Norges miljø- og biovitenskapelige universitet
NMBU Veterinærhøgskolen
Fakultet for Veterinærmedisin
Institutt for basal- og akvamedisin (BasAm)
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Early mortality in tilapia fingerlings on Lake Kariba in Zambia

Tidlig yngeldødelighet hos tilapia i Lake Kariba i Zambia

Christine Donbæk
Anniken Mork
Ingelin Ravlo
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Veiledere: Øystein Evensen og Stephen Mutoloki

Contents

Summary

Title: Early mortality in tilapia fingerlings on Lake Kariba in Zambia

Authors: Christine Donbæk, Anniken Mork and Ingelin Ravlo

Supervisors: Stephen Mutoloki and Øystein Evensen, NMBU Institutt for basal- og akvamedisin

Tilapia is the most popularly cultured fish in the world, 126 countries or regions reported cultured carp production to FAO in 2015. Tilapia is the second most important finfish species group cultured worldwide in terms of production. It is a robust fish that tolerates a wide range of environments and production systems and is therefore well suited for culturing in countries with climates in the warmer spectrum. The main objective of this thesis was to investigate causes of early mortality in tilapia fingerlings after transfer to cages at a grow-out farm in Lake Kariba in Zambia. This was accomplished by performing interviews and observations at four different tilapia farms during a fieldtrip in Zambia. Our focus during the visits on the different farms was on routines associated with production, and particularly the transport, of the fingerlings. Additionally, five water samples and 160 bacterial swabs from a total of 50 fingerlings were collected for further characterization and determination of agents in the laboratory. For characterization of the bacteria the following procedures were performed: seeding onto nutrient agar, sheep blood agar and MacConkey agar; Gram staining; oxidase and catalase testing; DNA-extraction by the CTAB-method; and PCR using the primer L. garvieae 167F and 1095R for sequencing. The observations and interviews from the farms show that the fingerlings are subjected to a considerable amount of stress during transport. The findings from the laboratory investigations indicate that there might be a series of different potentially pathogenic bacteria present on the farms.

Definitions and abbreviations

Abbreviations	**Definitions**
Amberjack	*Seriola dumerili*
BasAm	Institutt for Basalfag og akvamedisin
BLAST	Basic Local Alignment Search Tool
CFU	Colony forming units
CTAB	Cetyl Trimethyl Ammonium Bromide
DO	Dissolved Oxygen
FAO	Food and Agriculture Organization of the United Nations
GIFT	Genetically Improved Farmed Tilapia
Lake Kariba	One of the largest artificial lakes in the world, located on the border between Zambia and Zimbabwe (1)
Lactococcus garvieae	Causative agent of lactococcosis. Earlier named *Streptococcus garvieae* and *Enterococcus seriolicida*
Nile tilapia	*Oreochromis niloticus*
NMBU	Norges miljø- og biovitenskapelige universitet
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
Primer	Short strand of RNA and DNA that serves as a starting point for DNA synthesis (2)
Rainbow trout	*Onchorynchus mykiss*
TiLV	Tilapia lake virus
TNTC	Too numerous to count
UNZA	University of Zambia

UVP	UV transilluminator
Yellowtail	*Seriola quinqueradiata*

Introduction

Tilapia

Tilapia are freshwater fish belonging to the family *Cichlidae.* More than 70 tilapia species have been identified, most of them are native to Western rivers of Africa, but few are of aquaculture significance. The commercial production of tilapia is currently divided into three major groups, based largely on reproductive characteristics: *Tilapia* spp. (guard the developing eggs and fry in the nests), *Oreochromis* spp. (females incubate eggs and fry orally) and *Sarotherodon* spp. (males and females incubate eggs and fry orally) (3). However, global tilapia production is concentrated mainly on Nile tilapia (*O. niloticus*), Mozambique tilapia (*O. mossambicus*) and Blue tilapia (*O. aureus*) (4). Out of these three species Nile tilapia is responsible for about 72 % of the global tilapia production, mainly due to the Genetically Improved Farmed Tilapia (GIFT) project responsible for breeding improvements of farmed tilapia (5, 6). Nile tilapia is the species reared at the farm which is the object of our study and will therefore be the focus in this thesis.

Nile tilapia is a tropical species which prefers to live in shallow water. It is an omnivorous grazer that feeds on phytoplankton, periphyton, aquatic plants, small invertebrates, benthic fauna, organic debris and bacterial films associated with this. Its main source of nutrition is obtained by surface grazing on periphyton mats (7). In ponds, sexual maturity is reached at an

age of 5-6 months. Spawning begins when water temperature reaches 24 °C. Cold periods suppress spawning, and in absence of these, the females may spawn continuously. The breeding process starts when the male establishes a territory and digs a crater-like nest. The female spawns in the nest, and immediately after fertilization by the male, she collects the eggs in her mouth and swims off (7). The female incubates the eggs and broods the fry after hatching until the yolk sack is absorbed. Depending on temperature, incubating and brooding is accomplished in 1-2 weeks. After the fry are released, they may still swim back into the mother's mouth if danger is perceived (7). The number of eggs the female produces varies with weight, and ranges from a 100 to 15,000 eggs, in a 100 g female and a 600–1,000 g female, respectively. Nile tilapia can live longer than 10 years and reach a weight exceeding 5 kg (7).

Environmental requirements

Tilapia are a highly adaptable species to their environments, as reflected by their tolerance to a wide range of environmental conditions. This explains why they can live in various ecological water systems, including slow-moving rivers, swamps, small shallow lakes, large deep lakes, isolated crater lakes, soda lakes, thermal springs and brackish-water lakes etc. (8). The above-mentioned habitat diversity also represents a wide range of environmental conditions, including: temperature, photoperiod, depth, current velocity, turbidity, salinity, pH, dissolved oxygen, mineral and gas contents, competition, food availability and productivity (8). The water management is therefore a key factor for successful aquaculture practices (8).

Water temperature

Temperature is one of the most important factors affecting the physiology, growth, reproduction and metabolism in fish. Tilapia are thermophile fish and optimal temperature for growth in a controlled environment is shown to be approximately 27-30 °C (9). However, it has a high tolerance to a wide water temperature range, the lower and upper lethal temperatures being 11-12 °C and 42 °C, respectively (7).

Salinity

Most of tilapia grow, survive and reproduce at 0-29 ‰ depending on the species and acclimatization period. But the salinity tolerance of tilapia is also affected by fish sex and size. Adult fish are more salt-tolerant than fry and juveniles (8).

Dissolved oxygen (DO)

Levels of dissolved oxygen is one of the limiting environmental factors affecting fish feeding, growth and metabolism. DO fluctuation is affected by photosynthesis, respiration, temperature and diet fluctuation. Optimal DO levels for tilapia are over 6.0-6.6 mg/L (10). Tilapia are known to withstand very low levels of DO and can tolerate DO levels as low as 0.1-0.5 mg/l for varying periods of time. They can even survive at zero DO concentration for some time if they are allowed access to surface air. Stress caused by any means leads to significantly increased oxygen consumption by the fish, and monitoring of DO levels are therefore especially important during handling of the fish (8).

CO_2

Increased carbon dioxide in the water leads to decreased oxygen uptake, and increased ventilation rate. Tilapia can withstand very high levels of CO_2, ranging from 50 to 72.6 ppm (8). The concentration of CO_2 in the ponds is a result of respiration from the fish and the microscopic plants that are found there. Decomposition of organic materials is another source of carbon dioxide. During day time when the sun shines strongly, oxygen levels will increase in the ponds. This is due to photosynthesis of the algae and other aquatic plants. At night the photosynthesis ceases, and the algae, sediment and fish will consume the oxygen that is contained in the pond. The daily fluctuation of the CO_2 concentration is therefore in general the opposite of the amount of DO. During the day the CO_2 concentration will be at its lowest in the late afternoon, when DO is highest, and in the night it will accumulate to a maximum at dawn (11). Fish dispose CO_2 over the gills during expiration. If the CO_2 level in the water is too high, the fish will have problems with reducing the internal CO_2 concentration, resulting in an accumulation of CO_2 in the blood. This accumulation inhibits haemoglobin's ability to bind oxygen and can therefore cause the fish hypoxic stress. To prevent this, the farmers often add extra oxygen to the pond during the night (11).

Ammonia and nitrites

The toxicity of ammonia (NH_3) depends on the levels of DO, CO_2 and pH in the water. The toxicity increases with decreasing DO and decreases with increasing CO_2. Fish species and size, acclimatization time and culture systems also affect the toxicity of ammonia to the fish. The toxic level of NH_3 and its negative effect on the growth performance ranges from 0.07 to 0.14 mg/l. It is recommended that the NH_3 concentration should be maintained below 0.1 mg/l. Ammonia is oxidized into nitrite (NO_2) and then into nitrate (NO_3) through nitrifying bacteria growing in organic matter. The bacteria remove the organic matter from the system

by using it as food. Nitrate is relative non-toxic to tilapia. However, nitrite is highly toxic to fish, including tilapia, because it disturbs the physiological function in the fish leading to growth reduction. The tolerance of tilapia to nitrite is also influenced by fish size (8).

pH

Some tilapias are known to tolerate a very wide range of water pH. Nile tilapia can survive at a pH range of 4-11 (8).

Water turbidity

Water turbidity can be a major problem in fertilized freshwater ponds. The clay turbidity levels in earthen ponds should be kept below 100 mg/l. There is a number of sources that influence the water turbidity; such as rain water that run off from pond dykes and contains clay materials, or resuspension of bottom mud by water and fish movement. Fertilization of the water may also have an effect on the turbidity (8).

Tilapia farming

Tilapia production has been described as the aquatic chicken production, as it can be successfully farmed in any scale from small “backyard” farming in a few fish ponds to intensive aquaculture in corporation with several countries (3). This is because of their enormous ability to reproduce and live under a wide range of physical and environmental conditions (3). Surpassing the production of salmonids in 2006, tilapia became the second most important species in the world only after carp and are likely to become the most important of all cultured fish in the twenty-first century (3). In 2016 approximately 170,000 tons tilapia entered the international market (12). The increasing importance of tilapia as an

aquaculture candidate makes it necessary to understand their natural habitats and feeding regimes (8).

Farming systems

Many different culture systems exist for tilapia, this includes water-based systems and land-based systems. The choice of culture system is mainly based on the farmers economy and technological competence, the areas infrastructure, marketing capacity, and the circumstances around environmental conditions. Tilapia is known to tolerate a high stocking density and can withstand extreme crowding conditions. The relationship between stocking density and tilapia yield is generally positive (i.e. high fish densities leads to high yield), while negative correlations usually occurs between stocking density and individual fish growth (8). Tilapia are generally cultured semi-intensively or intensively, under different culture and environmental conditions, stocking densities and management strategies (8).

Fingerling production

Because of the increased demand for especially mono-sex (male) tilapia fingerlings, the fingerling production has expanded rapidly and become a significant bottleneck in the industry. Tilapia fingerlings can be produced in hapas, concrete or fiberglass tanks and earthen ponds. A hapa is a net cage constructed from fine (1.6 mm) mesh netting suspended in a lake, pond or tank (3). All commercial production systems use sex reversed fingerlings, this is because of the male's faster growth and that they are more uniform in size than females. Mono-sex male populations are achieved by either manual sorting, direct hormonal sex-reversal, hybridization or genetic manipulations (4). The most commonly technique for sex-reversal is using testosterone through oral administration. Most of the hatcheries that produce

fingerlings make their own sex-reversing feed. Synthetic steroids are not water soluble and are therefore dissolved in alcohol or oils before poured or sprayed over the feed. Tilapia eggs take 4 to 6 days to hatch depending on water temperature. At hatching, larvae are not well developed, and the gonads have not formed yet. The fry is therefore responsive to the hormone treatment. The fingerlings are treated with the sex-reversed feed for 21 to 28 days (3). After this they are moved to bigger grow-out ponds where they stay until they are 8 weeks old and have reached a weight around 1-2 g, before being sold on. The production cycle of tilapia is illustrated in Figure 1.

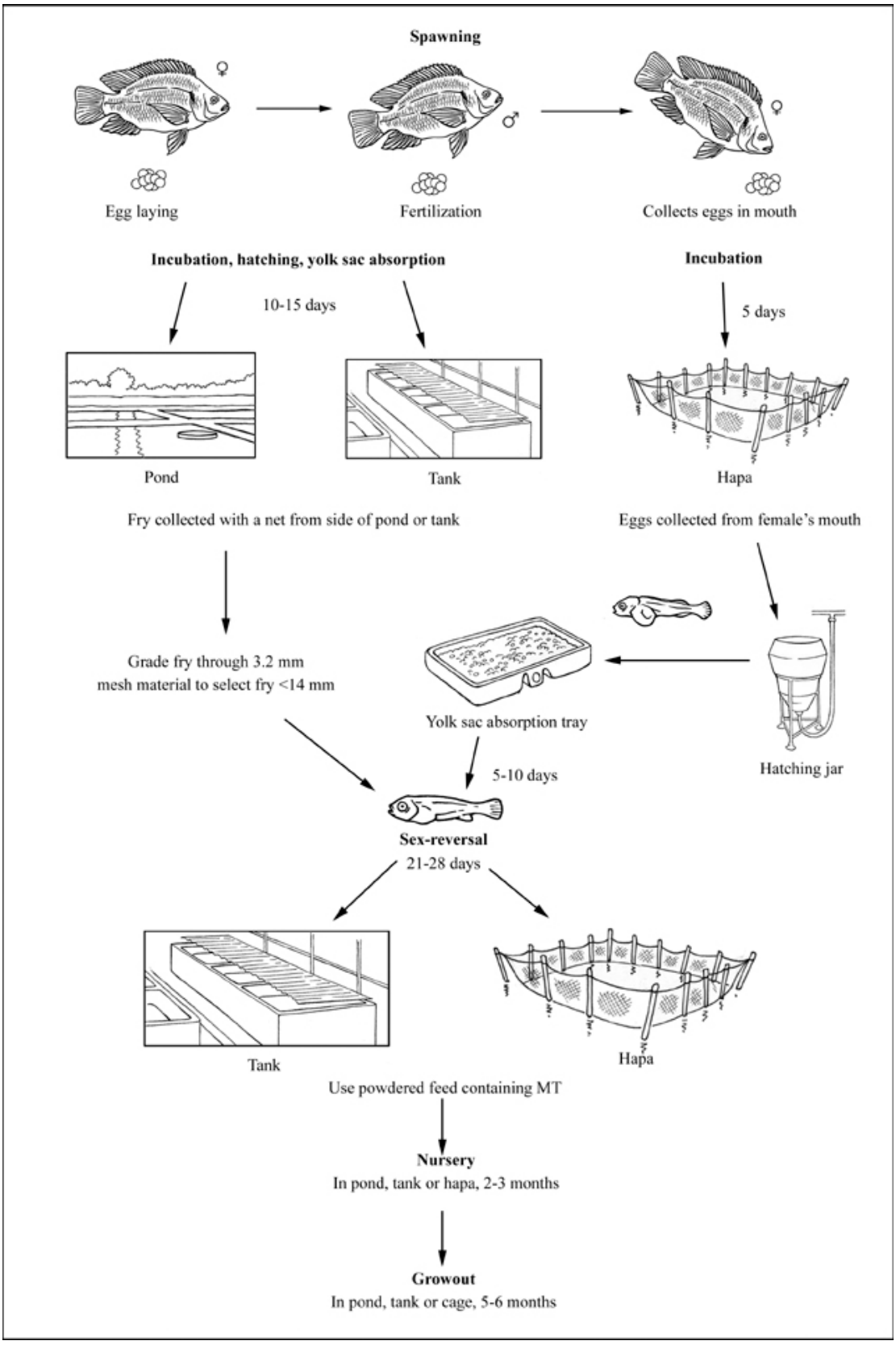

Figure 1. Production cycle of tilapia from spawning to nursery. (Rakocy (7))

Pond production

Pond culture of tilapia is the oldest production system among all systems used for tilapia culture (8). Nile tilapia being an omnivorous fish can be successfully raised in ponds that receive only fertilizer input. Both chemical and organic fertilizers can be used, either separately or as a combination. Organic fertilizers have traditionally consisted of animal manure, obtained by integrating farming of livestock (e.g. pigs, chickens, goats, ducks, geese, or cattle) with aquaculture. Pond management strategies progresses as production intensities increase, from extensive production (with earthen ponds with fertilization only), to semi-intensive productions (with fertilization plus feed (supplemental or complete) as shown in Figure 2, to intensive productions (with complete feeds only) and to hyper-intensive production (with lined ponds with feeds plus aeration and/or water exchange) (3). Semi-intensive production, in particular, has played an important part in contributing to hunger alleviation and food security in rural areas in developing countries, due to its low production costs and thereby low-cost fish product.

Figure 2. A semi-intensive pond with tilapia production. (source: private photo)

Cage production

Tilapia cage culture has been practiced experimentally and commercially since the early 1970's. Currently, commercial tilapia culture in cages is expanding at a very fast rate, especially in tropical and subtropical developing countries in Asia, Africa, and Latin America. The largest and most successful commercial farm for cage culture of tilapia in Zambia is located on Lake Kariba (8). Tilapia can be raised successfully in both sea cages, and pond cages.

Use of cages as a system for culturing of fish has several attributes, including

- Relatively low capital investment compared to other intensive culture systems
- Ease of observation and management and early detection of stress and disease
- Economic treatment of parasites and diseases
- The use of all the available water resources
- Ease of fish harvest and low cost of harvesting
- Minimum fish handling and reduction of mortality
- High stocking density, optimum feed utilization and improved growth rates

Cage culture also has some limits, including

- Risk of theft is high
- Risk of loss due to cage damage caused by predators or storms
- High risk of disease outbreaks and difficulty of disease control
- Complete dependence on high-quality feeds
- Rapid fouling of cage frames and nets
- The accumulations of faeces and metabolites underneath the cage has a negative environmental impact

The success of tilapia cage culture depends on a number of factors, including water quality, water level, tilapia species and strains, stocking density, stocking size, cage size and shape, feed quality and feed frequency. The sizes of tilapia cages vary considerably. Breeding cages and fingerling production cages are generally smaller than grow-out cages. Commercial production cages typically range from a medium size (6-20 m^3) to a very large size, up to 600 m^3. A typical cage like this is shown in Figure 3. (8)

Figure 3. Typical grow-out cage, from a cage production with tilapia. (Private photo)

Fish farming in Zambia

The Republic of Zambia is located in South-central Africa and covers a surface of 750,000 km^2 of which a great part is under water in forms of lakes, rivers and swamp lands. The climate is tropical to subtropical with three seasons:

- Cool dry period from May – August, with temperature 14-21 °C.
- Hot dry period from August – October
- Rainy season November – April, with temperature 27-38 °C.

With a population of 13.8 million, approximately 400,000 households are employed in the aquaculture (13). This gives us a small enlightenment in how important this sector is for the country. Zambia is the sixth largest producer of farmed fish in Africa, and some of the largest freshwater commercial farms in Africa operates in Zambia. The value chain consists almost exclusively of tilapia, and in recent times there have been large investments into the seed and feed sectors (14). Fish farming in Zambia goes back to the 1950s, when the first attempt to raise mainly tilapia in dams and earth ponds was made. In 2014 there was an estimated total of 12,010 small-scale fish farmers scattered around Zambia (14). These small-scale fish farmers are concentrated in the eastern, northern, Copperbelt and north-western provinces where most are using earthen ponds for fish production (13). Today there are more than 20 large commercial land-based fish farms distributed throughout the Copperbelt, Lusaka and Southern provinces, while most of the cage production is based on Lake Kariba (14).

