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Effect of salinity and feed sterilization in interactions between gut and water microbial communities in Nile tilapia (*Oreochromis niloticus*) larvae

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MSc thesis, Wageningen University, The Netherlands

To my wonderful family,

To my niece, Matilde

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Table XIV- Calculated means of Pearson correlation similarities, between system water and feed overtime in the different treatments: salinity (fresh and salt water) and feed sterilization (non-sterile and sterile feed). Each mean represents the average of water system similarity with feed in the different treatments per different sampling days.

Abstract

Some tilapia species, including *Oreochromis niloticus*, are good candidates for brackish water culture due to salinity tolerance and good growth performances. In this report, the effect of salinity on the bacterial dominance in fish gut, as well as their relation with water and feed microbial communities, were tested. The effect of a regular diet versus a sterile diet on the microbial community composition in the fish gut was also evaluated. This resulted in 4 treatments, randomly assigned to 12 aquaria active suspension systems. The experiment was had the duration of 42 days, from the moment of first feeding. The effects of these two factors (salinity (fresh water and salt water) and feed sterilization (non-sterile feed and sterile feed)) on the microbial composition in water and fish guts were evaluated in a 2x2 factorial design. Ten fish guts per aquaria and 1 water sample per aquarium were sampled on days 0, 7, 14, 28 and 42. Bacterial DNA was extracted and amplified by Polymerase Chain Reaction. Bacterial DNA profiles were obtained through Denaturing Gradient Gel Electrophoresis. All the samples were clustered based on band position and intensity (Pearson correlation) and the similarity values among sample profiles were obtained. The gut microbial communities were different between fishes from fresh and salt water systems on day 7 (88.2±9.52% vs 63.7±28.14%) and 28 (76.9±8.28% vs 70.2±17.58%). A significant increase of similarity between system water bacterial communities and fish gut over the different sampling days was observed. Feed also had a significant increase of similarity with fish gut over time, starting with 0.4±10.2% for fresh water and 3.5±2.94% for salt water, and ending with a significantly higher value of 21.7±11.09% for fresh water and 30.4±14.12% for salt water. On the other hand, feed sterilization presented significant differences in all sampling days between fresh and saltwater bacteria populations. However, gut microbiota from fish fed with sterile feed suffered less the effect of water and feed than the fish fed with regular feed. Clearly, this study is a starting point for the complete understanding of how microbial communities are established and what the goals of future research should be.

Keywords: *Microbial communities; Tilapia; Oreochromis niloticus; Salinity; Feed Sterilization; Active suspension systems*

Resumo

Algumas espécies de Tilápia, como *Oreochromis niloticus*, são consideradas boas candidatas à cultura em água salobra devido à sua tolerância à salinidade e às suas boas performances de crescimento. Neste relatório, foi testado o efeito da salinidade na dominância bacteriana no intestino de peixe, assim como a sua relação com as comunidades microbiológicas presentes na água e no alimento. O efeito de uma dieta livre de bactérias foi comparado ao de uma dieta regular, com objectivo de verificar a influência da esterilização do alimento nas populações bacterianas no intestino. Para atingir o objectivo principal, foi montado um conjunto de doze tanques de active suspension. A experiência teve a duração de 42 dias. Puderam ser distintos dois factores, salinidade (água doce e salgada) e esterilização de alimento (alimento não-estéril e alimento estéril). Foi amostrada água por aquário, assim como 10 intestinos de peixe por aquário em 5 pontos de amostragem distintos (dia 0, 7, 14, 28 e 42). O DNA bacteriano foi extraído e amplificado por Polymerase Chain Reaction. Os perfis de DNA bacteriano foram obtidos por Denaturing Gradient Gel Electrophoresis. Todas as amostras foram agrupadas com base na intensidade de banda (Pearson correlation) e assim os valores de similaridade entre estes perfis foram obtidos. A dominância bacteriana no intestino de peixe de água doce e salgada é significativamente diferente no ponto de amostragem 7 (88.2±9.52% vs 63.7±28.14%) e 28 (76.9±8.28% vs 70.2±17.58%). Observou-se um aumento significativo de similaridade entre as comunidades bacterianas da água do sistema e os intestinos de peixe, ao longo dos diferentes pontos de amostragem. O alimento também teve um aumento significativo de similaridade com o intestino de peixe ao longo do tempo, começando com 0.4±10.2% para a água doce e 3.5±2.94% para água salgada e acabando com um valor significativamente alto 21.7±11.09% para água doce e 30.4±14.12% para água salgada. Por outro lado, a esterilização de alimento apresentou diferenças significativas em todos os pontos de amostragem, entre populações bacterianas de água doce e salgada. No entanto, a microflora dos peixes alimentados com alimento estéril sofreu um efeito da água reduzido, quando comparado com os peixes alimentados com alimento regular. Claramente, este estudo revela-se um ponto de partida para o conhecimento completo de como as

comunidade bacterianas são estabelecidas e quais serão os objectivos para futuras investigações.

Palavras-chave: *Comunidades microbiológicas; Tilápia; Oreochromis niloticus; salinidade: esterilização de alimento; Active suspension systems*

1. Introduction

Gut microbial communities in animals are considered a complex and diverse system with importance in animal nutrition, physiology and pathology (Rastall, 2004). The research of microbial communities in gut started with the discovery of *Escherichia coli* in the human gut, and consequently this finding lead to more research and to the discovery of microbial communities in other groups of animals, such as in fish (Rastall, 2004).

A wide range of microorganisms colonizes the intestinal tract, coming from the surrounding environment (e.g water), sediment and feed (Ringø *et al*., 1995; Grisez *et al*., 1997; Ringø and Gatesoupe, 1998, Nayak, 2010). The major group of bacteria that colonize the gut are aerobic, facultative anaerobic and obligatory anaerobic bacteria (Sugita, 1991).

Gut microbiota of freshwater and saltwater organisms are distinct, an aspect related to the differential functioning of the intestine in these two environments (Ringø and Gatesoupe, 1998). While *Aeromonas spp*., *Pseudomonas spp*., and members of the *Flavobacterium* family are the most common group of microorganisms in the gut of freshwater fish, *Vibrio*, *Acinetobacter* and *Enterobacteriaceae* are the most common bacteria in marine fish (Ringø *et al*., 1995).

During the last decades much work has been done to identify and characterize the microbial communities in the gastrointestinal tract in different fish species (Limsuwan and Lovel, 1981; Ringø *et al*., 1995; Grisez *et al*., 1997; Ringø and Gatesoupe, 1998; Spanggaard *et al*., 2000; Ringø *et al*., 2003; Al-Harbi and Uddin, 2005; Hovda *et al.,* 2007); nevertheless, these studies focused on the relations between bacteria and environmental conditions, (Limsuwan and Lovel, 1981; Sugita, 1991; Ringø *et al*., 1995; Grisez *et al*., 1997; Ringø and Gatesoupe, 1998; Olafsen, 2001; Reitan *et al.,* 2001;) nutrition studies, (Moriaty, 1999; Al-Harbi and Uddin, 2005) improvement of systems such as active suspension ponds with microbial manipulation (Avnimelech, 2003, 2006, 2007; Avnimelech *et al.,* 2008; Azim and Little, 2008; Schryver *et al.,* 2008) and promotion of beneficial bacteria (probiotics) as a substitute of antibiotics (Gatesoupe, 1999; Moriaty, 1999; Olafsen, 2001).

The presence of gut microbiota in fish has long been recognized, but less is known about the stabilization and diversity of these communities and their role in fish health (Nayak, 2010).

The genus Tilapia belongs to the successful Cychlidae family (Nelson, 2006). These fish are the second most farmed species in the word, only surpassed by carps (Merrifiel *et al*., 2010).

The availability of freshwater is a major bottleneck to future fish production; subsequently, there has been an increase in competition for this resource between agricultural and urban activities (Verdegem *et al.,* 2009). Aquaculture industries have been forced to develop culture systems in brackish and seawater (Watanabe *et al.*, 1985 (b); Watanabe *et al.*, 1985 (a); El-Sayed *et al.*, 2005; El-Sayed, 2006; Rengmark and Lingaas, 2007). Tilapia was the first candidate for brackish water aquaculture, due to the low investment required when compared to other species such as turbot, sea bass and sea bream (Al-Harbi and Uddin, 2005; Rengmark *et al.,* 2007).

The main objective of this experiment is to compare the composition of the gut microbial communities of tilapia raised at different salinities and feed sterilized or non-sterilized feed.

2. Literature Review

2.1 Active Suspension System (AST)

During the last decades, much work has been done to improve the efficiency in water treatment, energy consumption and oxygen uptake in aquaculture systems. One example of merging water treatment with feed recycling in intensive ponds is the active suspension pond, ASP, also called *Bio floc* technology (BFT) (Avnimelech, 2006; Avnimelech, 2007). For that reason, in recent years, active suspension intensive ponds have been developed, as a mean to produce fish or shrimp at high densities (Milstein *et al*., 2001; Avnimelech, 2007).

2.1.1 Principle

The basic principle of the active suspension technology (AST) is the retention of waste and its conversion to *Biofloc* as natural food within the culture system. This is achieved through constant aeration and agitation of the water column to keep particles suspended, while maintaining a low water exchange rate (to about 10% per day). The addition of carbon sources as organic matter substrate, raise the C:N ratio, which favors microbial floc formation (Milstein *et al*., 2001; Avnimelech, 2006; Avnimelech *et al*., 2008; Azim and Little, 2008; Schryver *et al.,* 2008; Crab *et al.,* 2009). This floating *floc* (**Figure 1**), capable of reaching more than 1000µm in size (Schryver *et al.,* 2008), consists of phytoplankton, bacteria, aggregates of living and dead particulate organic matter, as well as grazers (Avnimelech, 1999). If carbon and nitrogen are well balanced, nitrogen will be immobilized into bacterial protein biomass (Avnimelech, 1999).

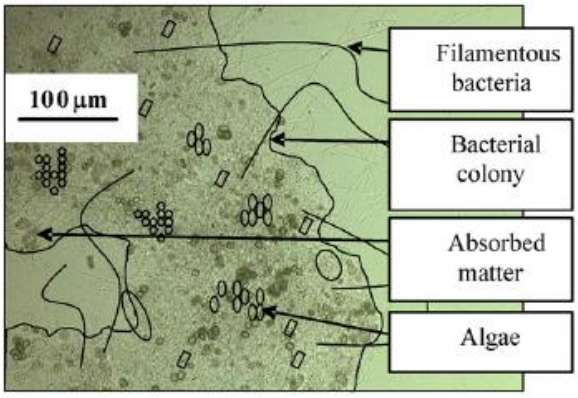


Figure 1- Floc structure within a ASP system and its composition (Schryver *et al.,* 2008).

2.1.2 AST and Microbial Control

Controlling aquaculture systems, like ASP, through manipulation of microbial activity, has become an important and regularly discussed technology arising from the efforts of improving intensive aquaculture (Avnimelech, 2003).

Microbial communities found in ASP contain algae, blue-green algae, bacteria, protists, zooplankton and fungi implanted in an extracellular polysaccharide matrix that develops in submerged surfaces (Avnimelech, 2007). Inside these communities, autotrophic or heterotrophic biomass dominate, depending on light, dissolved oxygen, and nutrient availability (Avnimelech *et al.*, 2008).

One serious problem in intensive ponds culture is the nitrogen disorder. Inorganic nitrogen accumulates in the pond for various reasons. For example, fish metabolize proteins as an energy source and feed residues have a long hydraulic retention in ASP (Avnimelech, 2006).

Proper manipulation of the microbial biomass enables the control of water quality, mostly through the conversion of the potentially toxic inorganic nitrogen forms to microbial protein. In turn, microbial protein may be utilized as a source of feed for the fish (Avnimelech, 2003; Avnimelech, 2006; Azim and Little, 2008; Schryver *et al.,* 2008). The major driving force is the intensive growth of heterotrophic bacteria that is dependent of the C/N ratio. They consume organic carbon (1.0 g of carbohydrate-C yields about 0.4 g of bacterial cell dry weight-C) and, depending on the bacterial C/N-ratio, they can also immobilize mineral nitrogen (Schryver *et al.,* 2008). Avnimelech (1999) calculated a carbohydrate requirement of 20 g to immobilize 1.0 g of NH_4-N, based on a microbial C:N-ratio of 4 and 50% C in dry carbohydrate. A proper C:N, as >10:1, is optimal for the *biofloc* production while regeneration of NH_4^+ is minimized (Avnimelech, 2006).

