Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Henrik Arntsen Pedersen

Growth kinetics and spoilage potential of *Aeromonas* strains isolated from seafood products.

Master's thesis in Food and Technology
Supervisor: Sunniva Hoel
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Preface

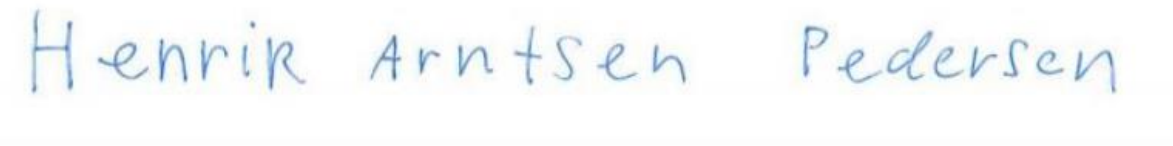
This master thesis is the final part of my Master education in Food and Technology at the Norwegian university of Science and Technology, Faculty of Natural sciences, Department of Biotechnology and Food Science. This has been a been both a challenging and extremely interesting assignment to work on.

I want to thank my main supervisor, Assistant professor Sunniva Hoel and co-supervisor, Associate Professor Anita Nordeng Jakobsen for the massive amount of help and counselling they have provided me during this period. I also want to express my gratitude for the help and assistance given to me by Head Engineer Torun Margareta Melø at the NMR lab that made parts of this thesis possible. Furthermore, I want to thank all my fellow students and friends that has made these last few years a joy, even in times effected by covid-19.

25th of May 2021

Author

Henrik Arntsen Pedersen



Abstract

As production of seafood is increasing and consumption of ready-to-eat products is becoming more common, knowledge about the different microorganisms existing on these products are becoming more relevant. Food spoilage is a result of microbial, chemical, or physical changes in a product. Bacteria being a major contributor to this, especially on protein rich foods such as fish. ATP degradation and TMAO reduction are some of the most important biochemical reactions that take place during spoilage. *Aeromonas spp.* is commonly isolated from spoiled seafood, but knowledge about their role in producing these spoilage metabolites are limited.

The aim of this study was to both determine the growth kinetics of eight different strains of *Aeromonas* and to investigate their spoilage metabolite production. The strains were cultivated in two different media; Tryptic soy broth (TSB) and Fish juice at 4, 8 and 15°C and their growth kinetics calculated using the database Combase. All strains were then cultivated on Iron Agar to determine their ability to produce H_2S. To analyze the development of Trimethylamine (TMA) and Hypoxanthine (Hx) all strains were cultivated in two enriched media, one enriched with 5,67g Trimetylamin-N-oksid (TMAO) the other with 3,2905g inosine monophosphate (INO). *A. piscicola, A.* bestiarum and *A. salmonicida* (SU2) were later used to contaminate both fresh and smoked vacuum-packed salmon and stored at 4°C for up to 21 days before being analyzed for TMA, Hx and biogenic amines. Hx and biogenic amines was measured using HPLC and TMA using NMR.

The results showed that fish juice inhibited growth compared to TSB. Cultivating in TSB resulted in all strains having a higher μ_{max} at all temperatures and was overall a more stable and reliable media for cultivating the bacteria. All strains except for *A. caviae* and *A. media* were able to produce H_2S at 15°C, but only *A. bestiarum* and *A. dhakensis* were able to the same at 4°C. All successfully studied strains produced Hx after six days with both strains of *A. salmonicida* together with *A. bestiarum* producing the largest concentrations.
The results also indicated that *A. salmonicida* produced higher concentrations of Hx and TMAO in Vacuum-packed salmon compared to the non-inoculated samples. The HPLC data for Biogenic amines was of poor quality and had to be discarded. The results obtained in this study indicate that *Aeromonas* can produce the spoilage metabolites TMA and Hx but there are some differences on strain or species level that need to be elucidated in more detail. Further studies are needed to determine the exact differences between the strains and to obtain more reliable data for both the Hx and TMA concentrations and especially biogenic amine production.

Sammendrag

Ettersom produksjon av sjømat øker og konsum av rå-ferdig prosesserte produkter blir mer vanlig, øker også behovet for kunnskap om de ulike mikroorganisme som eksisterer på slike produkter. Forringelse er definert som mikrobiologiske, kjemiske eller fysiske negative endringer på et produkt. Der bakterier er veldig relevant for proteinrike matvarer som sjømat. ATP-nedbrytning og TMAO-reduksjon er noen av de viktigste biokjemiske reaksjonene som finner sted under mikrobiell forringelse. *Aeromonas spp.* er ofte isolert fra forringet sjømat, men kunnskapen om deres evne til å produsere ulike forringelsesmetabolitter er begrenset.

Målet med denne oppgaven var å bestemme vekstkinetikken til åtte ulike *Aeromonas* stammer og undersøke deres evne til å produsere ulike forringelsesmetabolitter. Stammene ble dyrket i to forskjellige medier: Fiskejuice og TSB ved 4, 8 og 15°C og deres vekstkinetikk ble beregnet med databasen Combase. De samme stammene ble senere dyrket på Jern agar for å undersøke deres evne til å produsere H_2S. For å analysere utviklingen av Trimetylamin (TMA) og Hypoxantin (Hx) ble alle stammer dyrket i to berikede medium, et beriket med 5,67g Trimetylamin-N-oksid (TMAO) den andre med 3,2905g Inosin monofosfat (IMP), *A. piscicola*, *A.* bestiarum og *A. salmonicida* (SU2) ble senere brukt til å kunstig forurense både fersk og røkt vakuumpakket laks, kjølelageret i 4°C i opptil 21 dager, deretter ble konsentrasjonen TMA, Hx og biogene aminer analyser. Hx og biogene aminer ble målt med HPLC og TMA ved bruk av NMR.

Resultatene viste at fiskejuice hemmet veksten av alle stammene sammenlignet med oppdyrking i TSB, stammer dyrket i TSB hadde høyere μ_{max} ved alle inkubasjonstemperaturer og var generelt det mest stabile og pålitelige mediet for oppdyrkning. Alle stammen med unntak av *A. caviae* og *A. media* var i stand til å produsere H_2S ved 15°C, men bare *A. bestiarum* og *A. dhakensis* var i stand til det samme ved 4°C. Alle suksessfulle studere stammer produserte Hx etter seks dager, der begge stammene av *A. salmonicida* produserte de høyeste konsentrasjonene. Resultatene indikere også at *A. salmonicida* produserte høyere Hx og TMA konsentrasjoner i inokulert vakuumpakket laks sammenlignet med de ikke-inokulerte prøvene. HPLC-dataen for biogene aminer var av dårlig kvalitet og måtte forkastes.

Resultatene samlet fra denne oppgaven indikerer dermed at *Aeromonas* er delaktig i produksjonen av forringelsesmetabolittene TMA og Hx, men det er noen forskjeller fra stamme til stamme eller artsnivå som krever å bli studert nærmere. Videre forskning er nødvendig for å bestemme de spesifikke forskjellene mellom stammene og for å samle mer

pålitelig data både for Hx og TMA konsentrasjoner og viktigere produksjon av biogene aminer.

Innhold

1.0 Introduction

Seafood has been shown to be one of the fastest growing food sources in the world. For many nations, both developed and developing fishing and aquaculture is a large part of the national economy (Odeyemi, Burke, Bolch, & Stanley, 2018, p. 62). Consumption of seafood especially ready-to-eat (RTE) variant are becoming more common in Europe and the consumption of raw salmon is also increasing with the gaining popularity of homemade sushi and sashimi (Jakobsen, Shumilina, Lied, & Hoel, 2020). Fish being a highly perishable product makes it susceptible to spoilage causing economic losses for consumers and producers alike, as well as having the ability to cause food borne diseases.

Seafood is a product containing high amounts of non-protein nitrogenous compounds, this in combination with its slightly acidic pH (> 6) makes it susceptible to microbial growth. Especially relevant being growth of specific spoilage organisms that can, in combination with the naturally accruing postmortem processes produce a range of different spoilage metabolites. (Boziaris, 2014, p. 2)

Aeromonas spp. Are commonly found in spoiled seafood, implying that it has a role and partakes in the spoilage process. But knowledge of its growth and spoilage potential is limited (Jakobsen et al., 2020). Some studies have been conducted on the growth potential of and identification og *Aeromonas spp.* In different seafood products. For eksample (González-Rodríguez, Sanz, Santos, Otero, & García-López, 2002) has done a study about the growth potential of *Aeromonas* in rainbow trout and (Ullmann, Krause, Knabner, Weber, & Beutin, 2005) about isolation of different *Aeromonas* strains from a range of seafood products. But information about their role in spoiling these products are unknown.

The closest resembeling study was conducted by (Jakobsen et al., 2020), here investigating the spoilage potential of an enviromental strain of *Aeromonas salmonicida*. This made it of interest to investigate and compare different enviromental strains of *Aeromonas* with eachother to get a better picture of the species as a whole.

This thesis choose to focus on both the growth kinetics of 8 different strains of *Aeromonas* and their ability to produce spoilage metabolites both *in vitro* and in vacuumed packed salmon.

2.0 Background

2.1 Food spoilage

Food spoilage is the result of microbial, chemical, or physical changes in a product, to the point that it becomes unacceptable for human consumption. Microbial food spoilage specifically is the result of enzyme producing microorganisms that grow in the food, trough different biochemical reactions these enzymes produce unwanted bi-products that cause off-flavors, odors and/or compounds that can lead to foodborne diseases. (Bevilacqua, Corbo, & Sinigaglia, 2016, p. 1)

Bacteria can cause very rapid and evident spoilage in all kinds of food, but most importantly in protein rich foods like meat, fish, shellfish, and dairy. In these kinds of products bacteria is superior to yeast and molds as they can achieve a more rapid growth. The spoilage flora is partly determined by the natural microflora on the product, which is highly influenced by the environment it was sourced from. Fish collected from tropical waters will have a different microflora than one fished from artic waters, other factors like fishing season will also impact the natural flora on the product. Another maybe more important part is microorganisms that exist on the product because of cross contamination. These microbes are often transferred to the food during or after processing via processing equipment, humans or the surrounding atmosphere. (Bevilacqua et al., 2016, p. 2)

The microbial flora changes over time and a products initial flora will be vastly different than its flora at the end of its shelf life. There are usually just a small number of microorganisms that are responsible for the quality deterioration. These microbes are determined by the food characteristics, environmental conditions, and interaction between the microorganisms. The organisms that end up being responsible for a food's spoilage are known as specific spoilage organisms. The large range of microorganisms that can grow on food has evolved biochemical mechanisms to digest components that exist in the food. These mechanisms aid the organism in growth by providing a source of energy. But because of these mechanisms, it affects the food's sensory, chemical, and physical properties. What kind of compounds that are produced differ largely on the genetic capability of the microorganism as well as the characteristics of the product in question, the handling and the storage environment. (Bevilacqua et al., 2016, p. 2-3)

2.2 Amino acids and spoilage

Spoilage of meat is associated with microbial catabolism of carbon and nitrogen compounds, but muscle like fish contains low amounts of carbohydrates and high amounts of nitrogen compounds, the most important for spoilage being amino acids. Amino acids and oligopeptides are produced continuously in the fish muscle because of protein degradation caused by endogenous and microbial proteolytic enzymes. This makes amino acids highly important for the growth of spoilage bacteria in fish muscle. (Zhuang et al., 2021)

2.2.1 Deamination

Deamination (Figure 2.1) as well as decarboxylation are the two important biochemical reactions that takes place during fish spoilage. Deamination is a fundamental part of microbe's ability to utilize amino acids as a source of nutrients. Deamination produces carbon skeletons which the bacteria can use for its energy metabolism and substance synthesis. Deamination of amino acids can result in the production of ammonia that results in an increase of the muscle pH and produces off-flavors as well as unpleasant odors, which further contributes to quality deterioration of the fish. (Zhuang et al., 2021)

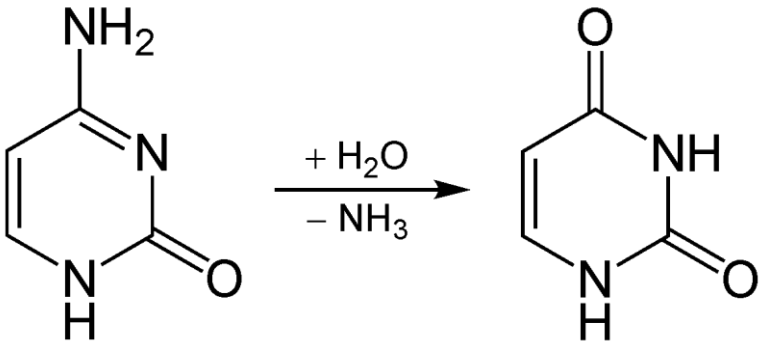


Figure 2.1: Example of deamination. Retrieved form: (Wikipedia, 2020a)

2.2.2 Decarboxylation

Decarboxylation (Figure 2.2) is another form of amino acid degradation that is caused by spoilage bacteria. In this degradation process corresponding biogenic amines are produced. In fish and other seafood products putrescine and cadaverine are important biogenic amines that cause off-flavors and odors. Decarboxylation can also result in the production of biogenic amines like histamine that can cause foodborne diseases. (Zhuang et al., 2021)

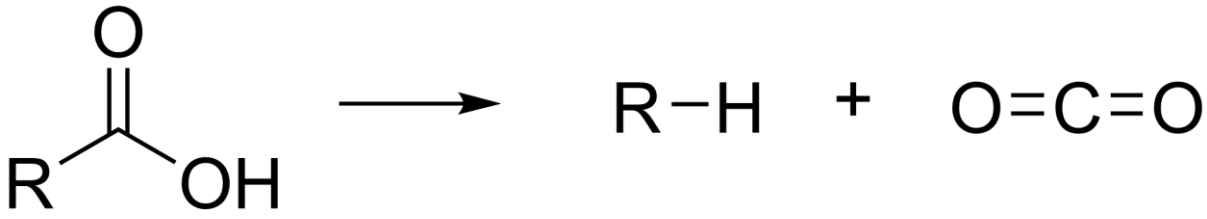


Figure 2.2: Example of decarboxylation. Retrieved from: (Wikipedia, 2020b)

2.2.3 Biogenic amines

Biogenic amines are a group of nitrogenous amines, there exists many but the most relevant for food spoilage and human disease are putrescine, cadaverine, histamine, tyramine, spermidine and spermine. They are commonly low in molecular weight with around 80-2000 Daltons. They are an active ingredient in biological organisms where they play an important physiological role. Biogenic amines are formed from some free amino acid's trough microbial decarboxylation. Fish, as well as wine, meat and dairy often contain these kinds of free amino acids, making them susceptible to this kind of formation. (Chengqiang Zhang et al., 2020)

Biogenic amines are in general harmless to humans as they become detoxified by amine oxidases in the intestine. Consuming large amounts of biogenic amines however can cause food poisoning where histamine has been shown to be the most toxic. There has also been shown that putrescine, cadaverine, spermidine and spermine could be potential carcinogens as they are converted to nitrosamines. Seafood has the potential to contain high amounts of biogenic amines, but this is strongly depended on the freshness of the seafood, as well as the storage conditions. The levels on biogenic amines in seafood are initially very low, but throughout its shelf life they are accumulated trough bacterial decarboxylation. (Chengqiang Zhang et al., 2020)

The names of different biogenic amines are often correlated to the amino acid they derive from, examples of this being histamine from histidine, tryptamine from tryptophan and tyramine from tyrosine, these all being examples of decarboxylation by removal of the α-carboxyl group. Other pathways exist were the same name correlation is not present, this being the case for decarboxylation of lysine to cadaverine or arginine to putrescine. (Özogul & Özogul, 2019)

Biogenic amines exists in three forms, these being aliphatic, aromatic and heterocyclic, as well as being classified as monoamines, diamines and polyamines depending on the number of amine groups that they have. (Özogul & Özogul, 2019)

2.3 ATP degradation

ATP degradation is a major part of fish spoilage and the overall quality of fish products. This is a biochemical change that takes place in post-mortem fish and shellfish muscle. After slaughter fish goes through 5 stages of change, these being Rigor mortis, resolution of rigor mortis, autolysis, and spoilage (Li et al., 2017).