The most important species being cultured in Zambia is the genetically improved fast growing and robust Nile tilapia. However, Nile tilapia is an imported species and is only allowed to cultivate in certain areas and after given permit (5). These restrictions were established as a

protective measure of local fauna, as Nile tilapia is considered an invasive species due to its aggressive spawning behaviour, the ability to spawn multiple broods during one season, and its broad diet. Potential impacts associated with the introduction of Nile tilapia may include the following: decreased abundance and extinction of native species due to habitat, trophic overlaps and competition for spawning sites; introduction of pathogens; habitat destruction; changes to water quality; and loss of genetic integrity and biodiversity due to hybridization (15). In areas where farming of Nile tilapia is banned, the farmers cultivate local species such as the Greenland bream (*Oreochromis macrochir*), the three-spotted bream (*Oreochromis andersonii*), the Tanganyika bream (*Oreochromis tanganicae*) and the redbreast bream (*Coptodon randalli*). The majority of these farmers grow these species because they are located in areas where cultivation of Nile tilapia is not permitted or because they are the only type of seed available (14). There is also some production of the common carp (*Cyprinus carpio*) (16).

While the three main types of production systems (extensive, semi-intensive and intensive) all exist in Zambia, the Zambian government characterizes them using a different typology, namely small-scale and large-scale production systems. This characterization creates a somewhat blurry line between extensive and intensive systems used by small to medium-sized enterprises and fails to account for fish that is cultivated for subsistence versus commercial purposes (14).

Ganschick et al (2018) defines the small-scale sector as *"…using a range of systems between extensive and semi- intensive, encompassing anything from rudimentary earthen pond systems that are extensive in nature (no intentional nutritional inputs to feed the system) to semi-*

intensive pond systems that—through fertilization and/or use of supplementary feed— provide a farmer with fish for household consumption and/or income." (14)

Large-scale commercial farmers in Zambia can be defined as those who employ an intensive production system (either in pond, tank or cages), stocking mono-sex seed at higher stocking densities and who rely on the use of artificial feed and make greater capital and labour investments (14). While small-scale farmers typically provide fish for consumption mainly within the household, the large-scale producers sell the fish to the consumers in the middle and upper class (14).

In the last 10-15 years the overall production of farmed fish in Zambia has expanded massively, with an estimated total of 4,240 tons in 2,000 and 30,285 tons in 2016 (5). There has been a striking shift in the source of farmed fish in Zambia during the last years, as 10 years ago the small-scale sector provided 75 % of the total aquaculture production in Zambia. Nowadays, however, the large-scale sector is the biggest contributor to the estimated overall aquaculture production, passing 70 % in 2014 (14).

Zambia has the potential for further development of aquaculture, and many donor programs have been made where the main target is to support the development of aquaculture in Zambia (16). Fisheries and aquaculture production contributes significantly to food security, provides an important protein source as well as contributes to economic development in Zambia (17).

The main species that are farmed in Zambia include breams, carp, Nile tilapia, catfish and crayfish (16).

Challenges with tilapia production in Zambia

- High mortality rates especially in the early phases of production.
- Insufficient amounts of animal manure (14).
- Insufficient extension services (14).
- Shortage of good-quality seed and not high enough production of fingerlings (8).
- Insufficient availability to affordable, high quality feed (18).
- Insufficient governmental financial resources allocated for aquaculture, low investments by the private sector and limited access to finance by farmers and traders in the aquaculture value chain. Lack of marketing strategy (8, 18).
- Infrastructure/electricity/cooling/production and transportation chain. Poor infrastructure, including poor storage, poor marketing channels and poor maintenance of farming facilities (8). Post-harvest losses due to inadequate transportation, storage and processing facilities. A lack of mobility to access markets from remote and often isolated pond sites (18).
- Climate changes: It is expected that global temperature will rise between 2 and 4 C° in the coming century, and that the global warming will have the greatest impact in Sub-Sahara Africa and India (19). The water levels in the rivers and lakes will decrease, the water temperatures will rise and in worst case scenario the water will drought out. This will also have an impact on the power production in the country, which is based on hydroelectric power from the Kariba dam.
- Lack of qualified and experienced labour; there is an inadequate capacity of training the staff in fish farming and production, and also a lack of people who can teach the staff about the nature and the needs of the fish they are farming (18).
- There is a critical lack of experts in aquaculture whom can design development programs, guide the implementation in the production system, and provide knowledge

of fish genetics, fish diseases and nutrition (18). This could for example be a marine biologist, fish health biologist or a veterinarian who specializes in fish farming. Lack of data and knowledge as well as shortage of expertise in several fields of aquaculture leads to a low esteem of the aquaculture sector, subsequently being graded as inferior to agriculture and therefore accorded little attention. Insufficient knowledge in the aquaculture sector also leads to inappropriate design of aquaculture research and development projects that often are not farmer driven (18).

- Uncoordinated promotion of aquaculture by various institutions, which include the Zambian government, research institutions and universities among others. The variation in approaches to promoting aquaculture compromises on the quality of information that eventually rolls out to the target beneficiaries who are the farmers (18).
- Inadequate entrepreneurship skills by farmers and managements, absence of recordkeeping and inaccurate data collection procedures leads to important information being lost (18).
- Fish losses due to theft, preying animals and disease (18).

Diseases

Nile tilapia has traditionally been considered to be a very robust species if living in high enough temperatures. It has been stated that at temperatures greater than 16 °C to 18 °C and in absence of severe environmental stress, tilapia rarely become diseased (3). Recently, this belief about tilapia being very disease resistant has been challenged. Today, scientists and producers are increasingly becoming aware of the disease problem in tilapia production (20).

Viral, bacterial, fungal and parasitic problems are more likely to emerge and cause clinical disease following stress due to handling, crowding, poor water quality (for example low circulation, low levels of DO, high levels of ammonium), poor feed quality, high organic loads or low water temperatures. Fungi infections, in particular saprolegnia infections, are widespread and are especially seen after handling when water temperatures are below 20 °C. Commonly isolated bacteria from sick fish include *Edwardsiella* sp*., Flavobaterium* sp., *Pseudomonas* spp. *Streptococcus* spp. and most commonly *Aeromonas* spp. (3). As of now there are only two viruses that are known to cause disease and mortality in tilapia; iridovirus and the newly described Tilapia Lake Virus (TiLV) (21). The most important parasites include *Ichtyophthirius multifillis* and other protozoans (e.g. *Tichodina, Trichodenella, Epistylus, Ichtyobodo*), parasitic crustaceans such as *Learnea*, *Argulus* and *Ergasilis,* and trematodes (8).

***Saprolegnia* spp.**

Saprolegniosis is a fungal disease of fish and fish eggs caused by *Saprolegnia* spp. and are commonly called “water moulds” (22). They are common in fresh and brackish water. Saprolegnia prefer temperatures between 15-30 °C. The fungi will attack any existing injury or breach in the skin barrier and can spread into healthy tissue. Stress of any kind to the fish will predispose to disease. Saprolegniosis is often first noticed as a cotton-like material, coloured white to shades of grey and brown on skin, fins, gills, eyes or on fish eggs. This can be scraped and mounted on a microscope slide for proper diagnosis. With progression of infection the fish usually become lethargic and less responsive to external stimuli (22).

***Edwardsiella* sp.**

Edwardsiella tarda is an opportunistic pathogen with a worldwide distribution and a wide host range. It is ubiquitous and healthy hosts can work as a reservoir. Edwardsiellosis in fish usually occurs under unbalanced environmental conditions. Infected fish usually develop septicaemia, focal suppurative or granulomatous lesions and cutaneous ulcerations (23). Clinical signs include altered behaviour, exophthalmia and loss of pigmentation, lethargy and altered swimming patterns (24). Gross external lesions vary between species, Nile tilapia typically develops swollen abdomen due to ascites (25).

***Flavobacterium* sp.**

Within the Flavobacterium genus the most important species in causing disease in tilapia is *Flavobacterium columnare,* which causes the disease columnaris, also called saddleback disease (23, 26). Columnaris is known to have a devastating effect on survival rate in tilapia farms throughout the world (27). Fish are susceptible to disease following some degree of stress. Once established the disease is highly contagious, especially at the fry and fingerling stages. It may spread horizontally from fish to fish, or from the environment through contaminated nets, specimen containers and food. Infected fish often display external lesions such as skin and gill erosion, and necrosis. In acute cases, these lesions may spread quickly and lead to high mortalities within a matter of hours (27). The presence of *F. columnare* may also lead to secondary infections and often precede a saprolegnia infection.

***Pseudomonas* spp.**

Both *Pseudomonas fluorescens* and *Pseudomonas anguilliseptica* are known to cause disease in Nile tilapia (23). Both are opportunistic pathogens with soil and water as the main

reservoir. Clinical signs include exophthalmia, dark skin and nodular lesions. These signs are very similar to the changes seen in fish infected with some *Aeromonas* species. Internal signs of infection include focal necrosis and granulomas in the liver, spleen, swim bladder, kidneys and gonads. The disease can, in addition to these findings, cause increased mortality. Infection with *Pseudomonas* spp. is more common in winter and spring when the water temperatures are low (8).

***Streptococcus* spp.**

Both *Streptococcus agalactiae* (earlier classified as *S. difficile*)*, S. dysglactiae* and *S. iniae* can cause disease in tilapia (23, 26). Streptococcosis is a devastating disease as it can cause massive kills of large size fish and is responsible for heavy economic losses. *S. agalactiae* is the major cause of streptococcosis in farmed tilapia (20). *S. iniae* also causes mortality but to a lesser extent. There is no obvious difference in clinical signs induced by one or the other of the streptococci. Clinical signs include abnormal behaviour such as swirling, lethargy, bent bodies and disorientation, abscesses which quickly burst and become non-healing haemorrhagic ulcers, skin haemorrhages around the mouth or at the base of the fins and ascites. In acute outbreaks it can in addition cause septicaemia and peritonitis. Outbreaks of streptococcosis usually occur when fish have been exposed to stress, such as increased or decreased water temperature, low levels of dissolved oxygen and overcrowding (6).

***Aeromonas* spp.**

Aeromonas spp. may cause a range of different diseases, the most important being “skin rot” or “fin rot” and Motile Aeromonas Septicaemia (MAS). Several bacteria can contribute to MAS, including *Aeromonas hydrophila, A. sobria, A. schubertii, A. caviae* and *Pseudomonas*

spp. Infection may lead to devastating mortalities (80-100%) in cultured tilapia (8). In tilapia culture *Aeromonas hydrophila* is considered the most important species within the genus. *A. hydrophila* is an opportunistic pathogen in both fish and humans (28). It is widely distributed in aquaculture and is probably the most common bacterial disease that infects wild and cultured tilapia (8). Infected fish usually have a dark colour, inappetence, ulcers and hyperaemia at the bases of the fins, ascites and exophthalmia. Internal signs include pale liver, and the presence of many focal haemorrhagic necrotic lesions in the liver, heart and skeletal muscles and over the visceral and peritoneal surfaces (8).

***Lactococcus* sp.**

Lactococcus garvieae is an important Gram positive bacterial pathogen in a variety of marine and freshwater fish worldwide. Infection with *L. garvieae* has also been seen in mammals such as dairy cows and water buffalos and is described as a potential emerging zoonotic agent, with particular importance in immunosuppressed individuals (29, 30). While it has long been known to cause disease outbreaks in cultured fish such as rainbow trout, yellowtail and amberjack, it was first isolated from Nile tilapia by Evans et al in 2009. *L. garvieae* causes lactococcosis which is defined as a systemic hyper acute infection with the occurrence of widespread haemorrhaging (30). It is in several articles defined as a subtype of streptococcosis (31) and *L. garvieae* is listed as a causative agent of said disease. It typically causes disease in big fish late in the production cycle and early symptoms of infection include anorexia, melanosis and erratic swimming (32). *L. garvieae* is very closely related to genus *Streptococcus* and *Enterococcus* and it can be hard to distinguish from streptococci species both by the clinical symptoms and the macroscopic- and histopathologic changes they cause, as well as by biochemical characteristics and morphologic appearance (30). Molecular

methods, such as DNA or RNA analysis, is the most reliable way to name the species with certainty (33).

Objective

Our main objective in this thesis was to investigate causes of early mortality in tilapia fingerlings after transfer to cages to a grow-out farm on Lake Kariba in Zambia. To respond to this objective, we had two sub aims:

1. To investigate the scope of early fingerling mortalities following seeding in juvenile cages at one selected grow-out farm (Farm Four) relative to handling and environmental factors associated with transportation from the hatcheries.

2. Compare fingerling mortalities from different hatcheries relative to bacterial diversity in the fingerlings and water from the different sources.

Materials and methods

Interviews

To gain a good understanding of the tilapia farming industry in Zambia, four different fish farms were visited where six employees in total were interviewed during the field trip. The farms visited included two hatcheries and two commercial farms. Among the commercial farms, one was a large-scale grow-out farm and the other one was a small-scale grow-out farm. The fish farms were chosen by Prof. Bernard Hang'ombe from UNZA, Prof. Stephen

Mutoloki from NMBU and Dr. Kunda Ndashe from UNZA. The fish farms were chosen based on the purpose of this thesis, which was to follow the fingerlings from the point of purchase (hatcheries) to juvenile cages at one selected grow-out farm (Farm Four) with a view to investigate possible reasons for early mortality of the fingerlings. In order to protect the farmers' privacy and to keep the analysis objective the actual names of the fish farms are replaced with numbers from one to four, see Figure 4 for an overview of the the relationship between the farms.

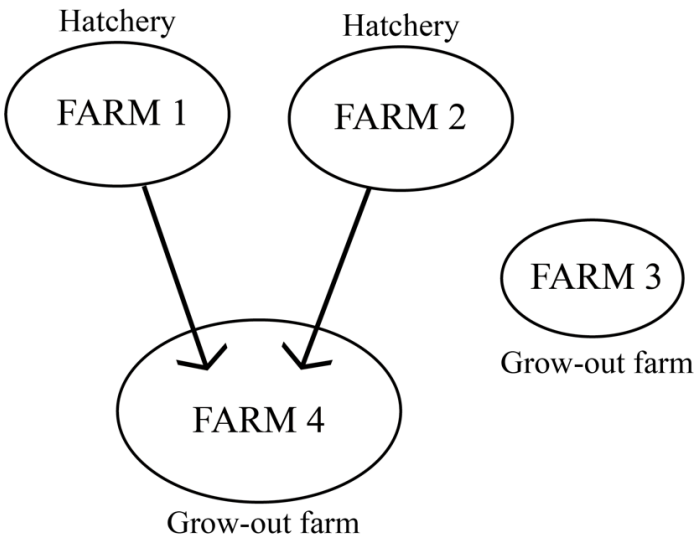

Figure 4. The relationship between the four farms visited in the present study.

Before the interview at each farm a tour of the facilities was done to gain insight of their operations. The interviews were based on a questionnaire consisting of 30 questions which was made prior to the field trip. The questions were divided into one general part and one part focusing on the fingerlings. In addition, some questions were prepared for the hatcheries. During the interviews the questionnaire was used as a guideline and more questions were asked when necessary. At all time during the interviews Prof. Hang'ombe, Prof. Mutoloki or Dr. Ndashe was nearby in case of any need for interpretation. During the interviews one

person conducted the interview while the other one was taking notes. The questionnaire is attached as Appendix I.

Collection of sample material in Zambia

Farm Four procures fingerlings from two hatcheries (Farm One and Two). Once purchased, the fingerlings are collected in large containers and transported by road to Farm Four where they are placed in juvenile cages on Lake Kariba. In these cages the fingerlings acclimatize and grow to about 20 g before they are transferred to grow-out cages. It is in the juvenile cages that mortalities of up to 60 % are observed. Collection of sample materials (both water and tissues) were done at three farms as described below. No samples were collected from Farm Three, information collected here was used for comparison purposes only.

Water samples

A total of 5 water samples were collected:

- Water sample 1: Was taken from Farm One, directly from the pond at the time of collection of the fingerlings.
- Water sample 2: Was taken from Farm Two, directly from the transportation box at the end of transportation of the fingerlings.
- Water sample 3: Was taken from Farm Four, before the fingerlings from Farm Two was transferred into the cage that already contained the fingerlings from Farm One.
- Water sample 4: Was taken from Farm Four at day 4 and 5 after the fingerlings from Farm One and Two was transferred into the cage at Farm Four.
- Water sample 5: Was taken from Farm Four at day 6 and 7 after the fingerlings from Farm One and Two was transferred into the cage at Farm Four.

Water sample collection technique

250 ml of water was collected into a sterile container. The container was submerged down to an elbow's depth (approximately 0.4 meters) before opened and then closed after the water had filled the container completely. This was done to avoid collecting the uppermost layer of water. After collection the water samples were stored at 4°C.

Water sample dilution technique

A dilution series was made by adding 1 ml of the water sample into a sterile tube containing 9 ml of natural saline (0,9% NaCl), making 10 ml of a 10^{-1} dilution. After thorough mixing using a vortex mixer, 1 ml was taken from the dilution and added into a new sterile tube containing 9 ml of natural saline and mixed. These steps were repeated five times for each water sample, reaching a dilution of 10^{-5}. The technique is illustrated in Figure 5.

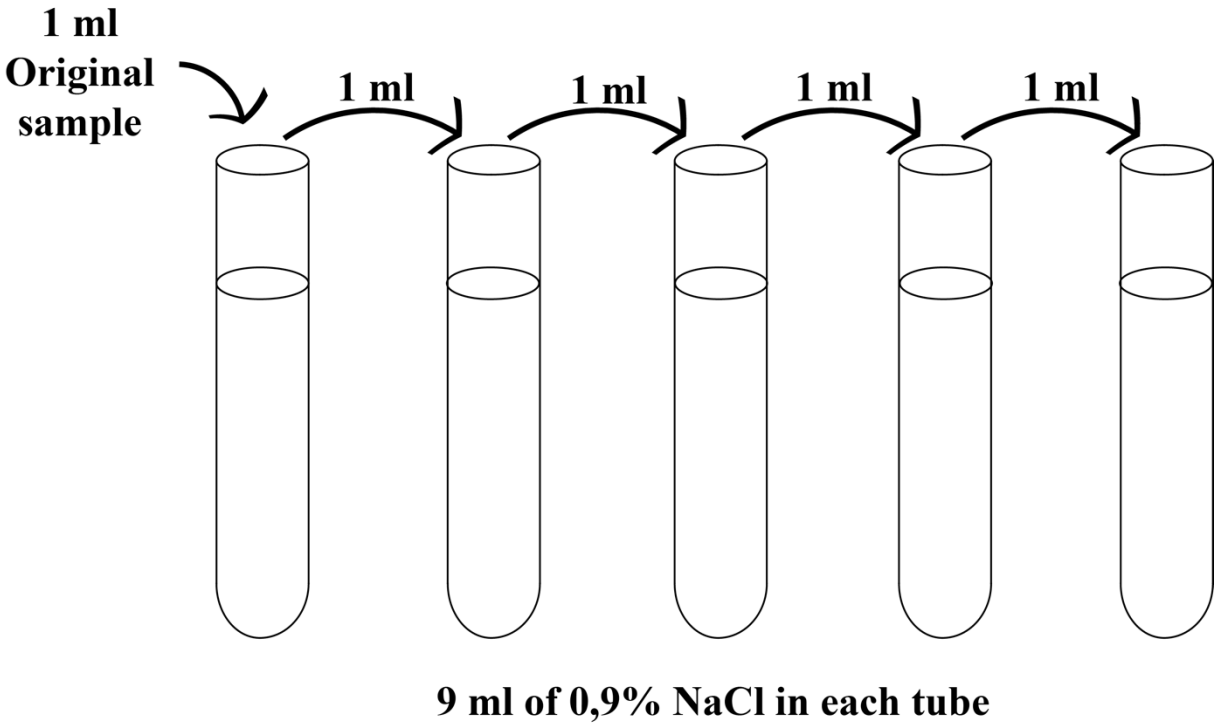

Figure 5. Water dilution technique.