In conclusion, microbially managed ponds provide stable control over the processes occurring in the ponds. This technology does not depend on light intensity and is not sensitive to population crashes. Also, microbial control leads to efficient degradation of waste materials, efficient nitrification and, through the manipulation

of C:N ratio, assists the control and recycling of nitrogen, thus doubling protein utilization.

2.1.3 AST Limitations

There are some limitations in the use of AST. Excessive turbidity may, however, have a negative effect on different fish species. Although for tilapia turbidity is not a problem, (Avnimelech 2006) it is not certain that all cultured fish species will easily adapt to growing in turbid water. Another problem is that above fish or shrimp densities of 10-20 kg m^{-3} the system becomes unstable.

2.2 Importance of Tilapia Culture in Aquaculture

Tilapia is one of the most farmed species in the world, (Merrifiel *et al*., 2010), with cultures occurring in more than 100 countries (El-Sayed *et al*., 2005). World production of cultured tilapia has increased from 28.260 metric tons in 1970 to 2.5 million metric tons in 2007 (Table I)(FAO, 2009).

Table I - World Aquaculture major production (in tons) of fresh water fish. (Adapted from FAO, 2009)

Specie group	1999	2001	2003	2005	2007
Carps and other cyprinids	13 514 192	14 601 849	15 585 483	17 548 264	18 944 071
Tilapias and other cichlids	1 037 156	1 303 425	1 577 367	1 980 450	2 505 465
Miscellaneous freshwater fishes	2 446 503	2 716 207	3 086 789	4 063 763	5 318 595

Characteristics such as fast growth, tolerance to a wide range of environmental conditions, resistance to stress and diseases, ability to reproduce in captivity, short generation time, feeding at low trophic levels and acceptance of artificial feeds immediately after yolk-absorption make tilapia an ideal candidate for aquaculture, particularly in developing countries (El-Sayed, 2006).

All tilapia species, including red tilapia, are cultured worldwide in tanks, ponds or cages (El-Sayed, 2006; He *et al.,* 2009), but *O. niloticus* is the most cultured species in this family (**Figure 2 & 3**) (Nelson, 2006).

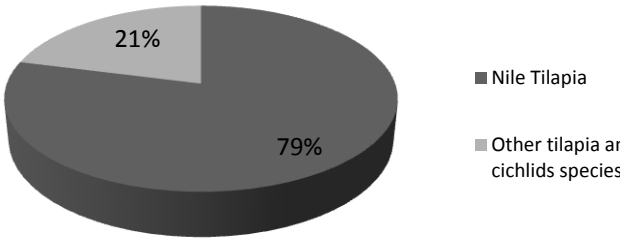


Figure 2- Percentage of Nile tilapia production of the total Cichlids group between 1950 and 2008. (Adapted from FAO, 2010)

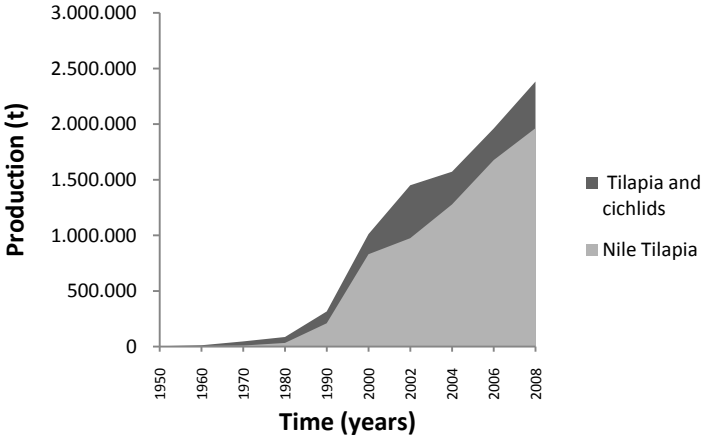


Figure 3- Word production of Nile tilapia over time. (Adapted from FAO, 2010)

2.3 Tilapia: A General Overview

2.3.1 Taxonomy and Distribution

Tilapia belongs to the *Cichlidae* family, representing a large number of freshwater species and considered the most successful fish family among teleosts (Nelson, 2006). Native to Africa (excluding Madagascar) and Palestine (Trewavas, 1982; Nelson, 2006), during the second half of the 20th century they were introduced (**Figure 4**) into several environments in tropical, subtropical and temperate regions (Pillary, 1990).

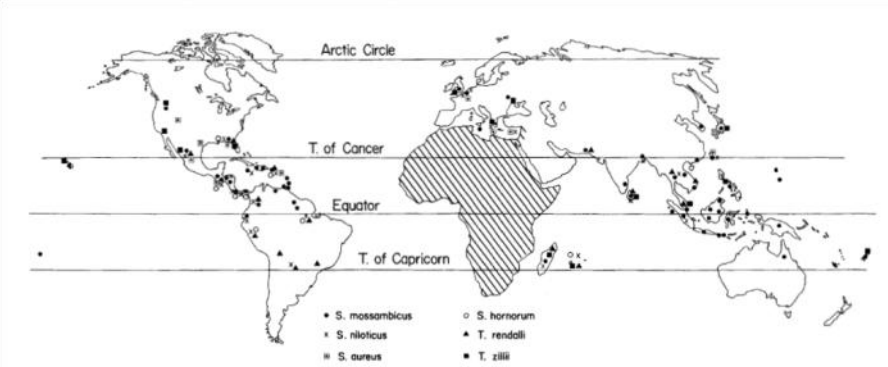


Figure 4- Introduction of tilapia (6 species) outside of Africa (Trewavas, 1982).

Due to the similarity among species (**Figure 5**) and overlapping of their morphological characteristics, taxonomic classification of tilapia is still confusing and a subject of discussion (El-Sayed, 2006; Nelson, 2006). Another confounding factor results from the free hybridization of these species in the natural environment (El-Sayed, 2006).

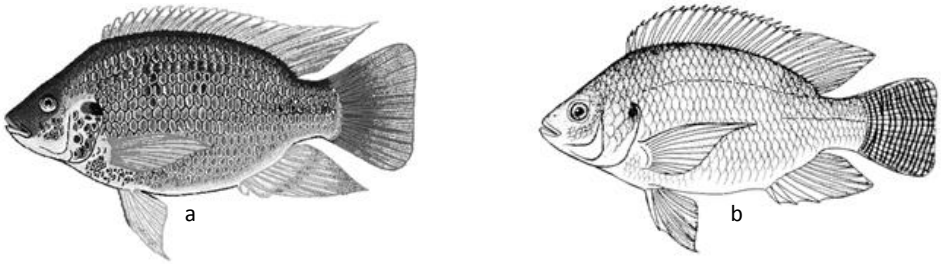


Figure 5- Mozambique Tilapia (a); Nile Tilapia (b). (FAO, 2010)

The development of hybrids like the red tilapia between stocks of *Oreochromis niloticus* and *Oreochromis mossambicus,* has the potential to combine the higher growth potential of the former with the salinity tolerance of the latter, therefore representing a potential commercial application for hybridisation in brackish water aquaculture (Kamal and Mair, 2005) that is of great interest to fish farmers.

2.3.2 Feeding Relationships

Tilapias are herbivorous/omnivorous fish with adaptable feeding habits (Fujimura and Okada, 2007) able to feed at a low level in the aquatic food chain (Philippart and Ruwet, 1982; El-Sayed, 2006). During larval stages, tilapia feed on zooplankton, especially crustaceans (copepods), while older stages feed on aquatic vegetation, phytoplankton, zooplankton, periplankton and detritus from plants (El-Sayed, 2006). The morphology of the feeding apparatus, including jaw and teeth, is amazingly diverse and therefore suitable for adaptation to their feeding behaviour (Fujimura and Okada, 2007).

2.3.3 Larvae History

Tilapia larvae can have various sizes according to the size of the egg that is related to the amount of yolk stored. The yolk sac is spherical and reasonably large for warm water fish such as Nile tilapia (**Figure 6**). At hatching, pigmented eyes and some chromatophores on the surface of the yolk sac are visible.

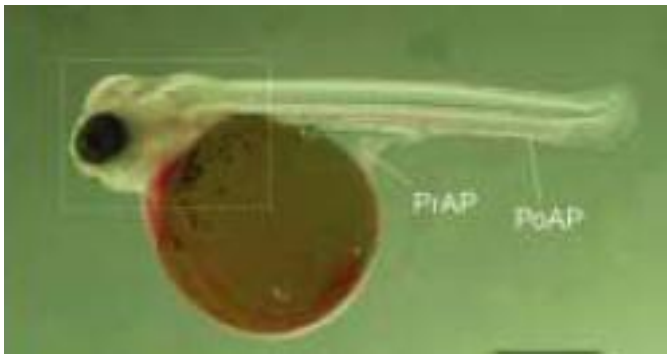


Figure 6- Newly hatched Nile tilapia larvae (Adapted from Fujimura and Okada, 2007)

Floatability control is achieved through the absorption of the yolk sac 8-12 days post hatch (dph) and the development of the swimming bladder. The mouth becomes visible 4-5 dph but feeding behaviour can be observed between 8 to 10 dph (Shelton and Popma, 2006).

Tilapia larvae can have several sizes, but usually cultured tilapia has similar sizes to those of natural populations. Size is about 5 mm for newly hatched and 8 mm for 12 dph individuals (Shelton and Popma, 2006).

2.4 Environment Factors

Tilapia is more tolerant than most commonly cultured fish to various "adverse" environmental factors such as salinity, high ammonia levels and water temperature and low dissolved oxygen (Shelton and Popma, 2006). The optimal environmental parameters for tilapia culture, described in Shelton and Popma, (2006) can be checked in **Table II**. Salinity tolerance and its effects on this group of fish will be analysed in more detail in sub-chapter 2.4.1.

Table II- Optimal environmental conditions for Nile tilapia culture (Adapted from Shelton and Popma, 2006)

Parameter	Level
Temperature	29ºC- 31ºC
Salinity	<10 ppt
pH	7-8
DO	>5 $mg.l^{-1}$
NH_3^-	<0.14 $mg.l^{-1}$
NH_4^+	<1.5 $mg.l^{-1}$
NO_2^-	<0.5 $mg.l^{-1}$

2.4.1 Salinity

Tilapias are euryhaline fish that can live and prosper in a large range of salinities, from freshwater to seawater, although some species tolerate a wider range of salinity when compared to others (Philippart and Ruwet, 1982). For instance, Nile tilapia can tolerate salinities up to 18 ppt, while Mozambique tilapia grows and reproduces in environments with salinities above 30 ppt (Chervinski, 1982(a), Watanabe *et al.*, 1985 (a).

For aquatic organisms like tilapia, salinity is considered one of the major environmental parameters exerting selective pressure. This fact can be illustrated observing the effect of salinity on tolerance to environmental parameters for different stages (Varsamos *et al*., 2005). According to Breves *et al.* (2010), salinity tolerance in tilapia species is related to divergence in their native geographical distribution. For instance, the continental *O. niloticus* is less tolerant to high salinity than the coastal *O. mossambicus*. Nevertheless, the major factor that regulates the salinity tolerance is the ability of fish to osmoregulate, which starts in early embryonic stages and is related to the presence and ion pumping activity of numerous integumental ionocytes: fish that adopt hypo-osmotic conditions face a permanent osmotic gain of water. Fish exposed to hyper-osmotic conditions face dehydration they release divalent and monovalent ions through kidneys and intestinal and brachial via respectively (Varsamos *et al*., 2005).

Table III compares different levels of salinity for different species of tilapia described by several authors. Chervinski (1982 (a)) demonstrated that, even though some species of tilapia tolerate high salinities but may not survive abrupt changes to it, they will respond well to gradual changes in salinity. On the other hand, it was verified that "California" *O. mossambicus* would successfully endure an abrupt change in salinity from 35 ppt to 60 ppt at 25ºC and 35ºC (Sardella *et al.,* 2004).

Research has also shown that salinity tolerance in fish is influenced by genetic factors, size, sex and several environmental factors, with particular emphasis on temperature (Suresh and Lin, 1992; Likongwe *et al.,* 1996; El-Sayed, 2006). Sardella *et al.* (2004) observed high survivability with both high salinity and temperature, which contrasted with increased mortality at low temperature (15ºC) and high salinity (35-60 ppt), leading to the conclusion that temperature and salinity act interdependently to have an effect on fish. Nevertheless, in a review by Suresh and Lin (1992) a high rate of survival to high salinities (for a fresh water fish) was attributed to low temperatures as some tilapia species exhibited better survivability at 11.5 ppt and low temperature (<15ºC). These different results, demonstrate that there is still work to do to find a common conclusion about this topic.