Glycogen in the dead fish is constantly being consumed and ATP is being produced rapidly. The ATP is then further degraded to adenosine diphosphate (ADP), then adenosine monophosphate (AMP) before being broken down to inosine monophosphate (IMP). IMP being different from the other compounds by being a wanted product as it is a flavor enhancer associated with the umami taste. The IMP is then further degraded to hypoxanthine ribonucleoside (HxR) and hypoxanthine (Hx) with help from bacterial enzymes that are being produced during spoilage, but enzymes that occur during autolysis has also been shown to be a factor in this degradation step (Li et al., 2017). An example if this degradation can be seen in figure 2.3.

Since IMP is a wanted compound due to its positive flavor and Hx being associated with spoiled fish these two compounds can be used as benchmarks for measuring both freshness and spoilage in fish (Li et al., 2017).

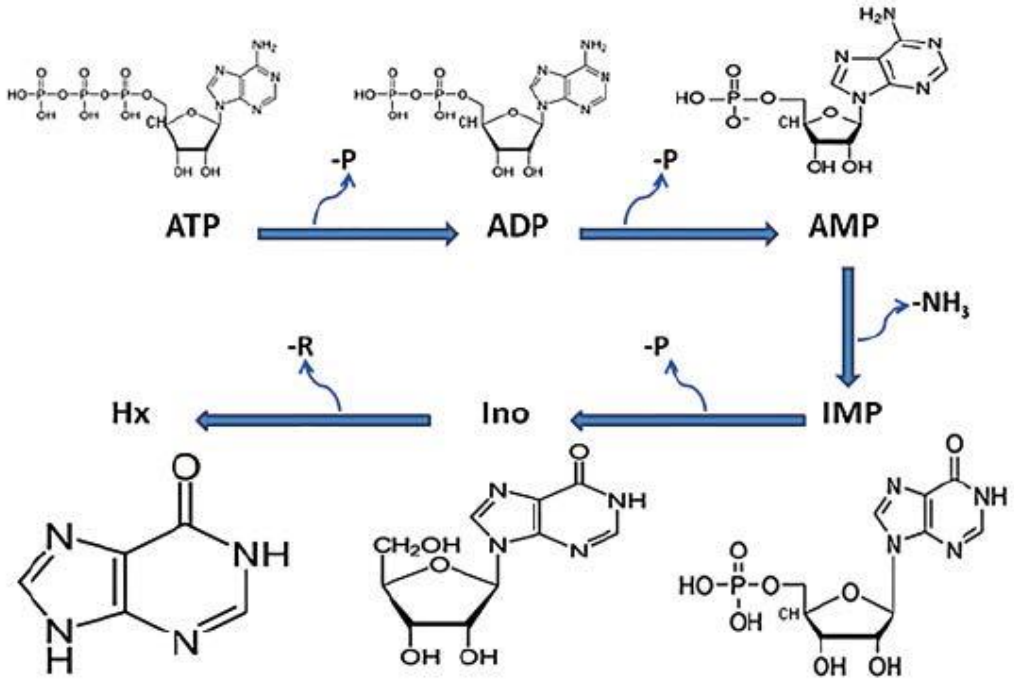


Figure 2.3. ATP degradation associated with spoilage. Retrieved from (Food Quality and Safety, 2015)

2.4 Spoilage of fish and specific spoilage organisms

Fish, like most other food products are highly susceptible to cross-contamination during processing. In the case of fish this processing happens right after slaughter and the fish flesh is rich in nonprotein low-molecular weight nitrogenous compounds Nonprotein nitrogen (NPN) as well as having a pH of >6.0. This favors growth of spoilage bacteria which further produce metabolic by-products. (Boziaris & Parlapani, 2017, p. 61)

The initial microbiota that exists on fish and seafood the start of its shelf life is a combination of the indigenous and exogenous microbiota. Where the indigenous microbiota is the bacteria that we naturally find in the fish's skin, gills and digestive tract, what type of bacteria that is a part of this indigenous microbiota is decided by the waters that the fish live in. The exogenous microbiota on the other hand is defined as the bacteria and other microorganisms that originates from contamination of the product, this being microorganisms that exists in the environment, contact surfaces, workers etc. Only a small part the initial microbiota prevails under the storage and processing condition that are set for a specific product, these microorganisms outgrow the other and becomes the dominant species. A part of the dominant microbiota can spoil food, this is the part that are referred as specific spoilage organisms (SSO). (Boziaris & Parlapani, 2017, p. 62)

What genera of bacteria that makes up the SSO varies and can be both Gram-negative and Gram-positive bacteria, but in the case of fish and seafood the most common gram-negative bacteria are *Pseudomonas, Shewanella, Psychrobacter, Pseudoalteromonas, Moraxella, Acinetobacter, Flavobacterium, Vibrio, Photobacterium* and *Aeromonas.* While the most common gram-positive bacteria include lactic acid bacteria (LAB), *Micrococcus, Corynebacterium, Vagococcus, Bacillus* and *Clostridium.* Other bacteria like *Enterobacteriaceae, Staphylococci* and *Listeria* can also be a part of the initial microbiota, but these are more likely to be a result of cross-contamination where the previously mention bacteria can all be found naturally on the fish, dependent on the environment they originate from. (Boziaris & Parlapani, 2017, p. 62)

In the spoilage microbiota, we only define the microorganisms that can produce metabolites in adequate quantities as the SSOs. The time it takes these SSOs to produce a concentration of metabolites to a point of sensory rejection determines its shelf life. Different fish, processing and storage conditions as well as different SSOs and their initial concentration all determine how long this is. (Boziaris & Parlapani, 2017, p. 63-70)

2.5 Autolysis

Autolysis is a post mortem process that results in degradation of muscle and skin that is onset by enzymes that are naturally a curing in the fish muscle. (Mukundan, Antony, & Nair, 1986)

The autolytic process happens shortly after death but the exact time varies from fish species to species (Jessen, Nielsen, & Larsen, 2014). The spoilage that is associated with the Autolysis process is far less impactful than the bacterial associated spoilage, but it is an important factor for flavor development and the onset of bacterial spoilage. Since bacteria cannot permeate through the skin of the fish and the availability of nutrients are limited, they have a difficult time to grow and multiply. But during and after autolysis this changes due to the skin being broken down as well as simple sugars, free amino acids and free fatty acids are being released (Mukundan et al., 1986).

2.6 Trimethylamine oxide reduction

Trimethylamine oxide (TMAO) is a naturally accruing compound that can be found in all marine fish and shellfish with varied concentration depending on the species, location, and season. TMAO is an important for the fishes' physiological function and at the same time it is an important factor in the spoilage process. (Summers, Wibisono, Hedderley, & Fletcher, 2017)

TMAO in live fish functions as an osmolyte meaning that it regulates osmotic pressure and maintain fluid balance. It is also speculated that the high concentration of TMAO in fish from polar regions keeps the liquids within the fish from freezing. (Summers et al., 2017)

Postmortem TMAO is a contributor to spoilage as it is degraded via non-enzymatic and enzymatic pathways to trimethylamine (TMA), dimethylamine (DMA) as well as formaldehyde. All these products contribute to decreased quality of the fish and seafood, were as TMA and DMA are associated with the "fishy" odor that occurs in old and/or spoiled fish. (Summers et al., 2017)

Bacteria can use TMAO as an energy source when bacteria grow anaerobically were TMAO is used as an alternative terminal electron acceptor. TMAO-reducing bacteria are in an abundance in marine environments and typical species include *Photobacterium, shewanella* and *vibrio* but enterobacteria also has the same TMAO reductase enzymes. (Dos Santos, Iobbi-Nivol, Couillault, Giordano, & Mejean, 1998)

2.7 H_2S producing bacteria and its role in spoilage.

Hydrogen sulfide (H_2S) is a colorless and irritant gas, it has a characteristic foul smell that can be described as the smell of rotten eggs. Different bacteria can convert sulfur-containing amino acids such as cysteine and methionine to H_2S as part of their natural metabolism. The most common H_2S-producing bacteria found in fish and meat are *Shewanella putrefaciens, Pseudomonas mephitica* and *Citrobacter freundii*, but other species and strains exists. (X. Liu, Gong, & Jiang, 2011)

H_2S-producing bacteria makes up a small percentage of the initial microbial flora of fish but has been shown to become more and more dominant during iced storage due to their psychotropic nature. (Birte Fonnesbech, Kasthuri, Masataka, & Lone, 2005)

2.8 *Aeromonas spp.*

Aeromonadaceae is a class of Gamma-proteobacteria. *Aeromonadaceae* is a fairly new family, being recognized as its own family for just over 25 years, were it was previously classified under the family *Vibrionaceae*. (Parker & Shaw, 2011)

As of 2002 there were 14 different confirmed species, these being: *A. allosaccharophila, A. Hyderophila, A. Bestiarum, A. caviae, A. encheleia, A. eucrionophila, A. jandaei, A. popoffii, A. media, A. salmonicida, A. schubertii, A. sobria, A. veronii* and *A. trota* (Isonhood & Drake, 2002). With modern tools like PCR and DNA-hybridization more and more species are being found and classified under the *Aeromonadaceae* class where it had grown to include 36 species as of 2019 (Hoel, Vadstein, & Jakobsen, 2019).

Aeromonas spp. are gram-negative bacteria. They are often motile, facultative anaerobic, non-spore forming, rod shaped with polar flagella. These bacteria live primary in water and can be found in both fresh and brackish waters. Aeromonas varies in size but are normally within the range of 0.4 to 1.0µm in Width and 1.0 to 4.4µm in length. *Aeromonas* spp. can tolerate a pH range of 4.0 – 10 and has a growth optimum between 20°C and 35°C (Kerry, 2012, p. 37). *Aeromonas* are catalase- and oxidase positive and has a fermentative and oxidative carbohydrate metabolism, with this being able to ferment D-glucose and produce a range of extracellular hydrolytic enzymes (Tang, 2015, p. 1101).

Not all *Aeromonas* species fit the description above, alternatively they can be classified as non-motile psychrophilic aeromonads, and motile mesophilic aeromonads. Psychrophilic strains include the fish pathogen *A. Salmonicida* which has a growth optimum between 22°C

and 28°C where a mesophilic strain grows best between 35°C and 37°C. There also exist strains that can grow between 4°C and 42°C. There is still some debate over mesophilic *Aeromonas*'s ability to cause disease in humans but considering available evidence we can say that several *Aeromonas* species are in fact human pathogens, particularly *Aeromonas hydrophila* with its ability to cause gastroenteritis in humans. Mesophilic *Aeromonas* are also linked with other conditions, including wound infections, septicaemia and soft tissue infections (Percival, 2014, p. 49). The species that are found the most in clinical samples are: *Aeromonas caviae* (29.9%), *A. dhakensis* (25,5%), *A. veronii* (22%) and *A. hydrophila* (18%). (Laviad & Halpern, 2016)

Aeromonas has been shown to make up a significant part of the spoilage microbiota in fish and seafood like sea bream, common carp, and whole tropical shrimps. But there is still little knowledge on what spoilage metabolites that are being produced as well as the differences in spoilage potential between the species and strains especially on products like Atlantic salmon (*salmo salar*), cod (*Gadus morhua*) and saithe (*Pollachius virens*). (Hoel et al., 2019)

Aeromonas salmonicida can also cause infections in fish called furunculosis which is a problem many places in the world especially in the aquaculture sector where is causes yearly economic losses (Bartkova, Kokotovic, Skall, Lorenzen, & Dalsgaard, 2017). This in combination with the genus being considered a major spoilage organism in fish *Aeromonas* has the potential to be a large problem for the aquaculture sector as a whole. (C. Zhang et al., 2020)

Given the fact that *Aeromonas* can cause decease in humans, their role in contamination of ready-to-eat products are important, especially since some species has been shown to produce exotoxins at low temperatures and up to 1M concentrations of sodium chloride. (Delamare, Costa, Da Silveira, & Echeverrigaray, 2000)

2.9 Kjeldahl

To measure and determine the total protein content of food the kjeldahl method is often used. This is a method that indirectly quantifies the total protein content by measuring the amount of nitrogen in the samples. Measuring nitrogen is done in 3 steps: digestion, distillation, and titration. The most common method today is by digesting the samples in sulfuric acid or potassium sulfate in combination with a catalyst that converts the nitrogen within the sample's protein to ammonium sulfate. The solution is then distillated and with excess sodium hydroxide, the ammonia is liberated and further absorbed in the acid and the titrated to quantitate ammonia. Since this is a very time-consuming method to do manually, automated systems have been made. (Wang, Pampati, McCormick, & Bhattacharyya, 2016)

2.10 High Preforming Liquid Chromatography

High preforming liquid Chromatography or sometimes referred to as High pressure liquid chromatography or HPLC for short (Hanai & Smith, 1999; Horvath, 1980). Is a process that aims to separate compounds within a liquid from each other, with this you can measure what compounds that exists within the samples as well as the concentration of said compound. (Snyder, Kirkland, & Dolan, 2010)

HPLC in its modern form is an automated process were computer systems picks out samples from a tray and injects them into a column. The system pumps solvent through this column and the separated compounds are continuously sensed by detectors within the HPLC system as they leave the column. (Snyder et al., 2010)

Chromatography exists in many forms and is often used as a tool to separate compounds from each other. HPLC and Gas chromatography (GC) share the same goal in separating high-molecular-weight biological substances but HPLC can detect more volatile compounds compared to GC because it can use lower temperatures as well as using a mobile and a stationary phase that compete whereas GC that only uses a stationary phase. In analysis where GC can detect the same compounds as the HPLC, the GC method is often preferred since it is a cheaper alternative. There are also other benefits to using the HPLC technique, mainly being that there exist automated methods that are not dependent on the operator's skill. (Hamilton & Sewell, 1982)

2.10 Nuclear Magnetic Resonance

Nuclear Magnetic Resonance or NMR for short is a form of spectroscopy in the radio area of the electromagnetic spectrum (Pedersen, 2017). NMR has become more popular in the field of food science, being a powerful and versatile analytic technique for analyzing liquid and/or solid materials. (Hatzakis, 2019)

The principle of NMR spectroscopy is using the magnetic properties of certain nuclei that have an odd mass number or in other cases an even mass number but an odd atomic number. These kinds of nuclei do a spin, or a rotation referred to as “S”. Dependent on the mass number and/or atomic number this spin generate energy in the form of magnetization that can be measured using the NMR instrument. (Hatzakis, 2019)

When placing a sample in the NMR system the samples are briefly irradiated with a strong radio radiation called a radio pulse, this pulse being determined by size of the magnetic field and the isotopes that are being studied. This causes the nuclei to change orientation, the NMR then measures a spectrophotometric value when these nuclei return to their original orientation. This being the signal that appears on the chromatograph to be further integrated and calculated. This pulse is repeated several times to generate a high quality spectrum. (Pedersen, 2017)

3.0 Materials and Methods

3.1 Preparation of growth media

Pre-rigor filleted salmon loins (Salma) were purchased from a local retail store and blended with distilled water in a ratio of 1:2 of salmon and distilled water in a high-power food processor (robot coupe, blixer 6 v.v.) until homogenized. The mixture was then boiled for 3-5 minutes before being passed through a sieve and later filtered into a Schott flask using a 185 mm paper filter (Schleicher & Schuell). The bottles of fish juice were later heated for 30 minutes at 100°C using an autoclave. After a brief cooldown, the mixture was poured into 50 ml sterile tubes and frozen at -45°C.

In total 3 batches of fish juice were made, where batch "14.09" was a mixture of Salma loins with slaughter date 03.09.2020 and 08.09.2020, batch "15.09" was only Salma loins with slaughter date 08.09.2020 and batch "04.10.2020" was made with salmon filets from a local slaughter facility frozen directly after slaughter.

3.1.1 Preparation of phosphate buffer

68,945g KH_2PO_4 and 87,09g K_2HPO_4 where added to two separate Erlenmeyer flasks and diluted with 500ml distilled water, making a 1M solution. The two solutions where then combined, pH was measured and adjusted to 6,7 before being sterilized at 121°C for 15 minutes.

