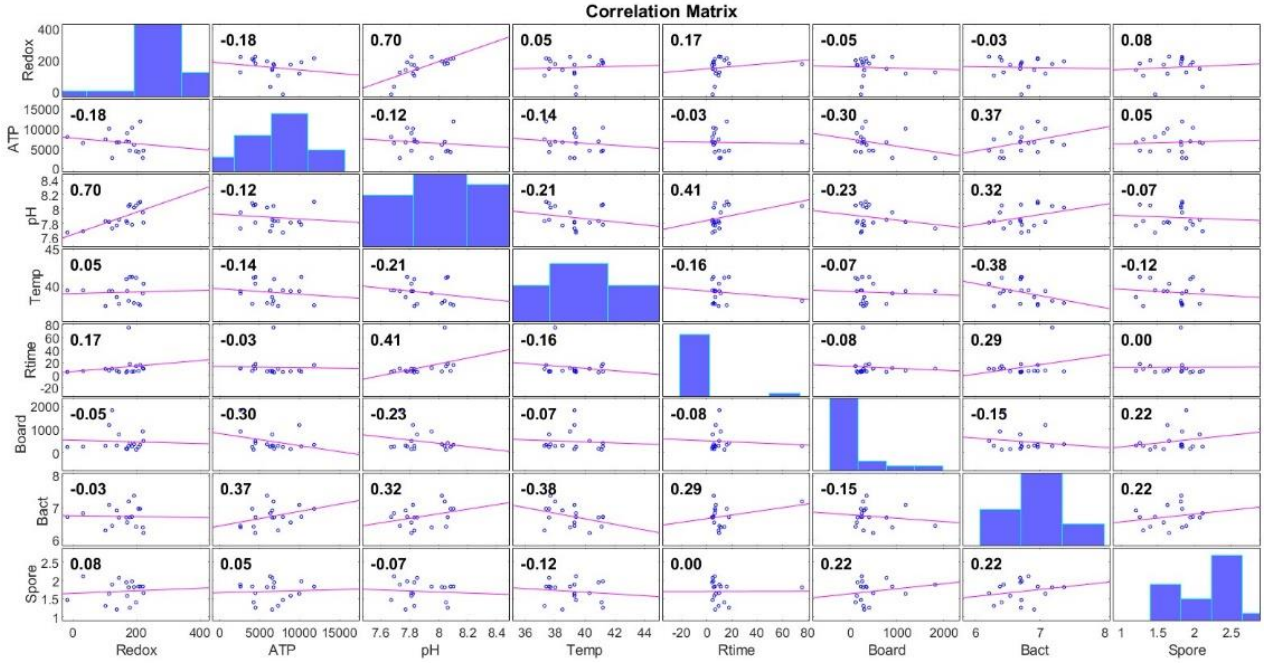
Appendix E. Correlogram of pulper 5.



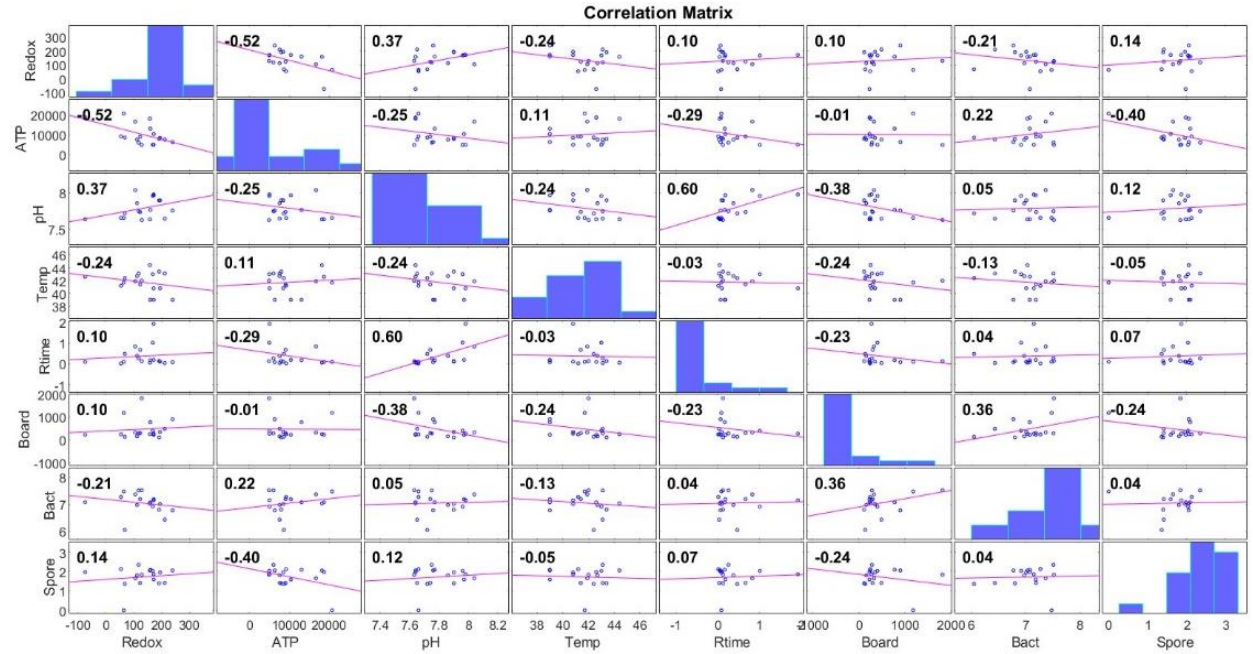
Appendix F. Correlogram of pulper 6.