Table III- Comparison of various salinity tolerance levels in some tilapia species.

Specie	Salinity (ppt)	Temperature (ºC)	Authors
O.mossambicus	45	27	El-Sayed (2006)
Hybrid tilapia	>20	35	Kamal and Mair (2005)
California *O.mossambicus*	60	35	Sardella *et al.* (2004)
California *O.mossambicus*	35	25	Sardella *et al.* (2004)
O. aureus	11	<15	Suresh and Lin (1992)
O. niloticus	18.9	27	Watanabe *et al.* (1985)

Several studies have revealed that salinity seems to affect reproduction in tilapia by seriously compromising female spawning (Watanabe and Kuo, 1985; Watanabe *et al.*, 1985 (b); Likongwe *et al*., 1996; Shelton and Popma, 2006). In these

same studies it is also mentioned that eggs or larvae acclimatized to increased salinity are more tolerance and have consequently more chance of surviving to the same or higher salinities in later stages of development. It is however worth noting that, despite the increase in survival rate, growth and reproduction remain below the values observed in fresh water.

2.5 Fish Performance Parameters

2.5.1 Growth

Growth is a straightforward concept to understand, as it can be easily determined by weighing or measuring fish (Sumpter, 1992). At cellular level, growth can be described as the deposition of mainly proteins and lipids (Van Weerd and Komen, 1998). Growth hormone (GH) plays a major role in growth regulation, which is under endocrine control (Bonga, 1997). Cortisol and catecholamines are key factors in the inhibition of somatic growth by stimulating energy consumption, gluconeogenis and lipolysis (Bonga, 1997).

Many factors can affect growth. Among these are included temperature, pH, salinity, pollutants and handling (Sumpter, 1992; Bonga, 1997). The reduction of growth under stress is linked with the relocation of metabolic energy from growth to other activities that require more energy to restore homeostasis (Bonga, 1997).

2.5.2 Growth and Salinity

Growth is significantly affected by salinity. Likongne *et al.* (1996) tested four different levels of salinity (0, 8, 12 and 16 ppt) and temperature (24, 28, 30 and 32ºC) in Mozambique tilapia. For the same temperature, different values of salinity were compared. Levels of salinity above the 8 ppt negatively affected growth. The effect of a stressor like salinity in fresh water fish destabilizes the osmoregulatory mechanisms. It increases the energy budget to ionic regulation (to keep homeostasis), which consequently affects growth (Likongne *et al.* 1996). The effects of salinity on growth can be changed by the ions concentrations, Ca^{2+} and Mg^{2+}, as by the non-osmoregulatory (e.g behavior) effects on metabolism (Watanabe *et al.* 1993).

2.6 Feed Sterilization

The use of gamma irradiation as a method of sterilizing feed is considered to be effective. This technique extends the storage life of certain feeds by controlling the microbial and parasitic activity (Ogbato, 1988). One important factor that must be taken into consideration is the penetrability of radiation into the feed. Thus, for sterilized feed, all parts of it must absorb sufficient radiation to kill all present spoilage microorganisms. Penetration of gamma rays is a function of their energy levels. The maximum energy limit of ***5*** MeV for these rays provides adequate penetration for practical applications. The maximum of 10 MeV for electrons, however, restricts applications to feeds less than ***5*** cm thick (Urbain, 1978).

For gamma ray sources, two radionuclides have been used in food irradiation: Cobalt- 60 (^{60}Co) or Cesium- 137 (^{137}Cs) (Urbain, 1984; Farkas, 1994).

The problems of food irradiation lies in changing smell and flavor of the products as well as the destruction of vitamin E, a natural antioxidant of lipids (Urbain, 1984; Farkas, 1994; Armstrong *et al.,* 1994).

2.7 Microbial Diversity

For some time now, researchers have been characterizing the existing differences in microbial composition through various environments. The knowledge about bacterial diversity is useful for understanding the nature of the sample being studied. Important questions were and still are raised when studying bacterial flora of environmental samples; e.g. in which way do bacterial communities respond to environmental changes? How does the microbiota interact and depend on the species? (Hovda, 2007)

2.7.1 The Larval Microbiota

Unlike fresh water fish, marine fish starts drinking water before the complete absorption of the yolk sac, so the bacterium enters in the digestive tract before the first feeding. This behavior can also be seen in more advanced larval stages, where

bacteria are ingested through grazing of suspended particles and eggs debris (Olafsen 2001). As a consequence, the microbiota of eggs and other organisms in the system will affect the primary microbiota of fish larvae (Hansen and Olafsen, 1999). Studies done in Atlantic halibut, concluded that mucus change from a predominantly neutral mixture to a more sulphated one, shifts the adhesion sites for bacteria during larval development, having implications in the changes of microbiota during larval stages (Olafsen 2001). Intestinal mucosa contains specific intestinal microbiota consisting of aerobic, facultative anaerobic and obligatory anaerobic bacteria. The composition may change with age, environmental conditions and nutritional status (Sugita *et al.,* 1991).

2.7.2 Gut Colonization

As the intestine of fish larvae is virtually sterile, the bacteria present in the water and initial feed are the first gut colonizers (Ringø *et al*., 1995; Grisez *et al*., 1997; Ringø and Gatesoupe, 1998). For this reason, gut environment can be influenced during the hatching process by the water in the incubators, which often contains a heavy bacterial load. In marine species, the drinking behavior may also contribute towards bacteria ingestion (Ringø *et al*., 1995).

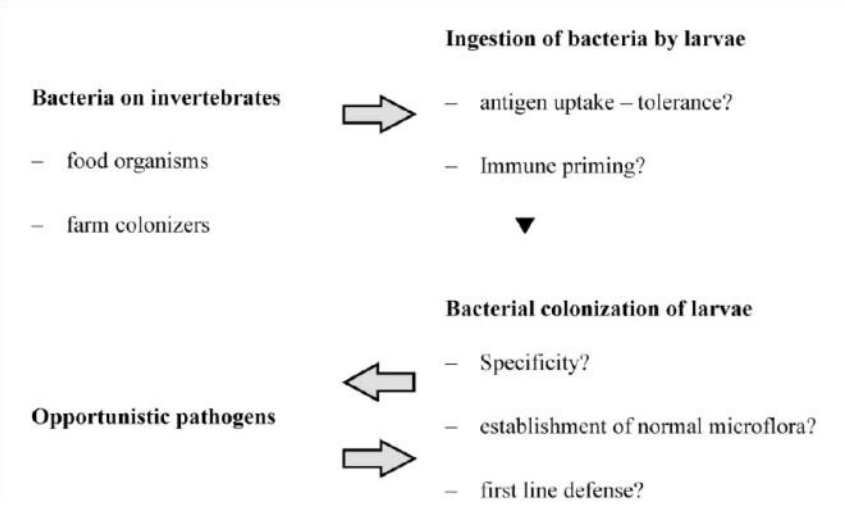


Figure 7- Steps and interactions between bacteria colonization and fish larvae (Adapted from Olafsen, 2001).

According to Ringø *et al*., (2003) there are several factors that can influence

adhesion and colonization of the microbiota in the digestive tract (**Figure 7**). These include: (a) peristalsis, (b) bile salts, (c) gastric acidity, (d) immune response, (e) digestive enzymes and (f) indigenous bacteria and the antibacterial compounds which they produce. These microbiota can be classified as autochthonous/indigenous (term used in endothermic animals to classify "normal" flora) or as allochthonous/ transient (incidental visitors of the gastrointestinal tract that are rejected after some time) (Ringø *et al*., 1995).

There are two phases of bacteria colonization in several marine species, such as turbot, *Scophthalmus maximus*, described by Ringø *et al*. (1995). Approximately $5x10^2$ CFU. $larvae^{-1}$ are established at day 1 after hatching, before introduction of live feed, corresponding to the first level of colonization. The second level is achieved between days 5 and 16 after hatching, with approximately 5 x $l0^4$ CFU. $Larvae^{-1}$. According to the same author, the increase in bacterial load and the differences in the intestinal microbiota during the first few days after hatching may be due to better capacity of bacteria to attach into the larval gut, as a result of the histological and functional development of larvae.

After colonization of the gut, bacteria present in the intestine of fish may either be beneficial in nutritional terms or in the prevention of colonization by pathogenic bacteria, may have no significant effect (neither beneficial nor harmful - commensalism process) or cause mass mortalities, depending on the species (Grisez *et al*., 1997).

2.7.3 Gut Microbial Communities vs Feed and Water Quality

Gut microbiota in fish has been regarded as fulfilling numerous functions (Spanggaard *et al*., 2000). It is accepted that aquatic microorganisms not only influence water quality but also are also associated with the physiological status of fish, disease incidence and post-harvest quality (Ringø *et al*., 2003; Al-Harbi and Uddin, 2005). Several authors (Ringø and Gatesoupe, 1998; Spanggaard *et al*., 2000; Ringø *et al*., 2003) suggested that these bacteria have, not only a nutritional role due to the way they break down ingested foods to individual components such as vitamins, (Limsuwan and Lovel, 1981) lipids or amino acids, but also act as a

protection barrier against diseases and pathogens. Owing to this nutritional function, fish with a diverse and abundant diversity in gut microbiota have a good capability of adapting to different nutritional substrates and incorporating food better, thus enhancing their adaptive potential (Al-Harbi and Uddin, 2005).

Olafsen (2001) found out that bacteria ingested by fish larvae by drinking water are primed with antigens before active feeding commences. This may result in the formation of an indigenous larval microbiota. Appropriate and cellular uptake of intact bacterial antigens by newly hatched larvae may affect the development of their immune system. The same author also affirmed that, as the aquatic invertebrates are natural food sources for fish larvae and are also co-inhabitants of larval ecosystems. The relationship between feed and fish imply that the establishment of a larval microbiota will also be influenced by the indigenous microbiota of invertebrates.

Some gastrointestinal bacteria have been demonstrated to enhance growth of catfish and, while this might be possible to extrapolate to other species (Limsuwan and Lovel, 1981), there is still some disagreement regarding this matter.

2.7.4 Gut Microbial Communities Diversity vs Salinity

Gut microbiota of organisms of freshwater and saltwater is distinct, an aspect related to the differential functioning of the intestine between these two environments (Ringø and Gatesoupe, 1998). While *Aeromonas spp*., *Pseudomonas spp*., and members of the *Flavobacterium* family are the most common group of organisms in the gut of freshwater fish, *Vibrio*, *Acinetobacter* and the *Enterobacteriaceae* are the most common bacteria groups in marine fish (Sakata *et al.,* 1980; Ringø *et al*., 1995).

The different bacterial communities may also be related with the drinking behavior (Ringø *et al*., 1995). Marine larvae must drink more water than fresh water fish to maintain their water balance, consequently the larvae due to the drinking process may take up dissolved and particulate compounds passively (Reitan *et al.,* 1998).

The predominant bacterial genera/species isolated from most fish gut have been aerobes or facultative anaerobes, (Ringø *et al*., 1995). Previous studies in salmonids demonstrated that even though Gram-negative bacteria appear to be dominant in fish gut, Gram-positive bacteria have also been found in the intestine, including different species of lactic acid bacteria (Ringø *et al*., 1995 Ringø and Gatesoupe, 1998). Some of the bacteria have been implicated in fish diseases and may be a problem for human health (Al-Harbi and Uddin, 2004).

2.7.5 Microbiota Manipulation in Aquaculture – Probiotics

The use of probiotics, also named microbial manipulation in aquaculture may have a profound impact in health management. This is based on the positive results with using probiotics rather then antibiotics in domestic animals (Moriarty, 1998; Olafsen, 2001). The term probiotic describes the beneficial health effects of living cells in the host, by improving the microbial balance of the indigenous microbiota (Gatesoupe, 1999). In aquaculture industries, beneficial bacteria are added to tanks and ponds because of their ability to change the microbiota composition of the water and sediment (Moriarty, 1998). These characteristics may be considered antagonistic or anti-colonization by pathogens, natural defenses stimulation or even health benefits from released factors (Olafsen 2001).