3.1.2 Preparation of enriched fish juice

The previously made fish juice were tawed overnight in a refrigerator, 900 ml was supplemented with 100 ml 1M phosphate buffer (pH 6.7), 10 g glucose (Merck) and 15g NaCl (Merck).

3.1.3 Preparation of Tryptone Soy agar and Tryptone Soy Broth

15.0 g Tryptone soy agar (TSA) (Oxoid) was combined with 500 ml distilled water in an Erlenmeyer flask and sterilized by autoclaving at 121°C for 15 minutes. After sterilization, the flasks were transferred to a 54°C water bath for 30 minutes before being poured into petri dishes.

20.0g Tryptone Soy Broth (TSB) (Oxoid) was dissolved with 500 ml distilled water in a Schott flask and sterilized at 121°C for 15 minutes.

3.2 Determination of nutrient composition in growth media

To compare the protein content in the fish juice and TSB media, a Kjeldahl analysis was performed using an Automated Kjeldahl system (KjelMaster K-375 and KjelDigester K-449 BUCHI) The preparation of samples was performed as previously described. The samples were set up as presented in table 3.1.

Table 3.1. Kjeldahl setup. Content of the different test-tubes

Tube:	**Content**
1 and 2	Two BUCHI Kjeldahl tablets, 15 ml sulfuric acid
3 and 12	Two BUCHI Kjeldahl tablets, 15 ml sulfuric acid and 0.1 g stearic acid
4 trough 11	Two BUCHI Kjeldahl tablets, 15 ml sulfuric acid, one “spatula tip” stearic acid and 10 ml sample.

2 parallels of each batch of fish juice were analyzed as well as 2 parallels of TSB All tubes (including tube 13 through 20 which were not used in this analysis) where placed into the automated Kjeldahl machine and the following program was set:

- Heating to 150°C
- 170°C for 15 minutes
- 220°C for 20 minutes
- 420°C for 115 minutes
- Cooling for 30 minutes

The analysis was done in duplicates with two different setups. The second round of analysis using 3 parallels of each batch of Fish Juice and no batches of TSB.

3.3 Analysis of free amino acids

To compare the free amino acid composition of the fish juice and TSB media, 3 parallels of each produced batch of Fish juice and 3 parallels of the TSB were diluted 1:50 and 1:500 with distilled water in preparation for HPLC analysis. The samples were filtered through a 0.2µm acetate filter (VWR) and 205 µl of each sample was transferred to a HPLC-tube and analyzed by the staff at the IBT Food Chemistry lab, campus Gløshaugen, Trondheim.

3.4 Temperature dependent growth kinetic parameters of *Aeromonas* strains

To assess the different strain's ability to grow in the two prepared media and to determine what media to use in further analysis a study of the strain's growth kinetics was performed.

Table 3.2. Eight Aeromonas strains isolated from different sources.

Strain (Strain ID)	**Source of isolation**
A. media	Sushi
A. salmonicida Nr. 21	Salmon
A. Bestiarum	Salmon
A. piscicola (SU58-3)	Retail Sushi
A. Caviae (SU3-2)	Retail Sushi
A. dhakensis (A536)	Retail Sushi
A. Hydrophila (A538)	Retail Sushi
A. Salmonicida (SU2)	Retail Sushi

The ability of the eight *Aeromonas* strains (Table 3.2.) to grow in Fish Juice and TSB was assessed at 4, 8 and 15°C over a period of 69 to 220 hours depending on the temperature and media.

Cultures of each strain was made by transferring 10 µl of bacteria and glycerol solution to a set plate of TSA before being incubated at 30°C for 20 hours. 1 colony of each was later added to 10 ml of TSB and incubated at 15°C on a platform shaker for another 20 hours to adapt the strains to lower temperatures. OD of these inoculums was measured at diluted until they reached an OD of <0.60.

30 ml of TSB was added directly to 50 ml sterile tubes, the fish juice mixture was passed through a 0,45 µm cellulose acetate filter (VWR) before being added to separate 50 ml tubes. 3 parallels of each bacteria strain were made for both the TSB and the fish juice in 3 sets of samples. 300µl of the inoculum was added to the parallels and the OD was measured right after inoculating. Three parallels were incubated at three different temperatures (4-, 8- and 15°C) and the OD was measured several times per day were the samples incubated at 15°C got measured more frequently for a shorter amount of time, while the sets incubated at 4°C was measured less frequently but over a longer time span (Table 3.2).

Table 3.2. Incubation time of strains grown in different media and temperatures.

Media	**Temperature**	**Incubation time**
Fish juice	4°C	220 Hours
	8°C	160 Hours
	15°C	81 Hours
TSB	4°C	147 Hours
	8°C	76 Hours
	15°C	69 Hours

All data gathered from this analysis was ran through the database Combase (2020) to calculate and model the growth parameters of the different stains and factors set. Combase data was then further investigated, and statistics were calculated using IBM Statistics 2.7.

3.5 Production of H_2S

To investigate if the strains used in this assignment can produce H_2S at different temperatures, tubes of Iron agar was prepared. 43.6 g of Iron agar (lyngby) (Oxoid, CM0964) was mixed with 1000ml of distilled water. The mixture was then heated up at 100°C before being sterilized at 121°C in an autoclave. After sterilization, the mixture was cooled down to 45°C in a water bath before 8ml, 5% solution of L-cystein was added after being run through a 0.2 µm cellulose acetate filter (VWR). 10ml of Iron agar was added to 15ml sterile tubes and stored at 4°C.

The 8 *Aeromonas*-strains were cultivated on plates of TSA for 24 hours at 15°C before a colony of each strain was transferred from the plate and "stabbed" halfway through the Iron Agar in the tubes. A total of 3 parallels per strain and per temperature was made, after transferring the strains to the Iron agar, the samples were incubated at 4, 8 and 15°C. A picture of the tubes was taken every 24 hours to see if the Agar had changed color. Examples of how this color change occurred can be seen in appendix 1.

3.6 Assessment of bacterial spoilage metabolite production in a liquid culture media

3.6.1 Experimental design.

Measurement of ATP degradation products

To further investigate the spoilage potential of *Aeromonas* in TSB an HPLC was performed with TSB inoculated with the 8 different *Aeromonas* species. The first HPLC was conducted with the aim to understand *Aeromonas's* ability to convert Inosine monophosphate (IMP) to hypoxanthine (Hx). 700ml of TSB was mixed with 3,2905 g of IMP resulting in a concentration of 9,397 mM before 25ml of the mix was divided into 50 ml sterile tubes. 250 µl of premade inoculums measured at an OD of 0.1 at 600nm were added to the tubes resulting in 3 parallels per strain. The strains were then incubated at 8°C over a period of 6 days.

Measurement of TMA conversion

The same method for TSB and inoculum preparation was conducted for the TMAO measurements as it was for ATP degradation. The only difference being that IMP was replaced with 0,567g of TMAO (concentration = 10,784 mM) to see if the strains could convert it into TMA. The inoculated samples were incubated at 8°C for a total of 5 days with

samples being taken out once every 24 hours. An overview of the experiments is presented under in figure 3.1.

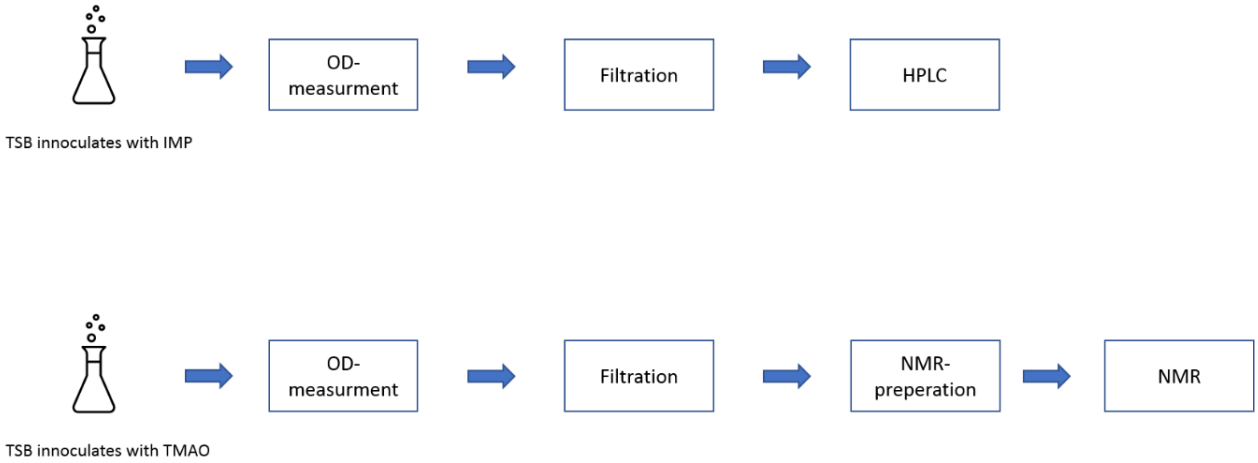


Figure 3.1. Experimental overview of metabolite assessment in liquid culture media.

3.6.2 Assessment of ATP degradation products using HPLC.

OD of the samples were measured every 24 hours for 6 days, with one set of samples being measured right after inoculation. 1,5 ml of the samples were pipetted into cuvettes and the OD were measured in a spectrophotometer, the content of the cuvette was then sterile filtered into a 2 ml Eppendorf tube and frozen at -45°C.

After thawing 50 µl of sample was added to HPLC-tubes and diluted 1:10 with distilled water. The tubes were then placed in the HPLC system (Agilent 2190) using an infinity diode array detector (Agilent Technologies) with an Poroshell 120 column (EC-C18 3,0 x 100 mm, pore size 2,7 µm). For the mobile phase Monopotassium phosphate ($(KH_2PO_4,)$ 0,215 M) and Tetrabutyammoniumhydrogensulfat ($[CH_3(CH_2)_3]_4N(HSO_4)$, 0,0023M) in 3,5% Acetonitrile with pH adjusted to 6.25 was used. The flow was set to 0,2 ml per minute for the first 2 minutes, then 0,8 ml per minute from minute 2-9 and back to 0,2 from minute 9-10. The column temperature was set at 20°C.

3.6.3 Measuring TMAO conversion with NMR.

Every sample had their pH adjusted to 7.00 ± 0.1 with 5 M NaOH and/or 1 M HCl. 495 µl of the adjusted samples were transferred over to a new Eppendorf tube together with 55 µl of 10 mM TSP (3-(trimethylsilyl) propionate-2,2,3,3-d4) (in 20 mM phosphate buffer in D2O, pH 7,0). The tubes where then centrifuged (Heraeus Megafuge 8R, 5 min, 8°C, 12000 rpm,) After centrifuging 530 µl supernatant was transferred to their individual NMR-tubes.

The NMR spectra was acquired at 300K using a Bruker Acance 600 MHz spectrometer with 5mm z-gradient TXI located at the NMR center of the Faculty of Natural Sciences and Technology (NTNU), campus Gløshaugen, Trondheim, Norway. The following settings where used: NS=128, SW=20,52 ppm. The phase and baseline of the spectra was later corrected using the software TopSpin 4.1.1pl 6 /Bruker, Germany). The TSP standard was set as an integrated standard of 9, and 0 ppm. The integrated values of TMA and TMAO was later divided by three, the same number of protons they have.

3.7 Assessment of bacterial spoilage metabolite production in vacuum packaged, refrigerated salmon fillet.

3.7.1 Experimental design.

3 inoculums were made from the strains *A. bestiarum*, *A. piscicola* and *A. salmonicida (SU2)* by transferring 1 colony of each strain into Erlenmeyer flasks containing 250ml TSB. The flasks were incubated at 8°C on a platform shaker set at 230 rpm for 48 hours.

Fresh and smoked salmon loins (Salma) was purchased from a local retailer. The loins were cut into 20 g pieces and placed onto an absorbent pad before being covered with plastic wrap. The previously made inoculums were diluted to an OD:600 of 0.1 before being further diluted with TSB in a ratio of 1:100. 200 µl of these inoculates were then dripped onto the fish samples and then distributed on the surface of the fish with a sterile plate spreader before being air dried for about 20 minutes. A total of 25 samples were prepared for each inoculum as well as 25 non-inoculated samples as control. All samples were then transferred to their own individual vacuum bags, vacuumed then stored in a cold room at 4°C. A total of 3 samples per inoculated strains as well as 3 control samples of fresh salmon were taken out every 2 days and the same set of samples of smoked salmon were taken out every 3 days in

the space of 3 weeks. These samples were then frozen at 45°C in preparation of an HPLC-analysis.

Day 0, 4, 8 and 14 of fresh Salma, and day 0, 8, 15 and 21 of smoke Salma was chosen to measure ATP degradation, TMAO conversion and biogenic amine production. The samples were grated using a box grater and 1,50 g of each was added to a centrifuge tube before 5 ml Trichloroacetic acid (TCA, 7%) was added and then homogenized using an Ultra turrax (T25 basic, IKA.werke, 13000 rpm). 1 ml potassium hydroxide was added to the tubes and then centrifuged (Rotina 420R, 4800rpm, 15mins). The supernatant was filtered and divided into 3 eppendorf tubes in preparation for further analysis. A simplified overview of the experiment is show in figure 3.2 and 3.3.

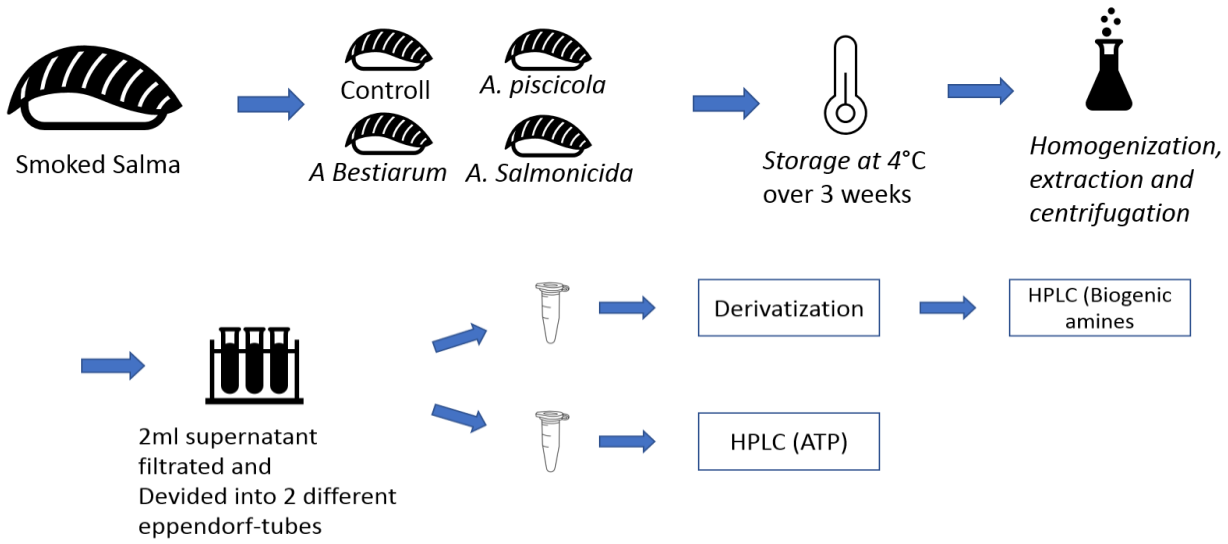


Figure 3.2. Experimental overview of metabolite assessment in smoked Salma loins.