0.1 ml of each dilution was pipetted onto the surface of a nutrient agar plate and spread evenly with a sterile single use plastic spreader until it was dry. This was done for the investigation of the amount of total coliform bacteria in the sample. In addition, 0.1 ml of each sample was pipetted onto the surface of a MacConkey agar plate and spread evenly with a sterile single use plastic spreader until it was dry. This was done for the investigation of the amount of faecal coliform bacteria in the sample. The plates with the water samples were incubated at 36.7 °C for 24 hours.

Plate count procedure

After incubation the number of colony forming units (CFU) were counted on each of the plates. A direct counting method with a counting chamber was used to count the number of colony forming units of the different dilutions, Figure 6 shows the counting chamber that was used. To determine the number of colonies forming units in the original sample the average

number of CFU's counted was divided on the volume dispensed on the plate and multiplied with the dilution factor.

$$CFU/ml = \frac{CFU}{volume} \times dilution\ factor$$

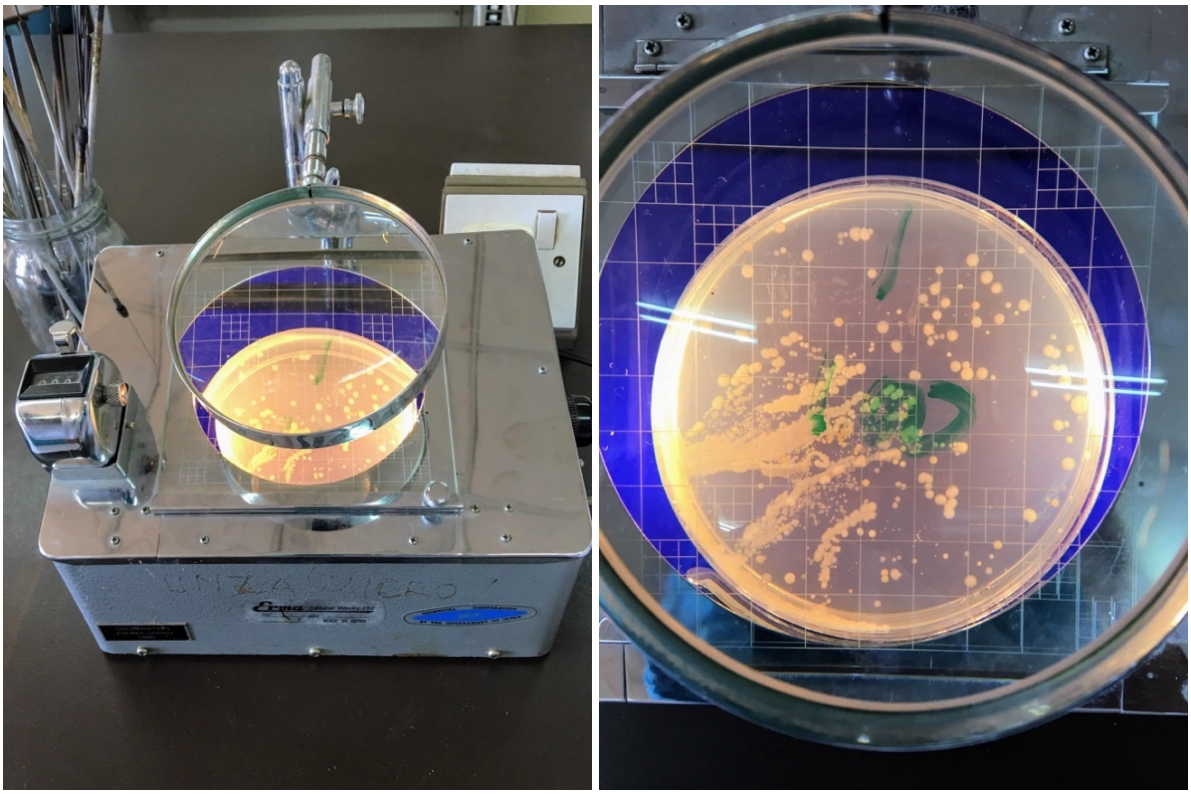

Figure 6. Counting chamber used at UNZA. (Private photo)

Necropsy sampling and sampling from fingerlings

First an external examination of the fingerling was performed to discover any abnormalities or lesions. Looking for poor body condition, exophthalmia, cloudy cornea or lens opacity, haemorrhaged lesions, damaged or missing fins, ulcerations, abscesses, cyst or tissue growth, body discolorations, body malformations, external foreign body such as fungus or parasites.

The fish was placed on its right side before disinfecting the outer surface, by wiping it with cotton that had been soaked with 70 % ethanol. Surgical instruments to be used were also disinfected (34).

First the operculum was removed, and the gills examined for abnormalities or other lesions. Using a small pair of surgical scissors, the first gill arch was removed before sampling from the gills left in the fish.

The abdominal cavity was entered by cutting into the abdominal wall at the base of the pectoral fin with a pair of small sterile scissors. The cut was continued dorsally to just below the lateral line. Starting again at the base of the pectoral fin and the incision was continued towards the posterior of the fish along the ventral abdominal wall to the vent. Puncturing of the abdominal tract, and thereby contaminating the tissues, was attempted to be avoided by staying slightly above the intestinal tract when making the incision. At the vent, the cut was continued dorsally to just below the lateral line and then continued anteriorly to connect with the first incision (34).

The flap of abdominal tissue was removed, thus exposing the internal viscera and abdominal cavity. Then a visual examination of the organs (heart, liver, gall bladder, kidney, pancreas, spleen, air bladder, GI tract) was performed, looking for abnormalities such as; discoloration, enlargement, haemorrhage or erythema, abscesses or cyst, ascites in the abdominal cavity. Figure 7 Shows necropsy of Nile tilapia.

Figure 7. Necropsy of Nile tilapia (Oreochromis niloticus). (Private photo)

Culturing on nutrient agar

Sterile swabs were used to swab the inside of the abdominal cavity of each fingerling and transferred into transportation tubes. Later the same day, the content on the swab was inoculated onto a nutrient agar plate. The swab was used to make the first streak on the plate. A sterile single-use loop was then used to make the four following streaks, as illustrated in Figure 8. The plate was closed with the lid and sealed with parafilm around the edge, covering the transition between the dish and the lid. The plates were kept with the bottom of the agar facing upwards at all times to avoid contamination of the samples. The plates were incubated at room temperature (approximately 28 °C) until clearly visual growth was seen and then moved into a fridge and kept at 4 °C.

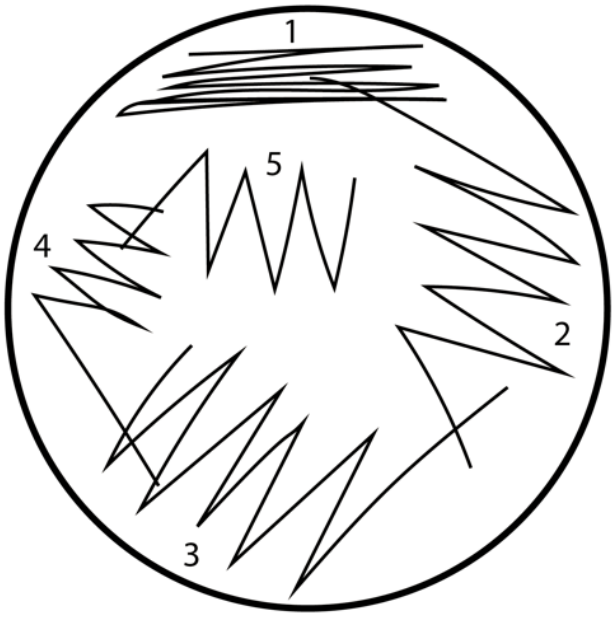

Figure 8. The pattern of the bacteria inoculation on the agar plates. The lines show how the inoculation loops were streaked along the surface of the agar. The numbers show in which order the streaks were made.

Characterization of bacteria in the laboratory

Sub culturing and purification

The different types of bacteria on each agar plate were allocated to different groups based on the morphology of the colonies on the plate. Each type of colony was transferred onto a fresh nutrient agar plate to make pure colonies. A metal inoculation loop was used to streak the bacteria onto the plate using the streaking pattern as shown Figure 8. Between each streak the metal loop was properly burned using the flame of a Bunsen burner to kill all bacteria on the loop. The plates with the pure colonies were then incubated at room temperature (approximately 32 °C) for 24 hours. A total of 170 plates were inoculated.

Transportation of the isolates from Zambia to Norway

Sterile Eppendorf tubes were prepared with 0.7 ml of nutrient agar. A metal inoculation loop was used to pick a generous colony from the agar plates. The loop was then used to streak the agar surface in the tube and then stabbed into the agar. This process was repeated for all samples. Between each sample, the needle was burned properly in the flame of a Bunsen burner. The tubes with the isolates were incubated at room temperature (approximately 32 °C) until visible growth was seen in the tubes and were then moved to a fridge (4 °C). The tubes were then packed in a hard case suitcase and transported by airplane to Norway. The transportation time was 20 hours. Immediately after arriving in Norway, the samples were placed in a cool room at 4 °C.

Laboratory work in Norway

Culturing on nutrient agar

Shortly after arrival in Norway, the isolates were transferred from the transport tubes onto nutrient agar plates. This was done by immersing a single-use loop into the agar in the transport tube, making sure to hit the area where there was visual bacterial growth in the agar. Then the bacteria were inoculated onto the agar plate by streaking the loop along the surface of the agar in the pattern shown in Figure 8. The plates were then put into separate zip-lock bags (see Figure 9) and incubated at room temperature (approximately 22 °C) until bacteria colonies had formed on the agar surface (24-144 hours). After that, the plates were moved into a cool room (4 °C).

Figure 9. Bacteria inoculated onto nutrient agar plate in a zip-lock bag. (Private photo)

Bio bank

To be able to store the isolates for a long time, a bio bank was made (Figure 10). The bacteria isolates were inoculated into 1.8 ml thermostable Cryo tubes containing 500 µl nutrient broth incubated at room temperature for 24 hours. Then 250 µl of sterile 100 % glycerol was carefully added one drop at a time to the broth. The tubes were then frozen at -80 °C for long time storage.

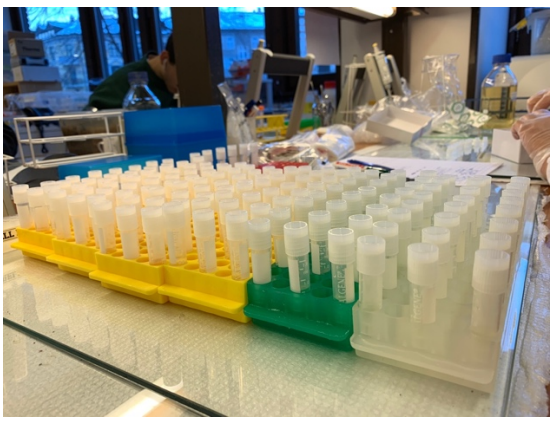

Figure 10. Bio bank in the making. (Private photo)

Grouping

Based on the morphology of the colonies on the nutrient agar plates, the 170 isolates were sorted into 20 groups. Representative isolates from each group were chosen, in total 60 isolates. These made the basis for further bacteriological testing.

Gram staining

Fresh bacterial isolates grown on nutrient agar that had been incubated at room temperature (approximately 22 °C) for 24-96 hours were used. A drop of PBS was placed on a glass slide and a colony of bacteria added and mixed using a sterile disposable inoculation loop. The smear was then air-dried and then gently heated by passing the slide over a flame to fix the bacteria. The glass slides were then dipped into crystal-violet for 40 seconds, and then rinsed gently with water. Next, the slides were dipped into iodine solution for 60 seconds, then washed with water again. Excess water was shaken off, and the slides washed with 95% ethanol until clear. Finally, the slides were dipped into Safranin solution for 60 seconds to counterstain the smear. The slides were washed with water one last time before left to airdry. 60 smears were made, with three different colonies on each slide, giving a total of 20 glass slides. Each smear was examined using light-microscope to categorise them into Gram negative or Gram positive rods, cocci or spirochetes. Each sample were photographed through the microscope for later references.

Positive controls

Three known bacteria isolates were used in addition to the 60 samples for the following biochemical tests. These were used as positive controls. The isolates used were:

- *Pseudomonas aeruginosa*

- *Streptococcus agalactiae* Bio 1
- *Streptococcus agalactiae* Bio 2

Culturing on blood agar

Culturing on 5% sheep blood agar was used to further characterize the bacteria. The same streaking technique as described earlier was used, and the bacteria was incubated at 37 °C for 24 hours until clearly visible growth. The bacteria and the agar were then closely examined to look for alpha-, beta- or gamma haemolysis.

Culturing on MacConkey agar

MacConkey agar is a selective and differential medium used to differentiate between lactose fermenting and non-lactose fermenting bacteria. Lactose fermenting bacteria colonies will appear pink on the agar, whereas non-lactose fermenting bacteria will produce yellow colonies. To test the bacteria's ability to ferment lactose the isolates were inoculated onto MacConkey agar plates using the same streaking technique as earlier described and incubated at 37 °C for 24 hours. The colour of the colonies was then noted for each isolate and recorded in a Microsoft Excel document. Each sample was photographed with a mobile phone camera for later documentation of the results.

Oxidase test

An oxidase test was used to determine if the bacteria produced the cytochrome oxidase enzyme. The isolates from Zambia were inoculated onto nutrient agar plates. After incubating the plates at 37°C for 24 hours a filter paper was placed in an empty petri dish and one drop of oxidase reagent was added onto the filter paper. Thereafter a generous colony of bacteria was

picked from the nutrient agar and placed onto the filter paper. A blue/dark purple colour change within 30 seconds marked a positive oxidase test.

Catalase test

To determine if the bacteria produced catalase enzyme, a catalase tests was done. The isolates from Zambia were inoculated onto nutrient agar plates and incubated at 37 °C for 24 hours. Then one drop of H_2O_2 was added to the bacteria on the slide and carefully covered by the lid of a petri dish to protect from aerosols forming during a positive catalase reaction. Bubbles forming on the slide after adding the H_2O_2 indicated a positive reaction. If no bubbles were forming the test was negative. The reaction was graded from 0 to 3 based on how fast the reaction started and how strong the reaction was. A grade 0 reaction indicated no reaction (ie. negative) whereas a grade 3 reaction indicated a very strong reaction that started immediately after applying the H_2O_2 to the bacteria on the microscope slide. These procedures were repeated for all 60 isolates.

Motility test

Glass tubes containing a semi-solid 0.3 % nutrient agar were prepared for motility testing of the bacteria. The bacteria were carefully stabbed/immersed in a straight line approximately 5 cm down into the agar. After inoculation, the tubes were incubated at 37 °C for 24 hours, whereupon the distribution of the bacterial growth in the tubes were carefully examined. If the bacteria had only grown in the stabbing line the bacteria were noted as non-motile, whereas as motile bacteria appeared cloudy in the area around the stabbing line or throughout the whole tube.

Determination of bacteria

The bacteria characteristics based on Gram staining, morphological appearance in the microscope, culturing on MacConkey and blood agar were used to classify the bacteria according to "Metode-kompendiet" by Anette Wold, Trine L'abee-Lund. (Translation: “The Method Compendium for Bacterial Testing") (Appendix II)

DNA extraction by the CTAB method

Based on the biochemical tests, 21 of the isolates were chosen for DNA extraction and further analysis. To extract the DNA the CTAB (Cetyl Trimethyl Ammonium Bromide) method was used. Bacteria was grown in nutrient broth and 1.5 ml of the culture was added to a 1.8 ml Eppendorf tube and centrifuged at 10,000 rounds per minute for 10 minutes. Then the supernatant was removed, and the pellet was resuspended in 567 µl of 1x TE buffer, 30 µl of 10% SDS and 3 µl of proteinase K and incubated at 37 °C for one hour. Thereafter, 100 µl of 5 M NaCl and 80 µl of CTAB-NaCl was added. The sample was mixed and incubated at 65 °C for 10 minutes. The volume in the Eppendorf tube was measured and an equal volume of Chloroform-Isoamylalcohol (24:1) was added and mixed gently with the sample before centrifuged at 10,000 rounds per minute for 10 minutes. After centrifuging, the aqueous layer was carefully removed and transferred into a fresh Eppendorf tube. The volume of the aqueous layer was measured and an equal volume of Phenol-Chloroform-Isoamylalcohol (25:24:1) was added. The sample was mixed and centrifuged at 10,000 rounds per minute for 10 minutes. Thereafter the supernatant was removed into a fresh Eppendorf tube and the DNA in the sample was precipitated by using 0.6 times the volume in the tube of isopropanol (2-propanol). Then the sample was centrifuged at 12,000 rounds per minute for 10 minutes. The supernatant was removed, and 0.8 ml of 70 % ethanol was added to the tube and mixed to

wash the DNA pellet. Thereafter the tube was centrifuged at 12,000 rounds per minute, the supernatant was removed, and the DNA pellet was dried at 37 °C. After drying, the pellet was resuspended by adding 50 µl of TE-buffer. The DNA concentration was then measured by using the Epoch Microplate Spectrophotometer (BioTek). See Appendix III for full protocol.

Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a common laboratory technique used to amplify a specific DNA segment. In the present study, it was done to verify the results obtained by biochemical results. As different primers are used to identify different bacteria, the selection of primers in the present study was based purely on convenience and availability.

Components of PCR:

- DNA template: The sample DNA that contains the target sequence. Initially high temperature is applied to the DNA molecule to separate the original double-stranded DNA into single strands.
- DNA polymerase: An enzyme used to synthesize new strands of DNA complimentary to the target sequence.
- Primers: Short strands of single-stranded DNA complimentary to the target sequence. The primers are used to make an initiation site at which the DNA polymerase can bind, allowing the DNA polymerase to synthesize new DNA from the end of the primer.
- Nucleotides (dNTP): Single units of the bases adenine (A), cytocine (C), guanine (G) and thymine (T), which are the “building-blocks” needed to make new DNA strands.