There are several reasons to use probiotics (such as *Bacillus* species) instead of antibiotics to control the growth of unwanted bacteria (e.g *Vibrio*). *Bacillus* species can naturally secrete several antibiotic compounds and enzymes that can penetrate and degrade the biofilms. Also, this group of bacteria competes for nutrients and space (e.g. gut hall) avoiding the fast growth and reproduction of resistant bacteria. The antibiotics are used to kill bacteria, although several pathogens are carrying resistant genes that can be transmitted to future generations. More, unwanted bacteria can reenter the tanks through biofilms on water pipes, air lines or even in contaminated fish guts, exchange genetic information with the resistant bacteria and consequently survive further doses of antibiotics (Moriarty, 1998). Nevertheless, the risk to select probiotic resistant pathogens must not be underestimated (Gatesoupe, 1999). The bacteria used must

be carefully chosen for specific functions that are amenable to bioremediation, introduced in high enough densities and in the right environmental conditions (Moriarty, 1998). Diversified antagonistic properties must be investigated, in a way that can decrease the possibility of multi-resistance. For example, antagonistic behavior can be caused by competition for nutrients that favors the intensification of probiotics, or the expression of their inhibitory effects (Gatesoupe, 1999).

2.8 Molecular Methods Describing Microbial Diversity

Nowadays, molecular techniques can provide an exceptional tool for identification and characterization of microorganisms found in the environment, food and other complex ecosystems (Ercolini, 2004; Hovda, 2007).

Traditionally, the analyses of the microbial communities in fish were carried out using conventional culture-based techniques followed by isolation and phenotypic characterization. With the advances in molecular technology these culture-based methods are slowly been surpassed by molecular methods based on Polymerase Chain Reaction (PCR), 16S rRNA gene sequencing and Denaturing Gradient Gel Electrophoresis (DGGE) (**Figure 8**)(Hovda *et al.,* 2007; Nayak, 2010). The

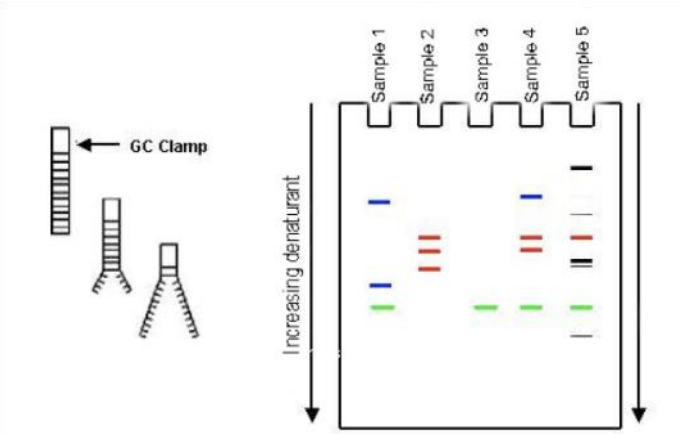


Figure 8 - The principle of denaturing gradient gel electrophoresis (DGGE) (Hovda, 2007).

major reason for the use of culture-independent methods relates to the required knowledge about the conditions under which most bacteria develop in their natural habitat and the complexity of developing media for cultivation resembling these exact conditions (Ercolini, 2004).

Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) is a technique that combines two methods in one: the amplification of DNA by PCR and electrophoresis in acrylamide gel in denaturing conditions (Tatsadjieu *et al.,* 2010)**.** Separation of PCR products in DGGE is based on the decrease of the electrophoretic mobility of partially melted doubled stranded DNA molecules in polyacrylamide gels containing a linear gradient of DNA denaturants like formamide and urea at 60 ºC. Molecules with different sequences will have a different melting performance and will cease migration at a singular position in the gel (Muyzer *et al.,* 1993). The 16S rRNA gene can describe both cultivable and uncultivable bacteria by phylogenetic relationship (Pond *et al.*, 2006; Hovda *et al.,* 2007).

PCR and DGGE are frequently used in environmental microbial ecology assessments (**Figure 9**) (Ercolini, 2004), in studying food and in analyses of gut microbiota (Muyzer *et al.,* 1993). These two genetic fingerprinting methods can be used to identify the genetic diversity of dominant populations from amplified DNA products (Muyzer *et al.,* 1993).

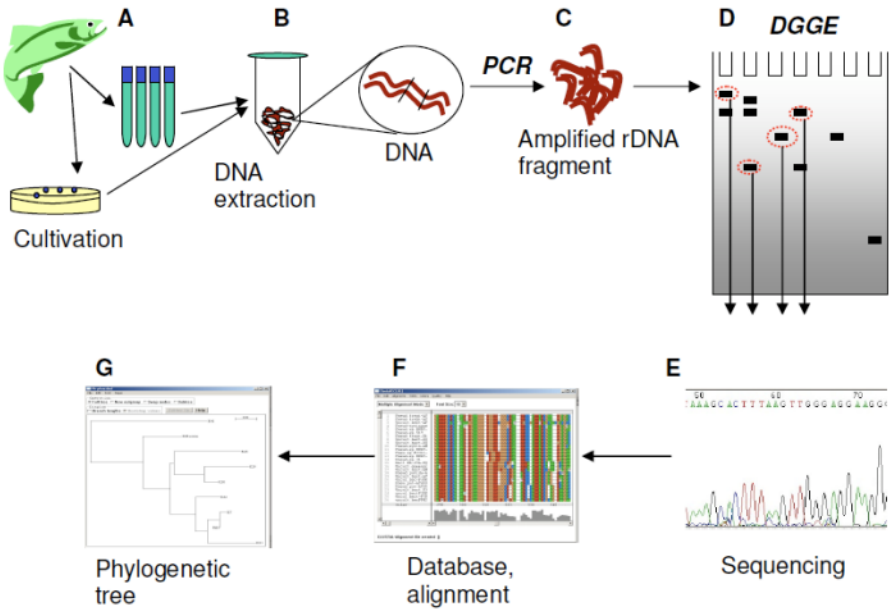


Figure 9 - Flowsheet of the process form fish sampling to bacterial detection and identification. Samples from the fish are taken directly for DNA extraction (A). In B, bacterial DNA is extracted using standard procedures, or kits, before the DNA is amplified using PCR (C). The PCR products are separated on a denaturing gradient gel (D), and bands of interest are excided and sequenced (E). For further comparison of the bands, the sequences can be aligned in suitable programs, such as ClustalX (F), and a phylogenetic tree can be made to display similarities graphically (G). (Hovda, 2007)

3. Study aims and Hypothesis

3.1 Main Objective

The aim of the present experiment is to study the influence of water and feed microbial communities on gut of Nile tilapia larvae. The present study also aimed to evaluate the influence of sea and freshwater systems and non-sterilized and sterilized feed on the gut biota.

3.2 Research Questions and Hypothesis

Question 1: Does salinity affect the microbial communities in Nile tilapia (*O. niloticus*) gut?

H0: Salinity does not affect the microbial communities in Nile tilapia (*O. niloticus*) gut.

Question 2: Does feed sterilization affect the microbial communities in Nile tilapia (*O. niloticus*) gut?

H0: Feed sterilization does not affect the microbial communities in Nile tilapia (*O. niloticus*) gut.

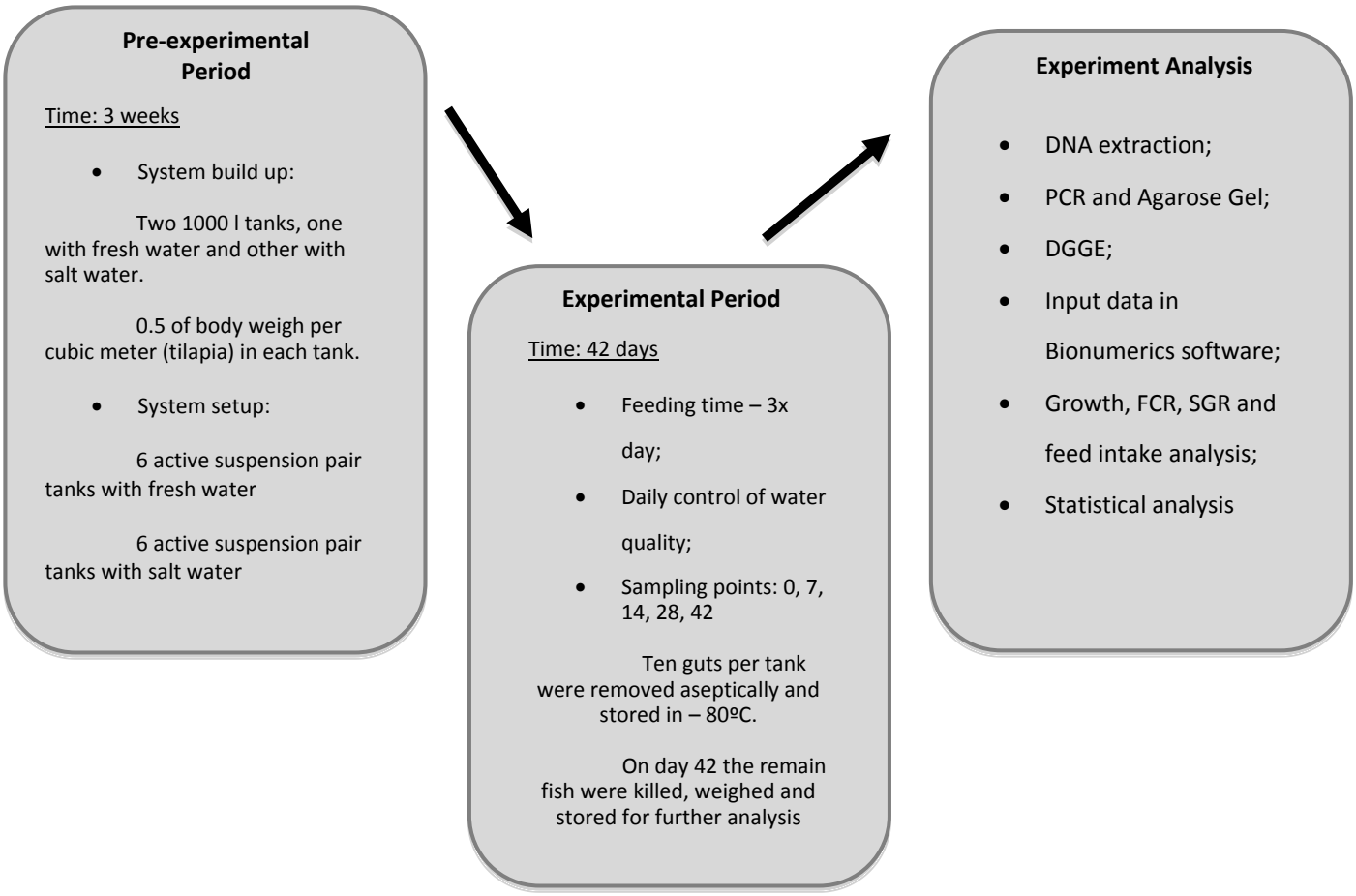
Question 3: Are the microbial communities of water and feed related?

H0: Microbial communities of water and feed are not related.

4. Material and Methods

This experiment was approved by the Ethical Committee for Animal Experiments (DEC), Wageningen University with the following protocol number: 2010032.b.

4.1 General Overview of the Experiment



4.2 Pre-Experimental Period

In order to establish microbial communities in both treatments, fresh, saltwater (water maturation), and 3 active suspension tanks (one of 500l and two of

1000l of capacity) were arranged for this experiment. The maturation period (pre-experimental period) had the duration of 3 weeks.

Bacterial load was achieved with the introduction of feed (0.5% of 5 kg of body weight per cubic meter) and fish (5 kg/m^3) until the end of the pre-experimental period.

For the saltwater tank, four fishes were introduced (± 400/500 g per fish) and the salinity was gradually increased 5 ppt every two days, until it reached 25 ppt. For each freshwater tank, nine fishes with the similar body weight were introduced in order to have a density of 5 kg/m^3.

Fish were fed 0.5% of their body weight (15 g) and were maintained at a constant temperature of 28ºC.

The water quality parameters (pH, temperature, O_2, NH^+_4, NO^-_3 and NO^-_2) were monitored daily.

4.2.1 Water Quality:

- pH: 6.5-8.0
- TAN-N: < 2 mg/l
- NO_2^--N: < 1.5 mg/l
- NO_3^--N: < 113 mg/l
- Temperature: 26-28 ºC
- Dissolved Oxygen (DO): > 6.5 mg/l
- Salinity: 0 and 25ppt

When the readings of any of the parameters were abnormal, 10% of the total water volume of the tank was exchanged to avoid potential fish stress and not compromise water maturation. Hydrochloric acid (HCl) was also added as a way of quickly lowering pH.

Every two days, three samples of water were collected and analyzed in the spectrophotometer to measure absorbance, with the objective of checking the optimal density of bacterial communities in each tank.

4.3 Experimental Animals: Tilapia Larvae

Tilapia 6-8 days old larvae were acquired from the company *Til-Aqua*©. Two different batches of tilapia larvae were received, one acclimated in fresh water and the other acclimated in salt water (25 ppt), both during the incubation period.