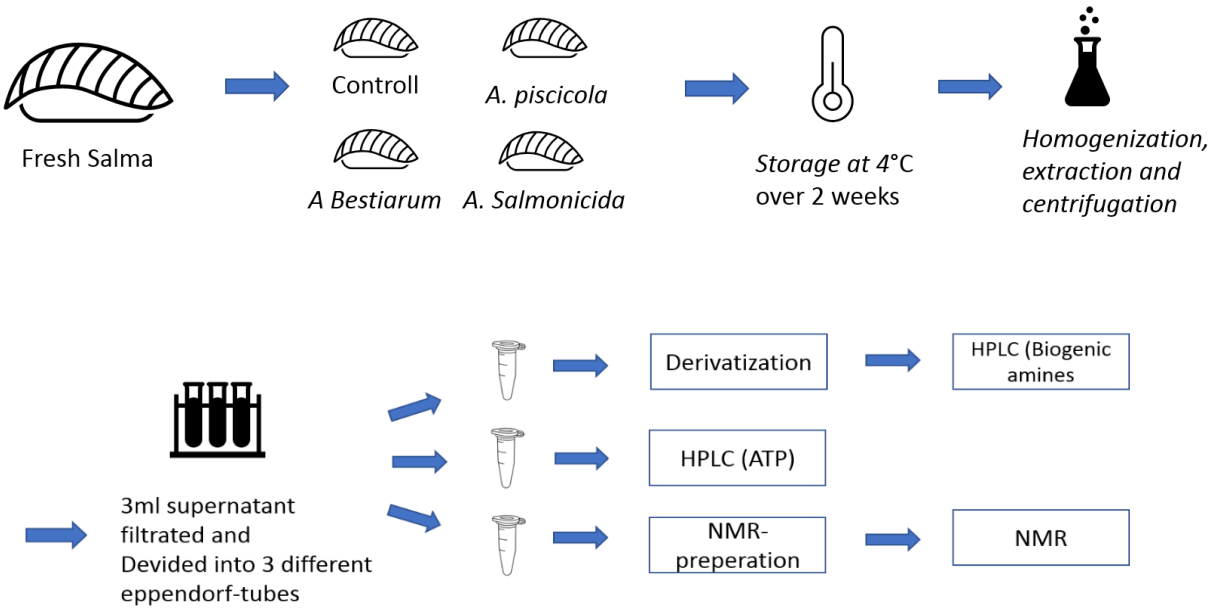


Figure 3.3. Experimental overview of metabolite assessment in Fresh Salma loins.

3.7.2 Measuring ATP degradation in vacuumed packaged refrigerated salmon filets.

For measuring ATP degradation with HPLC in the vacuumed packed fish, the same method as previously described in chapter 3.6.2 “Assessment of ATP degradation products using HPLC”.

3.7.3 Measuring TMA conversion in vacuumed packaged refrigerated salmon filets.

The same process and instrumental setup used in chapter 3.6.3 Measuring TMAO conversion with NMR Was done with samples deriving from day 0, 4, 8 and 15 of refrigerated vacuum-packed Salma.

3.8 Measuring the content of Biogenic Amines using HPLC.

The previously frozen supernatants were thawed and 1 ml of each parallel was added to their individually marked Kimax tube. 500 µl of 2% Benzoylchlorid and 2 M NaOH. The mixture was then vortexed for 60 seconds before being left out at room temperature for 20 minutes. 1 ml of a saturated NaCl solution was added and the upper organic phase was transferred into a clean Kimax tube before 1 ml Diethyl ether was added to the original tube and the new upper organic phase was transferred from the original tube to the new one. The Diethyl ether step

was then repeated once more. The samples were stored on ice before being placed in a sample concentrator (Stuart) set at 50 °C with continuously flow of Nitrogen until all visible liquid had evaporated. 500 µl of 100% acetonitrile was added to the tubes and vortexed before being transferred into Eppendorf tubes and frozen at -45°C.

The content of the Eppendorf tubes was later tawed and filtered into HPLC tubes and diluted 1:1 with distilled water before being placed into the HPLC system (Agilent), using an YMC HPLC column (YMC co., LTD) (TPF12SP9-1002PT, 100 x 2.0 mm, S – 1.9 µm). The mobile phase was a sample dependent mixture of 100% acetonitrile and water.

4.0 Results

4.1 Growth kinetic parameters.

To investigate the eight strains growth kinetics at different temperatures and to determine what media was best suited to use in subsequent analysis, an in-depth analysis was preformed where all strains were grown at 4, 8 and 15°C in both Fish Juice and TSB. The analysis was done over a period of 3 weeks, and the incubation time varied from 69 to 220 hour depending on the set incubation parameters.

The results of the growth kinetic parameters (Table 4.1) show that all strains where able to grow on both 15 and 8°C on both media with the only exception being *A. caviae* at 8°C in fish juice. *A. hydrophila, A. dhakensis* in addition to *A. caviae* did not grow on 4°C on either media.

A. caviae have a significantly lower μ_{max} than *A. salmonicida (SU2), A. media* and *A. piscicola* when cultivated in TSB ($P<0,05$). *A. caviae* also have a significantly lower μ_{max} than *A. piscicola, A. dhakensis, A. bestiaraum* and *A, media* after cultivation in Fish juice ($P<0,05$). The lag phase differences between the strains showed that *A. salmonicida* and *A. caviae* both had significantly lower values than *A. media* and *A. hydrophila* in TSB cultivated samples. In the case of Fish juice *A. caviae* were only significantly different from *A. hydrophila* ($P= 0,003$).

In the case of Y_{max} all strains are significantly different form each other when grown in TSB ($P<0,05$), except for *A. piscicola* and *A. salomonicida (*Nr.21), *A. piscicola* and *A. bestiarum* as well as *A. bestiarum* and *A. salmonicida* (SU2). The Fish juice cultivated strains were also significantly different from each other, except for the following combinations: *A. media* and *A. caviae, A. caviae* and *A. piscicola, A. salmonicida* (SU2*)* and *A. piscicola.*

The strains cultivated in TSB have a significantly higher growth rate than in Fish Juice, at all temperatures ($p<0,05$ for all strains). For example, *A. piscicola* showing an μ_{max} of 0.058 ± 0.004 at 15°C in Fish juice and 0.105 ± 0.0164 at the same conditions but cultivated in TSB. The difference between the medias is also illustrated in figure 4.1.

The TSB cultivated strains also has a shorter lag phase than their counterpart cultivated in Fish Juice, as an example *A. salmonicida SU2* at 4°C in Fish Juice had a Lag phase (hours) of 17.8 ± 14.0 and 2.5 ± 3.0 in TSB, this is most prevalent at 4°C and less so at 8 and 15°C as illustrated in figure 4.2. There is also a larger number of cases of "No Lag" in the fish juice cultivated samples.

Temperature does influence on the growth parameters, especially the growth rate. In all cultivated strains the μ_{max} increased 2 – 5x from 4 to 15°C, with a significant difference between the three different temperature levels (P= <0,05) at both media. But all successfully cultivated strains reach similar Y_{max} given enough time (Figure 4.3).

Table 4.1. Growth kinetic parameters (maximum growth rate (μ_{max}, day^{-1}), lag phase duration in days and maximum population density (Y_{max} log CFU per g) of Aeromonas species in both TSB and fish juice at 4, 8 and 15°C. Estimated using the primary growth model of Baranyi and Roberts (1994). "NG" representing no growth and "NL" meaning No lag phase. R^2 being the models fit to the dataset and SE being the standard error of the estimate. n=3

Strain	Growth temperature	Growth media	μmax (h^{-1})	Lag phase (h)	Y_{max}(log CFU per g)	R^2	SE
A. media	4°C	Fish juice	0.019 ± 0.003	31.4 ± 14.8	8.83 ± 0.09	0.97	0.164
	8°C	Fish juice	0.030 ± 0.003	6.4 ± 5.1	8.91 ± 0.05	0.98	0.099
	15°C	Fish juice	0.079 ± 0.006	0.6 ± 2.2	8.92 ± 0.04	0.99	0.088
	4°C	TSB	0.026 ± 0.002	13.3± 3.3	8.64 ± 0.03	0.99	0.046
	8°C	TSB	0.041 ± 0.005	1.5 ± 3.6	8.81 ± 0.04	0.99	0.071
	15°C	TSB	0.106 ± 0.022	6.4 ± 3.4	9.02 ± 0.01	0.98	0.116
A. bestiarum	4°C	Fish juice	0.020 ± 0.002	17.4 ± 9.0	8.96 ± 0.05	0.99	0.103
	8°C	Fish juice	0.029 ± 0.002	NL	9.01 ± 0.56	0.98	0.113
	15°C	Fish juice	0.075 ± 0.002	NL	9.12 ± 0.03	0.99	0.062
	4°C	TSB	0.026 ± 0.002	9.9 ± 5.4	8.90 ± 0.44	0.99	0.077
	8°C	TSB	0.045 ± 0.003	3.6 ± 2.2	9.01 ± 0.03	0.99	0.058
	15°C	TSB	0.083 ± 0.003	1.5 ± 0.6	9.12 ± 0.02	0.99	0.027
A. piscicola	4°C	Fish juice	0.019 ± 0.002	19.5 ± 7.7	8.87 ± 0.04	0.99	0.079
	8°C	Fish juice	0.028 ± 0.001	NL	8.90 ± 0.03	0.99	0.061
	15°C	Fish juice	0.058 ± 0.004	NL	8.96 ± 0.05	0.98	0.118
	4°C	TSB	0.028 ± 0.002	8.7 ± 4.6	8.90 ± 0.04	0.99	0.068
	8°C	TSB	0.043 ± 0.002	4.7 ± 1.7	9.08 ± 0.02	0.99	0.042
	15°C	TSB	0.105 ± 0.0164	5.4 ± 2.7	9.12 ± 0.05	0.98	0.106
A. salmonicida SU2	4°C	Fish juice	0.014 ± 0.002	17.8 ± 14.0	8.86 ± 0.09	0.98	0.119
	8°C	Fish juice	0.024 ± 0.001	2.4 ± 5.3	9.05 ± 0.05	0.99	0.079
	15°C	Fish juice	0.044 ± 0.004	NL	8.95 ± 0.09	0.97	0.137
	4°C	TSB	0.024 ± 0.001	2.5 ± 3.0	8.99 ± 0.02	0.99	0.039

	8°C	TSB	0.045 ± 0.003	3.6 ± 2.3	9.07 ± 0.03	0.99	0.056
	15°C	TSB	0.098 ± 0.018	4.2 ± 3.3	9.13 ± 0.06	0.97	0.138
A. salmonicida Nr. 21	4°C	Fish juice	0.015 ± 0.001	4.4 ± 8.4	9.10 ± 0.06	0.99	0.075
	8°C	Fish juice	0.026 ± 0.001	6.3± 2.9	9.08 ± 0.03	0.99	0.047
	15°C	Fish juice	0.054 ± 0.004	NL	8.97 ± 0.06	0.98	0.127
	4°C	TSB	0.021 ± 0.001	NL	8.93 ± 0.03	0.99	0.057
	8°C	TSB	0.038 ± 0.003	2.8 ± 2.5	9.02 ± 0.03	0.99	0.057
	15°C	TSB	0.091 ± 0.009	2.8 ± 1.8	9.03 ± 0.04	0.99	0.082
A. hydrophila	4°C	Fish juice	NG	NG	NG	-	-
	8°C	Fish juice	0.028 ± 0.001	22.9 ± 2.7	8.79 ± 0.02	0.99	0.046
	15°C	Fish juice	0.060 ± 0.006	0.3 ± 3.0	8.95 ± 0.04	0.99	0.082
	4°C	TSB	NG	NG	NG	-	-
	8°C	TSB	0.038 ± 0.001	14.9 ± 0.4	8.80 ± 0.01	1	0.011
	15°C	TSB	0.098 ± 0.01	3.2 ± 2.2	9.07 ± 0.05	0.98	0.105
A. dhakensis	4°C	Fish juice	NG	NG	NG	-	-
	8°C	Fish juice	0.020 ± 0.001	10.2 ± 6.8	8.76 ± 0.06	0.99	0.084
	15°C	Fish juice	0.071 ± 0.003	NL	8.98 ± 0.04	0.99	0.086
	4°C	TSB	NG	NG	NG	-	-
	8°C	TSB	0.028 ± 0.004	1.7 ± 6.8	8.73 ± 0.10	0.97	0.011
	15°C	TSB	0.114 ± 0.050	7.8 ± 6.7	9.06 ± 0.08	0.95	0.178
A. caviae	4°C	Fish juice	NG	NG	NG	-	-
	8°C	Fish juice	NG	NG	NG	-	-
	15°C	Fish juice	0.066 ± 0.004	NL	8.91 ± 0.05	0.98	0.111
	4°C	TSB	NG	NG	NG	-	-
	8°C	TSB	0.034 ± 0.003	6.7 ± 1.7	8.80 ± 0.02	0.99	0.038
	15°C	TSB	0.081 ± 0.014	NL	8.88 ± 0.06	0.97	0.128

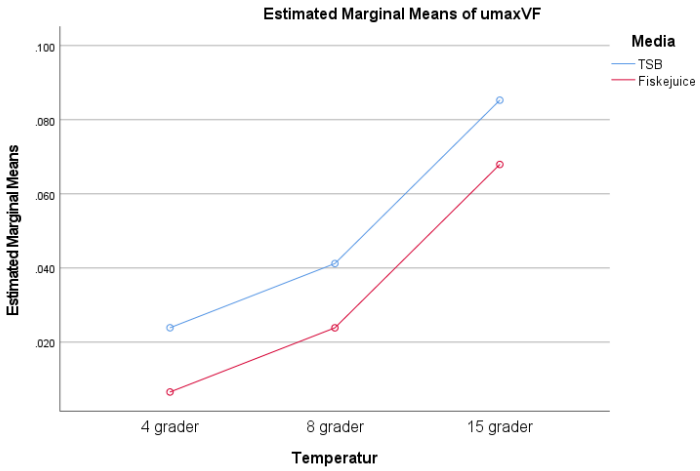


Figure 4.1. The combined average umax-values of strains grown in TSB compared to Fish Juice at the different temperatures. Figure made with IBM SPSS statistics 2.7.

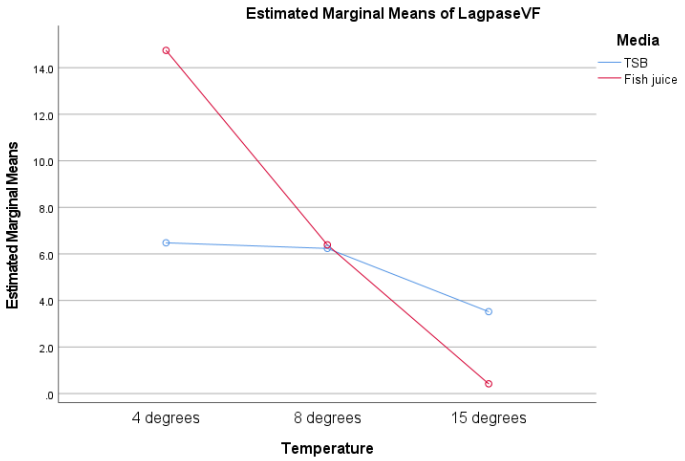


Figure 4.2. The combined average Lagpase -values of strains grown in TSB compared to Fish Juice at the different temperatures. Figure made with IBM SPSS statistics 2.7.

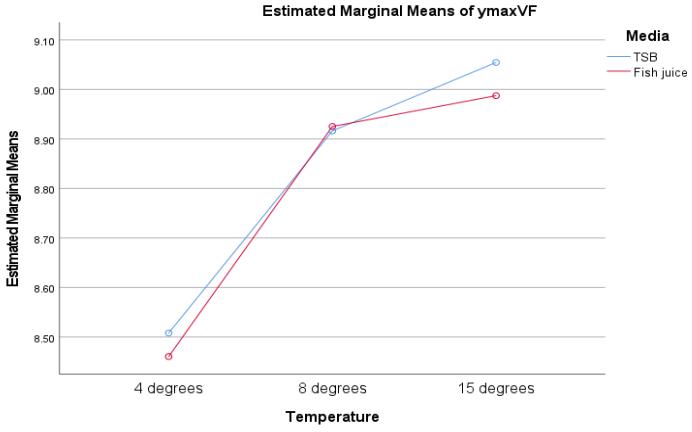


Figure 4.3. Statistical differences between Lag phase at different temperatures within the same growth media. Figure made using IBM SPSS statistics 2.7.

4.2 Amino acid and nitrogen composition of the media.

Because of the differences found between the two media, especially in growth rate an HPLC-analysis of free amino acids was performed to both determine the nutrient composition of the media and to compare the batches of fish juice to the TSB (Table 4.2.).

This analysis demonstrated that the nutrient composition varies significantly between the batches of fish juice as well as between the fish juice and the TSB. However, this variation was not consistent between the different batches.