- Buffer: Providing an optimal chemical environment to promote the stability and activity of the DNA polymerase (35).

In the present study a master mix was prepared by mixing 5 µl of HF buffer, 1 µl of dNTP, 1 µl R-primer (1095R), 1 µl F-primer (L. garviae 167F) and 0.5 µl enzyme. The primers used were designed in-house on the basis of the 16S ribosomal RNA of *Lactococcus garvieae* and were (LG-167F-TACCGCATAACAATGAGAATC and LG-1095-CTTAACCCAACATCTCACGAC for forward and reverse primers, respectively). 8.5 µl of the master mix was allocated for each of the 24 samples, including 2 positive and 1 negative control). About 100-200 µg of the DNA sample was used as starting material for PCR. Sterile water was added to each reaction to a total volume of 25 µl per reaction. The positive controls used in the present study were *Lactococcus garvieae* isolates previously isolated from Zambia. As for the negative control no template was added to the master mix. The samples were then placed in a thermal cycler (Bio-rad T100) and a three-step protocol as shown in Table 1. was used to amplify the DNA for each sample.

Table 1. The thermal cycling protocol used to amplify the DNA during the polymerase chain reaction.

Cycle step	Temperature	Time (minutes)	Cycles
Initial denaturation	98 °C	3:00	1
Denaturation Annealing Extension	98 °C 55 °C 72 °C	0:30 0:30 0:30	34
Final extension	72 °C	5:00	1

Gel electrophoresis

2 % agarose gel was made by adding 10 g agarose (Sigma Aldrich) to 500 ml 1x TAE-buffer and microwave heated until nearly boiling. 250 ml of the solution was then gently mixed with 25 µl of SYBR-safe (Invitrogen) before poured into an agarose gel tray with 2 x 15 combs.

Each sample was then mixed with 2.5 µl of 10x Blue Juice Gel Loading Buffer (Invitrogen) before loaded into a well in the agarose gel. 5 µl of TrackIt 1 Kb Plus DNA Ladder (Invitrogen) was loaded in the first well in the agar and used as a reference as it contains DNA fragments of known lengths. An electric current of 70 V was then applied to the gel for 45 minutes. Due to the negatively charged phosphate group on the sugar-phosphate backbone of the DNA fragments, the DNA fragments will move towards the positive electrode in the gel. All DNA molecules have the same amount of charge per mass, resulting in that the speed the fragments move through the gel is determined by the size of the fragments. Smaller fragments move faster through the gel than longer fragments and will therefor move further towards the positive electrode within a certain amount of time than the longer fragments. To visualize the distribution of the DNA fragments in the gel an UV light imaging machine GeneFlash (Syngene) was used. Due to the DNA loading buffer groups of DNA fragments could be seen as bands in the gel when exposed to UV light. By comparing the bands from the samples to the bands of the DNA ladder the approximate size of the DNA fragments in the sample could be determined (36). The bands were then cut out from the gel by using a scalpel and stored in separate Eppendorf tubes at 4°C. During the cutting process a UV transilluminator (UVP) was used do visualize the bands in the gel. The samples were sequenced commercially at EUROFINS in Germany.

BLAST-ing

BLAST is an acronym for Basic Local Alignment Search Tool and is a tool for finding regions of local similarity between biological sequences (37). The nucleotide sequences we received after sending our bands from PCR and gel electrophoresis for sequencing were plotted into NCBI's database for BLAST-ing (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The program compared the nucleotide sequences with sequences in it's database and calculated the statistic significance of local similarity.

Results

Interviews

Table 2. A summary of the visited farms.

Farm	Water source	Production	Employees	Type of production	Production system
Farm One	Zambezi River	Medium-scale hatchery	18	Intensive	Ponds
Farm Two	Lake Kariba	Large-scale hatchery	500	Intensive	Ponds
Farm Three	Lake Kariba	Small commercial farm	16	Intensive	Cages
Farm Four	Lake Kariba	Large-scale commercial farm	74	Intensive	Cages

Farm One

Farm One is a medium-scale hatchery located approximately 150km (two hours drive) from Siavonga, close to the Zambezi River. It was established in 2002 and had a total of 18 employees. The interview was done with the fish department manager who worked at the farm on a daily basis. The main product of the farm is Nile Tilapia (*Oreochromis niloticus*) fingerlings. Their production capacity is 6,000,000 fingerlings per season (9 months) and their

production cycle is 8 weeks from eggs until fingerlings ready for sale. The size of the fingerlings when sold is 1.5 g. The fingerlings are kept in ponds and the water source is the Zambezi River. The farm has its own breeding lines and rotates the breeders every second week to ensure that genetic variation is upkept. The fertilized roe is incubated in a house separated from the ponds. After hatching the yolk sack fry are immediately moved over to a separate tank where they are kept until the yolk sack is completely absorbed. The fry is then transferred to hapas in the ponds and fed with sex reversal feed for 3 weeks before released into the ponds.

Farm Two

Farm Two is a large-scale hatchery located in Zimbabwe with the water source coming from Lake Kariba. The farm was established in 1997 and has 500 employees on site. The interview was done with the person in charge of the fingerling production. The farm produces Nile Tilapia (*Oreochromis niloticus*) and have a capacity of 70-80,000,000 fingerlings per season. At this farm they have an advanced hatchery with strict hygiene control and also regulations on the people going in and out. The fertilized roe is disinfected before going into the incubator, and the yolk sac fry goes into tanks directly after hatching. This is a separate part of the farm, which has a recirculating water system and the inlet water is decontaminated through several steps. When the yolk sack is absorbed the fry is moved into a nursery tub before going into the ponds. One week before they are sold, the fingerlings are sorted by a grading machine and placed into different hapas based on their size.

Farm Three

No sampling was performed at Farm Three, but the farm was included in the interviewing process to help us get a better insight into fish farming in Zambia. The farm is located in Siavonga and is a small commercial farm with 16 employees. The interview was done with the director of finance. Their cages are located on Lake Kariba and they farm Nile Tilapia (*Oreochromis niloticus*). The business was established in 2015. They build the fish cages themselves. In the beginning the cages were squared but have now changed to round cages due to better weather resistance. They transport the fingerlings from the hatcheries in plastic bags inside a cooled van. At arrival at the farm they put the bags in the water in the cages for 10 minutes to let the fingerlings acclimatize before they open the bags and let the fish swim out on their own. They have 25,000 fingerlings in each grow-out cage for two months whereupon they count the fish and move them over to production cages. They are hoping for a production capacity of 100 tons per year. Their production cycle is between 5 and 7 months, depending on the stocking density.

Farm Four

Farm Four was our main focus in this study. It is a large commercial farm located in Siavonga with cages on Lake Kariba divided on two different sites. The business was established in 2013 and has today a total of 74 employees. The interview was done with the lake supervisor. The farm has a production capacity of 1,500 tons per year and a production cycle of 32 weeks (8 months). They buy fingerlings from three different hatcheries including Farm One and Two. They have production all year but stop moving fingerlings into the farm during winter. When the fingerlings arrive at the farm they are moved into juvenile cages (6 x 6 x 3 meters) on the lake and stay there until they reach the size of 20 grams. Then they are moved into big production cages (6 meters in diameter and 6 meters deep) and kept there until they are sold at

400 grams. In the juvenile cages they keep 500,000 fingerlings maximum while in the production cages they keep 100,000 fish. The farm has frequent contact with a veterinarian whom they can call in case of disease outbreaks and increased mortality.

Fieldwork in Zambia

Findings associated with transportation of fingerlings

Findings associated with transportation of fingerlings from Farm One and Farm Two are listed and compared in Table 3.

Table 3. A comparison between the observational findings from Farm One and Farm Two associated with transportation of the fingerlings.

	Farm One	Farm Two
Size of fingerlings at time of collection	2 g	1,5 g
Total number of collected fingerlings	200,000	200,000
Number of fingerlings in each transportation box	26,000	26,000
Time used from pond to transportation box	10 minutes	10 minutes
Total time out of the water	5 seconds	~ 1 minute
Amount of water in each transportation box at beginning of the transport	1000 L	1000 L
Total transportation time	5 hours	8 hours
Highest water temperature recorded during the transport	23.7 ºC	28.2 ºC
Lowest water temperature recorded during the transport	16.7 ºC	26.3 ºC

Highest DO levels recorded during the transport	19.5 mg/L	12.1 mg/L
Lowest LO levels recorded during the transport	3.3 mg/L	0.1 mg/L
Observation of gasping fingerlings during the transport	No	Yes

Measurements during transport

The measurements of water temperature and dissolved oxygen levels during the transport at Farm One shows that the pond where the fingerlings were collected from had a water temperature of 28.3 ºC. The water in the transportation boxes had a maximum water temperature of 23.7 ºC and a minimum of 16.7 ºC during the transport and a dissolved oxygen level varying between 19.5 mg/L and 3.3 mg/L. The water in the cage had a temperature of 26.9 ºC at time of arrival.

At Farm Two the water temperature in the pond was measured to be 27.1 ºC. The variations in water temperature in the transportation boxes varied between a maximum of 28.2 ºC and a minimum of 26.3 ºC during the transport of the fingerlings. The highest recorded dissolved oxygen level in the transportation boxes during the transport was 12.1 mg/L, whereas the lowest level was recorded at 0.1 mg/L.

An overview of the variations in temperature and dissolved oxygen can be seen in Figure 11, 12 and 13.

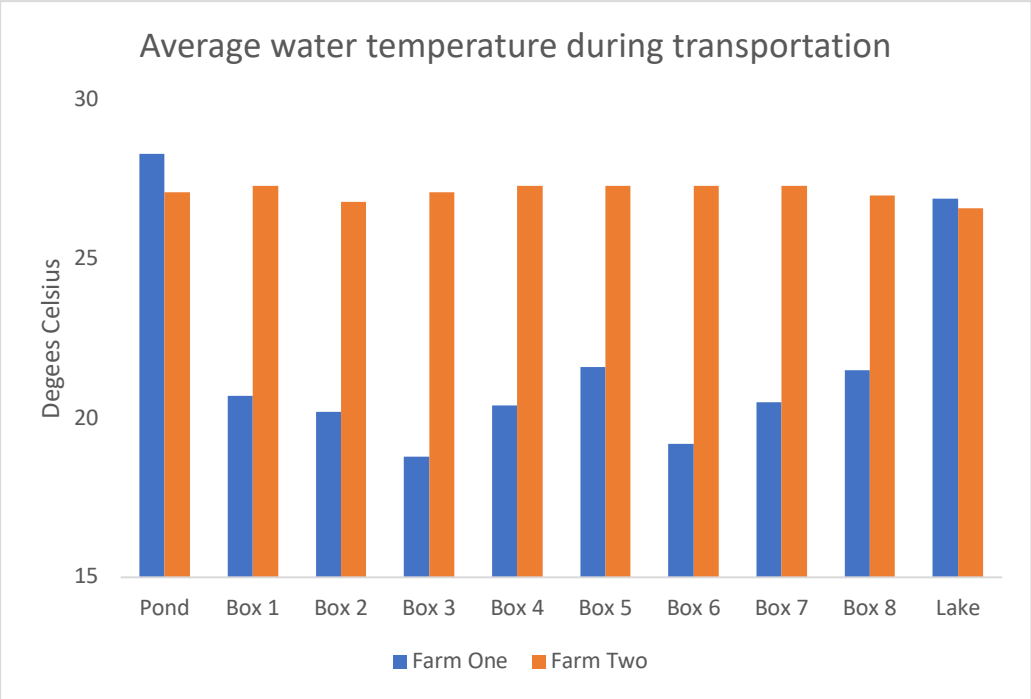

Figure 11. Average temperature during transportation of the fingerlings from Farm One and Two to Farm Four. The column to the left shows the water temperature in the pond of Farm One and Two respectively at the time of collection of the fingerlings. The column to the right shows the water temperature in the lake at Farm Four at the time of arrival. The columns named "Box 1-8" show the average water temperature in the eight transportation boxes during the transport of the fingerlings.

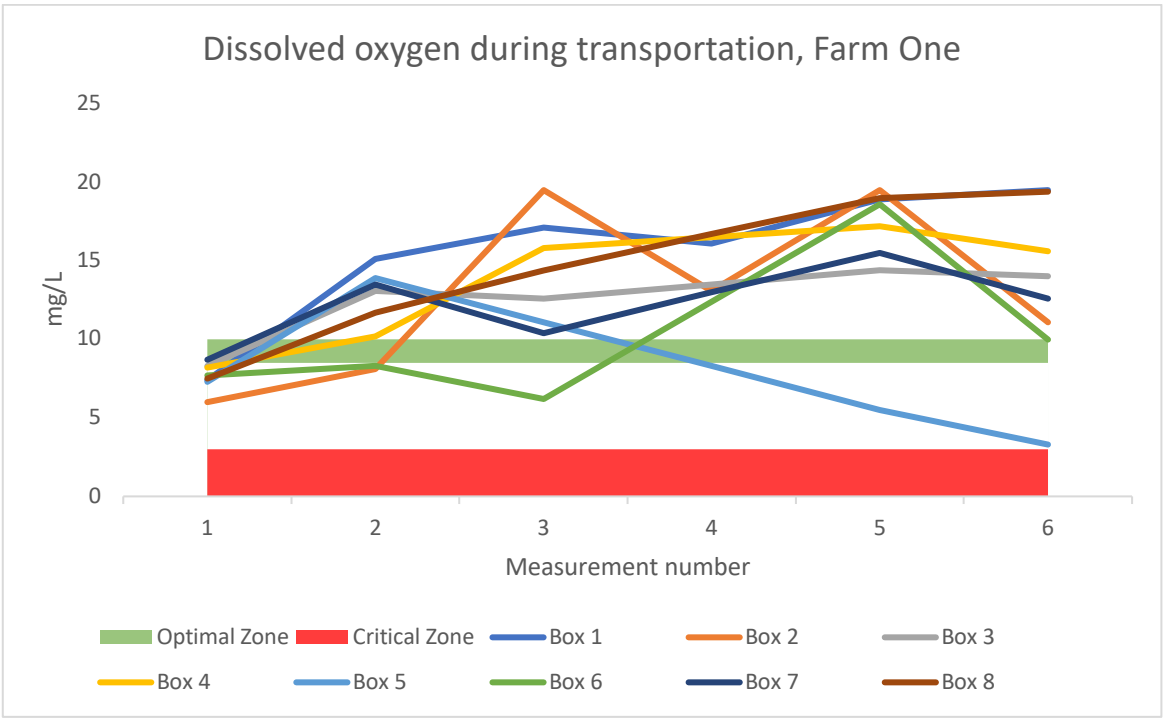

Figure 12. The variations in dissolved oxygen in the eight transportation boxes during transportation of the fingerlings from Farm One. The first measurement represents the first registration of dissolved oxygen done during the transport. Measurement number six represents the registration of dissolved oxygen done at the end of the transport. The green area shows the preferred level of dissolved oxygen during transportation (8,5-10 mg/L), whereas the red area represents critically low levels of dissolved oxygen (< 3 mg/L).

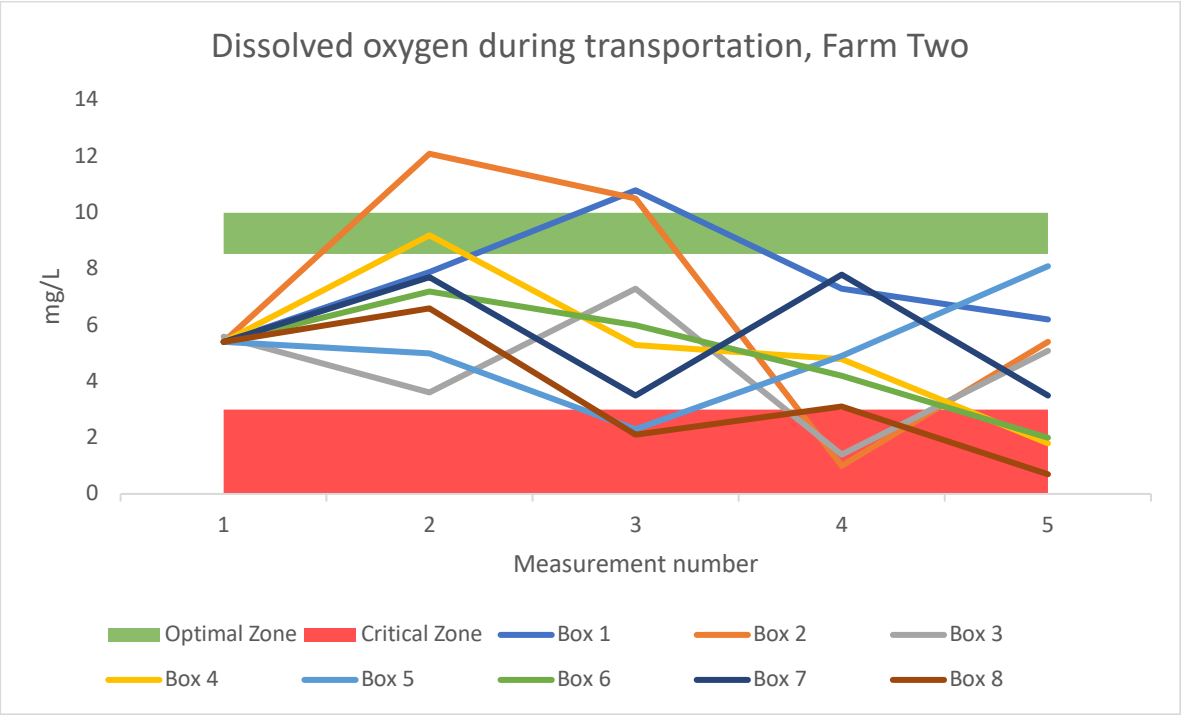

Figure 13. The variations in dissolved oxygen in the eight transportation boxes during transportation of the fingerlings from Farm Two. The first measurement represents the first registration of dissolved oxygen done during the transport. Measurement number five represents the registration of dissolved oxygen done at the end of the transport. The green area shows the preferred level of dissolved oxygen during transportation (8,5-10 mg/L), whereas the red area represents critically low levels of dissolved oxygen (< 3 mg/L).

Necropsy findings

In general, there were very few findings in the first three rounds of sampling (fish number 1-29 from Farm One and Two) except for some minor skin erosions. In the fourth round of sampling (day 4-5 after transfer to cages) there was evidence of pale gills and fin rot on 3 out of 10 fingerlings. In the last sampling (day 6-7 after transfer to cages) 9 out of 10 fingerlings had pathologic changes, including a pale and enlarged liver (n=7) (Figure 14), skin and fin lesions (hyperaemia, erosions and ulcerations), and petechial bleeding on the surface of the liver. See Appendix IV for complete results.

Figure 14. Fingerling with an enlarged and pale liver. (Private photo)

Laboratory work at UNZA

Water samples

Complete results from the quantitative analysis, colony counting on nutrient agar and MacConkey agar plates are shown in Appendix V. Bacteria was found in all the water samples. The total number of colonies forming units (CFU) ranged from 2,300 total coliform CFU per ml from water sample 4 (taken from cage on Farm Four 4-5 days after transfer) to 870,000 total coliform CFU per ml from water sample two (taken from transportation box from Farm Two at the end of the transport).