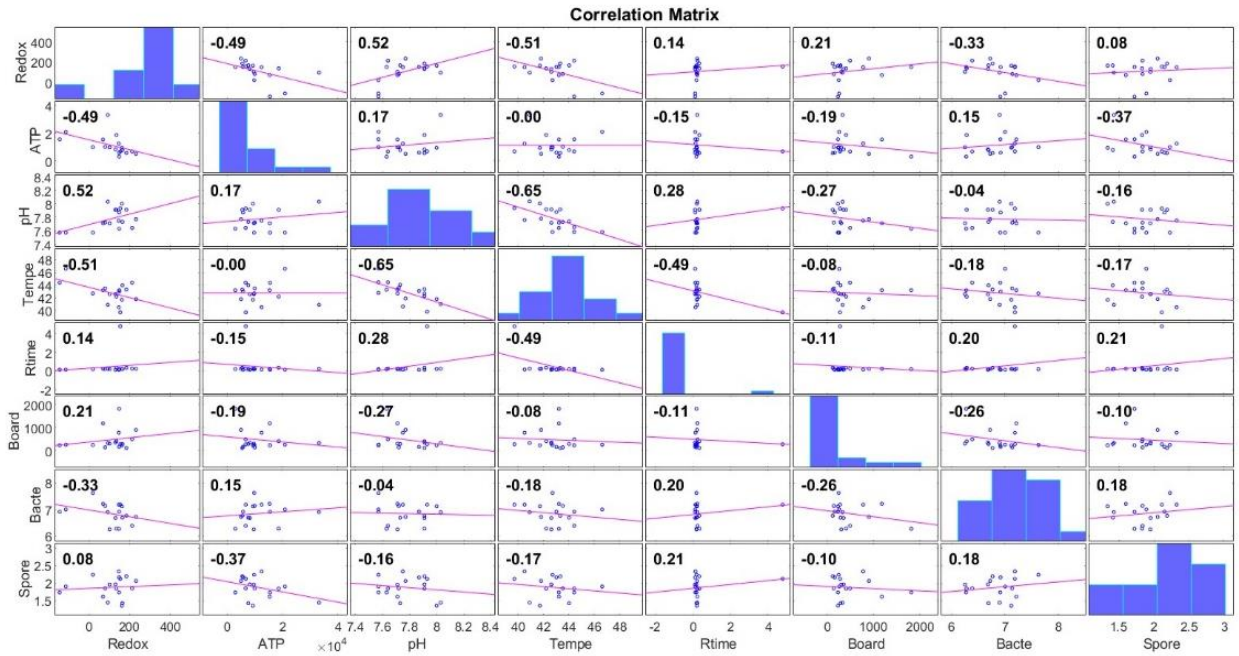
Appendix G. Correlogram of broke tower.