Moreover, the concentration of each amino acid was higher in TSB than in the fish juice for all amino acids ($p<0.05$) except for aminobuteric acid, alanine, and threonine There was no significant difference in the total nitrogen content of the three batches of fish juice, the nitrogen content of TSB was higher, though not significant ($P>0,05$)

Tabell 4.2. Free amino acid in three batches of fish juice and one batch of TSB. Different superscript letters indicate statistical difference in concentration of each amino acid between the different media and batches ($p<0.05$). n=3 (amino acids), n=5 (Nitrogen in fish juice), n=2 (Nitrogen in TSB).

Amino acid	**Fish Juice 15.09 (µmol/l)**	**Fish Juice 04.10 (µmol /l)**	**Fish Juice 14.09 (µmol /l)**	**TSB (µmol /l)**
Aspartic acids (asp)	$30,0 \pm 0,0^{a}$	$238,3 \pm 7,6^{b}$	$253,3 \pm 5,8^{b}$	$316,7 \pm 10,4^{c}$
Glutamic acid (glu)	$511,7 \pm 32,5^{a}$	$556,7 \pm 16,1^{a}$	$670,0 \pm 5,0^{b}$	$901,7 \pm 42,5^{c}$
Aspergine (Asn)	$5,0 \pm 0,0^{a}$	$5,0 \pm 0,0^{a}$	$5,0 \pm 0,0^{a}$	$396,7 \pm 7,6^{b}$
Histidine (His)	$975,0 \pm 56,8^{a}$	$978,3 \pm 41,9^{a}$	$995,0 \pm 50,0^{a}$	$315,0 \pm 39,1^{b}$
Serine (Ser)	$211,7 \pm 31,8^{a}$	$313,3 \pm 29,3^{b}$	$556,7 \pm 29,3^{c}$	$828,3 \pm 20,2^{d}$
Glutamine (glu)	$200,0 \pm 21,8^{a}$	$181,7 \pm 5,8^{a}$	$306,7 \pm 11,5^{b}$	$31,7 \pm 15,3^{c}$
Glysine / Arginine (Gly / Arg)	$453,3 \pm 33,3^{a}$	$678,3 \pm 23,6^{b}$	$718,3 \pm 20,2^{b}$	$1811,7 \pm 38,2^{c}$
Threonine (Thr)	$680,0 \pm 65,4^{a}$	$433,3 \pm 11,5^{b}$	$980,0 \pm 5,0^{c}$	$740,0 \pm 35,0^{b}$
Alanine (Ala)	$1590,0 \pm 50,0^{a}$	$2071,7 \pm 68,3^{b}$	$2830,0 \pm 30,4^{c}$	$1220,0 \pm 30,0^{d}$
Methionine (Met)	$75,0 \pm 0,0^{a}$	$110,0 \pm 5,0^{a}$	$161,7 \pm 7,6^{b}$	$1610,0 \pm 39,7^{c}$
Aba (Aminobuteric acid)	$28,3 \pm 7,6^{ab}$	$41,7 \pm 2,9^{b}$	$35,0 \pm 0,0^{ab}$	$15,0 \pm 17,3^{a}$
Tyrosine (Tyr)	$120,0 \pm 10,0^{a}$	$213,3 \pm 12,6^{b}$	$280,0 \pm 0,0^{c}$	$1246,7 \pm 15,3^{d}$
Valine (Val)	$278,3 \pm 22,5^{a}$	$320,0 \pm 10,0^{a}$	$591,7 \pm 2,9^{b}$	$1495,0 \pm 47,7^{c}$
Phenylalanine (Phe)	$80,0 \pm 5,0^{a}$	$201,7 \pm 7,6^{b}$	$271,7 \pm 2,9^{c}$	$2653,3 \pm 22,5^{d}$
Isoleucine (Ile)	$116,66 \pm 7,6^{a}$	$111,66 \pm 2,9^{a}$	$241,66 \pm 2,9^{b}$	$1105,66 \pm 15,3^{c}$
Leucine (Leu)	$186,7 \pm 11,5^{a}$	$325,0 \pm 10,0^{b}$	$493,3 \pm 10,4^{c}$	$5910,0 \pm 86,7^{d}$
Lysine (Lys)	$438,3 \pm 25,2^{a}$	$568,3 \pm 17,6^{b}$	$490,0 \pm 10,0^{ab}$	$6183,3 \pm 75,2^{c}$
Totalt nitrogen (N)	$4,7 \pm 1,49^{a}$	$4,1 \pm 1,40^{a}$	$4,2 \pm 0,8^{a}$	$6,5 \pm 0,1^{a}$

4.3 Production of H_2S on Iron agar at different temperatures.

Because H_2S is a major contributor to microbiological spoilage of seafood, the strains' ability to produce this compound was of interest. To analyze this all eight *Aeromonas* strains was grown on Iron agar at 4, 8 and 15°C over a period of 5 days and graded after the amount of black coloration on the media (Table 4.3, 4.4 and 4.5).

All strains, except for *A. caviae* and *A. media* were able to produce H_2S after incubation at 15°C (Table 4.3), there was speculations if this was due to the limited availability of O_2 in the tubes as the agar was incubated in tubes, with the cap on. This was disproven with a separate analysis were the same agar and strains were tested but grown on a petri dish instead of being grown in tubes (data not shown). All other species did produce H_2S with some variance between strains in the amount of coloration on the media on day 1, but all strains except the two previously mentioned ended up with high amounts of discoloration on the media within 48 hours. indicating high amount of H_2S production.

Table 4.3. The strains' ability to produce H_2S when grown on Iron agar at 15°C. "–" representing no coloration on the media where as "+" representing some coloration. "++" representing a substantial amount of coloration and "+++" representing a large amount of coloration. All sample sets are a result of 3 parallels.

Strain	**24 Hours**			**48 Hours**			**72 Hours**			**96 Hours**			**120 Hours**		
	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3
A. caviae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. hydrophila	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. salmonicida (Su-2)	++	++	++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. salmonicida (Nr. 21)	+	+	+	++	+++	++	++	+++	++	++	+++	+++	++	+++	+++
A. piscicola	+++	+++	++	+++	+++	++	+++	+++	++	+++	+++	++	+++	+++	++
A. bestiarium	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. dhakensis	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
A. media	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

A. caviae and *A. media* did not produce any discoloration on the media at 8°C (Table 4.4) either. *A. salmonicida* (both strain Nr. 21 and SU2) were shown to need more time before the discoloration took place as well as being less prevalent after 5 days of incubation. *A. piscicola* and *A. bestiarum* did not seem to be affected greatly by the change in temperature but needed an extra 24 hours before reaching the same visual values as seen after only 24 hours at 15°C.

Table 4.4. The strains' ability to produce H_2S when grown on Iron agar at 8°C. "–" representing no coloration on the media where as "+" representing some coloration. "++" representing a substantial amount of coloration and "+++" representing a large amount of coloration. All sample sets are a result of 3 parallels.

Strain	**24 Hours**			**48 Hours**			**72 Hours**			**96 Hours**			**120 Hours**		
	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3
A. caviae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. hydrophila	-	+	+	+	++	++	+	+	++	+	++	++	+	++	++
A. salmonicida (Su-2)	-	-	-	-	+	+	-	+	-	-	+	+	+	+	+
A. salmonicida (Nr.21)	-	-	-	-	+	-	-	+	-	-	+	-	+	+	+
A. piscicola	++	+	+	+++	++	++	+++	++	++	+++	++	++	+++	++	++
A. bestiarum	+	+	+	+++	+++	+++	++	++	++	+++	+++	+++	++	++	++
A. dhakensis	+	+	-	++	++	+	+	++	+	++	++	+	++	++	++
A. Media	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Incubation at 4°C (Table 4.5) resulted in less H_2S production compared to the other temperatures. Visible growth (but no coloration) was observed in all tubes after 5 days, the expectation being *A. piscicola* and *A. bestiarum* that produced some discoloration after 48 hours but never reaching the same values as shown for the higher temperatures.

Table 4.5. The strains' ability to produce H_2S when grown on Iron agar at 4°C. "–" representing no coloration on the media where as "+" representing some coloration. "++" representing a substantial amount of coloration and "+++" representing a large amount of coloration. All sample sets are a result of 3 parallels.

	24 Hours			**48 Hours**			**72 Hours**			**96 Hours**			**120 Hours**		
Strain	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3	P1	P2	P3
A. caviae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. hydrophila	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. salmonicida (Su-2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. salmonicida (Nr.21)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. piscicola	-	-	-	+	+	+	+	+	+	+	++	+	+	++	+
A. bestiarum	-	-	-	+	-	-	+	+	+	++	+	+	++	++	+
A. dhakensis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. media	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

4.4 Measurement of ATP degradation products caused by Aeromonas *In vitro*.

The strains that were later used to analyze the development of Hx was first cultivated in a TSB media enriched with IMP at 8°C over 148 hours. The OD was measured once per day before being frozen in preparation for the coming HPLC.

All eight strains grew in the enriched media (Figure 4.4). Some differences can be seen in both the lag phase and the endpoint OD measurement. The strains appear to group up in two OD ranges, one ranging from 0,700 to 0,988, the other from 1,677 to 1,858. The strains *A. piscicola*, *A. salmonicida* (SU2) and *A. salmonicida* (Nr.21) are all in the upper range of endpoint OD, the rest belonging to the other OD range.

The strains that had the lowest endpoint OD, often also had longer lag phases, this can especially be observed on *A. dhakensis*, *A. caviae* and *A. Hydrophila*. *A. bestiarum* and *A. media* on the other hand has their exponential growth around the same time as the upper OD range group, but the growth stagnates earlier.

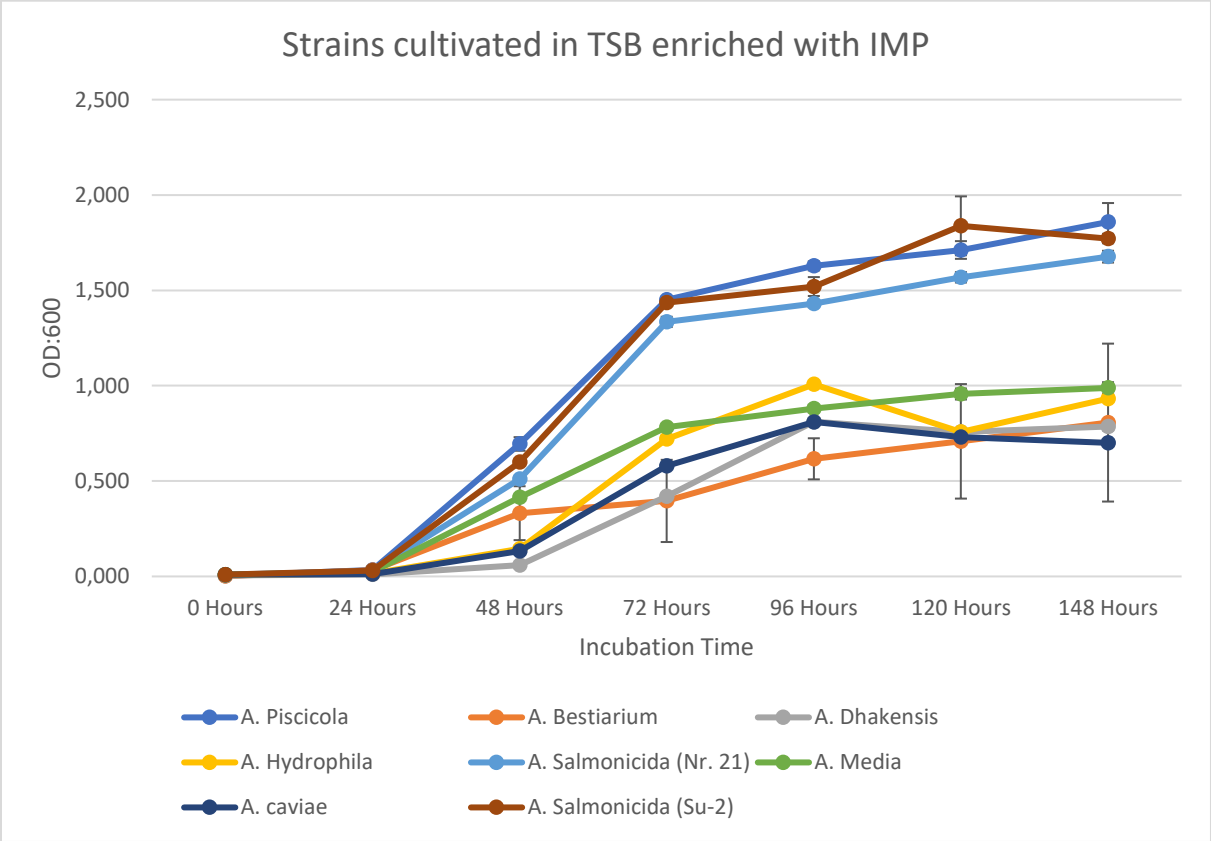


Figure 4.4. Growth curves of all eight Aeromonas strains cultivated in TSB enriched with IMP. Incubation temperature 8°C. Error bar indicate ± Standard deviation. N= 3 for all sample point.

As an attempt to determine and compare the strains' ability to convert IMP to Hx (Table 4.6) inoculated TSB with an added known IMP concentration (9,397 mM) was investigated using HPLC over a period of 6 days. Because of the large overlap of the chromatographic peaks representing IMP and INO, the concentrations of these metabolites have been added together.

The initial IMP/INO concentration was similar between all strains, this also being shown by the fact that no strain is significantly different from each other (P>0.05). All strains except *A. piscicola* had a noticeable decrease in their IMP/INO concentration over the incubation time. There were however differences in the endpoint concentration (Day 6) between the strains. As an example, *A. salmonicida* SU2 has an endpoint concentration of 12,27 ± 1,13 mg/g whereas *A. caviae* shows 513,71 ± 49,39 mg/g. Both strains of *A. salmonicida* reach very low endpoint concentrations and are significantly different from all other strains (P<0,05). *A. dakensis, A hydrophila* and *A. media* reach somewhat similar concentrations being significantly different from *A. caviae* (P<0,05) and the *A. salmonicida* strains (P<0,05), but not from each other (P>0,05). in the case of *A. hydrophila* this value is linked to some uncertainties given its high standard deviation and the fact that the day 1 value is 0.

Day 6 Hx concentrations shows that the two *A. salmonicida* strains and *A. bestiarum* reach the highest concentrations and *A. caviae* the lowest (ignoring *A. piscicola*). There can also be seen a pattern where strains show a low IMP/INO on day 6 and showing a high Hx concentration on the same day.

Table 4.6. concentration of Inosine monophosphate (IMP), Inosine (INO) and hypoxanthine (Hx) in TSB inoculated with 8 different strains of Aeromonas. Different superscript letters indicate statistical difference between the INO/IMP and the Hx concentration between the sample types on that specific day (p<0,05). Shown as value ± SD. n = 3.