Samples from fingerlings

Key to numbering/naming of the samples:

- **Sample 1-10:** Farm One, samples were taken from the pond at the time of collection of the fingerlings

- **Sample 11-20:** Farm Four, day 1 after transportation the fish samples was taken before the arrival of the fingerlings from farm two.
- **Sample 21-30:** Farm Two, fish samples was taken from the transportation box at end of transportation
- **Sample 31-40:** Farm Four, samples was taken day 4 and 5 after transportation. Fingerlings from Farm One and Farm Two were now mixed in the same cage.
- **Sample 41-50:** Farm Four, samples was taken day 6 and 7 after transportation fingerlings from Farm One and Two, still mixed in the same cage.

During sub culturing of the samples each type of colony was assigned one letter from the roman alphabet in addition to the number of the sample, e.g.: “1a”, “1b”, “1c” etc. For some of the samples further sub culturing was necessary to obtain pure cultures. The samples were then assigned an additional number after the letter, e.g.: “1a-1”, “1a-2” etc

The water samples were named following the same numbering system, but with “W” at the beginning e.g.: “W1a”, “W1b” and “W2a-1”, “W2a-2” etc.

Laboratory work in Norway

Grouping

The bacteria were grouped based on the morphology, such as colour, shape and size, of the colonies grown on nutrient agar plates (Figure 15). Appendix VI shows the distribution of the isolates into the different groups. A total of 54 representative isolates were chosen for further biochemical testing.

Figure 15. Grouping of bacteria based on colony morphology. The picture to the left shows bacteria from group 2. The picture to the right shows bacteria from group 8. (Private photos)

Gram staining and biochemical tests

A complete summary of the results from the Gram staining and the biochemical tests can be found in Appendix VII.

Flow chart

Based on the results from the biochemical tests an estimate of the bacteria genera was done using a flow chart for characterization of bacteria (see Appendix II). The bacteria isolated may belong to the following genera/families: *Actinobacillus, Actinomyces, Aeromonas, Bacillus, Bordetella, Brucella, Citrobacter, Corynebacterium, Enterobacter,* Enterobacteriaceae*, Enterococcus, Erysipelothrix, Haemophilus, Klebsiella, Listeria, Lactobacillus, Micrococcus, Moraxella, Neisseria, Pasteurella, Proteus, Pseudomonas, Rhodococcus, Salmonella, Shigella, Staphylococcus, Streptococcus, Trueperella, Vibrio,*

Yersinia. No difference in diversity between the farms was found. Appendix VIII shows the distribution of possible bacteria genera in each sample. In Appendix IX the distribution of possible bacteria genera in the different sampling rounds is enumerated.

DNA extraction

See Appendix X for the full results from the DNA extraction. The 260/280 ratio is a measurement for the purity of the DNA in the sample, and a ratio of 1.8 being the least acceptable (38). In the DNA extraction of the present study, most of the samples ended up with a 260/280 ratio of more than 2. The concentration number should preferably be as high as possible. Seven of the samples had a concentration less than 100 ng/µL, considered as a low concentration.

Polymerase chain reaction (PCR) and gel electrophoresis

As Table 4 and Figure 16 shows, the results from the PCR and gel electrophoresis with the primers L. garvieae 167F and 1095R showed clear DNA bands in the gel for the samples in wells number 11, 12 and 13 on the upper row and for the samples in wells number 8 and 12 on the lower row. In addition, a weak band was seen in well number 5 on the upper row.

Table 4. shows the distribution of the samples in the gel electrophoresis agarose gel. The highlighted samples are the ones that showed DNA bands after the gel electrophoresis. NK = negative control, PK = positive control.

Upper row													
Well number	1	2	3	4	5	6	7	8	9	10	11	12	13
Sample name	DNA ladder	NK	27b	16c	3d	W5b	41a	17a	W5c	W2c	27c	23a	Bio2

Lower row													
Well number	1	2	3	4	5	6	7	8	9	10	11	12	13
Sample name	DNA ladder	27a	4c	10b	19d	34b	33a	13c	1c	50a	46b	PK	PK

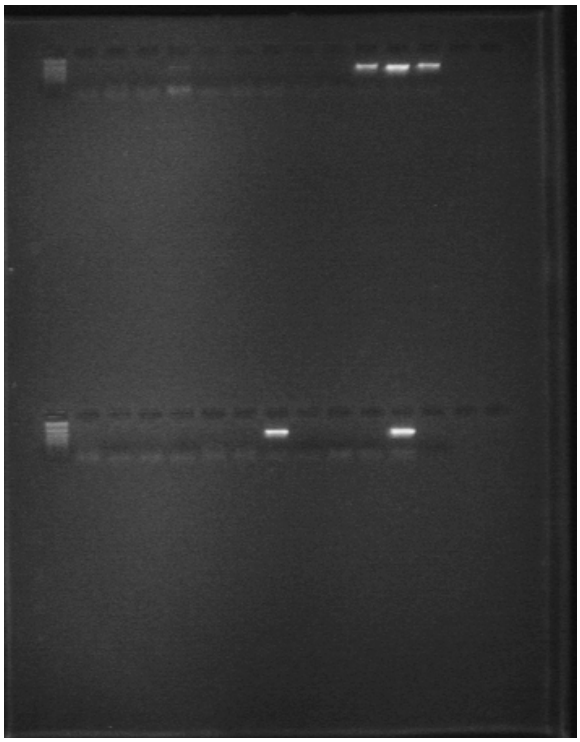

Figure 16. Results from the PCR and gel electrophoresis. Bands can be seen in well number 5 (weak), 11, 12 and 13 on the upper row and in well number 8 and 12 on the lower row. These bands correspond to the samples named: 3d (weak), W5b, 23a, Bio2 (upper row) and 27a and PK (lower row).

Sequencing and BLAST-ing of DNA

Out of the 5 DNA bands sequenced and BLAST-ed, 2 bands showed a 99.72 % and 99.64 % correspondence to the 16S rRNA sequence of *Lactococcus garvieae*. The last 3 samples did not match with the sequence and were therefore categorized as inconclusive.

Discussion

Findings from interviews and observations in Zambia

When looking at fingerling mortality and its possible causes it is natural to bring in the physiological effects of stress on the fish. Stress is defined as a psychological and physiological response to perceived danger to avoid the noxious stimuli (39). When it comes to fish the term, stress is often used to describe an acute physiological stress response with the endocrine and biochemical reactions that occur in the fish when exposed to changes in the environment. These are equivalent to the stress response what we find in mammals. When experiencing stress cortisol plasma levels rise, catecholamines are released and oxygen consumption rises (39).

Some of the fingerlings from Farm Two showed skin lesions before transportation. The fingerlings had been sorted by a sorting machine 2-3 days before the transport, which was closer up to the transportation date than the usual 5-7 days according to the interview subject from Farm Two. The skin lesions might have come as a result of mechanical stimuli caused by the sorting machine. The shortened time lap between the sorting and transportation is probably not ideal as the stress following both the sorting and the wounds lower the immunological competence of the fingerlings. Rough mechanical stimuli is known to cause loss of scales and heightened stress levels in fish, which predisposes for skin lesions like erosions, ulcers, damages to fins and secondary fungal or bacterial infections (40). Sorting the fingerlings by size also has some beneficial effects. These include less competition for feed and more uniform growth rate of the batch which leads to decreased stress levels, less cannibalism and fewer loser fish (smaller, less active fish with lower chances of survival) (41, 42). Sorting of the fingerlings also gives more control and predictability for both the hatchery

and their customers. At Farm One, where they also raise fingerlings, no sorting based on size was performed before transportation. The fish sampled from this farm did not show any signs of skin lesions. One might argue that by not being sorted in such short time before transportation the overall stress exposure, and by that the predisposition for infection, is less than what is the case for the fingerlings from Farm Two. This could give them a better starting point after transfer to the cage and the new environment in the lake.

Both hatcheries practice starving of the fingerlings before handling. Farm One states that they cease the feeding two days before they collect the fingerlings into the hapas. After collection the fingerlings get two days of rest, still without feed, before transportation to the grow-out farms. At Farm Two they starve the fingerlings for 24 hours before transportation. They did not mention any starving of the fingerlings before other kinds of handling. However, we can not exclude that they had protocols for starving also before and after sorting.

After arrival at the grow-out farm (Farm Four) and transfer into the cage, the fingerlings are rested two more days before feeding starts. This gives a total of 6-7 days of starvation for the fingerlings from Farm One and somewhat shorter for the fingerlings from Farm Two. Studies show that starvation of juvenile tilapia before handling is beneficial. It leads to lower plasma cortisol levels after handling compared to fish that has not been starved (43). Starvation has also been showed to lower the resting metabolic rate and post exercise oxygen consumption, which decrease the demand for dissolved oxygen in the water for the fish and slows the development of hypoxia (44). It also improves the hygienic quality of the transportation water by lowering the levels of nitrogenous waste (39). Although the starvation might be perceived as stressful for the fish, the long-term benefits are shown to outweigh the temporary

discomfort experienced (39). Starvation of juvenile tilapia beyond 7 days may have adverse effects and should be avoided (44).

Transport is a stressful event for the fingerlings due to several reasons. These include, but are not limited to, stressors such as change in water temperature, low DO levels, high density of fish, movement leading to mechanical trauma, higher infection pressure, change in salinity and high turbidity. As a consequence of earlier handling (crowding, sorting, weighing etc.) the fingerlings are already under strain and their threshold value for sudden changes in the environment are considerably lowered.

As mentioned earlier, water temperature is a key factor affecting the physiology, metabolism and immune system of the fish. Being a thermophile species Nile tilapia thrives within water temperatures ranging from 27 °C to 30 °C, but is also known to survive within a wide range of temperatures (lower and upper lethal temperatures are 11 °C and 42 °C, respectively) (7). They are, however, not adapted to withstand very sudden changes in water temperatures, even if these changes are within the optimum temperature range of the fish (45).

The water temperature in the individual transportation boxes was measured and noted down at regular intervals during the transport from the hatcheries to the grow-out farm at Lake Kariba. Ice was added to the boxes if the temperature rose above 28 °C. The registrations from the transport from Farm Two show that the water temperatures were kept at a relatively stable level with a maximum temperature difference of 6.5 °C (26.3 °C – 28.2 °C). The registrations from Farm One on the other hand show that there were considerable variations in temperature during transport and that the average temperature in the water tanks was quite low. The fingerlings started out with 28.3 °C in the pond at the hatchery. The highest temperature

measured in the transport tanks at the start of transport was 21.8 °C, while the lowest was 16.7 °C. This results in a sudden temperature drop of 6.5 °C and 11.6 °C in the two tanks respectively. The temperature in the cage in Lake Kariba was measured to 26.9 °C at arrival. These water temperature changes in such a short time puts the fish under considerable stress (45).

The second variable measured in the water tanks during the transport was levels of dissolved oxygen. It was stated that they aimed at keeping the DO level at 8.5-10.0 mg/L. At 5.3-6.0 mg/L measures were taken, namely adding oxygen into the water. DO levels < 3.0 mg/L should never occur. Despite this we observed gasping fish in the water surface at the end of the transport from Farm Two, and the registrations showed DO levels down to 0.7 mg/L. This is low even for tilapia, particularly for very young fish that's already been subjected to considerable amount of stress due to handling and transport.

Other important stressors during transport include variations in salinity, accumulation of waste material and a heightened infection pressure. In an effort to lower the infection pressure by *Saprolegnia* spp. salt was added to the transport boxes. The sudden change in salinity the fish experience when moved from the pond with low salinity to the transport box with a relatively higher salinity means that the fish needs to upregulate the amount of energy and oxygen spent on osmoregulation in order to maintain homeostasis. There was a high occurrence of saprolegnia lesions observed on the fish from Farm Two at arrival at Lake Kariba, see Figure 17, even though none such lesions were observed on the same batch of fish at departure from the hatchery eight hours earlier. Hence the salt treatment did not have the intended outcome, and one might ask whether the effectiveness of the salt treatment in

lowering the occurrence of saprolegnia makes up for the additional strain it inflicts upon the fingerlings.

Figure 17. Fingerlings with saprolegnia lesions. (Private photo)

Laboratory findings

Part of our aim was to compare fingerling mortalities from different hatcheries relative to bacterial diversity in the fingerlings from the different sources. However, this was not possible as the batches of fingerlings from the two different hatcheries were mixed into the same production cage upon arrival at the grow-out farm at Lake Kariba. According to the interview subjects at said farm, batches from different fingerling producers were usually kept apart in separate cages. When we asked why they chose to mix the batches in this case, there was no clear answer, except perhaps that one production cage was available at the time. One advantage of doing this though, is that there is one step less of handling of the fish later on in the production cycle. Being thwarted of our original aim to compare the fingerling mortality between the two hatcheries, we chose to still perform the necropsies and sample from the same number of fingerlings as originally planned to investigate what bacterial agents that might be present before transport and the days just after transport.

Necropsy

Progressively more lesions were found from the first two samplings through to the last sampling. During the first three rounds of necropsy and sampling there were few and mostly minor pathologic findings, and almost all of these were external lesions. In the last sampling (Farm Four, day 6 and 7) 9 out of 10 fingerlings had pathologic lesions. These included pale and/or enlarged liver, petechial haemorrhages, hyperaemia, erosions and ulcerations. Several bacterial agents can cause these lesions in tilapia, including *Aeromonas* spp., *Streptococcus* spp. and *Lactococcus* sp. (31). The stress and mechanical trauma related to the transport of the fingerlings may have led to higher susceptibility for diseases due to weakened skin barriers and decreased immune defence. See Figure 18 for a causal diagram where we have tried to summarize how stress, disease and death may relate to each other. Another plausible explanation for such an increase in number of findings are the steep learning curve for the samplers (the authors). As we got more experienced at performing the necropsy, we got better at actually finding pathologic lesions.

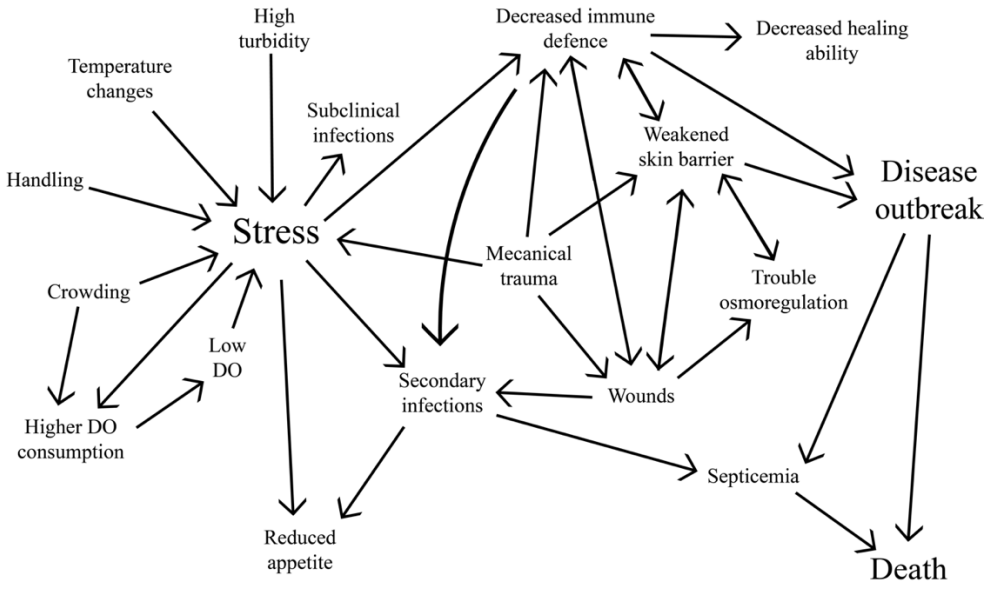

Figure 18. Causual diagram showing the relations between stress and different exertions it may lead to.

Water samples

Bacteria was found in all the water samples. The water sample from Farm Two had to be diluted five times for the CFU to be countable, giving a total of 870,000 CFU/mL. This water sample was collected from one of the transport boxes at the end of the eight hours long transport from Farm Two to Lake Kariba. The water had a visibly high turbidity, and large amounts of faeces was observed. The short starving period at Farm Two might have influenced the turbidity and amount of waste material in the transportation water. No exchange of water during the transport and high temperatures can partly explain the high number of CFU. The water sample from Farm One was taken from the pond before transport. The fingerlings from Farm One was already transferred from the transportation boxes to the cage in Lake Kariba when we arrived. Therefore, no sample was collected from the water the fingerlings was transported in. Hence the level of bacteria in the water from the two hatcheries can not be compared.

Four of the water samples are likely to be members of the Enterobacteriaceae family. Enterobacteriaceae contains faecal bacteria species which are partly ubiquitous and partly normal microflora, opportunistic or pathogenic in mammals, reptiles, birds and fish (46). As there is seepage into the ponds from the surroundings, the ponds are fertilized with manure as well as the fact that there are members of this family that are normal microbiota, isolation of faecal bacteria from the water is not surprising.

Bacterial isolates from fingerlings

It is nearly impossible to accurately name the species or even the genus of the isolates without further testing of the biochemical characteristics or using molecular methods such as PCR. The number of tests we could perform were limited because of restricted time available. Due to the fact that we are far from experienced in bacteriological diagnostics, we found it difficult both to interpret and to trust some of our results from the Gram staining. The results from the biochemical testing, Gram staining and growth on MacConkey and blood agar showed large variations within the groups we originally had placed the isolates in based on the appearance of the colonies grown on nutrient agar. We were hence reminded that morphology, neither macroscopic nor microscopic, is sufficient information to determine a bacterial species or genus.

We can find no significant variation in distribution of the different bacteria between the different samplings. Among the possible genera we have isolated bacteria from, the following genera contain potentially pathogenic species to fresh water fish: *Aeromonas, Pseudomonas, Citrobacter, Moraxella, Neisseria, Streptococcus, Pasteurella, Enterobacter, Listeria, Yersinia, Vibrio, Edwardsiella* (26). The flowchart we used did not include *Lactococcus,* which is also a genus containing species that are known fish pathogens. *Lactococcus* is however phenotypically very similar to *Streptococcus,* and these two genera are difficult to distinguish from each other based only upon biochemical tests. To obtain a higher degree of specificity and more conclusive results, several additional tests would be needed to be performed. Without a greater specificity it is nearly impossible to draw any conclusions on frequency or occurrence of specific agents on the different farms. When looking at the table in Appendix IX which shows the number of samples possibly belonging to each specific genus, it must be empathized that these are not the actual number of findings of bacteria, but rather

an enumeration of how many different possible genera we get when using the flow chart. Hence a high number of genera listed per sample in Appendix VIII does not reflect that we have found and isolated a large number of bacteria, but rather that we have a high degree of uncertainty in the characterization and therefore there are many different potential candidates.

21 isolates were chosen for further testing with PCR. In prioritized order, this was based on Gram staining, catalase test, oxidase test, haemolysis and growth on MacConkey agar. To extract DNA from the isolates we used the CTAB protocol. Recommended 260/280-ratio for PCR amplification is ~1.8, and indicates that the extracted DNA has a high degree of purity free of proteins (38). From some of the isolates we got high concentration of DNA with a high degree of purity. From a few of the isolates we struggled to achieve sufficiently high concentration, despite several retakes of extraction. The CTAB protocol is a well documented method to produce DNA for use in PCR amplification. Some of the disadvantages are that it is a time consuming method which require long incubations, has multiple precipitation steps and demand high precision to produce RNA-free genomic DNA with a high degree of purity (38). Today many alternative laboratory protocols and commercial DNA extraction kits exists that both are quicker and have higher grades of sensitivity than the CTAB protocol (47). The CTAB was in our case used for educational purposes.