The tilapia larvae were immediately transferred into 12 aquaria in order to achieve full acclimation to system conditions avoiding stress. This period lasted for 2 days, time at which the experiment begun (Day 0). With the mouth open (Day 0), gut samples were taken for microbial analysis (10 larvae x 12 tanks).

4.4 Experimental Period

4.4.1 Accommodation

For this experiment, 20-l tanks were arranged and each one was individually connected to a 120l active suspension tank. Good mixing and aeration of the water was provided within the active suspension tanks, thus ensuring efficient swimming and feed uptake by the larvae in the 20l aquaria.

Six of these paired tanks were filled and worked as a salt water system (25 ppt) while the other six worked as a fresh water system (0 ppt).

For the global design (**Figure 10)** of the experiment there are three couples representing two variables within two variables:

- 3 tanks with fresh water and fed with non-sterilized feed (FN),
- 3 tanks with fresh water and fed with sterilized feed (FS),
- 3 tanks with salt water and fed with non-sterilized feed (SN),
- 3 tanks with salt water and fed with sterilized feed (SS).

In each aquarium, 120 swim-up fry of Nile tilapia (before first feeding) were stocked (total: 1440 larvae). From these 1440 larvae, 120 were sampled for initial weight determination at the beginning of the experiment. The remaining 1200 larvae were considered as the experimental animals for the main experimental period. This experimental period lasted 42 days.

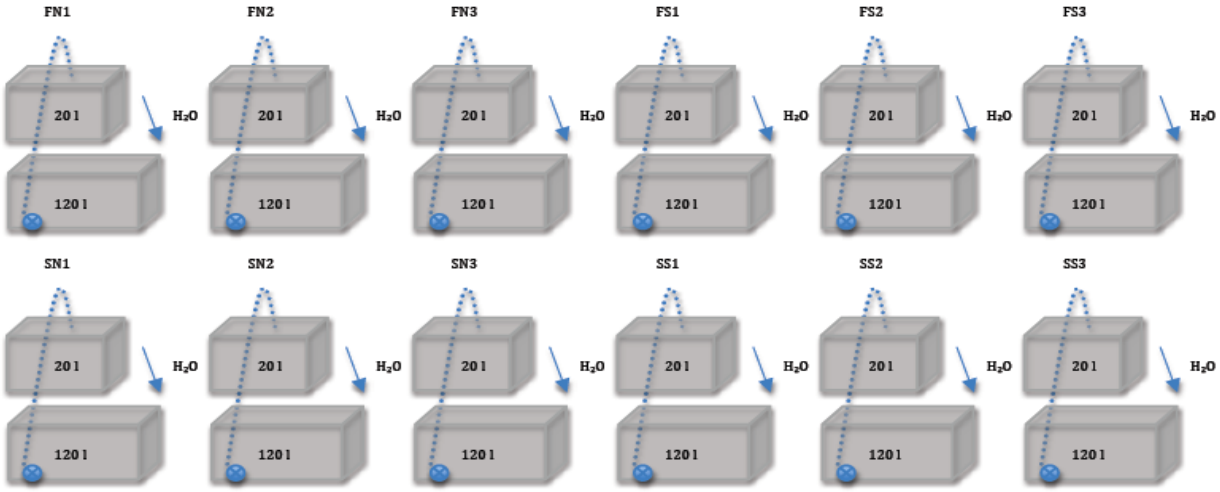


Figure 10- System design. Six tanks running with fresh water (top system). Six tanks running with salt water, 25 ppt, (bottom system). The arrows indicate the water flow from the pump (blue circle) in the bottom tank until the fish tank (top) and vice-versa.

4.4.2 Environmental Conditions:

Photoperiod – 12L: 12D

Water quality:

- pH: 6.5-8.0
- TAN-N: < 2 mg/l
- NO_2^--N: < 1.5 mg/l
- NO_3^--N: < 113 mg/l
- Temperature: 26-28 ºC
- Dissolved Oxygen (DO): > 6.5 mg/l
- Salinity: 0 and 25ppt

Daily water quality parameters were checked in order to maintain the levels mentioned above. In case of higher levels of nitrogen compounds, water was exchanged (10% of total volume). For that reason a 1000l tank filled with freshwater was prepared and maintained, and a 300l tank filled with seawater (seawater was prepared by mixing freshwater with *InstantOcean* sea salt) throughout the experiment. Water was treated with ozone produced by 2 ozone generators Aqua Forte KOIZO_3 Ozone Cell (three cells), producing O_3 through water electrolysis and

with UV.

Also from the third week until the end of the experiment, four water samples from each system were taken to analyze the carbon/nitrogen ratio.

On a daily basis, while the routines were being executed, the ozone devices were disconnected and the UV generator was connected. Redox potential was also measured every day in order to control the level of ozonation; above 700 mV the water was considered sterile.

4.4.3 Feeding Method and Regime:

The feed was sent to be sterilized (*Isotron*©, Netherlands) before starting the experiment and was sealed in plastic containers in daily portions per tank. Non-sterilized feed was also sealed in containers. Each day the left-overs of feed given to the larvae were stored for further analyses. Two types of feed were given: In half the salt water and fresh water tanks, larvae were fed with non-sterilized feed, while in the remaining six tanks they were fed with gamma irradiated, sterilized feed. Samples of feed were randomly collected in order to analyze microbial community composition from every sampling point of the experimental period.

A commercial starter, sterilized and non-sterilized feed (*F-0.5 GR Pro Aquabrut*- Trow Nutrition©, Germany) and was fed *ad libitum* from first feeding (7-11 days post fertilization) to juvenile (42 days). Fish were fed 3 times a day (9.00, 12.30 and 16.00) by hand, during a maximum of 30 minutes each time. Fish growth and feed utilization was also monitored.

Feed recipe
Fishmeal - 67.6%
Starch – 6.8%
Fish oil – 6.3%
Fish soluble – 6.7%
Gluten – 5%

4.4.4 Sampling Days

Days 0, 7, 14, 28, and 42 were scheduled as sampling points. Ten individuals per tank were sampled on each sampling day, (50 individuals in total). The remaining 50 individuals were collected at the end of the experiment for proximate analysis (approximately 60g) (Protocols in Appendix 1, 2, 3, 4 and 5).

4.5 Sampling and Storage

4.5.1 Feed Sampling and Storage

Feed microbial communities were analyzed during the pre-experimental period and experimental period, to assess if the feed-MC varied over time (in both sterilized and non-sterilized feed). Samples were stored at -80ºC until further microbial analyses.

4.5.2 Water Sampling and Storage

Homogeneous 250 ml water samples from each tank were analyzed. These samples were collected at the beginning of the experiment and in every sampling point. The water samples were filtered using 0.45 and 0.22 µm filters through a vacuum apparatus. The filters were stored at -80ºC for further microbial analyses.

4.5.3 Gut Sampling and Storage

At each sampling point, larvae were euthanatized by anaesthetic overdose (MS222). Only 10 individuals per tank (12* 10=120 larvae) per sampling were euthanatized (no dead larvae were maintained in the water or in the refrigerator/freezer until the time of sampling). Each larva was washed (held downwards) first with 70% ethanol and then with 18M-Ohm Milli Q water. Under the microscope, larvae were dissected by holding the abdominal cavity upwards and by aseptically removing the gut. All micro-dissecting tools and surfaces were sterilized. These tools were also rinsed with 70% ethanol and held over a Bunsen burner flame before sampling procedure, as well as between different gut extractions. Gut samples were transferred into 2.5ml cryopreservation tubes and were snapped

freeze in dry ice before storage at -80^{0}C until further microbial analyses.

4.6 Samples Analysis

Feed, water and gut microbial communities were analyzed by DNA extraction, PCR and DGGE and selected identified samples were sequenced, to profile the composition of the microbial communities.

4.6.1 DNA Extraction

Gut samples' DNA was extracted according to "WUR-modified Norwegian University of Science and Technology (NTNU) protocols". DNA from water and feed samples were set based on "FastDNA SPIN kit" for soil and the kit protocol was followed.

4.6.2 Polymerase Chain Reaction (PCR)

For this step, the primers were chosen in order to amplify only bacterial DNA. As so, the primers used were *968GC* (forward) and *1401* (reverse).

In a UV chamber, the Eppendorf tubes and PCR water were sterilized and the entire master mixes and dilutions were prepared, to avoid contaminations. Each master mix had the following components, with the following concentrations:

- dNTP: 1 µL
- 968GC: 2 µL
- 1401: 2 µL
- Phire: 1 µL
- Phire Buffer: 10 µL
- PCR Water: 33 µL

Total Reaction Volume - 50 µL: 49.0 µl + 1 µl DNA of sample

The DNA concentration in each sample was different according to the value obtained in the *Nanodrop* analysis (nucleic acid concentration). For this experiment the range of DNA concentrations was from 20 to 100 ng/µl.

The PCR program used in this work was completed in 35 cycles. Each cycle consisted of the following steps:

Pre-denaturation - 2 minutes at 95ºC

Denaturation - 30 seconds at 95ºC

Hybridization - 40 seconds at 56ºC

Elongation - 1 minute at 72ºC

Cool down - 4ºC

Storage temperature - 12 ºC

In order to visualize the PCR products, an Electophoresis in Agarose Gel was performed.

4.6.3 Denaturing Gradient Gel Electrophoresis (DGGE)

The PCR products were analyzed by the DGGE that was based on standard operating procedure "DGGE Version 25 January 2010, Laboratory of Microbiology, Wageningen University" (Appendix,8). DGGE products were, afterwards, analyzed by *BioNumerics* software to profile bacterial diversity.

4.7 Calculations

For the analysis of growth and feed intake, *Excel* software was used and the following calculations were done.

4.7.1 Growth:

- Growth Rate (GR):

  $GR = (W_t - W_0)/ t$ $(g.d^{-1})$ t = time (in days)

  W_t = body weight at time t

  W_0 = body weight at time 0

- Specific growth rate (SGR):

  $SGR = (\ln W_t - \ln W_0)/ t \times 100$ % (% bw.d^{-1})

- Geometric mean body weight (W_g):

  $W_g = e^{((\ln Wt + \ln W0)/2)}$ (g)

- Metabolic growth rate (MGR)

$$MGR = (W_t - W_0)/W_g^{0.8}/t \qquad (g.kg^{-0.8}.d^{-1})$$

4.7.2 Feed:

- Feed given (F):

F = amount of feed given during the measuring period. (g)

- Metabolic ration (R_m)

$$R_m = F/t/W_g^{0.8} \qquad g.kg^{-0.8}.d\text{-}^{1}$$

- Feed intake (FI):

$$FI = FW_i - FW_f \qquad (g)$$

4.7.3 Growth Efficiency:

- Feed conversion ratio (FCR):

$$FCR = (R_m/MGR) \qquad (g.g^{-1})$$

4.8 Statistical Analysis

All statistical analyses were performed using SPSS™ 19.0. The water quality parameters, FCR, SGR, feed intake and proximate analysis were compared within systems and treatments using two factor ANOVA ($p > 0.5$).

For the estimation of the similarity together with bacterial communities in larvae gut, water and feed, the profiles were analyzed by *BioNumerics* software based on Pearson correlation. Further, the Pearson values were compared within treatments using one way and two factor ANOVA ($p > 0.5$) and a *T-test* ($p > 0.5$).

5. Results

5.1 Water Quality

The means of temperature, pH, dissolved oxygen (DO), conductivity, total ammonia nitrogen (TAN), nitrite (NO_2^-), nitrate (NO_3^-), urea and phosphate, measured during the experimental period in different factors, salinity and feed sterilization, are represented in **Table IV.**

Temperature was not significantly different between factors (two factor ANOVA). Despite the similar values, there were significant differences in temperature during the 6 week experimental period (**Table V**). Nevertheless, no interaction was found between factor salinity and factor week (Repeated measurements ANOVA), feed sterilization and week (Repeated measurements ANOVA) and salinity, feed sterilization and week (Repeated measurements ANOVA). The calculated means for pH are equal, showing no variation of this parameter between or within treatments (**Table IV**). The maximum and minimum value of pH during week 1 was 8.3 and 7.6, respectively.