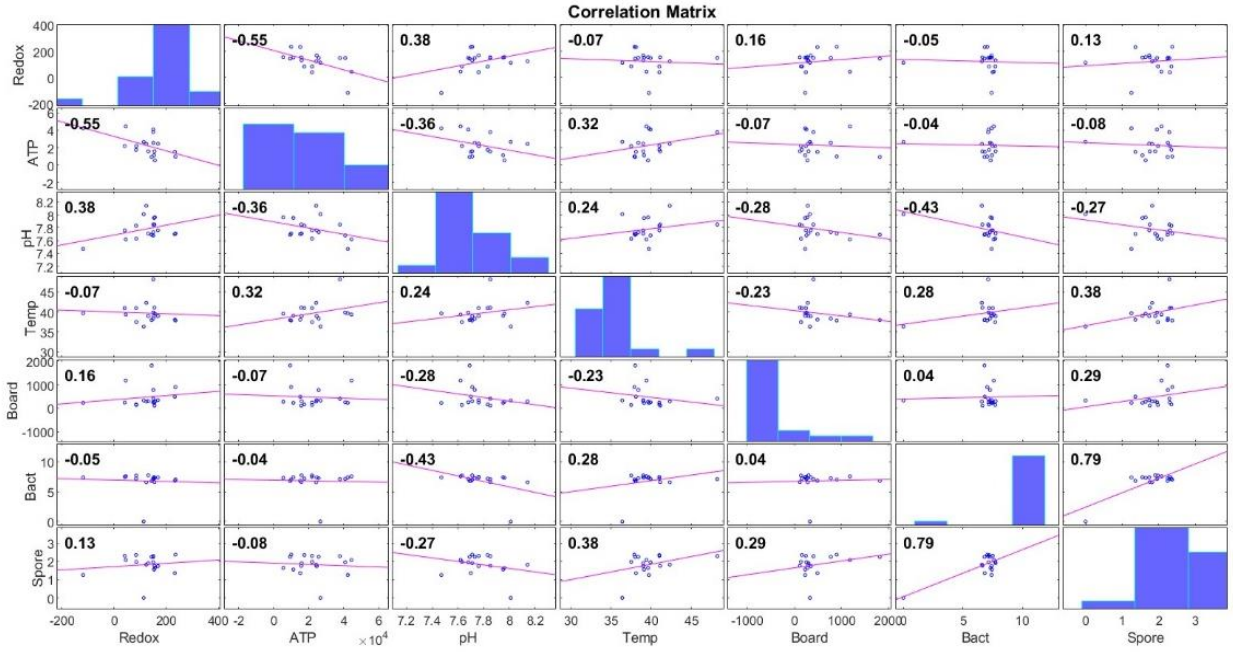
Appendix H. Correlogram of broke chest 1.



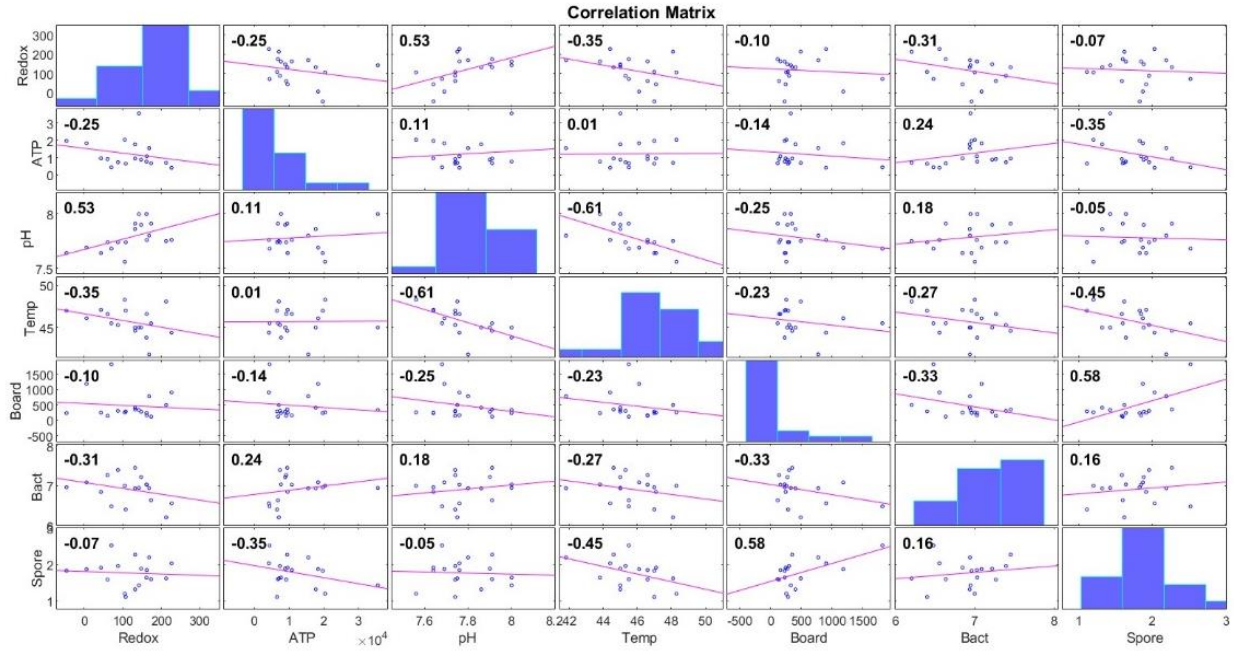
Appendix I. Correlogram of broke chest 2.