Due to a tight time schedule only one primer was used for the PCR amplification. As lactococcosis could be suspected in the fingerlings based on the necropsy findings and earlier isolation of *L. garvieae* from fish in the grow-out farm, a primer designed by Professor Mutoloki from the 16s rRNA sequence in *L. garvieae* was chosen for the PCR amplification. 16S rRNA sequences are by far the most common housekeeping gene marker used to study bacteriological phylogeny and taxonomy (48). There are several reasons for this, some of the

most important being (i) it's presence in almost all bacteria, often existing as operons; (ii) the function of the 16S rRNA gene has not changed over time, indicating that random changes in the sequences can be an accurate mean of measuring time (evolution); (iii) the 16S rRNA gene consists of about 1,500 base pairs and is big enough for further processing and analysis. A major disadvantage with the use of the 16S rRNA sequence is that has a relatively low resolution. This leads to low phylogenic power at species level and poor discriminating powers for certain genera. Hence many investigators experience problems with resolution at genus and/or species level with this gene sequence (48).

PCR products on gel electrophoresis revealed only 5 bands with expected length. These were excised and sent for sequencing. Three out of five samples came back as inconclusive, while two came back as sequences corresponding to *L. garvieae* when BLAST-ed against known sequences in the NCBI database. Both of these samples were extracted from isolates that were used as positive controls, while none of the samples from Zambia tuned out positive. Interestingly, one of them (isolate "Bio 2") was stated to be *Streptococcus agalactiae* and was used as positive control for the biochemical testing. The other was stated to be *L. garvieae* and was used as the positive control for the PCR. When looking closer on the bands from the three inconclusive samples we can see that these deviate slightly from the two bands confirmed to be *L. garvieae.* If we had run the electrophoresis for some more time these deviations would probably have been more obvious, and we would not have suspected them to be *L. garvieae* in the first place. Another possible reason for inconclusive results is that there were not enough DNA in the bands excised or that the DNA was not of sufficient quality.

Conclusion

Through the work with this thesis we have seen that there are high mortality rates among the fingerlings during the first days after arrival at the grow-out farm. Based on the interviews and our observations, we suggest that some of these mortalities might be associated with the transport of the fingerlings, which is clearly a source of stress to the fingerlings. It is well known that both handling and crowding leads to increased stress levels in the fish. Stress in general compromises the immune system leading to higher susceptibility to disease (3). A lot of effort is already done to keep the transport as calm as possible for the fingerlings. Still there are some measures that could be easily be improved and which probably would make a considerable difference in mortality. Examples of these are:

- Better conditioning: Making sure that the fingerlings get sufficient amount of time to rest between each stress/handling event, and that the starvation period is long enough, but not too long, before the fingerlings are transported.
- Better routines in keeping the DO and temperature levels stable during the transport of the fingerlings. Making sure that the actions done to keep the water environment stable match the measurements that are done.
- Plan the transport precisely and making sure that everything is working before loading the fingerlings onto the truck to keep the transportation time as short as possible.

As described earlier the bacteria samples were collected by swabbing the inside of the abdomen during the necropsy of the fingerlings. Typically, one would expect to find little to no bacteria from abdominal swabs in healthy individuals and it's therefore somehow surprising that several different bacteria was isolated from each fingerling. There might be several causes for this finding. The fingerlings were very small (< 2 g) and contamination could easily arise. The abdominal cavity and the sample could have been contaminated

internally from punctured intestines, or from the outside due to insufficient disinfection of the surface of the fish, the equipment and work space during the necropsy.

Our impression after performing interviews at several farms is that disease is not considered a big issue in tilapia farming except for seasonal saprolegniosis when water temperature is lower. Saprolegnia is easy to detect even on small fish. Most other diseases can be difficult to spot on such small fish. Even though it is not visible to the naked eye, diseases can still be present. The high mortality numbers during the first days after transfer to the cages, in combination with our bacteriological findings, could indicate that there are pathogenic bacteria at the farms. Further scientific research would be necessary to confirm this suspicion. A good measure to improve the current health situation could be to develop and use effective vaccines. Exchange of experience between different farms, higher focus on biosecurity and better registrations of diseases and mortality would also probably be a contributing factor in lowering the mortality numbers.

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We would like to thank our supervisors Associate Professor Stephen Mutoloki and Professor Øystein Evensen for good guidance through the entire work with our thesis, both in the preparations and during the fieldwork in Zambia as well as in the laboratory work in Norway and during the writing process. We would also like to thank Associate Professor Bernard Hang'ombe and Dr. Kunda Ndashe for showing us tilapia farming in Zambia and for letting us use the laboratory at UNZA. We are also grateful for the help from Racheal Amono, Mustapha Lamkhannat, Maron Mubanga, Henning Sørum, Sandra Radunovic and Florin Asavei during our work in the laboratory in Norway and in Zambia. We also thank the farmers in Zambia for allowing us visit.

Sammendrag

Tittel: Tidlig yngeldødelighet etter utsett hos tilapia i Lake Kariba

Forfattere: Christine Donbæk, Anniken Mork, Ingelin Ravlo

Veiledere: Stephen Mutoloki, Øystein Evensen, NMBU Institutt for basalfag og akvamedisin

Tilapia er, etter karpe, den fisken som det blir produsert mest av på verdensbasis. Den er en robust fisk som tåler en rekke miljøer og oppdrettssystemer, og egner seg derfor godt som oppdrettsfisk i land med varmere klima. Hovedformålet med oppgaven var å undersøke mulige årsaker til tidlig yngeldødelighet etter utsett hos niltilapia i Lake Kariba i Zambia. Dette ble løst gjennom intervju og observasjoner ved fire anlegg i Zambia. Fokus ved besøkene var på rutiner knyttet til produksjonen, og da særlig transport, av yngelen. I tillegg ble det samlet inn fem vannprøver, samt 160 bakterieprøver fra tilsammen 50 yngel for videre karakterisering og bestemmelse av agens i laboratoriet. For karakterisering av bakteriene ble det utført utstryk på ulike vekstmedier, gramfarging, biokjemiske tester, DNA-ekstraksjon med CTAB-metoden og PCR med primer L. garvieae 167F og 1095R for sekvensering. Observasjonene fra besøk på anleggene viser at yngelen blir utsatt for store mengder stress i forbindelse med transport. Funnene fra laboratorieundersøkelsene indikerer at det kan være en rekke potensielt sykdomsfremkallende bakterier til stede.

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Attachments

Appendix I: Questionnaire for tilapia farmers

Appendix II: Flow chart for bacteria identification

Appendix III: DNA extraction protocol - CTAB

Appendix IV: Necropsy findings

Appendix V: Results from water sampling

Appendix VI: Bacterial grouping

Appendix VII: Bacteria analysis of isolates

Appendix VIII: Distribution of bacteria in the different samples

Appendix IX: Distribution of bacteria in the different sampling rounds

Appendix X: DNA extraction results

Appendix I. Questionnaire for tilapia farmers

Generally:

1. Name:
2. Farm
3. Location:
4. Contact information:
5. What do you work with/what is your title here?
6. What are your main responsibilities?
7. For how long have you been working here?
8. Did you have any experience in fish farming before this job?
    - Yes, what did you do before?
    - No
9. Number of employees on this site?
10. When was the business/farm/unit established?
11. Do you come here on a regular basis?
    - Daily
    - Every other day
    - Twice a week
    - Every week
12. Fish generations: Do you keep generations of fish separated or mixed?
13. What are the sources of your fish? If more than one at the same time; how do you handle them once they have arrived?
14. For how long are the ponds/cages without fish before new fish come in?
15. How large is your production per year? (tons/number of fish)
16. How long is the production cycle?
17. For how long are the fish at this site?
18. What species do you farm/grow?
19. Farming type:
    - Ponds
    - Cages
    - Hapas
    - If cages – what size:

20. Water Source:
    - River
    - Borehole
    - Lake

21. Type of water supply:
    - Continuous flow or not?
    - If not - then replenishment how often?

Fingerlings:

22. What size are the fingerlings at arrival?

23. Routines for handling fingerlings at arrival?
    - Mixed with batches from different hatcheries?
        - Yes
        - No
    - Sorting and grading process?
    - Age and size differentiation?
    - Sex differentiation?
    - Temperature and pH in water they are transferred to?
        - Temperature:
        - pH:
    - Is there a big difference from the water they arrive in (quality, temperature, particles/turbidity)?
        - Yes
        - If yes, do you think this is a problem?
        - No
    - Feeding routines
        - Monitoring of appetite
            - Yes, what do you observe?
            - No
        - What kind of feeding system do you use/how do you feed your fish?
        - Do you have automatic feeding?
            - Yes, what kind:
            - No

24. How many fingerlings do you lose in the time period immediately after transfer to nursery?

25. For how long are the fingerlings in the nursery dams (hapas)?

26. Monitoring and control of water quality and fish welfare and -health?
    - Frequency?
    - Which variables?
    - Documentation?

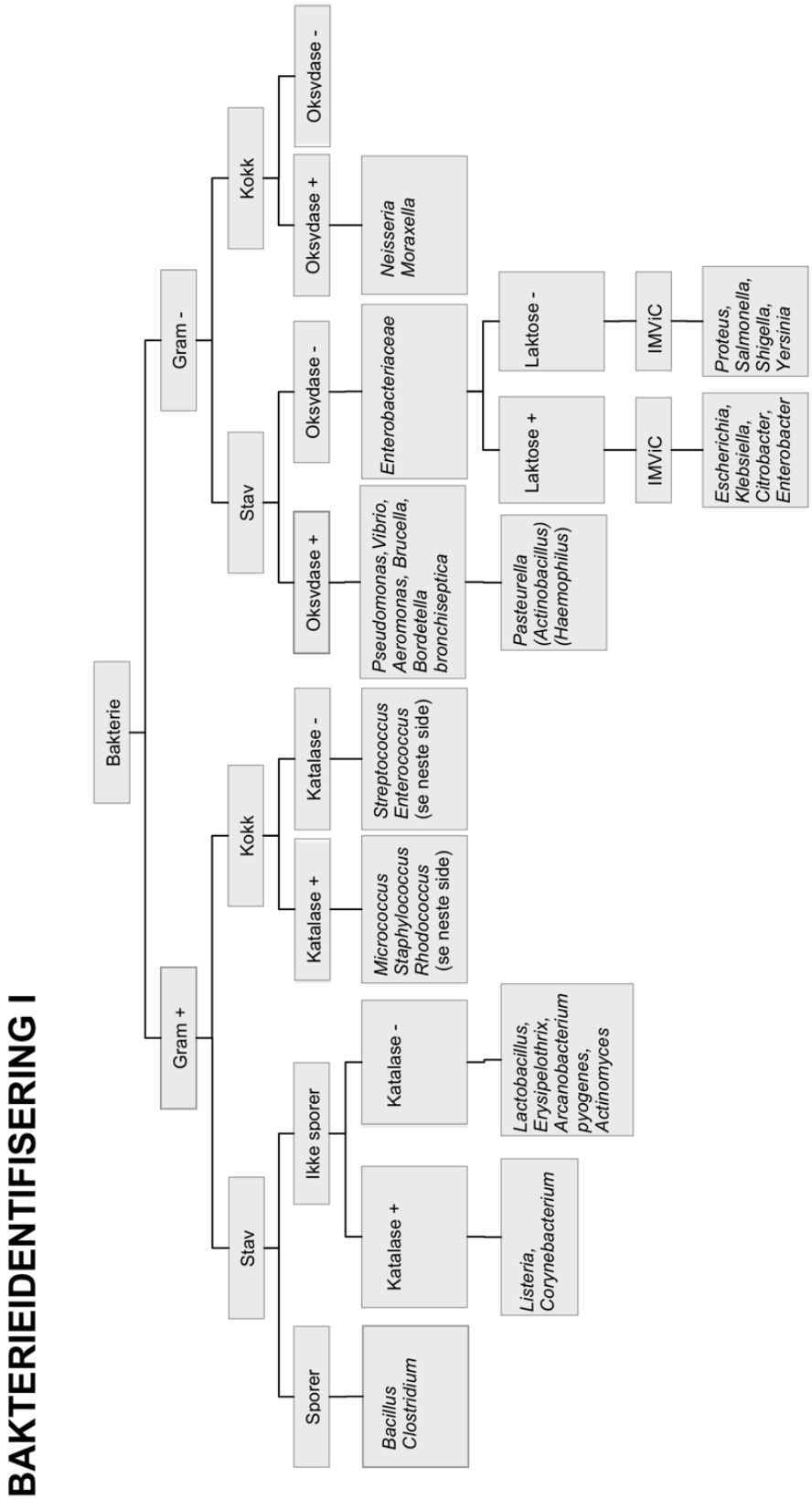
27. How do you transfer them from nursery to grow-out cages?

28. Are you experiencing any specific problems in the production?
    - What is the nature of these?
        - Infections?
        - Fungus?
    - Do they appear at certain times of the year? Or are they unrelated to season?
    - Have you seen any relation between different providers and the scale and type of problems?
    - How do you handle sick fish?
    - Do you sample from sick fish – how often?
    - Do you contact the veterinarian when you have sick fish?
    - How big are the fish when you notice disease problems?
    - Has anything been done to target these problems? If so, what?
        - Medical treatment?
        - Salinization of water?
        - Vaccines?
        - Other?

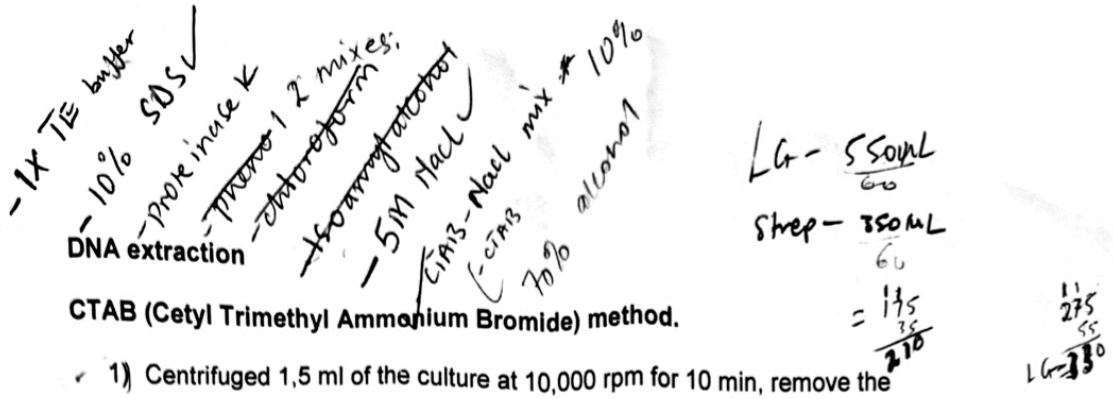
29. What are the survival percentage at this farm?
    - How are they registered?
    - How is the mortality monitored?
    - Difference between cages?
    - Difference between providers?
    - How do you handle dead fish?
    - How is it quantified?
    - Do you count dead fish daily, weekly?

30. In your opinion: what are the main challenges with tilapia production at this farm and in Zambia in general?

Appendix II. Flow chart for bacteria identification



Appendix III. DNA extraction protocol - CTAB



DNA extraction

CTAB (Cetyl Trimethyl Ammonium Bromide) method.

1) Centrifuged 1,5 ml of the culture at 10,000 rpm for 10 min, remove the supernatant and resuspend the pellet in 567 µl of 1x TE buffer, 30 µl 10% SDS and 3 µl of proteinase K.
2) Incubate the sample at 37°C for 1 hour.
3) Add 100 µl of 5 M NaCl and 80 µl of CTAB-NaCl, mix and incubate at 65°C for 10 minutes.
4) Measure volume in the eppendorf tube, and add an equal volume of Chloroform-Isoamylalcohol (24:1). Mix gently and centrifuge at 10,000 rpm for 10 minutes. After centrifuging the tubes, carefully remove the aqueous layer at the top and transfer into a fresh eppendorf tube.
5) Measure the volume and add an equal volume of Phenol-Chloroform-Isoamylalcohol (25:24:1), mix and centrifuge at 10,000 rpm for 10 minutes.
6) Remove the supernatant (aqueous phase) into a fresh eppendorf tube, and precipitate the DNA in the sample by using 0,6 times the volume in the tube of isopropanol, then centrifuge the sample at 12,000 rpm for 10 minutes to pellet the DNA.
7) After centrifuging the sample, remove the supernatant and washe the DNA pellet with 70% ethanol, mix and centrifuged at 12,000 rpm for 10 minutes.
8) Finally, remove the supernatant and dry the pellet at 37°C with the eppendorf tube open. After drying the DNA, resuspend by adding 50 µl TE buffer and measure the DNA concentration using the Nanodrop

Appendix IV. Necropsy findings

Findings from the necropsy of the fingerlings.

** Number of days from the transfer of the fingerlings from the hatcheries (Farm One and Two) to the grow-out farm (Farm Four).*

Farm	Fish number	Time of sampling*	Necropsy findings
One	1	Day 1	Ulcers on abdomen
One	2	Day 1	None
One	3	Day 1	None
One	4	Day 1	Haemorrhages on operculum
One	5	Day 1	None
One	6	Day 1	Haemorrhages on head and operculum
One	7	Day 1	Haemorrhages and ulcers on pectoral fins, caudal fin erosions, ascites, enlarged gall bladder
One	8	Day 1	None
One	9	Day 1	None
One	10	Day 1	None
One	11	Day 2	None
One	12	Day 2	None
One	13	Day 2	None
One	14	Day 2	None
One	15	Day 2	None
One	16	Day 2	None
One	17	Day 2	None
One	18	Day 2	None
One	19	Day 2	None
One	20	Day 2	None
Two	21	Day 1	None
Two	22	Day 1	None
Two	23	Day 1	None
Two	24	Day 1	None
Two	25	Day 1	Saprolegnia lesions
Two	26	Day 1	Saprolegnia lesions
Two	27	Day 1	Saprolegnia lesions
Two	28	Day 1	Saprolegnia lesions
Two	29	Day 1	Saprolegnia lesions
Two	30	Day 1	Saprolegnia lesions
Four	31	Day 4-5	Caudal fin rot
Four	32	Day 4-5	Caudal fin rot

Four	33	Day 4-5	None
Four	34	Day 4-5	None
Four	35	Day 4-5	None
Four	36	Day 4-5	Scales sloughing off
Four	37	Day 4-5	None
Four	38	Day 4-5	None
Four	39	Day 4-5	Very small fish
Four	40	Day 4-5	Caudal fin rot
Four	41	Day 6-7	Pale gills, ulcer on abdomen, scales coming off cranial to pectoral fins
Four	42	Day 6-7	Erosion on caudal fin, pale gills, pale and enlarged liver, pp haemorrhages on liver
Four	43	Day 6-7	Enlarged and pale liver
Four	44	Day 6-7	Pale gills, enlarged and pale liver
Four	45	Day 6-7	Haemorrhages on mandible, enlarged liver, pp haemorrhages on liver
Four	46	Day 6-7	Caudal fin erosion, pale gills, pale and enlarged liver
Four	47	Day 6-7	None
Four	48	Day 6-7	Caudal fin erosion, haemorrhage on ventral abdomen, pale liver
Four	49	Day 6-7	Nodule caudal to dorsal fin, pale gills, pale liver, caseous exudate around intestines
Four	50	Day 6-7	Erosion on caudal fin, pale liver

Appendix V. Results from water sampling

Results from quantitative analysis, colony counting on nutrient agar and MacConkey agar plates.