Conductivity was significantly higher in salt water (3.94±4.68E3 µS/cm) than in fresh water (351.4±54.9 µS/cm) (**Table IV**). This parameter demonstrates similar values in non-sterile and sterile feed (1.9E4 ±1.98E4 µS/cm and 1.9E4 ±1.85E4 µS/cm respectively) (two factor ANOVA). Still, there is no interaction among salinity and feed sterilization for conductivity (two factor ANOVA). Notwithstanding, when the week means are analyzed, an interception between week and salinity can be observed (Repeated measurements ANOVA) (**Table V**). In general, dissolved oxygen did not differ among the treatments (**Table IV**). Again, note that there is no interaction between factors (two factor ANOVA). Despite the similar values in each week, there is statistical difference between the first, fifth and sixth weeks. Furthermore, a significantly different value of the interception of salinity and week is present (Repeated measurements ANOVA) (**Table V**).

For the nitrogen compounds, TAN, NO_2^--N, NO_3^--N and urea-N, different results are presented in **Tables IV** and **V**. Despite TAN values keeping close to 0.1 mg/l-N in all treatments, the values of NO_2^--N, NO_3^--N and urea-N vary. A significant variation was observed within salinity, but there is no significant interaction between the factors in the all nitrogen compounds (two factor ANOVA). **Table V** shows the weekly variance of each parameter. For TAN, there are differences between weeks and an interaction between salinity and week (Repeated measurements ANOVA). NO_3^--N concentrations increased significantly over time, from 0.2±0.13 mg/l in week

one to 7.4 ±1.74mg/l in week six. An interaction can also be observed between factor salinity and feed sterilization (two factor ANOVA). NO_2^--N and urea-N followed a similar trend as NO_3^--N, there is an increase of concentrations over time and there is an interaction between salinity and week (Repeated measurements ANOVA).

Phosphate concentrations increased significantly over time and there can be observed a significant difference between fresh and salt water (two factor ANOVA). There is no significant interaction between the factors salinity x feed sterilization (two factor ANOVA) (**Table IV**), and the interaction between feed sterilization x week, but there are statistical differences for the interaction salinity x week (Repeated measurements ANOVA) (**Table V**).

Table IV
Values of water parameters, Temperature, pH, dissolved oxygen (DO), total ammonia nitrogen (TAN-N), nitrite (NO_2^--N), nitrate (NO_3^--N), urea-N and phosphate, during the experimental period in the treatment salinity (S) and feed sterilization (FS).

	Salinity		**Feed sterilization**		S x FS *p-value***
	Fresh	Salt	Non-Sterile	Sterile	
Temperature (ºC)*	26.7[a] ±0.48	26.6[a] ±0.41	26.5[a] ±0.34	26.7[a] ±0.49	0,961
pH*	8.1 ±0.09	8.1 ±0.06	8.1 ±0.08	8.1 ±0.08	
Conductivity (µS/cm)*	351.4[a]±54.9	3.9E4[b] ±4.68E3	1.9E4[a] ±1.98E4	1.9E4[a] ±1.85E4	0,637
DO (mg/l)*	7.8[a] ±0.48	7.8[a] ±0.45	7.8[a] ±0.46	7.8[a] ±0.46	0,420
TAN (mg/l-N)*	0.1[a] ±0.05	0.1[a] ±0.05	0.1[a] ±0.08	0.1[a] ±0.06	0,802
NO_2^- (mg/l-N)*	0.6[a] ±0.53	4.3[b] ±2.16	2.6[a] ±2.54	2.3[a] ±2.34	0,588
NO_3^- (mg/l-N)*	5.0[a] ±3.17	1.7[b] ±2.07	3.5[a] ±3.35	3.2[a] ±2.90	0,264
Urea (mg/l-N)*	0.2[a] ±0.22	0.2[b] ±0.10	0.2[a] ±0.17	0.2[a] ±0.2	0,582
Phosphate (mg/l- P)*	0.1[a] ±0.09	0.2[b] ±0.10	0.1[a] ±0.11	0.1[a] ±0.09	0,540

*Values are given as mean ± SD. Means followed with different subscript letter (**row**) are statistically different (p<0.05)
***p-values* of the interception of factor salinity and treatment feed sterilization(two factor ANOVA (p<0.05)).

The carbon nitrogen (C:N) ratio was measured during the last three weeks of the experiment in each treatment. The higher values for C:N ratio found were 2.5:1 in fresh water, 4.5:1 in salt water, 3.3:1 in non-sterile feed and 3.1:1 in sterile feed, occur in the fourth week (Appendix 8). With time, the concentration of carbon becomes lower and the concentration of nitrogen compounds increases. There can be observed a significant difference among fresh and salt water systems in total carbon and total nitrogen (two factor ANOVA) (**Table IV**). This disequilibrium limits carbon and the C:N ratio consequently decreases, especially in the fresh water system (**Table IV**). The values of C:N ratio during the last experimental week are 0.8:1 for fresh water, 2.1:1 for salt water, 1.4:1 for non-sterile feed and 1.1:1 for sterile feed (Apendix 8).

Table V

Calculated means of water parameters, Temperature, pH, dissolved oxygen (DO), total ammonia nitrogen (TAN-N), nitrite (NO_2^--N), nitrate (NO_3^--N), urea-N and phosphate, during the six experimental weeks.

	Week						Salinity x Week	Feed Steri. x Week	Salinity x Feed Steri. x Week
	1	2	3	4	5	6	*p-value***	*p-value***	*p-value***
Temperature (Cº)*	26.5^{a} ±0.39	26.7^{b} ±0.36	26.7^{b} ±0.33	26.6^{ab} ±0.32	26.6^{ab} ±0.23	26.8^{c} ±0.29	0.955	0.857	0.888
pH	7.6- 8.3	7.9- 8.2	8- 8.2	7.9- 8.2	8- 8.4	7.9 - 8.2			
Conductivity (µS/cm)*	1.9E4^{a} ±1.96E4	1.9E4^{a} ±2.01E4	2.1E4^{b} ±2.08E4	2.1E4^{b} ±2.05E4	1.9E4^{ac} ±1.90E4	1.9E4^{bc} ±1.91E4	**0**	0.973	0.760
DO (mg/l)*	7.8^{a} ±0.08	7.4^{a}±0.04	7.8^{a} ±0.11	7.9^{a} ±0.05	8.0^{b} ±0.07	8.1^{c} ±0.04	**0**	0.109	0.714
TAN (mg/l-N)*	0.1^{ab} ±0.03	0.0^{a} ±0.0	0.1^{ab} ±0.03	0.1^{b} ±0.05	0.1^{b} ±0.03	0.2^{ab} ±0.1	0.076	0.375	0.721
NO_2^- (mg/l-N)*	1.5^{a} ±0.23	1.6^{a} ±1.20	2.1^{b} ±2.05	2.5^{b} ±2.39	3.5^{c}±3.33	3.5^{c} ±3.30	**0.002**	0.289	0.324
NO_3^- (mg/l-N)*	0.2^{a} ±0.13	1.1^{b} ±1.02	3.1^{c} ±1.86	3.6^{d} ±2.77	4.7^{e} ±3.31	7.4^{f} ±1.74	**0**	0.344	0.347
Urea (mg/l-N)*	0.1^{a} ±0.16	0.1^{a} ±0.05	0.1^{a} ±0.04	0.2^{a} ±0.07	0.3^{a} ±0.15	0.4^{b} ±0.24	**0**	0.514	0.489
Phosphate (mg/l- P)*	0.1^{a} ±0.04	0.1^{b} ±0.05	0.1^{ab} ±0.07	0.1^{a} ±0.05	0.2^{c} ±0.07	0.3^{d} ±0.07	**0**	0.386	0.876

*Values are given as mean ± SD. Means followed with different subscript letter (**row**) are statistically different (p<0.05).

***p-values* of the interception of factor salinity and treatment feed sterilization and week (repeated measurements ANOVA(p<0.05)). The values in bold have statistical differences.

Table VI

Measured values of inorganic carbon (IC), non-purgeable organic carbon (NPOC), total carbon (TC), total ammonia nitrogen (TAN-N), nitrite (NO_2^--N) + nitrate (NO_3^--N), total nitrogen (TN) and Carbon Nitrogen ratio in three different weeks in the two factors, salinity (fresh and salt) and feed sterilization (non-sterile feed and sterile feed).

	Salinity		Feed sterilization		S x FS
	Fresh	Salt	Non-Sterile	Sterile	*p-value***
IC (mg/l)*	12.2^{a} ±3.85	17.1^{a} ±2.17	15.3 ^{a}±3.77	14.0^{a} ±3.95	0.346
NPOC (mg/l)*	4.6 ^{a}±1.00	2.8^{b} ±1.13	3.7^{a} ±0.50	3.7^{a} ±2.00	0.11
TC (mg/l)*	17.1^{a} ±2.24	21.3^{b} ±2.72	19.7^{a} ±3.56	18.7^{a} ±3.14	0.262
$NH4^+$ (mg/l-N)*	0.1^{a} ±0.03	0.1^{a} ±0.04	0.1^{a} ±0.04	0.09^{a} ±0.04	0.814
(NO_3^- + NO_2^-)(mg/l-N)*	10.8^{a} ±4.90	6.7^{b} ±2.46	8.6^{a} ±4.30	8.9^{a} ±4.60	0.82
NT (mg/l)*	12.1^{a} ±5.27	7.4^{b} ±2.67	9.5^{a} ±4.46	9.9^{a} ±5.31	0.971
Ratio C:N	1.4 :1	2.9:1	2.1 :1	1.9 :1	

*Values are given as mean ± SD. Means followed with different subscript letter (**row**) are statistically different (p<0.05).

***p-values* of the interception of factor salinity, factor feed sterilization (two way ANOVA (p<0.05)).

5.2 Growth Parameters

Feed intake, metabolic ration (Rm), feed conversion ratio (FCR) and metabolic growth ratio (MGR) were not influenced by salinity or feed sterilization (two factor ANOVA). The mean value is 13.4 g, 15.5 g/kg$^{0.8}$/aquaria, 0.7 and 24.5 g/kg$^{0.8}$/aquaria, respectively **(Table VII)**. This means that the fresh water treatment fish grew as much as salt water treatment ones. It can also be observed in the same table that for all growth parameters, no interaction between factors exists during the entire-experimental period (two factor ANOVA).

Table VII
Calculated means for fish performance, feed intake, metabolic ration (Rm), feed conversion ratio (FCR) and metabolic growth ratio (MGR) in each factor (salinity (S) and feed sterilization (FS)).

	Salinity		Feed Sterilization		S x FS *p-value***
	Fresh	Salt	Non-Sterile	Sterile	
Feed intake (g/aquaria)*	13.4^{a} ±1.70	13.1^{a} ±1.66	13.4^{a} ±1.69	13.1^{a} ±1.66	0,504
Rm (g/kg$^{0.8}$/aquaria)*	15.7^{a} ±1.99	15.6^{a} ±1.42	15.5^{a} ±1.74	15.8^{a} ±1.70	0,317
FCR (g/g/aquaria)*	0.7^{a} ±0.08	0.7^{a} ±0.11	0.7^{a} ±0.09	0.7^{a} ±0.08	0,295
MGR (g/kg$^{0.8}$/aquaria)*	24.3^{a} ±3.48	24.3^{a} ±2.93	24.7^{a} ±3.68	23.9^{a} ±2.62	0,893

*Values are given as mean ± SD.
***p-values* of the interception of factor salinity and factor feed sterilization (two factor ANOVA (p<0.05)).

The growth performance changed over time (**Table VIII).** Feed intake values increase per week, from 1.4±0.04 g/week to 52.5±1.09 g/week, and are statistically different. Also note that salinity (fresh and salt) and feed-sterilization (non-sterile and sterile) has a significant interaction within the period (Repeated measurements ANOVA) for both cases.

The metabolic ration obtained in this study shows a low value in days one (14.2±0.47 g/kg$^{0.8}$/week) and a significantly high value on period three (17.5±0.98 g/kg$^{0.8}$/week). No significant interaction was found between either each factor or both factors against period (Repeated measurements ANOVA).

Growth expressed in metabolic weight follows an increasing curve. The lowest values are observed in period one (20.0±1.74 g/kg$^{0.8}$/week) to four (24.8±2.60g/kg$^{0.8}$/week). However, there are no statistical differences in growth during the same period. Additionally, no significant differences between factor and period are apparent, despite the low *p-values* obtained (Repeated measurements ANOVA).

The FCR values shown are those with less variation compared to the previously described values. Nevertheless, significant differences between the highest value (0.7g/g/week ±0.07) and the lower value (0.6g/g/week ±0.05) can be observed in periods four and three, respectively. In this case, note an interception between factor feed sterilization and period (Repeated measurements ANOVA).

Table VIII
Calculated means for fish performance: feed intake, metabolic ration (Rm), feed conversion ratio (FCR) and metabolic growth ratio (MGR) for the four growth periods.