	A. piscicola	***A. bestiarum***	***A. dhakensis***	***A. hydrophila***	***A. salmonicida Nr. 21***	***A. media***	***A. caviae***	***A. salmonicida SU2***
IMP/INO (mg/g) Day 0	MV	556,53 ± 36,72^{a}	567,37 ± 24,40^{a}	598,57 ± 114,90^{a}	659,57 ± 33,23^{a}	603,61 ± 13,60^{a}	654,55 ± 40,33^{a}	611,91 ± 20,54^{a}
IMP/INO (mg/g) Day 1	567,96 ± 16,05^{a}	541, 34 ± 53,93^{a}	589,04 ± 5,47^{a}	585,48 ± 8,28^{a}	585,98 ± 7,11^{a}	587,34 ± 5,67^{a}	585,17 ± 4,50^{a}	576,31 ± 6,09^{a}
IMP/INO (mg/g) Day 2	250,90 ± 244,08^{a}	602,68 ± 107,37^{ab}	679,13 ± 198,58^{b}	713,35 ± 132,49^{b}	448,07 ± 8,88^{ab}	590,79 ± 228,78^{ab}	577,22 ± 29,63^{ab}	445,22 ± 23,94^{ab}
IMP/INO (mg/g) Day 3	305,47 ± 141,29^{a}	284,46 ± 60,41^{a}	427,31 ± 27,99^{ab}	310,12 ± 94,19^{a}	295,74 ± 118,25^{a}	433,12 ± 84,45^{ab}	598,72 ± 60,93^{b}	199,41 ± 19,94^{a}
IMP/INO (mg/g) Day 4	628,10 ± 20,80^{d}	246,16 ± 57,70^{b}	284,69 ± 34,06^{b}	264,08 ± 27,23^{b}	56,40 ± 3,17^{a}	250,76 ± 15,11^{b}	448,47 ± 80,96^{c}	53,40 ± 2,59^{a}
IMP/INO (mg/g) Day 5	501,85 ± 5,39^{e}	123,40 ± 31,03^{b}	217,81 ± 8,12^{c}	MV	161,58 ± 46,37^{bc}	304,13 ± 10,86^{d}	490,29 ± 49,80^{e}	3,75 ± 6,50^{a}
IMP/INO (mg/g) Day 6	598,69 ± 53,99^{d}	60,88 ± 87,93^{ab}	213,60 ± 27,76^{bc}	248,64 ± 17,83^{c}	0,00 ± 0,00^{a}	125,74 ± 84,78^{abc}	513,71 ± 49,39^{d}	12,27 ± 1,13^{a}
Hx (mg/g) Day 0	0,00 ± 0,00^{a}	0,00 ± 0,00^{a}	0,00 ± 0,00^{a}	15,36 ± 13,87^{b}	0,00 ± 0,00^{a}	0,00 ±0,00^{a}	0,00 ± 0,00^{a}	0,00 ± 0,00^{a}
Hx (mg/g) Day 1	3,14 ±2,73^{a}	0,00 ± 0,00^{a}	0,00 ± 0,00^{a}	0,00 ± 0,00^{a}	0,00 ± 0,00^{a}	0,00 ± 0,00^{a}	0,00 ± 0,00^{a}	2,56 ± 2,23^{a}
Hx (mg/g) Day 2	61,64 ±34,22^{b}	22,92 ± 14,91^{a}	4,34 ± 1,29^{a}	6,73 ± 0,48^{a}	25,14 ± 1,82^{a}	16,84 ± 0,00^{a}	2,77 ± 0,18^{a}	25,22 ± 2,41^{a}
Hx (mg/g) Day 3	60,64 ±95,28^{ab}	83,70 ± 34,94^{ab}	35,46 ± 4,40^{ab}	83,70 ± 47,01^{ab}	125,86 ± 48,02^{ab}	46,61 ± 4,18^{ab}	22,90 ± 2,60^{a}	148,07 ± 11,19^{b}
Hx (mg/g) Day 4	0,58 ±0,83^{a}	136,98 ±46,75^{c}	95,38 ± 13,36^{bc}	106,91 ± 14,10^{bc}	280,03 ± 6,70^{d}	143,35 ± 25,85^{c}	27,63 ± 3,51^{ab}	283,62 ± 13,78^{d}
Hx (mg/g) Day 5	2,63 ±0,27^{a}	204,42 ±67,07^{cd}	114,04 ± 2,54^{bc}	258,58 ± 59,10^{d}	111,16 ± 35,25^{bc}	36,55 ± 69,02^{ab}	40,35 ± 4,21^{ab}	269,50 ± 6,39^{d}
Hx (mg/g) Day 6	2,63 ± 0,26^{a}	276,53 ± 65,49^{c}	150,44 ± 22,67^{bc}	157,35 ± 5,30^{bc}	273,77 ± 24,51^{c}	129,10 ± 1,02^{ab}	50,93 ± 4,97^{ab}	270,20 ± 23,96^{c}

4.5 Measurement of ATP degradation products in Fresh and smoked Vacuumed packed salmon.

Samples of Smoked and fresh Salma loins were artificially contaminated with the *Aeromonas* stains *A. piscicola, A. bestiarum* and *A. salmonicida* (SU2) to investigate their effect on the ATP degradation. The contaminated samples were together with control samples vacuum-packed and stored at 4°C over a period of 21 days.

The HPLC analysis of ATP degradation products in fresh Salma (Table 4.7) shows that the concentration of INO/IMP varies between the types of samples that were investigated in this study. But there is only a significant difference between the control samples and A. *salmonicida SU2* on day 0 ($P<0,05$). But the overall pattern of INO/IMP concentration indicates that the concentration is being reduced from day 0 to day 14, the exception to this being "Fresh control", But the interpretation of the results is difficult because of large deviations in the measured concentration of metabolites between the three parallels at the same sample point.

The Hx concentration is also higher in the inoculated samples compared to the control, again the only significant difference being on day 0 ($P<0,05$). The control samples also here suffer from large standard deviation giving some uncertainties in the results. "*Bestiarum"* and *"Salmonicida"* both having higher endpoint concentration compared to both *"piscicola"* and the control. All "day 14" values are significantly different from their day 0 counterparts ($P<0,05$)

Table 4.7. Inosine monophosphate (IMP), Inosine (INO) and hypoxanthine (Hx) concentration of both the inoculated fresh Salma samples and the non-inoculated control samples. Different superscript letters indicate statistical difference between the INO/IMP and the Hx concentration between the sample types on that specific day (p<0,05). Shown as concentration ± SD. n = 3.

	Control	**Piscicola**	**Bestiarum**	**Salmonicida SU2**
INO/IMP (mg/g) Day 0	11,88 ± 7,40^{a}	20,57 ± 1,24^{ab}	20,13 ± 2,62^{ab}	24,19 ± 3,87^{b}
Hx (mg/g) Day 0	1,20 ± 1,87^{a}	3,38 ± 0,28^{ab}	3,46 ± 0,46^{ab}	4,03 ± 0,68^{b}
INO/IMP (mg/g) Day 4	14,17 ± 5,52^{a}	19,75 ± 3,38^{a}	19,22 ± 4,30^{a}	19,79 ± 4,89^{a}
Hx (mg/g) Day 4	2,59 ± 2,43^{a}	4,55 ± 0,53^{a}	4,45 ± 0,90^{a}	4,88 ± 0,92^{a}
INO/IMP (mg/g) Day 8	17,40 ± 11,67^{a}	16,70 ± 2,37^{a}	23,27 ± 2,99^{a}	20,06 ± 5,26^{a}
Hx (mg/g) Day 8	4,60 ± 4,58^{a}	5,28 ± 0,44^{a}	7,04 ± 0,92^{a}	5,97 ± 0,96^{a}
INO/IMP (mg/g) Day 14	14,52 ± 14,52^{a}	17,61 ± 2,32^{a}	17,14 ± 3,92^{a}	19,28 ± 0,74^{a}
Hx (mg/g) Day 14	5,76 ± 1,90^{a}	5,91 ± 0,87^{a}	10,02 ± 3,58^{a}	9,80 ± 1,31^{a}

Looking at the results generated for smoked Salma (Table 4.8) the reduction in INO/IMP concentration that was shown in the Fresh salmon samples cannot be seen. As an example, the control samples have an initial INO/IMP concentration of 24,67 ± 4,08 mg/g and an end-point concentration of 31,68 ± 5,12, a similar increase can be seen on all inoculated samples as well. No samples are significantly different from another when comparing the samples analyzed at the same day (P>0,05).

The Hx concentration on the other hand does follow the same pattern as for fresh salma. The Hx concentration was higher at day 21 compared to day 0 for all studied samples. Salmon inoculated with *A. salmonicida* had the highest endpoint concentration with 9,80 ± 0,53 mg/g compared to the control having 6,34 ± 1,21. In the case of the smoked samples the control does have a higher average Day 21 value than both samples inoculated with *A. piscicola* and *A. bestiarum* but at the same time having a high standard deviation. All “day 21” values are significantly different from their day 0 counterparts (P<0,05)

Table 4.8. Inosine monophosphate (IMP), Inosine (INO) and hypoxanthine (Hx) concentration of both the inoculated smoked Salma samples and the non-inoculated control samples. Different superscript letters indicate statistical difference between the INO/IMP and the Hx concentration between the sample types on that specific day (p<0,05). Shown as value ± SD. n = 3.

	Control	Piscicola	Bestiarum	Salmonicida SU2
INO/IMP (mg/g) Day 0	24,67 ± 4,08[a]	25,71 ± 4,06[a]	16,76 ± 1,32[a]	27,67 ± 2,60[a]
Hx (mg/g) Day 0	2,41 ± 0,81[a]	2,39 ± 0,67[a]	2,08 ± 0,19[a]	2,54 ± 0,25[a]
INO/IMP (mg/g) Day 8	23,21 ± 1,31[a]	21,17 ± 1,31[a]	24,34 ± 1,61[a]	25,60 ± 0,75[a]
Hx (mg/g) Day 8	3,34 ± 0,45[a]	2,91 ± 1,26[a]	3,11 ± 0,52[a]	3,24 ± 0,35[a]
INO/IMP (mg/g) Day 15	28,65 ± 3,53[a]	26,79 ± 3,53[a]	30,70 ± 3,55[a]	27,99 ± 0,72[a]
Hx (mg/g) Day 15	4,98 ± 0,26[a]	5,02 ± 0,39[a]	4,88 ± 1,00[a]	5,35 ± 0,47[a]
INO/IMP (mg/g) Day 21	31,68 ± 5,12[a]	29,18 ± 5,12[a]	22,34 ± 6,01[a]	28,56 ± 3,09 [a]
Hx (mg/g) Day 21	6,34 ± 1,21[a]	5,91 ± 0,34[a]	5,72 ± 0,45[a]	9,80 ± 0,53[a]

4.6 TMA concentration of inoculated and non-inoculated Fresh Salma.

To gather more data on the strains ability to spoil both salmon, an NMR of the vacuum packed artificially contaminated Fresh Salma was conducted. This time to analyze the development of TMA concentration over the storage time.

TMA concentration on day 0 were shown to be similar between the different samples (P<0,02). The concentration of all samples increased over the duration of the storage time. The inoculated samples were shown to generate much higher endpoint values compared to the control, though some uncertainties are present given high standard deviation especially on samples type "*salomicida"* and "*Bestiarum"* on day 14. Both "*piscicola"* and "*bestiarum*" have significantly different values at day 14 compared to day 0 (P<0,05).

Table X.X: TMA concentration of the Fresh inoculated and non-inoculated Salma samples over a two-week timespan. Values gathered using NMR. n = 3.

** 2 parallels*

	Mg TMA/g fish Day 0	**Mg TMA/g fish Day 4**	**Mg TMA/g fish Day 8**	**Mg TMA/g fish Day 14**
Fresh Control	0,009 ± 0,001[a]	0,021 ± 0,012[a]	0,010 ± 0,038[a]	0,018 ± 0,006[a]
Fresh Piscicola	0,006 ± 0,001[a]	0,014 ± 0,014[a]	0,027 ± 0,012[a]	0,185 ± 0,046[b]
Fresh Bestiarum	0,009 ± 0,000[a]	0,026 ± 0,026[a]	0,042 ± 0,022[a]	0,215 ± 0,104[b]
Fresh Salmonicida SU2	0,008 ± 0,00[a]	0,060 ± 0,070[a]	0,073 ± 0,043[a]	0,354 ± 0,253*[a]

4.7 TMAO reduction and TMA development of eight strains of *Aeromonas in vitro*

The chromatographs where not able to be read and no peaks could be integrated. Results are therefore not presented here.

5.0 Discussion

In this study, the growth kinetics of eight different strains of *Aeromonas* have been investigated in two different media and at three different temperatures to both compare the differences of these medias and to determine what media was best suited for further analysis. Another important aspect was to evaluate the differences in the strain's growth kinetics. One of the main objectives of this study was also to determine differences between the strains ability to produce spoilage metabolites both in controlled and natural environments. The metabolites that were chosen to focus on was Trimethylamine (TMA), hypoxanthine (Hx), biogenic amines as well as the strains' ability to produce H_2S. Because of poor data, the results of biogenic amine production are lacking, but discussion on that topic will follow.

Temperature was an important factor to include when examining the two medias, as there where uncertainties if the strains would behave different in the medias on all or just some temperature levels, and there was of interest to see if some strains could handle the colder temperatures better than the others.

Aeromonas, as described by (Jakobsen et al., 2020) and (Hoel, Mehli, Bruheim, Vadstein, & Jakobsen, 2015) are common bacteria that are isolated from both marine environments and seafood. But knowledge about its role in spoilage are limited, especially on Atlantic fish species. This made it of interest to study their ability to produce these metabolites in salmon, especially when combined with the inhibiting factor of vacuum packaging and smoke.

5.1 Growth kinetic parameters.

(Jakobsen et al., 2020) stated in their study that determining TMA and biogenic amine concentrations is difficult when analyzing inoculated salmon because of the complex microbial background flora that exists on the product. Making it more relevant to study these parameters in a controlled environment.

Many studies, including (Jia et al., 2020; Lillebjerka, 2019) have used a fish juice system as a media for determining and/or analyzing both growth and spoilage of different microorganisms. The reasoning for using this system is primarily to emulate salmon as a product but in a sterile environment. But knowledge about its nutrient composition and how it effects growth parameters compared to traditional media is limited.

All strains where able to grow in both media, but not at every temperature level. Overall, the strains seem to be more inhibited when cultivated in Fish Juice compared to TSB. This can be seen especially on the duration of the lag phase (table 4.1) where the fish juice values are in general higher than the TSB values or in some cases, a lag phase was not detected. The strains in general achieve a higher μ_{max} when grown in TSB. Both factors indicates that the strains are less inhibited in their growth in the TSB media, this is also reflected on the amount of time needed to reach the stationary growth phase during the experiment.

It has also been demonstrated that if given enough time, strains grown on in Fish juice can achieve the same or comparable Y_{max}. Some strains were more inhibited by temperature than others, this being especially prevalent on *A. hydrophila*, *A. dhakensis* and *A. caviae* as they were not able to grow in 4°C on either media, *A. caviae* not being able to grow in 8°C Fish juice as well. Krovacek, Faris, and Månsson (1991) has demonstrated in their study that *A. hydrophilla* can grow at low temperatures, but only 50% of their cultivation attempts were successful at 4°C.

Strains grown in TSB often have about a 2x increase in μ_{max} compared to their Fish juice counterpart. The length of the lag phase is also much more stable in TSB when comparing the three temperatures. This, combined with all other parameters indicates that Fish Juice cannot substitute TSB as a traditional growth medium as it inhibits growth comparably. Lerke, Adams, and Farber (1963) however has shown that it does follow a similar pattern to the growth kinetics that are present in natural fish muscle. What media to choose might then be depended on what information one would want from an experiment.

5.2 Amino acid and nitrogen composition of the media.

An important factor in determining the differences between the fish juice and the TSB and to be able to determine the remake ability of the juice was gathering data of the amino acid composition. The original amino acid composition is highly important when analyzing for spoilage metabolism, therefor it is of high value that the media in use has a somewhat similar composition from batch to batch.

Judging by the results from this Assignment there are some batch-to-batch differences as well as differences between the fish juice and the TSB. Most notably the overall amino acid concentration in TSB is higher than in Fish Juice. Interestingly Batch 15.09 has an overall lower amino acid concentration than batch 14.09, these batches are produced with raw

material from the same producer. Even further indicating that the differences are random. Random differences indicates that it is not an easy media to remake with a set amino acid profile and could provide different growth kinetics and spoilage results depending on the batch.

Given the values presented here and because of factors mentioned above, fish juice does not qualify as a substitute for TSB, but it might still be useful as a media to simulate spoilage that occurs in a natural environment. But for the sake of the following experiment TSB was the preferred media going forwards as it was both a less time investment to make and the importance of having a stable media that is standardized when studying strain-to-strain differences.

5.3 Production of H_2S on Iron agar at different temperatures.

To Further investigate the *Aeromonas* strains spoilage potential and their sensitivity to temperature an analysis of H_2S production was conducted. Many studies including (Carriero, Mendes Maia, Moro Sousa, & Henrique-Silva, 2016) has shown that *Aeromonas*, in this case a strain of *A. dhakensis* can produce H_2S, but information about the specific strains used in this study is unknown. Most studies also determine H_2S production after incubation on much higher temperatures (as an example Parlapani, Kormas, and Boziaris (2015) used 25°C) than relevant in this specific instance.