Water sample 1 (Farm One, pond).

	10^{-1}	10^{-2}	10^{-3}
Nutrient agar (Total coliform)	132	3	0
MacConkey agar (Faecal coliform)	17	0	

$$\text{CFU/ml} = \frac{\text{CFU}}{\text{volume}} \times \text{dilution factor} = \frac{132\ \text{CFU}}{0.1\ \text{ml}} \times \frac{1}{10^{-1}} = 13{,}200 \text{ total coliform CFU per ml}$$

$$\text{CFU/ml} = \frac{\text{CFU}}{\text{volume}} \times \text{dilution factor} = \frac{17\ \text{CFU}}{0.1\ \text{ml}} \times \frac{1}{10^{-1}} = 1{,}700 \text{ fecal coliform CFU per ml}$$

Water sample 2 (Farm two, transportation box). Number of colony forming units counted on the agar plates. TNTC: Too numerous to count.

	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Nutrient agar (Total coliform)	TNTC	TNTC	87	58	3
MacConkey agar (Faecal coliform)	TNTC	130	35		

$$\text{CFU/ml} = \frac{\text{CFU}}{\text{volume}} \times \text{dilution factor} = \frac{87\ \text{CFU}}{0.1\ \text{ml}} \times \frac{1}{10^{-3}} = 870{,}000 \text{ total coliform CFU per ml}$$

$$\text{CFU/ml} = \frac{\text{CFU}}{\text{volume}} \times \text{dilution factor} = \frac{130\ \text{CFU}}{0.1\ \text{ml}} \times \frac{1}{10^{-2}} = 130{,}000 \text{ fecal coliform CFU per ml}$$

Water sample 3 (Farm four, day 1-2, cage. Number of colony forming units counted on the agar plates.

	10^{-1}	10^{-2}	10^{-3}
Nutrient agar (Total coliform)	53	6	3
MacConkey agar (Faecal coliform)	2	0	

$$\text{CFU/ml} = \frac{\text{CFU}}{\text{volume}} \times \text{dilution factor} = \frac{53\ \text{CFU}}{0.1\ \text{ml}} \times \frac{1}{10^{-1}} = 5{,}300 \text{ total coliform CFU per ml}$$

$$\text{CFU/ml} = \frac{\text{CFU}}{\text{volume}} \times \text{dilution factor} = \frac{2\ \text{CFU}}{0.1\ \text{ml}} \times \frac{1}{10^{-1}} = 200 \text{ fecal coliform CFU per ml}$$

Water sample 4 (Farm four, day 4-5, cage). Number of colony forming units counted on the agar plates.

	10^{-1}	10^{-2}	10^{-3}
Nutrient agar (Total coliform)	23	3	0
MacConkey agar (Faecal coliform)	4	1	

$$\text{CFU/ml} = \frac{\text{CFU}}{\text{volume}} \times \text{dilution factor} = \frac{23\ \text{CFU}}{0.1\ \text{ml}} \times \frac{1}{10^{-1}} = 2{,}300 \text{ total coliform CFU per ml}$$

$$\text{CFU/ml} = \frac{\text{CFU}}{\text{volume}} \times \text{dilution factor} = \frac{4\ \text{CFU}}{0.1\ \text{ml}} \times \frac{1}{10^{-1}} = 400 \text{ fecal coliform CFU per ml}$$

Water sample 5 (Farm four, day 6-7, cage) Number of colony forming units counted on the agar plates.

	10^{-1}	10^{-2}	10^{-3}
Nutrient agar (Total coliform)	192	23	2
MacConkey agar (Faecal coliform)	26	4	

$$\text{CFU/ml} = \frac{\text{CFU}}{\text{volume}} \times \text{dilution factor} = \frac{192\ \text{CFU}}{0.1\ \text{ml}} \times \frac{1}{10^{-1}} = 19{,}200\ \text{total coliform CFU per ml}$$

$$\text{CFU/ml} = \frac{\text{CFU}}{\text{volume}} \times \text{dilution factor} = \frac{26\ \text{CFU}}{0.1\ \text{ml}} \times \frac{1}{10^{-1}} = 2{,}600\ \text{fecal coliform CFU per ml}$$

Appendix VI. Bacterial grouping

Table 5: grouping of the samples. Both fingerling samples and water samples are included. The grouping is based on morphology of the colonies grown on nutrient agar plates. The underlined samples (54 samples) indicates which samples that were chosen for further testing

Group Number	**Colony Morphology on Nutrient Agar Plate**	**Samples**	**Metodekompendiet-findings**
Group 1	Creamy, shiny, round, 2-5 mm	1a-1, 1b, 5a, 35a, 13a-1, 14b, 21b, 23c, 23d, 26b, 33b, 36c, 41a, 41b, 48a	*Pseudomonas, Aeromonas, Vibrio, Brucella, Pasteurella, Actinobacillus, Bordetella, Haemophilus, Bacillus, Listeria, Corynebacterium*
Group 2	Creamy, shiny, round, 1-2 mm	2d-2, 3b, 11b, 12a, 15a, 16b, 17a, 17d, 19c, 20a, 22a, 24b, 24c-2, 25e, 26a, 26c, 27b, 28a, 28b, 29a, 30a, 36a, 36b, 37a, 39a, 42a, 43a, W2b, W2d, W3d, W3e, W4e, W5b, W5a-2	*Pseudomonas, Aeromonas, Vibrio, Brucella, Bordetella, Pasteurella, Actinobacillus, Haemophilus, Neisseria, Moraxella, Proteus, Salmonella, Shigella, Yersinia,*
Group 3	➔ 8		
Group 4	Transparent, round, 2-4 mm	W4a	*Micrococcus, Staphylococcus, Rhodococcus*
Group 5	➔ 15		
Group 6	Water-like. Slightly creamy	32a, 32c, 36a-2, 50a	*Bacillus, Listeria, Corynebacterium, Micrococcus, Staphylococcus, Rhodococcus*
Group 7	Transparent edges with denser centre (creamy), swarming, not possible to see single colonies	3d, 3e-1, 19d, 20b	*Pseudomonas, Aeromonas, Vibrio, Brucella, Pasteurella, Bordetella, Actinobacillus, Haemophilus*

Group 8	Yellow/orange, round, shiny, 1-3 mm	1c, 2a, 3a, 3c, 4a-1, 9a, 9b, 11a-1, 14b-2, 18a, 18c-2, 19a, 21a, 23a, 24a, 25b, 25d, 27a, 28c, 30b, 30d, 34a, 35c, 38a, 40a, W2a	Enterobacteriaceae*, Bacillus, Listeria, Corynebacterium, Micrococcus, Staphylococcus, Rhodococcus, Lactobacillus, Erysipelothrix, Trueperella, Actinomyces*
Group 9	Transparent edges with denser centre (creamy), swarming, but still possible to see single colonies	20d, 25c, W2c, W4c, W5a-1	*Pseudomonas, Aeromonas, Vibrio, Brucella, Bordetella, Pasteurella, Actinobacillus, Haemophilus, Moraxella, Neisseria,* Enterobacteriaceae*, Listeria, Corynebacterium, Micrococcus, Staphylococcus, Rhodococcus,*
Group 10	Red, shiny, round, 1-2 mm	4c, 9c, 10b	*Micrococcus, Staphylococcus, Rhodococcus, Bacillus, Listeria, Corynebacterium*
Group 11	Yellow, dry, 1-3 mm	27c	*Micrococcus, Staphylococcus, Rhodococcus*
Group 12	Bright yellow, shiny, round, 1-2 mm	1d, 6a, 6b, 6c, 7a, 10a, 13a-2, 14c, 17c, 24c-1, 25a, 34c-1, 34b	*Micrococcus, Staphylococcus, Rhodococcus*
Group 13	Creamy, dull, 5-8 mm	2c, 22b, W4b	*Bacillus, Listeria, Corynebacterium, Micrococcus, Staphylococcus, Rhodococcus*
Group 14	Creamy/white, round, 1-2 mm, concave	2b, 2d-1, 4d, 15f, 23b	*Bacillus, Listeria, Corynebacterium, Micrococcus, Staphylococcus, Rhodococcus*
Group 15	Creamy/white pinpoint-1 mm, shiny	1a-2, 13b, 14d, 15e, 16c, 18c-1, 40a-2, 45a, 46b, W4d	*Pseudomonas, Aeromonas, Vibrio, Brucella, Pasteurella, Actinobacillus, Bordetella, Haemophilus, Bacillus, Listeria, Corynebacterium,*

			Micrococcus, Staphylococcus, Rhodococcus, Streptococcus, Enterococcus, Neisseria, Moraxella
Group 16	Creamy/transparent edges, convex centre	20c, 35b	*Bacillus, Listeria, Corynebacterium, Micrococcus, Staphylococcus, Rhodococcus*
Group 17	Fungi. No further testing was done as the main focus of this thesis is on bacteria	3e-2, 5d, 12b, 13d, 15b-2, 15d, 27a, 38c	
Group 18	Transparent (/white), pinpoint-1mm, round	4b, 11a-2, 32b, 33a, 40b, 46a, 47a, W5c,	*Pseudomonas, Aeromonas, Vibrio, Brucella, Pasteurella, Actinobacillus, Bordetella, Haemophilus, Neisseria, Moraxella, Escherichia, Klebsiella, Citrobacter, Enterobacter*
Group 19	White edge, white centre, transparent between centre and edge	34c-2, 38b	Enterobacteriaceae
Group 20	Creamy/transparent, rough appearance	12c, 13c, 14a, 18b, 22c, 44a, 29b, W3c	*Escherichia, Klebsiella, Citrobacter, Enterobacter, Pseudomonas, Aeromonas, Vibrio, Brucella, Bordetella, Pasteurella, Actinobacillus, Haemophilus, Neisseria, Moraxella*
Group 21	White pinpoint	8a	*Micrococcus, Staphylococcus, Rhodococcus*
Group 22	Creamy, oval, shiny 1-4 mm	45b	*Micrococcus, Staphylococcus, Rhodococcus*

Appendix VII. Bacteria analysis of isolates

Isolate	Colony Morphology	Group	Gram staining	Shape	Hemolysis	Oxydase	Katalase	Lactose fermentation	Colony morphology on MacConkey agar	Bevegelighet halvflytende
Pseudomonas aeruginosas			-	rods	γ	+	+	-	Transparent colonies, flat, shiny. Yellow agar	+
41a	Creamy, shiny, round, 2-5 mm	1	-	rods	β	+	+	-	Yellow/transparent colonies, slightly convex, shiny, 0,2-0,5 mm. Yellow agar	+
16c	Creamy/white pinpoint-1 mm, shiny	15	-	rods	γ	+	+	-	Yellow/transparent colonies, pp-0,2 mm, slightly shiny. Yellow agar	?
W4-d	Creamy/white pinpoint-1 mm, shiny	15	-	?	γ	+	+	-	Transparent(slightly pink) colonies, Convex, shiny, 0,5 mm. Yellow agar. Color change (yellow) thougout the whole agar	+
33a	Transparent (/white), pinpoint-1mm, round	18	-	?	γ	+ (weak)	+	NG		+
W5-c	Transparent (/white), pinpoint-1mm, round	18	-	?	γ	-	+	+	Pink colonies, very shiny, pp-0,5 mm, utflytende, convex. Pink agar	+
11a-2	Transparent (/white), pinpoint-1mm, round	18	-	coccus	γ	-	+	-	Yellow/transparent colonies, pp, medium opak, tett vekst, flat.Yellow agar	+
38b	White edges, white center, transparent between (som en blink)	19	-	rods	γ	-	+	NG		+
W5-b	Creamy, shiny, round, 1-2 mm	2	-	?	β	+	+	-	Pink/purple/yellow colonies, 0,2-0,5 mm, convex, shiny. Yellow agar	?
W4-e	Creamy, shiny, round, 1-2 mm	2	-	?	β	+	+	-	Yellow/transparent colonies, slightly convex,shiny, 0,4-1 mm. Yellow agar	+
19c	Creamy, shiny, round, 1-2 mm	2	-	?	γ	-	+	-	Yellow/white colonies, pp, flat. Yellow agar	?
W3-e	Creamy, shiny, round, 1-2 mm	2	-		α/β	+	+	-	Creamy/slightly pink colonies, slightly transparent, convex, shiny but a bit dry, 0,2-0,5 mm. Yellow agar	+
17a	Creamy, shiny, round, 1-2 mm	2	-		γ	-	+	-	Sligthly pink/transparent colonies, 0,2-0,5 mm, shiny. Yellow agar surrounding first streak	+
W3-d	Creamy, shiny, round, 1-2 mm	2	-	rods	α	+	+	-	Slightly pink/transparent colonies, shiny, convex, 0,7 mm. Yellow agar	?
28a	Creamy, shiny, round, 1-2 mm	2	-	?	β	+	-	-	Yellow/ transparent colonies, pp-0,2 mm, slightly shiny. Yellow agar	+
27b	Creamy, shiny, round, 1-2 mm	2	-	rods	β	+	+	-	Yellow/creamy colonies, pp, shiny, convex, slightly dry, slightly opak. Yellow agar	+

Isolate	Colony Morphology	Group	Gram staining	Shape	Hemolysis	Oxydase	Katalase	Lactose fermentation	Colony morphology on MacConkey agar	Bevegelighet halvflytende
W5-a2	Creamy, shiny, round, 1-2 mm	2	-	rods	β	+	+	-	Yellow/transparent colonies, pp-utflytende, convex. Yellow agar	+
W2-d	Creamy, shiny, round, 1-2 mm	2	-	rods	γ	-	+	-	Pink/transparent colonies, 0,5 mm, shiny, convex. Yellow agar	-
W3-c	Creamy/transparent, kladdete	20	-	?	α	-	+	+	Pink colonies, 0,5-07 mm, shiny, convex. Pink agar	-
22c	Creamy/transparent, kladdete	20	-	?	γ	+	+	-	Yellow/slightly pink colonies, 0,7mm. Yellow agar	+
13c	Creamy/transparent, kladdete	20	-	coccus	γ	-	-	-	Slightly pink centered, yellow/transparent edged colonies, 1-1,5 mm, convex, shiny. Yellow agar	?
3d	Transparent edges with denser center (creamy), utflytende, not possible to see single colonies	7	-	rods	β and α?	+	+	-	Creamy colonies, 0,5-1 mm, shiny, medium opak, convex. Yellow agar	?
19d	Transparent edges with denser center (creamy), utflytende, not possible to see single colonies	7	-	rods	β and α?	+	+	-	Yellow colonies, 1 mm, shiny, convex. Yellow agar	?
W2-a	Yellow/orange, round, shiny, 1-3 mm	8	-	?	γ	-	+	NG		?
W2-c	Transparent edges with denser center (creamy), utflytende, but possible to see single colonies	9	-	?	α?	+	-	-	Slightly pink/transparent colonies, 2mm, shiny, convex. Yellow agar	?
20d	Transparent edges with denser center (creamy), utflytende, but possible to see single colonies	9	-	rods	α	-	+	NG		?
HBiB + Gly 2-1a			+	c	γ	-	+	-	Slightly pink/transparent colonies, pp, shiny, convex. Yellow agar	+
Bio1 s. Agalact			+	c	β	-	-	NG		-

Isolate	Colony Morphology	Group	Gram staining	Shape	Hemolysis	Oxydase	Katalase	Lactose fermentation	Colony morphology on MacConkey agar	Bevegelighet halvflytende
Bio2 s. Agalact			+	c	α	-	-	NG		-
36c	Creamy, shiny, round, 2-5 mm	1	+	rods	γ	-	+	NG		-
10b	Red, shiny, round, 1-2 mm	10	+	coccus	γ	-	+	NG		-
4c	Red, shiny, round, 1-2 mm	10	+	rods	γ	-	+	NG		-
27c	Yellow, dry, 1-3 mm	11	+	coccus	γ	+	+	-	Yellow colonies, 1 mm, dull, uneven surface and edges, "tree rings" in single colonies, opak. Yellow agar. Color change (yellow) thougout the whole agar	?
10a	Bright yellow, shiny, round, 1-2 mm	12	+	coccus	γ	-	+	NG		-
13a-2	Bright yellow, shiny, round, 1-2 mm	12	+	coccus	γ	-	+	NG		-
25a	Bright yellow, shiny, round, 1-2 mm	12	+	coccus	α?	-	+	NG		-
34b	Bright yellow, shiny, round, 1-2 mm	12	+	coccus	γ	-	+	NG		-
W4-b	Creamy, dull, 5-8 mm	13	+	?	β	-	+	NG		+
2c	Creamy, dull, 5-8 mm	13	+	?	γ	-	+	NG		?
23b	Creamy/white, round, 1-2 mm, concave	14	+	rods	γ	-	+	NG		-
2d-1	Creamy/white, round, 1-2 mm, concave	14	+	?	β and α?	-	+	NG		?
46b	Creamy/white pinpoint-1 mm, shiny	15	+	?	γ	-	+	-	Yellow/slightly pink colonies, 0,5 mm, shiny, convex. Yellow agar	+
20c	Creamy/transparent edges, convex center (speilegg)	16	+	?	γ	-	+	NG		?
8a	White pinpoint	21	+	coccus	α	-	+	NG		
45b	Creamy, oval, shiny 1-4 mm	22	+	coccus	γ	-	+	NG		-
W4-a	Transparent, round, 2-4 mm	4	+	coccus	α	-	+	NG		-

Isolate	Colony Morphology	Group	Gram staining	Shape	Hemolysis	Oxydase	Katalase	Lactose fermentation	Colony morphology on MacConkey agar	Bevegelighet halvflytende
50a	Water like + touch of creamy	6	+	?	β	-	+	NG		?
32c	Water like + touch of creamy	6	+	coccus	β	-	+	NG		-
38a	Yellow/orange, round, shiny, 1-3 mm	8	+	rods	α	-	+	NG		-
27a	Yellow/orange, round, shiny, 1-3 mm	8	+	?	γ	-	+	NG		+
23a	Yellow/orange, round, shiny, 1-3 mm	8	+	coccus	γ	-	+	NG		?
34a	Yellow/orange, round, shiny, 1-3 mm	8	+	coccus	γ	-	+	NG		-
9a	Yellow/orange, round, shiny, 1-3 mm	8	+	coccus	γ	-	+	NG		+
18c-2	Yellow/orange, round, shiny, 1-3 mm	8	+	coccus	γ	-	+	-	Slightly pink colonies, 2 mm, concave. Yellow agar surrounding first streak	+
40a-2	Creamy/white pinpoint-1 mm, shiny	15	?	coccus	α	+	-	NG		?
1c	Yellow/orange, round, shiny, 1-3 mm	8	?	rods	α	-	-	NG		?
2a	Yellow/orange, round, shiny, 1-3 mm	8	?	rods	α	-	-	NG		-
W4-c	Transparent edges with denser center (creamy), utflytende, but possible to see single colonies	9	?	?	β and α?	+	+	-	Yellow/creamy/ slightly transparant colonies , 0,3-0,5 mm, shiny, convex. Yellow agar	+