	Period				Water x Period *p-value***	Feed x Period *p-value***	Water x Feed x Period *p-value***
	1	2	3***	4***			
Feed intake (g/week)*	1.4^{a} ±0.04	3.6^{b} ±0.35	22.1^{c} ±1.20	52.5^{d} ±1.09	**0,02**	**0,02**	0,77
Rm (g/kg$^{0.8}$/week)*	14.2^{a} ±0.47	14.9^{b} ±1.24	17.5^{c} ±0.98	16.1^{bc} ±1.68	0,20	0,15	0,26
FCR (g/g/week)*	0.7^{b}±0.08	0.6^{a}±0.05	0.7^{bc}±0.08	0.7^{c}±0.07	0,74	**0,02**	0,07
MGR (g/kg$^{0.8}$/week)*	20.0^{a} ±1.74	25.8^{b} ±1.72	26.2^{b} ±2.50	24.8^{b} ±2.60	0,07	0,07	0,47

*Values are given as mean ± SD. Means followed with different subscript letter (**row**) are statistically different (p<0.05).
***p-values* of the interception of factor salinity and factor feed-sterilization and period (repeated measurements ANOVA(p<0.05)). The values in bold have statistical differences.
***Period 3 corresponds to week 3 and 4; Period 4 corresponds to week 5 and 6.

5.3 Proximate Analysis

After the end of the experiment the fish that remained in the aquaria were collected and their content analyzed in terms of: dry matter (DM), ash, crude protein, energy and fat. The results are shown in **Table IX**.

The dry matter content does not have significant variation within treatments; the higher value is observed in treatment fresh water (195.5±4.16 g/kg), as well as the lower (182.2±9.80 g/kg). Also note a *p-value* higher that 0.05 (two factor ANOVA), which suggests no interaction between factors. Ash has a higher value in treatment fresh water as well (120.9±0.82 g/kg DM), which is significantly different from the value of the other treatments. Once more there is no interaction among salinity and feed sterilization (two factor ANOVA). Crude protein, energy and fat do not have significant differences among the factors salinity and feed sterilization, having the higher values of 558.7±4.00 g/kg DM, 25.6±0.29 g/kg DM and 320.6±3.27g/kg DM, respectively.

Table IX
Values of dry matter content (DM), ash, crude protein, energy and fat in fish larvae from the different treatments: Salinity (S) and feed sterilization (FS).

Factor	Salinity		Feed Sterilization		S x FS *p-value***
	Fresh	Salt	Non-Sterile	Sterile	
Dry Matter g/kg*	195.5^{b} ±4.16	182.2^{a} ±9.80	192.8^{a} ±6.22	184.3^{a} ±11.69	0,275
Ash g/kg DM*	120.9^{b} ±0.82	116.8^{a} ±1.52	120.2^{a} ±1.07	117.6^{a} ±1.85	0,333
C.Protein g/kg DM*	553.0^{a} ±2.58	555.9^{a} ±5.57	558.7^{a} ±4.00	555.2^{a} ±6.64	0,707
Energy g/kg DM*	25.4^{a} ±0.12	25.4^{a}±0.22	25.3^{a} ±0.18	25.6^{a} ±0.29	0,345
Fat g/kg DM*	312.0^{a} ±1.71	320.6^{a} ±3.27	312.0^{a} ±2.20	320.6^{a} ±3.58	0,418

*Values are given as mean ± SD. Means per factor followed with different subscript letter (**row**) are statistically different (p<0.05).
***p-values* of the interception of factor salinity and factor feed sterilization (two ways ANOVA (p<0.05)).

The content in dry matter (DM), ash, crude protein, energy and fat was also analyzed for the feed (**Table X**).

Significant (*t-test)* high values for ash, (114.1±0.34 g/kg DM) crude protein, (618.7±5.18 g/kg DM) energy, (23.3±0.16 g/kg DM) and fat (184.4±0.49 g/kg DM) in the non-sterile feed compared with sterile feed can be seen in **Table X** On the other hand the dry matter content is significantly higher in sterile feed (876.5±0.21 g/kg), compared to non-sterile feed (868.2±0.02 g/kg).

Table X
Values of dry matter content, ash, crude protein, energy and fat in non-sterile feed and sterile feed.

	Feed	
	Non-sterile	Sterile
Dry Matter g/kg*	868.2^{a} ±0.02	876.5^{b} ±0.21
Ash g/kg DM*	114.1^{b} ±0.34	113.8^{a} ±0.20
C.Protein g/kg DM*	618.7^{b} ± 5.18	617.0^{a} ±0.53
Energy g/kg DM*	23.3^{b} ±0.16	23.2^{a} ±0.14
Fat g/kg DM*	184.4^{b} ±0.49	183.0^{a} ±0.11

*Values are given as mean ± SD. Means followed with different subscript letter (**row**) are statistically different (*T-test p-value <0.05)*

5.4 Microbial Communities

All of the following results are presented based on the similarity values obtained by the Pearson correlation matrixes.

5.4.1 Microbial Communities in the gut:

Over time

The means of similarity within individuals within tanks (FN, FS, SN) are high (above 75%) and similar over time as can be observed in **Figure 11**. The SS treatment is the only one that does not have this trait, having two values out of the range observed at sampling point 2 (27.2±21.00%) and sampling point 4 (60.1±18.79%). Significant differences (one factor ANOVA) at particular sampling points can be seen, such as in the FN treatment at sampling points 1 (69.2±13.80%) and 3 (39.9±22.16%), in the FS treatment at sampling points 0 (69.5±15.01%) and 3 (78.1±17.81%), in the SN treatment at sampling points 1 (92.7±3.15%) and 2 (79.0±11.98%) and finally in the SS treatment at sampling points 1 (64.2±19.80%) and 2 (44.5±16.86%) (Appendix 10). As a result, the fresh water treatments start to diverge in similarity in a later sampling point, compared with salt water treatments. From that point forward three situations are relevant enough to highlight: the similarity inside the system at the end of the experiment (sampling point 5) is statistically equal to sampling 0, as

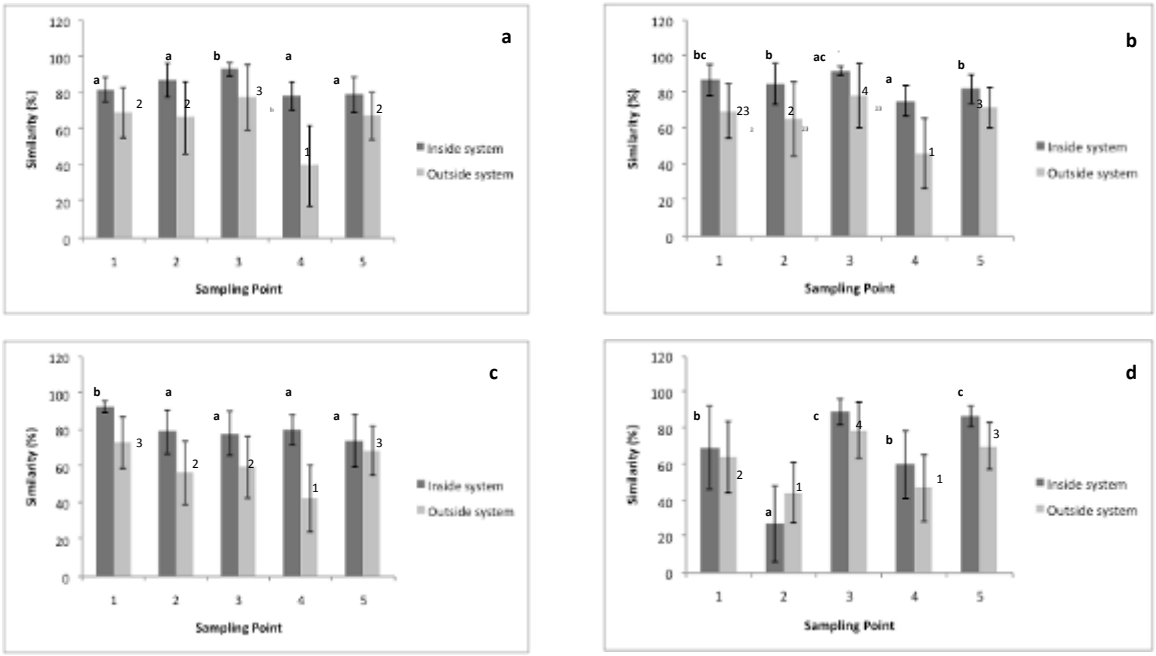


Figure 11- Similarities inside and outside systems, based on Pearson correlation similarity values. Means followed with different subscript letter are statistically different inside treatments, over time (p<0.05). Means followed with different subscript number are statistically different outside treatments, over time (p<0.05). a. Fresh water non-sterile feed treatment (FN); b. Fresh water sterile feed treatment (FS); c. Salt water non-sterile feed treatment (SN); d. Salt water sterile feed treatment (SS).

the cases of treatments FN and FS. The similarity stabilizes (SN treatment) until the end (sampling point 5) or even have significantly different oscillations until sampling point 5 (SS treatment).

The results obtained by similarities outside of the treatment are low and the minimum values observed in all treatments correspond to sampling point 3. They are significantly different (one way ANOVA) from the others, apart from treatment SS (44.5±16.86% is equal to 47.0±18.34%) (Appendix 10). Fresh water treatments have an increase of similarity from sampling point 1 to 3, while saltwater treatment (SN) has a decrease of similarity in the same period. SS treatment is a particular case, since on sampling point 2 were removed three fish guts, due to bad profiles on the DGGE. Can be made the assumption, that SS follows the same trait as the fresh water treatments. At the end of the experiment is observed an increase of similarity in all treatments. Was also compared the values of similarity inside and outside each treatment and as a result there are differences between these two values in each sampling point (*t-test* p<0.05*)*.

Between Treatments

Table XI shows the means of gut similarity between treatments. Over time, the gut profiles, in the different treatments have some significant variability. In fresh water treatment, the maximum value occurs on day 7 (88.2±9.52%) and the minimum, on day 28 (76.9±8.28%). Contrarily, in salt water treatment the higher value occurs on day 14

(83.7±11.40%) and the lower on day 7 (63.7±28.14%). Salinity does have a significant effect in some sampling points, namely on day 7 (two factor ANOVA p=0.000*)* and on day 28 (two factor ANOVA p=0.001).

For non-sterile feed the higher similarity is occurs on day 0 (89.6±6.57%) with the lower similarity occurring on day 42 (76.6±12.43%). Sterile feed high and low similarities are 84.2±7.32% and 64.7±16.35% for days 42 and 7, respectively. Contrarily to factor salinity, factor feed sterilization shows a significant effect on gut profiles in all sampling points (two factor ANOVA p<0.05).

Table XI
Calculated means of Pearson correlation similarities of fish gut overtime by treatment. Each mean represents the average of gut similarity within the different treatments per different sampling days.