The strains of *A. caviae* and *A. media* used in this study were not able to produce H_2S at 15, 8 or 4°C. Datta, Menon, and Varughese (2017) also demonstrated that *A. caviae* was not able to produce H_2S even at higher temperatures, but a different strain was used.

At 15°C H_2S production was observed after overnight cultivation and with a large area of coloration on the media. This not being the case at 8°C when strains needed more time to produce the same amount or in some cases any coloration on the media. *A. hydrophila, A. piscicola, A. bestiarum* and *A. dhakensis* was shown to be less effected by the temperature reduction compared to both *A. salmonicida* strains. The only strains that were able to produce H_2S at 4°C were *A. piscicola* and *A. bestiarum*.

Interestingly both Shotts and Rimler (1973) and Topic´ Popovic´, Teskeredz˘ic´, Strunjak-Perovic´, and C˘oz˘-Rakovac (2000) has described *A. hydrophila* as a non-H_2S producing bacteria in their studies, the fact that it contradicts what is found in this experiment might be

because of strain differences or the fact that there have been used different media to determine this. Specifically Shotts and Rimler (1973) used a modified Iron media referred to as the "Rimler-shotts medium" (incubation at 37°C) where Topic´ Popovic´ et al. (2000) used a triple sugar Iron agar (TSI, Difco) (incubated at 22°C)

The fact that *A. bestiarum* was able to produce H_2S at such a low temperature indicates that even though it registered no growth in the previous analysis, it still may have a role to play in spoilage even at refrigerated temperatures.

5.4 Measurement of ATP degradation products caused by *Aeromonas In vitro.*

All 8 *Aeromonas* strains were inoculated in a mixture of TSB and IMP (9,397 mM) at 8°C. The HPLC results showed that there was a lot of overlap between the IMP and INO, this made it difficult to determine with certainty where the IMP had been converted to INO. These values have therefor been added together as the conversion from INO to Hx is of most interest. *A. piscicola* is also being overlooked in this discussion as most of the data is of poor quality.

All strains (except for *A. piscicola)* has been shown to reduce IMP, some better than others. *A. caviae* has the highest IMP/INO concentration after 6 days, indication that its reducing abilities are less than the other strains. Both strains of *A. salmonicida* as well as *A. bestiarum* have converted all or most of the added INO (final concentration ranging from 0,00 to 60,88). The same strains also have a significantly higher Hx value at day 6 than the other strains.

A. media, *A. hydrophila* and *A. dhakensis* are on the intermediate level, all having reduced a substantial amount of IMP/INO, final concentration ranging from 125,74 ± 84,78 to 248,64 ± 17,83 mg/g, though some differences can be seen within this subgroup, where *A. media* is not significantly different from *A. salmonicida* og *A. bestiarum*, at the same time as not being significantly different from *A. hydrophila* and *A. dhakensis*. The same pattern can be seen on their Hx concentration on day 6. All being statistically similar to each other at the same time as *A. caviae* not being significantly different from *A. salmonicida* and *A. bestiarum.*

The results indicate that *A. caviae* does not have the same ability to convert INO to Hx as the other strains, but some Hx was detected meaning that might have a role in the spoilage process as well.

A. Salmonicida (both SU2 and Nr.21) does seem to convert INO at a high pace and together with *A. bestiarum* they express themselves as dominant spoilers.

Jakobsen et al. (2020) have also described *A. salmonicida* as a potential spoilage organism in vacuum packaged cold stored salmon and found that the Hx production was increased in its presence. This study used the same strain (SU2) as was used in this assignment, but it is unknown if all *A. salmonicida* stains have the same ability as shown for SU2 and Nr.21. Information about *A. bestiarum* as a spoilage organism and its ability to convert INO is however limited but it is commonly isolated from food as described by (Hoel et al., 2015).

Both strains of *A. salmonicida* had higher endpoint OD values than all other strains, except for *A. piscicola*. There might be a link between the endpoint OD values and the amount of Hx produced. This link might have been clearer had HPLC results for *A. piscicola* been of better quality. At the same time, *A. bestiarum* generated relatively high Hx concentrations on day 6 but was in the lower OD range at the same day, not following the pattern described for *A. salmonicida* SU2 and Nr.21.

Judging by the results obtained in this thesis, it can be speculated that *A. bestiarum* requires lower overall growth to convert similar amounts of IMP to Hx. But further studies are needed to conclude this.

5.5 Measurement of TMAO conversion by *Aeromonas In vitro*

The NMR results gathered from the *In vitro* analysis of TMAO conversion was of poor quality and therefore not presented in this assignment. The NMR chromatographs (Presented in Appendix 2) where not able to be read or integrated as there was too much signal detected, this overshadowing both the parameters of interest and the TSP.

It is speculated that the amount of salts and other compounds was to concentrated for the sensitivity of the NMR. It is therefore suggested to optimize the sample preparation protocol in future NMR experiments.

5.6 Measurement of ATP degradation products in Fresh and smoked Vacuumed packed salmon.

IMP and INO was also investigated in both Fresh and Smoked vacuumed packed salmon stored at 4°C. In this case focusing on a few strains that had over the course of the experiments indicated to spoil products in some capacity or because more information was needed of that specific strain. *A. pisciola* and *A. bestiarum* were both able to produce H_2S at 4°C indicating an ability to spoil even at lower temperatures. *A. salmonicida SU2* was chosen as it has been shown *In vitro* to be the superior strain when investigating their ability to convert IMP to Hx. In this experiment there were even more overlap between INO and IMP in the chromatographs, so these values have again been merged.

The strain-to-strain differences are not as prevalent in this experiment as they were in the *in vitro* analysis. *A. salmonicida* and *A. bestiarum* both have a higher day 14 Hx concentration compared to the control and *A. piscicola* samples. The control however has a smaller initial IMP/INO concentration, making it harder to compare the endpoint Hx values as it has less IMP to convert. Nevertheless, there are no significant differences between Hx concentration on day 14. As there are still detected a fair amount of INO/IMP on day 14, there would have been of value to extend this experiment to see if the strains were able to convert all the remaining IMP to Hx, more clear differences between the strains might have been observed.

P.-f. Liu, Du, Meng, Li, and Liu (2016) have also monitored Hx development in Salmon kidneys infected my *A. salmonicida*. In this study there could not be seen a clear difference between the control and the inoculated samples. But interestingly their experiment also showed that the Hx concentration increased drastically between day 7 and day 14.

The smoked vacuum-packed salmon follow a similar trend to the fresh salmon, *A. salmonicida* has the highest endpoint Hx concentration, but also the highest initial IMP/INO concentration. Even after 21 days there are still higher concentrations of IMP/INO compared to Hx. Again, there might be of interest to extend the storage time to get better data on strain-to-strain differences.

For both Fresh and smoked vacuumed packed salmon there is an increase in Hx on all sample types, how much this is impacted by the inoculated bacteria is however difficult to determine as the control follow similar patterns to the inoculated samples.

5.7 TMA concentration of inoculated and non-inoculated Fresh Salma.

To determine the strains' ability to convert TMAO to TMA the inoculated Fresh Salma samples was investigated using NMR. There were some troubles with the chromatographs, but in this case not overshadowing the parameters of interest. There was however difficult to determine the changes in TMAO concentration because this specific compound was overshadowed by other unknown substances (Appenix 2, page 2).

There are strong indications that the strains used in this assignment can produce TMA under the applied conditions. There are clear differences between the day 14 values comparing the control to the inoculated samples. There are uncertainties connected to the "Fresh *A. salmonicda"* day 14 results as there are both a very high standard deviation and only two parallels. But the concentration is however higher at both day 4 and day 8 indicating that it has a significant role in reducing TMAO. Day 14, Interpretation of the results for *A. bestiarum* is also challanging because of the large deviations between the parallels. But day 8 results are also indication that there are differences in TMA concentrations with samples inoculated with *A. bestiarum* compared to the control.

In the study done by Beaz-Hidalgo, Agüeria, Latif-Eugenín, Yeannes, and Figueras (2015) they also showed that *A. salmonicida* as well as *A. hydrophila* reduced TMAO effectively. Information about *A, bestiarum* and *A. piscicola's* ability to to reduce TMAO is however very limited.

The results indicates that there are strain-to-strain differences in spoilage potential, but because of factors above it is difficult to conclude this. But judging by the Day 8 *A. salmonicida SU2* and *A. bestiarum* results both indicate to reduce TMAO at a faster pace than *A. piscicola*.

5.8 Biogenic amines in fresh and smoked vacuum-packed salmon.

As described earlier in the assignment an extraction, derivatization and HPLC was preformed to determine the content of different Biogenic amines in both fresh and smoked vacuum-packed salmon. The results were however of poor quality and was subsequently discarded. The reason for this is unknown, but it is likely that the HPLC system in use was affected by some contaminants in the samples, as there was seen some precipitation in the HPLC tubes.

6.0 Conclusion

From the results presented in this thesis there are speculations about the viability of using Fish Juice as a culture media when measuring spoilage metabolites, as there was shown some batch-to-batch differences on its nutrient composition which may affect the metabolite production.

The results obtained in this study also indicates that there are some strain-to-strain differences in their ability to produce the spoilage metabolites Hx and TMA. This was indicated both in an enriched culture media and in vacuum-packed Fresh salmon. The exact differences on strain and even species level however needs to be elucidated in more detail as more data is needed to conclude this. It was found that all analyzed strains, except for *A. caviae* and *A. media* were able to produce H_2S at 15°C, with *A. bestiarum* and *A. piscicola* being able to produce H_2S even at 4°C, indicating that they have a role in the spoilage of refrigerated food.

It is advised to study the spoilage potential of these strains in further detail, especially their ability to produce biogenic amines as the results generated in this thesis had to be discarded.

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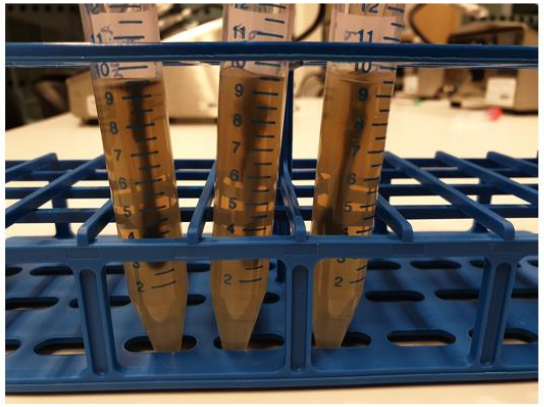
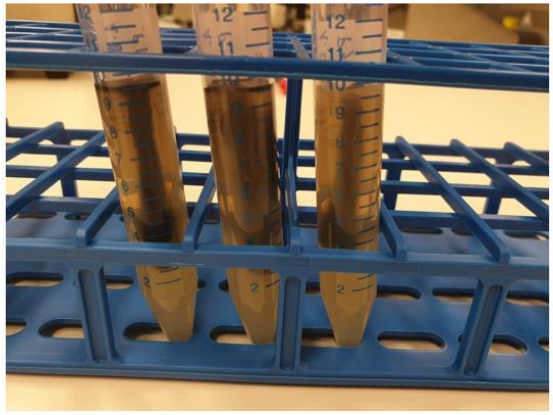
Appendix 1. Coloration changes in Iron agar at 15, 8 and 4°C