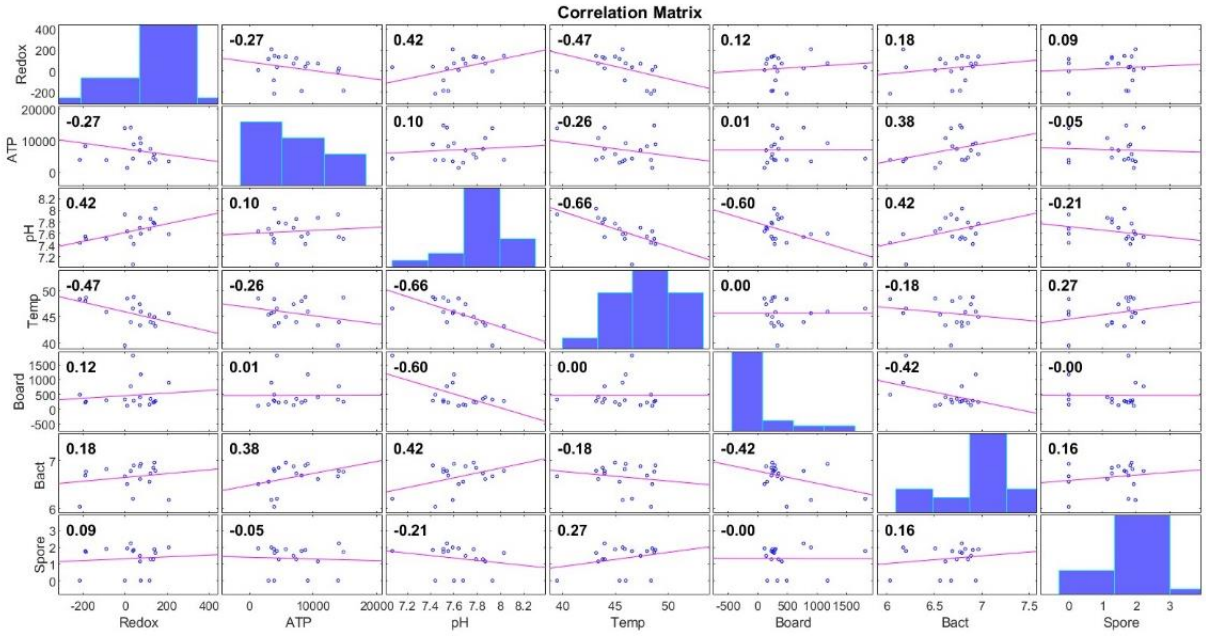
Appendix J. Correlogram of disc filter.