Appendix VIII. Distribution of bacteria in the different samples

Farm	Sample	Bacteria genera/family
Fish from Farm 1, before transport	Fish 1	*Actinobacillus, Actinomyces, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium,* Enterobacteriaceae*, Enterococcus, Erysipelothrix, Haemophilus, Lactobacillus, Listeria, Micrococcus, Moraxella, Neisseria, Pasteurella, Pseudomonas, Rhodococcus, Staphylococcus, Streptococcus, Trueperella, Vibrio*
	Fish 2	*Actinobacillus, Actinomyces, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium,* Enterobacteriaceae*, Erysipelothrix, Haemophilus, Lactobacillus, Listeria, Micrococcus, Moraxella, Neisseria, Pasteurella, Proteus, Pseudomonas, Rhodococcus, Trueperella, Salmonella, Shigella, Staphylococcus, Vibrio, Yersinia*
	Fish 3	*Actinobacillus, Actinomyces, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium,* Enterobacteriaceae*, Erysipelothrix, Haemophilus, Lactobacillus, Listeria, Micrococcus, Moraxella, Neisseria, Pasteurella, Proteus, Pseudomonas, Rhodococcus, Salmonella, Shigella, Staphylococcus, Trueperella, Vibrio, Yersinia*
	Fish 4	*Actinobacillus, Actinomyces, Aeromonas, Bacillus, Bordetella, Brucella, Citrobacter, Corynebacterium, Enterobacter,* Enterobacteriaceae*, Erysipelothrix, Escherichia, Haemophilus, Klebsiella, Lactobacillus, Listeria, Micrococcus, Moraxella, Neisseria, Pasteurella, Pseudomonas, Rhodococcus, Staphylococcus, Trueperella, Vibrio*
	Fish 5	*Actinobacillus, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium, Haemophilus, Listeria, Pasteurella, Pseudomonas, Vibrio*

	Fish 6	*Micrococcus, Rhodococcus, Staphylococcus*
	Fish 7	*Micrococcus, Rhodococcus, Staphylococcus*
	Fish 8	*Micrococcus, Rhodococcus, Staphylococcus*
	Fish 9	*Actinomyces, Bacillus, Corynebacterium,* Enterobacteriaceae*, Erysipelothrix, Lactobacillus, Listeria, Micrococcus, Rhodococcus, Staphylococcus, Trueperella*
	Fish 10	*Micrococcus, Rhodococcus, Staphylococcus*
Fish from Farm 1, day one after transfer to Farm 4	Fish 11	*Actinobacillus, Actinomyces, Aeromonas, Bacillus, Bordetella, Brucella,* Enterobacteriaceae*, Erysipelothrix, Listeria, Corynebacterium, Lactobacillus, Haemophilus, Micrococcus, Moraxella, Neisseria, Pasteurella, Proteus Pseudomonas, Staphylococcus, Rhodococcus, Salmonella, Shigella, Trueperella, Vibrio, Yersinia*
	Fish 12	*Actinobacillus, Aeromonas, Pseudomonas, Brucella, Bordetella, Citrobacter, Enterobacter, Escherichia, Haemophilus, Klebsiella, Moraxella, Neisseria, Pasteurella, Proteus, Salmonella, Shigella, Vibrio, Yersinia*
	Fish 13	*Actinobacillus, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium, Enterococcus, Haemophilus, Listeria, Micrococcus, Moraxella, Neisseria, Pasteurella, Pseudomonas, Rhodococcus, Staphylococcus, Streptococcus, Vibrio*
	Fish 14	*Actinobacillus, Actinomyces, Aeromonas, Bacillus, Bordetella, Brucella, Citrobacter, Corynebacterium, Enterobacter Erysipelothrix, Escherichia, Haemophilus, Klebsiella, Lactobacillus, Listeria, Micrococcus, Moraxella,*

		Neisseria, Pasteurella, Pseudomonas, Rhodococcus, Staphylococcus, Trueperella, Vibrio
	Fish 15	*Actinobacillus, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium, Enterococcus, Haemophilus, Listeria, Micrococcus, Moraxella, Neisseria, Pasteurella, Proteus, Pseudomonas, Rhodococcus, Salmonella, Shigella, Staphylococcus, Streptococcus, Vibrio, Yersinia*
	Fish 16	*Actinobacillus, Aeromonas, Bordetella, Brucella, Haemophilus, Moraxella, Neisseria, Pasteurella, Proteus, Pseudomonas, Salmonella, Shigella, Vibrio, Yersinia*
	Fish 17	*Actinobacillus, Aeromonas, Bordetella, Brucella, Haemophilus, Micrococcus, Moraxella, Neisseria, Pasteurella, Proteus, Pseudomonas, Rhodococcus, Salmonella, Shigella, Staphylococcus, Vibrio, Yersinia*
	Fish 18	*Actinobacillus, Actinomyces, Aeromonas, Bacillus, Bordetella, Brucella, Citrobacter, Corynebacterium, Enterobacter,* Enterobacteriaceae*, Erysipelothrix, Escherichia, Haemophilus, Klebsiella, Lactobacillus, Listeria, Micrococcus, Moraxella Neisseria, Pasteurella, Pseudomonas, Rhodococcus, Staphylococcus, Trueperella, Vibrio*
	Fish 19	*Actinobacillus, Actinomyces, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium,* Enterobacteriaceae*, Erysipelothrix, Haemophilus Lactobacillus, Listeria, Micrococcus, Pasteurella, Proteus, Pseudomonas, Rhodococcus, Trueperella, Salmonella, Shigella, Staphylococcus, Vibrio, Yersinia*
	Fish 20	*Actinobacillus, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium,* Enterobacteriaceae*, Haemophilus, Listeria, Micrococcus, Moraxella, Neisseria, Pasteurella, Pseudomonas, Proteus, Rhodococcus, Salmonella, Shigella, Staphylococcus, Vibrio, Yersinia*

Water from Farm 4, day one after transfer of fish from Farm 1	Water sample 3	*Actinobacillus, Aeromonas, Bordetella, Brucella, Citrobacter, Enterobacter, Escherichia, Haemophilus, Klebsiella, Moraxella, Neisseria, Pasteurella, Pseudomonas, Vibrio*
Fish from Farm 2, taken from transportation box at the end of the transport	Fish 21	*Actinobacillus, Actinomyces, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium,* Enterobacteriaceae*, Erysipelothrix, Haemophilus, Lactobacillus, Listeria, Micrococcus, Pasteurella, Rhodococcus, Trueperella, Staphylococcus, Vibrio*
	Fish 22	*Actinobacillus, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium, Haemophilus, Listeria, Micrococcus, Moraxella, Neisseria, Pasteurella, Proteus, Pseudomonas, Rhodococcus, Salmonella, Shigella, Staphylococcus, Vibrio, Yersinia*
	Fish 23	*Actinobacillus, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium, Haemophilus, Listeria, Micrococcus, Pasteurella, Pseudomonas, Rhodococcus, Staphylococcus, Vibrio*
	Fish 24	*Actinobacillus, Actinomyces, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium,* Enterobacteriaceae*, Erysipelothrix, Haemophilus, Lactobacillus, Listeria, Micrococcus, Moraxella, Neisseria, Pasteurella, Proteus, Pseudomonas, Rhodococcus, Salmonella, Shigella, Staphylococcus, Trueperella, Vibrio, Yersinia*
	Fish 25	*Actinomyces, Aeromonas, Actinobacillus, Bacillus, Bordetella, Brucella, Corynebacterium,* Enterobacteriaceae*, Erysipelothrix, Haemophilus, Lactobacillus, Listeria, Micrococcus, Moraxella, Neisseria, Pasteurella, Proteus, Pseudomonas, Rhodococcus, Salmonella, Shigella, Staphylococcus, Trueperella, Vibrio, Yersinia*

	Fish 26	*Actinobacillus, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium, Haemophilus, Listeria, Moraxella, Neisseria, Pasteurella, Proteus, Pseudomonas, Salmonella, Shigella, Vibrio, Yersinia*
	Fish 27	*Actinobacillus, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium, Haemophilus, Listeria, Micrococcus, Pasteurella, Pseudomonas, Rhodococcus, Staphylococcus, Vibrio*
	Fish 28	*Actinobacillus, Actinomyces, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium,* Enterobacteriaceae*, Erysipelothrix, Haemophilus, Listeria, Lactobacillus, Micrococcus, Moraxella, Neisseria, Pasteurella, Proteus, Pseudomonas, Rhodococcus, Salmonella, Shigella, Staphylococcus, Trueperella, Vibrio, Yersinia*
	Fish 29	*Aeromonas, Actinobacillus, Bordetella, Brucella, Citrobacter, Enterobacter Escherichia, Haemophilus, Klebsiella, Moraxella, Neisseria, Pasteurella, Proteus, Pseudomonas, Salmonella, Shigella, Vibrio, Yersinia*
	Fish 30	*Actinobacillus, Actinomyces, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium,* Enterobacteriaceae*, Erysipelothrix, Haemophilus, Lactobacillus, Listeria, Micrococcus, Moraxella, Neisseria, Pasteurella, Proteus, Pseudomonas, Rhodococcus, Salmonella, Shigella, Staphylococcus, Trueperella, Vibrio, Yersinia*
Water from Farm 2, taken from transportation box at the end of the transport	Water sample 2	*Actinobacillus, Aeromonas, Bordetella, Brucella,* Enterobacteriaceae*, Haemophilus, Moraxella, Neisseria, Pasteurella, Pseudomonas, Proteus, Salmonella, Shigella, Vibrio, Yersinia*
Fish from farm 1 and 2, day four and five after	Fish 32	*Actinobacillus, Aeromonas, Bacillus, Bordetella, Brucella, Citrobacter, Corynebacterium, Enterobacter, Escherichia, Haemophilus, Klebsiella, Listeria, Micrococcus, Moraxella, Neisseria, Pasteurella, Pseudomonas, Rhodococcus, Staphylococcus, Vibrio*

transfer to Farm 4	Fish 33	*Actinobacillus, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium, Haemophilus, Listeria, Moraxella, Neisseria, Pasteurella, Pseudomonas, Vibrio*
	Fish 34	Enterobacteriaceae*, Micrococcus, Rhodococcus, Staphylococcus*
	Fish 35	*Actinobacillus, Actinomyces, Aeromonas, Bacillus, Brucella, Bordetella, Corynebacterium,* Enterobacteriaceae*, Erysipelothrix, Haemophilus, Lactobacillus, Listeria, Micrococcus, Pasteurella, Pseudomonas, Rhodococcus, Staphylococcus, Trueperella, Vibrio*
	Fish 36	*Actinobacillus, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium, Haemophilus, Listeria, Neisseria, Micrococcus, Moraxella, Pasteurella, Proteus, Pseudomonas, Rhodococcus, Salmonella, Shigella, Staphylococcus, Vibrio, Yersinia*
	Fish 37	*Actinobacillus, Aeromonas, Bordetella, Brucella, Haemophilus, Moraxella, Neisseria, Pasteurella, Proteus, Pseudomonas, Salmonella, Shigella, Vibrio, Yersinia*
	Fish 38	*Bacillus, Corynebacterium,* Enterobacteriaceae*, Listeria*
	Fish 39	*Actinobacillus, Aeromonas, Bordetella, Brucella, Haemophilus, Moraxella, Neisseria, Pasteurella, Proteus, Pseudomonas, Salmonella, Shigella, Vibrio, Yersinia*
	Fish 40	*Actinobacillus, Actinomyces, Aeromonas, Bacillus, Bordetella, Brucella, Citrobacter, Corynebacterium, Enterobacter,* Enterobacteriaceae*, Enterococcus, Erysipelothrix, Escherichia, Haemophilus, Klebsiella, Lactobacillus, Listeria, Micrococcus, Moraxella, Neisseria, Pasteurella, Pseudomonas, Rhodococcus, Staphylococcus, Streptococcus, Trueperella, Vibrio*
Water from Farm 4, day four and five after transfer	Water sample 4	*Actinobacillus, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium,* Enterobacteriaceae*, Haemophilus, Listeria, Micrococcus, Moraxella, Neisseria, Pasteurella, Pseudomonas, Rhodococcus, Staphylococcus, Vibrio*

Fish from Farm 1 and 2, day six and seven after transfer to Farm 4	Fish 41	*Actinobacillus, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium, Haemophilus, Listeria, Pasteurella, Pseudomonas, Vibrio*
	Fish 42	*Actinobacillus, Aeromonas, Bordetella, Brucella, Haemophilus, Moraxella, Neisseria, Pasteurella, Proteus, Pseudomonas, Salmonella, Shigella, Vibrio, Yersinia*
	Fish 43	*Actinobacillus, Aeromonas, Bordetella, Brucella, Haemophilus, Moraxella, Neisseria, Pasteurella, Proteus, Pseudomonas, Salmonella, Shigella, Vibrio, Yersinia*
	Fish 44	*Actinobacillus, Aeromonas, Bordetella, Brucella, Citrobacter, Enterobacter, Escherichia, Haemophilus, Klebsiella, Moraxella, Neisseria, Pasteurella, Pseudomonas, Vibrio*
	Fish 45	*Actinobacillus, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium, Enterococcus, Haemophilus, Listeria, Micrococcus, Moraxella, Neisseria, Pasteurella, Pseudomonas, Rhodococcus, Staphylococcus, Streptococcus, Vibrio*
	Fish 46	*Actinobacillus, Aeromonas, Bordetella, Bacillus, Brucella, Citrobacter, Corynebacterium, Enterobacter, Escherichia, Haemophilus, Klebsiella, Listeria, Micrococcus, Moraxella, Neisseria, Pasteurella, Pseudomonas, Rhodococcus, Staphylococcus, Vibrio*
	Fish 47	*Actinobacillus, Aeromonas, Bordetella, Brucella, Citrobacter, Enterobacter, Escherichia, Haemophilus, Klebsiella, Moraxella, Neisseria, Pasteurella, Pseudomonas, Vibrio*
	Fish 48	*Actinobacillus, Aeromonas, Bacillus, Bordetella, Brucella, Corynebacterium, Haemophilus, Listeria, Pasteurella, Pseudomonas, Vibrio*
	Fish 50	*Bacillus, Corynebacterium, Listeria, Micrococcus, Rhodococcus, Staphylococcus*

Water from Farm 4, day six and seven after transfer	Water sample 5	*Actinobacillus, Aeromonas, Bordetella, Brucella, Citrobacter, Corynebacterium, Enterobacter,* Enterobacteriaceae*, Escherichia, Haemophilus, Klebsiella, Listeria, Micrococcus, Moraxella, Neisseria, Pasteurella, Pseudomonas, Rhodococcus, Staphylococcus, Vibrio*

Appendix IX. Distribution of bacteria in the different sampling rounds

	Fish from farm1			Fish from Farm 2		Mixed fish (Farm 4)			
Bacteria	**Sampling I**	**Sampling II**	**Water sample 3**	**Sampling III**	**Water sample 2**	**Sampling IV**	**Water sample 4**	**Sampling V**	**Water sample 5**
Actinobacillus	5	10	1	10	1	7	1	8	1
Actinomyces	5	4	0	5	0	2	0	0	0
Aeromonas	5	10	1	10	1	7	1	8	1
Bacillus	6	7	0	9	0	6	1	5	0
Bordetella bronchiseptica	5	10	1	10	1	7	1	8	1
Brucella	5	10	1	10	1	7	1	8	1
Citrobacter	1	3	1	1	0	2	0	3	1
Coryonebacterium	6	7	0	9	0	6	1	5	1
Enterobacter	1	3	1	1	0	2	0	3	1
Enterobacteriaceae	5	4	0	5	1	4	1	0	1
Enterococcus	1	2	0	0	0	1	0	1	0
Erysipelothrix	5	4	0	5	0	2	0	0	0
Haemophilus	5	10	1	10	1	7	1	8	1
Klebsiella	1	3	1	1	0	2	0	3	1
Lactobacillus	5	4	0	5	0	2	0	0	0
Listeria	6	7	0	9	0	6	1	5	1
Micrococcus	9	8	0	8	0	5	1	3	1
Moraxella	4	9	1	7	1	6	1	6	1
Neisseria	4	9	1	7	1	6	1	6	1
Pasteurella	5	10	1	10	1	7	1	8	1
Proteus	2	7	0	7	1	3	0	2	0
Pseudomonas	5	10	1	9	1	7	1	8	1
Rhodococcus,	9	8	0	8	0	5	1	3	1
Salmonella	2	7	0	7	1	3	0	2	0
Shigella	2	7	0	7	1	3	0	2	0
Staphylococcus	9	8	0	8	0	5	1	3	1
Streptococcus	1	2	0	0	0	1	0	1	0
Trueperella pyogenes	5	4	0	5	0	2	0	0	0
Vibrio	5	10	1	10	1	7	1	8	1
Yersinia	2	7	0	7	1	3	0	2	0

Appendix X. DNA extraction results

Results from the DNA extraction done by using the CTAB protocol. 260/280 is a measurement of the purity of the sample.

Extraction number	Sample number	260/280	ng/µL	Mean concentration ng/µl	CV (%)
1	27b	2,432 2,491	887,744 897,33	892,537	0,759
2	16c	2,183 2,161	178,507 161,373	169,94	7,129
3	3d	2,166 2,201	549,816 542,682	546,249	0,923
4	W5b	2,162 2,157	430,265 436,325	433,295	0,989
5	41a	2,212 2,208	896,525 855,406	875,966	3,319
6	17a	2,175 2,165	106,891 103,279	105,085	2,431
7	W5c	2,224 2,222	587,65 594,469	591,06	0,816
8	W2c	2,201 2,179	507,626 519,13	513,378	1,585
9	27c	2,245 2,21	304,905 314,509	309,707	2,193
10	23a	2,154 2,153	270,741 274,544	272,643	0,986
11	Bio2	1,463	28,7	28,7	8,834
12	27a	2,179 2,168	251,604 249,222	250,413	0,672
13	4c	2,043 2,099	51,796 51,524	51,66	0,372
14	10b	1,862 1,595	21,097 7,97	14,533	63,872
15	19d	2,157 2,198	303,485 307,325	305,405	0,889
16	34b	1,385 1,159	5,844 8,913	7,379	29,416
17	33a	2,179 2,155	105,675 106,884	106,28	0,804
18	13c	2,134 2,165	83,931 84,284	84,107	0,296
19	1c	1,464 1,501	58,983 63,498	61,24	5,213
20	50a	1,519 1,498	11,592 9,59	10,591	13,361
46b	46b	1,731 1,737	297,7 297,656	297,678	0,01

Norges miljø- og
biovitenskapelige
universitet

Postboks 5003
NO-1432 Ås
67 23 00 00
www.nmbu.no