Aeromonas Dhakensis 15°C

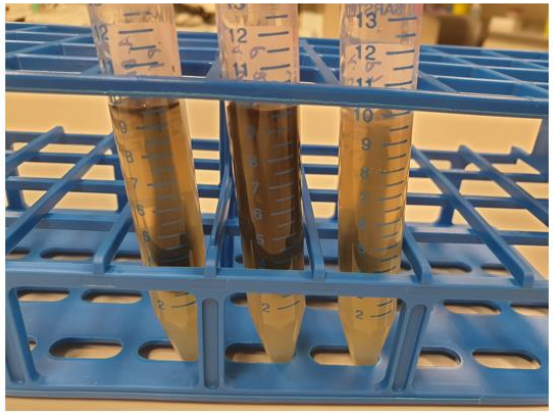
24 Hours



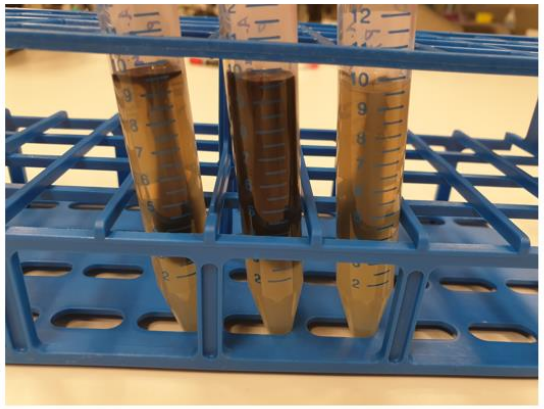
48 Hours



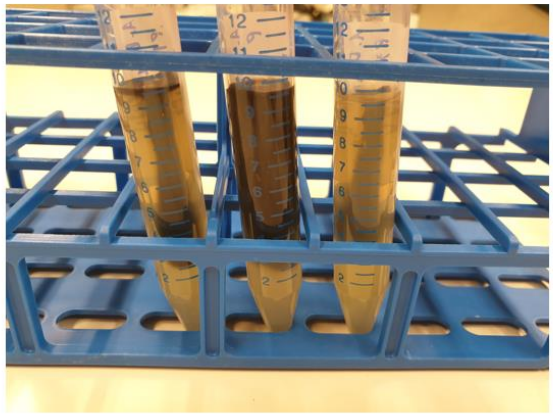
72 Hours



96 Hours

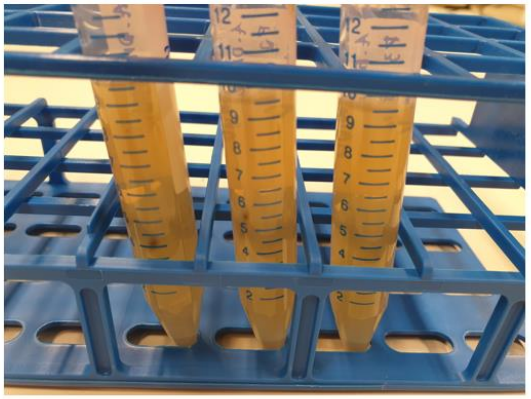


120 Hours

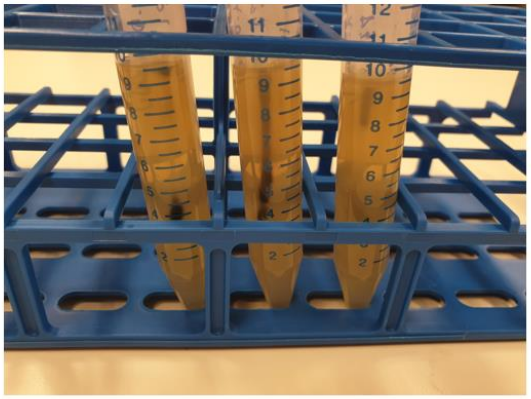


Aeromonas Dhakensis 8°C

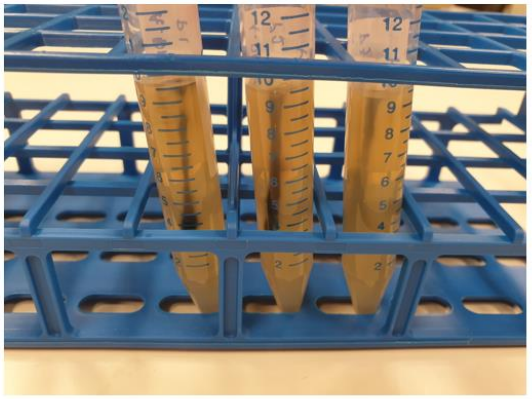
24 Hours



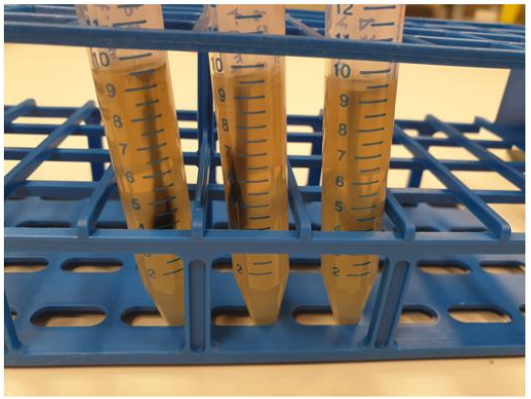
48 Hours



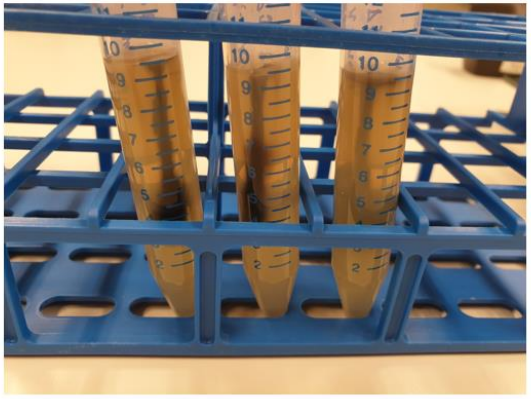
72 Hours



96 Hours

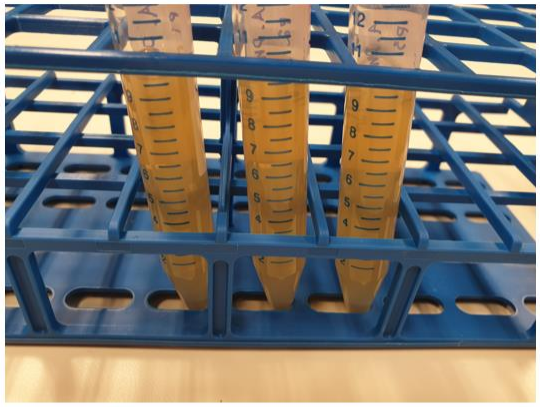


120 Hours

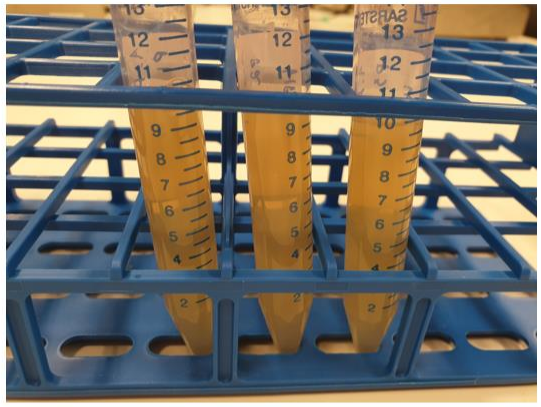


Aeromonas Dhakensis 4°C

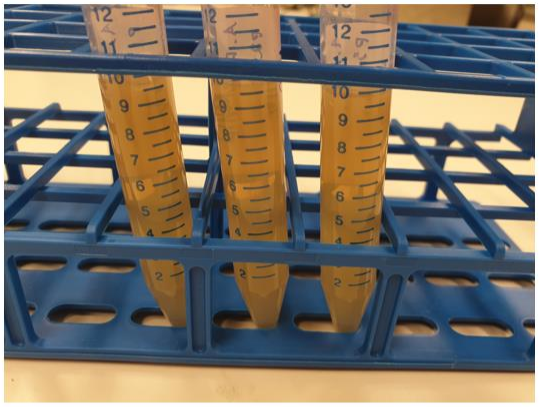
24 Hours



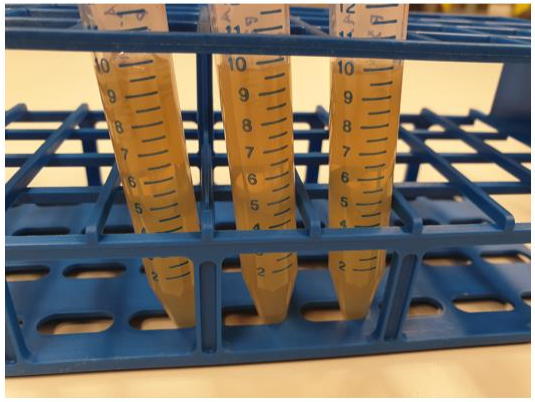
48 Hours



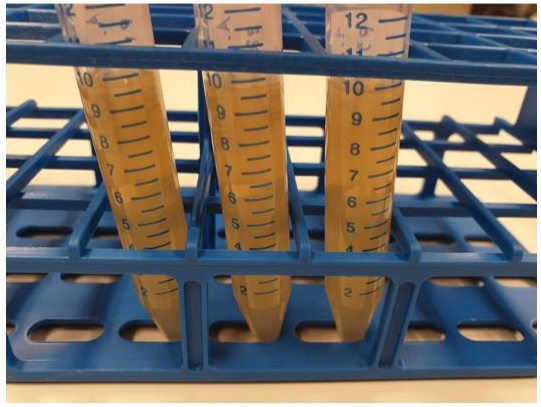
72 Hours



96 Hours



120 Hours



Appendix 2. NMR spectra

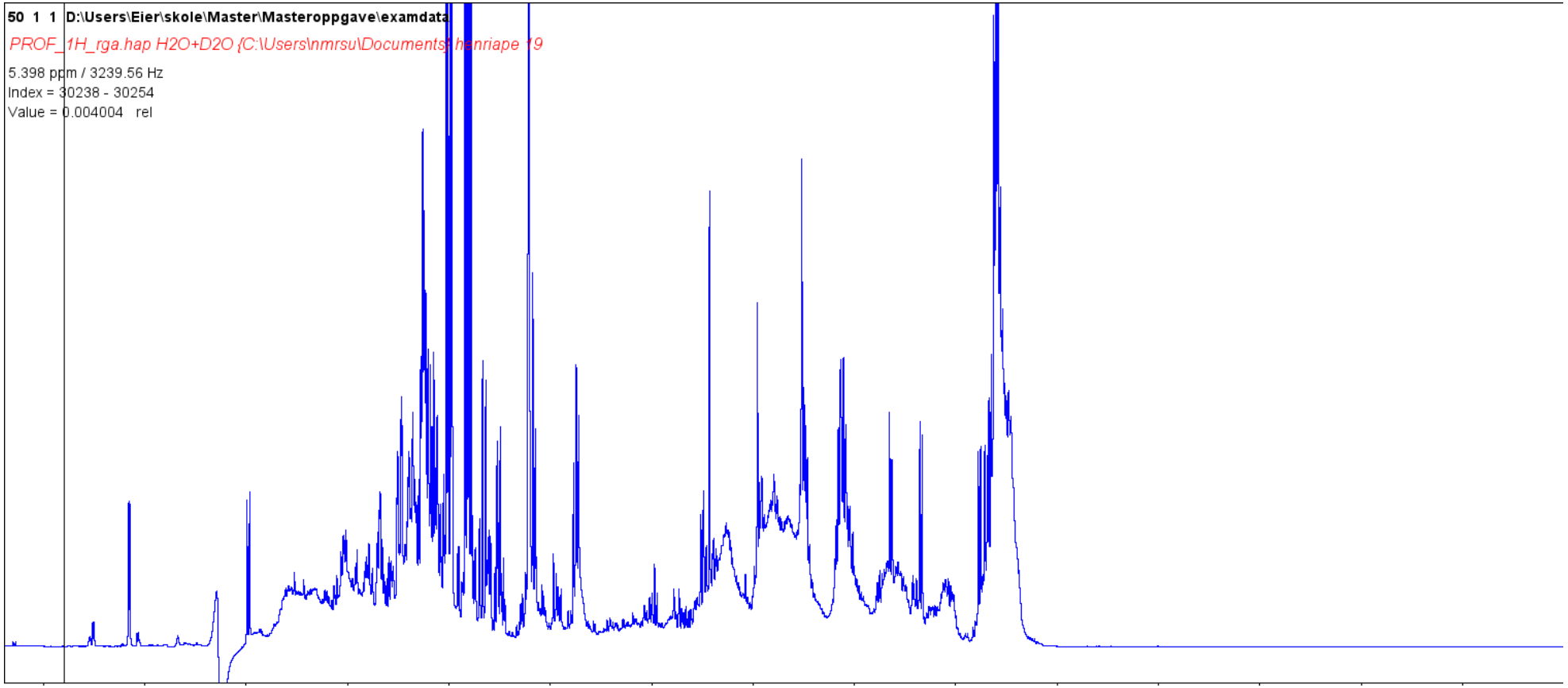


Figure 1. NMR spectrum of TSB inoculated with *Aeromonas*. Entire spectrum shown.

Appendix 2 NMR spectra

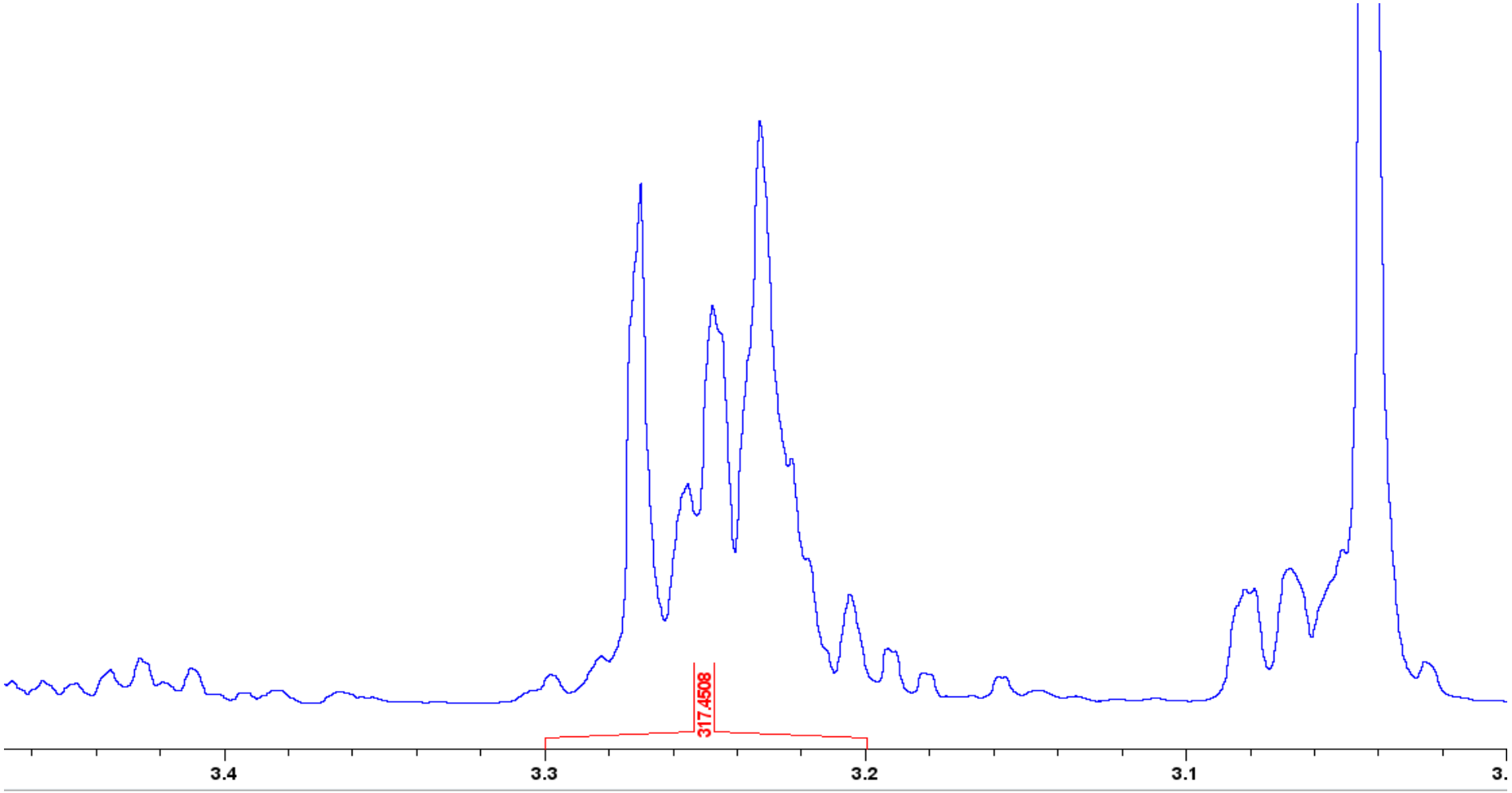
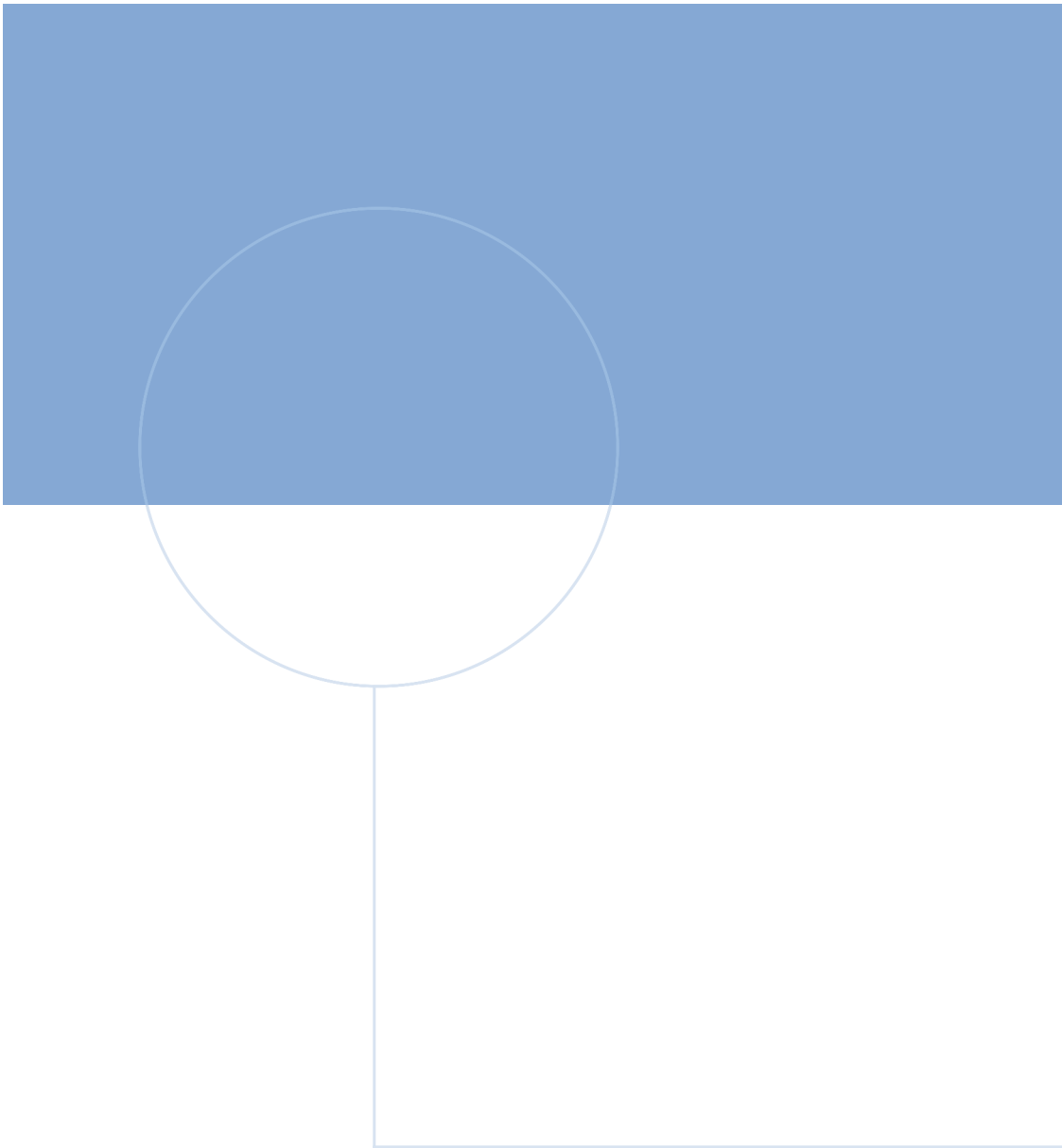


Figure 2. NMR spectrum of inoculated vacuum-packed Salmon. Spectrum has been zoomed in on the area where TMAO should be observed. But is in this case overshadowed by one or more compounds.



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