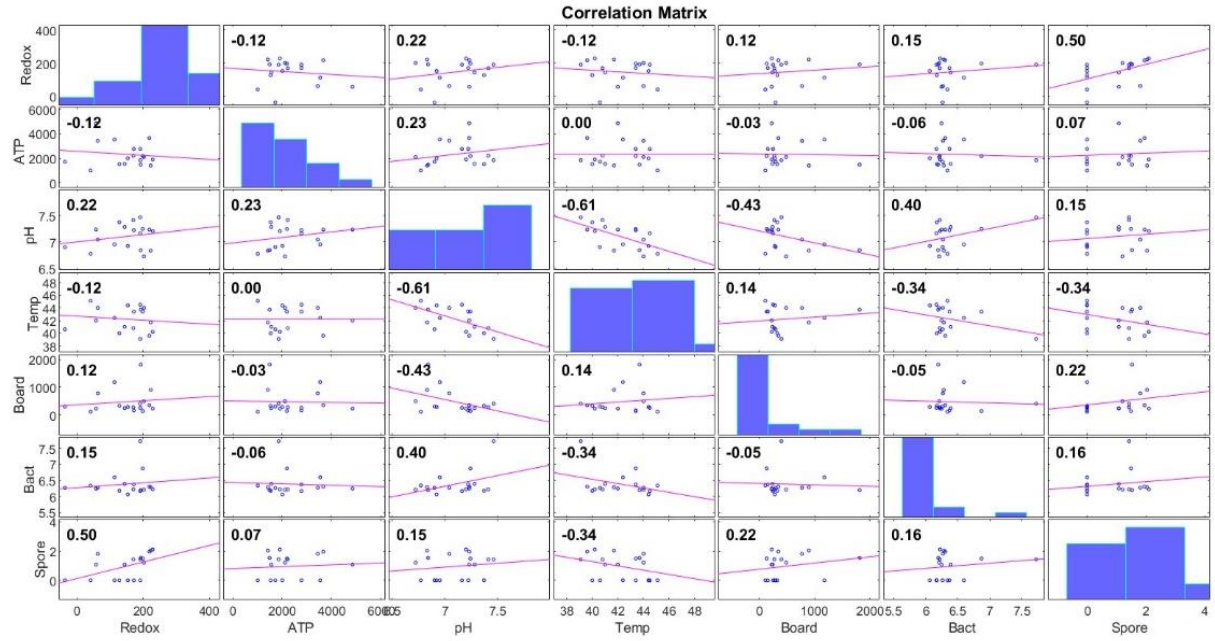
Appendix K. Correlogram of broke refiner.



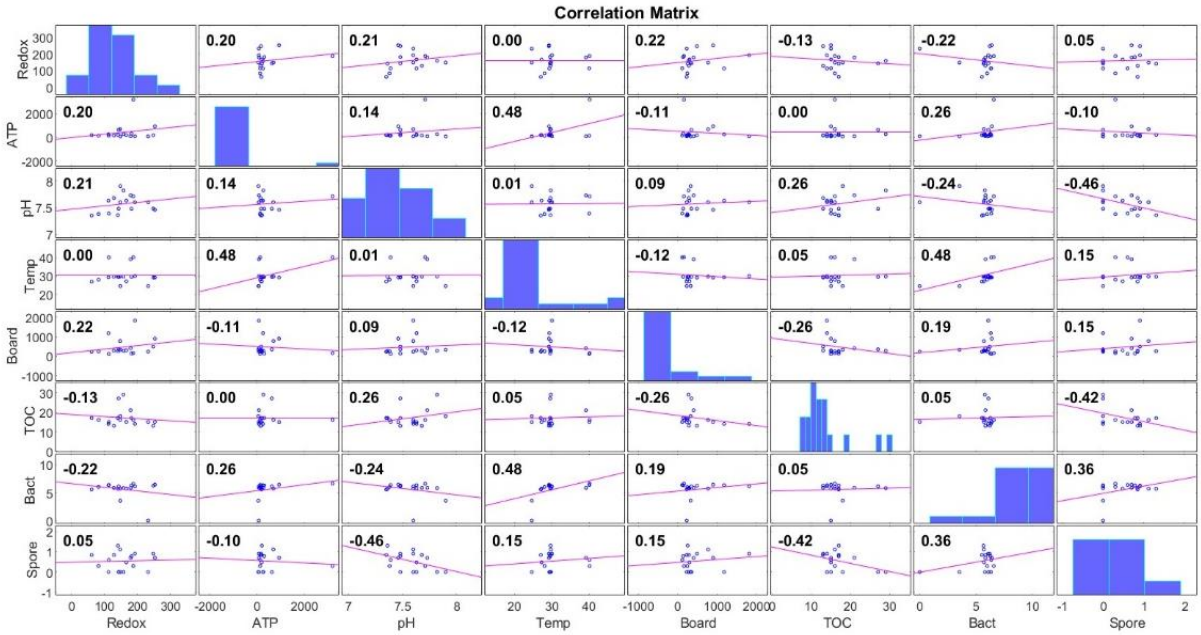
Appendix L. Correlogram of machine chest 1.



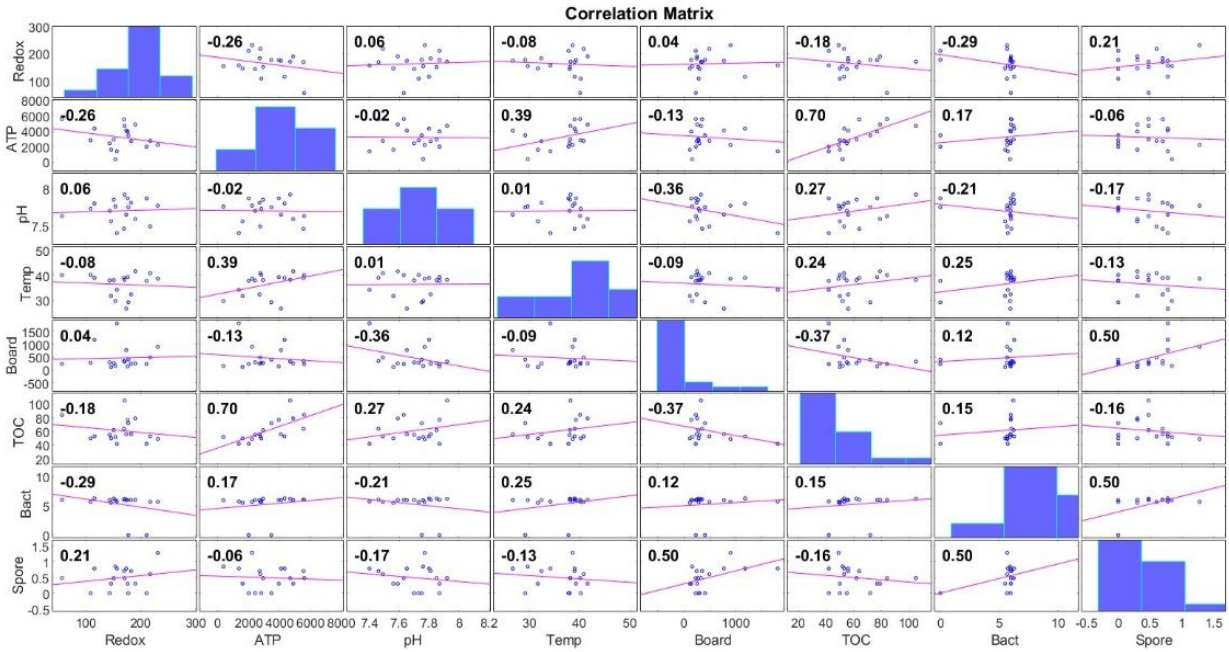
Appendix M. Correlogram of machine chest 2.



Appendix N. Correlogram of white water tower 1.



Appendix O. Correlogram of white water tower 2.



Appendix P. Raw data from broke tower simulation.

Day/Total ATP (RLU)	Average	Confidence interval
0	7736,166667	766,7568961
1	10715,16667	992,3217067
2	10592	730,6005749
3	7715	1136,208901
6	9248,333333	827,9450867
7	1727,333333	258,015245
8	2096,666667	259,9505081

Day/Redox potential (mV)	Average	Confidence interval
0	151,06	5,296064
1	-71,1	8,96073
2	-94,9667	152,6557
3	-204,15	268,1414
6	-320,033	10,03793
7	-274,167	6,024407
8	-295,967	14,97257

Day/pH	Average	Confidence interval
0	7,891666667	0,048053

1	7,196666667	0,016195807
2	6,708333333	0,022426188
3	6,876666667	0,028676041
6	6,675	0,018477221
7	6,676666667	0,01433802
8	6,516666667	0,024536754

Day	**Total bacterial conc. (CFU/mL)**	**Log 10(total bact.)**	**Spore conc. (CFU/mL)**	**Log 10(spore conc.)**
0	7918400	6,898637	16	1,20412
1	15940800	7,20251	123	2,089905
2	736000	5,866878	32	1,50515
3	3,18E+08	8,502705	115	2,060698
6	1654400	6,218641	32	1,50515
7	577363	5,761449	77	1,886491
8	355074	5,550319	67	1,826075

Appendix Q. Formulas for coefficient of variation