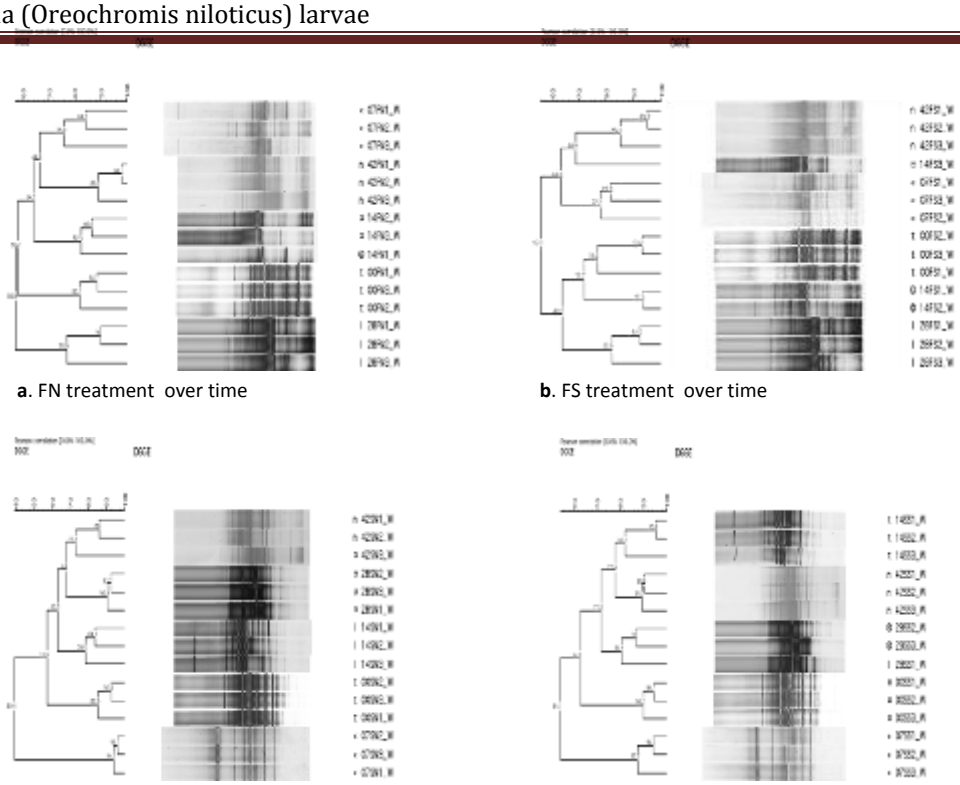
	Sampling day				
	0	**7**	**14**	**28**	**42**
Fresh Water*	84.3^{ab} ±7.88	88.2^{b}±9.52	85.3^{b} ±8.85	76.9^{a} ±8.28	80.4^{a} ±9.02
Salt Water*	83.4^{b} ±18.40	63.7^{a} ±28.14	83.7^{b} ±11-40	70.2^{a} ±17.58	80.5^{b} ±12.51
Non-sterile F.*	89.6^{b} ±6.57	86.1^{b} ±11.37	78.3^{a} ±10.09	79.4^{a} ±7.93	76.6^{a} ±12.43
Sterile F.*	75.9^{ab} ±20.43	67.7^{a} ±3.20	90.7^{c} ±5.49	67.7^{a} ±16.35	84.2^{bc} ±7.32

*Values are given as mean ± SD. Means followed with different subscript letter (**row**) are statistically different (one factor ANOVA p<0.05)

5.4.2 Microbial Communities in Water

Over time

Figure 12 a, b, c and d show the clustered water profiles per treatment (FN, FS, SN and SS) and over time. Correlation values of similarity were calculated for all means of similarity within and between treatments (Appendix 10), based on Pearson correlation. Except for the FS treatment in day 14, all water samples from the same treatment are clustered together and demonstrate a high value of similarity (above 75%). Nevertheless, over time these values are not significantly different (one factor ANOVA p>0.05). There is a significant increase of similarity outside treatments over time (one factor ANOVA p<0.05), with a common maximum value on day 42 71.0±18.52% for FN treatment, 70.0±16.84% for FS treatment, 77.7±9.85% for SN treatment and 67.2±11.19% for SS treatment. Due to an increase of similarity outside treatments, over time the difference between similarities inside and outside treatments ceases to be significant (*t-test p*>0.05).



a. FN treatment over time

b. FS treatment over time

c. SN treatment over time

d. SS treatment over time

Figure 12- DGGE profiles of water in 3 tanks per treatment over time, based on Pearson correlation matrixes. a. Fresh water non-sterile feed treatment (FN); b. Fresh water sterile feed treatment (FS); c. Salt water non-sterile feed treatment (SN); d. Salt water sterile feed treatment (SS).

5.4.3. Relation Between Gut and Water Over time

Water has a significant effect in gut over time, despite the low values of similarity presented. **Table XII**, shows a different trait between treatments. At the beginning of the experiment, day 0, water had a low effect in fish guts profiles in both fresh and saltwater treatments (37.6±7.39% and 10.5±7.31%). The two factors ANOVA analysis shows a significant difference between salt and fresh water treatments (p=0.000) at the same sampling point. Within time the similarities of system water and fish gut increase significantly in both treatments. At day 7 the fresh water treatment achieved maximum similarity (54.2±14.30%), while only on day 42 (the end of the experiment) the salt water reaches the maximum (30.2±14.56%). Note that in all sampling days, 0, 7, 14, 28 and 42, the similarities of fresh and salt water are significantly different, which points to an effect of salinity in the microbial communities of water and fish gut.

A different trait is present in factor feed sterilization. ANOVA shows not significant similarity in non-sterile feed and sterile feed in days 0 and 7. The highest and lowest similarities in this factor correspond to the same sampling days (42 and 28), as can be observed in **Table XII**. Feed sterilization factor presents high similarities over time between water and fish gut, when compared to salt water.

Table XII
Calculated means of Pearson correlation similarities, between fish gut and water system over time in the different treatments: salinity (fresh and salt water) and feed sterilization (non-sterile and sterile feed). Each mean represents the average of gut similarity with water system in the different treatments per different sampling days.

	Sampling days				
	0	**7**	**14**	**28**	**42**
Fresh water*	37.6^{a} ±7.39	54.2^{c} ±14.30	51.0^{c} ±13.90	41.6^{ab} ±11.00	43.7^{b} ±11.81
Salt Water*	10.5^{a} ±7.31	12.8^{ab} ±11.88	23.4^{c} ±16.00	17.2^{b} ±8.83	30.2^{d} ±14.56
Non-sterile F.*	25.5^{a} ±16.30	34.3^{ab} ±25.52	40.4^{c} ±22.80	24.7^{a} ±17.39	39.2^{b} ±17.54
Sterile F.*	24.2^{ab} ±20.21	27.1^{ab} ±23.34	31.2^{b} ±24.85	19.4^{a} ±15.48	32.9^{ab} ±19.66

*Values are given as mean ± SD. Means followed with different subscript letter (**row**) are statistically different (one factor ANOVA $p<0.05$)

5.4.4 Relation Between Gut and Feed Over time

At the first sampling point, day 0, feed similarity with fish gut is almost null in all treatments (**Table XIII**). No significant increase (one factor ANOVA) of similarity from day 0 to day 14 is apparent in factor salinity and non-sterile feed treatment. Nevertheless, despite this non-significant increase in similarity between fish gut and feed, a significant effect between non-sterile feed and sterile is present in days 7 and 14. Feed continues to have a significant effect (one factor ANOVA) on fish gut from the fresh water system until the end of the experiment, while in the saltwater treatment this effect ceases on day 14 until the end of the experiment. There is a significant effect of salinity in days 0, 14, 28 and 42. There is no interaction between factors on days 0, 28 and 42 (two factor ANOVA *p>0.05)*. The oddest results appeared when each type of feed, non-sterile and sterile, was plotted with the respective gut profiles. A significant difference was expected between the pairs *non-sterile feed–gut*, and *sterile feed–gut*. In day 0 and 28 this did not happen (two factor ANOVA p >0.005), meaning that either there was no feed sterilization or there was contamination during daily feeding or during posterior analysis.

Table XIII
Calculated means of Pearson correlation similarities, between fish gut and feed overtime in the different treatments: salinity (fresh and salt water) and feed sterilization (non-sterile and sterile feed). Each mean represents the average of gut similarity with feed in the different treatments per different sampling days

	Sampling day				
	0	**7**	**14**	**28**	**42**
Fresh water*	0.4^{a} ±1.02	9.4^{a} ±5.83	23.7^{b} ±20.40	49.0^{d} ±5.18	21.7^{c} ±11.09
Salt Water*	3.5^{a} ±2.94	10.1^{a} ±8.65	33.7^{b} ±27.00	18.8^{b} ±15.11	30.4^{b} ±14.12
Non-sterile F.*	3.5^{a} ±3.88	12.8^{a} ±7.34	51.3^{c} ±10.35	35.7^{b} ±17.21	32.0^{b} ±11.46
Sterile F.*	0.9^{a} ±1.73	6.27^{a} ±5.51	6.1^{a} ±2.84	32.3^{b} ±20.88	16.1^{b}±16.11

*Values are given as mean ± SD. Means followed with different subscript letter (**row**) are statistically different (one factor ANOVA $p<0.05$)

5.4.5 Relation Between Water with Feed Over time

Table XIV presents the percentage of similarity between water system and feed. Water from fresh and salt water systems against feed, over time, is always significantly different between which other (two factor ANOVA $p< 0.005$). Fresh water similarity with feed increases significantly at day 28 (61.6±7.90%). It is also at this point that the similarity starts to stabilize (no significant differences between and 28 and 42). Salt water has a different pattern, which starts with the second maximum of similarity at day 0 (39.9±3.96%), then the similarity drops significantly at day 14 (13.0±4.02%) and increases again on day 42 (46.7±8.74%).

In factor feed sterilization, over time, there are significant differences of non-sterile and sterile feed, from day 14 to day 42. Non-sterile treatment suffers a significant decrease of similarity from day 0 (27.6±9.40%) to day 7 (14.5±1.98%) and increases at day 28 (50.6±19.37%). For sterile feed there are no relevant significant differences between sampling points.

All treatments have a dominant tendency: within time, the similarity between water system and feed given becomes more similar. The two factors ANOVA results support this through a significant interception between factors on the first 3 sampling points.

Table XIV

Calculated means of Pearson correlation similarities, between system water and feed overtime in the different treatments: salinity (fresh and salt water) and feed sterilization (non-sterile and sterile feed). Each mean represents the average of water system similarity with feed in the different treatments per different sampling days.

	Sampling day				
	0	**7**	**14**	**28**	**42**
Fresh water*	23.7^{a} ±7.93	13.2^{a} ±4.18	38.4^{a} ±30.42	61.6^{b} ±7.90	65.8^{b} ±11.77
Salt Water*	30.9^{a} ±3.96	19.5^{ab} ±6.96	13.0^{b} ±4.02	25.2^{ab} ±9.16	46.7^{c} ±8.74
Non-sterile F.*	27.6^{ab} ±9.40	14.5^{a} ±1.98	41.1^{ab} ±27.62	50.6^{b} ±19.37	57.2^{b} ±11.72
Sterile F.*	26.9^{abc} ±6.44	18.2^{ab} ±8.84	10.3^{a} ±1.62	36.2^{bc} ±20.97	45.3^{c} ±6.66

*Values are given as mean ± SD. Means followed with different subscript letter (**row**) are statistically different (one factor ANOVA $p<0.05$)

6. Discussion

In this study the factors salinity and feed sterilization were tested. Salinity does not have a clear effect on fish gut microbial communities, contrary to that was observed in literature. In the other hand, feed sterilization has a significant effect on gut microbiota over time. Although the microbial communities were different, fish performance was similar between treatments.

6.1 Microbial Communities in Fish Gut

6.1.1 Gut Over time

Similarity of DGGE bacteria profiles within FN, FS, SN treatments is high, meaning that fish were subjected to the same bacterial load within systems (**Figure 11**). However, significant differences were found between sampling points over time (one factor ANOVA $p<0.05$). This conclusion is supported by studies from Al-Harbi and Uddin (2004) and Varsamos *et al*. (2005), who also found significant differences in gut microbiota over time. For the SS treatment this pattern was not applicable. On day 7, three out of nine SS guts were removed from the results due to poor DGGE profiles. Taking into account the similarity indexes in other sampling points, it can be concluded that, had there not been a problem with the DGGE, this treatment would have followed the same tendency as the others.

6.1.2 Effect of Salinity

Despite the discrepancies observed between saltwater and freshwater treatments in **Figure 11**, salinity only had a significant effect in the microbial communities in two out of five sampling points, day 7 and day 28 (**Table XI**). The results on sampling point 1 (day 0) can be explained by the reduced amount of DNA products on some gut profiles, which means that most of the sampled larvae had a sterile gut due to their mouths being closed (Ringo *et al.,* 1995). As so, samples might not have been illustrative of the system reality. On the other hand, and in the same sampling point larvae, which already had their mouth open, were subjected to a bacterial load before first feeding. Verner-Jeffreys *et al.* (2003), counted

approximately 10^2 CFU $larva^{-1}$ in the early stages of larval Atlantic halibut, *Hippoglossus hippoglossus* development. This load mostly derives from the effect of the surrounding environment (Ringo *et al.,* 1995; Grisez *et al*., 1997; Ringø and Gatesoupe, 1998).

Because the subject is fresh and salt water, it would be expected that the two environmental conditions had different effects (Sakata *et al.* 1980; Ringo *et al.,* 1995). However, this was not the case, and the high similarity in the bacterial communities of the fish gut, in both fresh and salt water treatments (**Table XI**), can be explained by an identical genetic composition in the bacterial flora fresh and salt system waters (**Figure 12**), as was observed by Sakata *et al.* (1980). These authors concluded that, in terms of diversity, water samples profiles were very similar, changing only when it came to the dominance of bacteria.

Because marine fish face dehydration, water ingestion begins before the total absorption of the yolk sac in order to maintain homeostasis (Reitan *et al.,* 1998; Olafsen, 2001; Varsamos *et al.,* 2005). As such, a higher influence of water on fish gut was expected in the early developmental stages in the saltwater treatment, due to the high drinking behavior observed in marine species. In sampling point 1, day 0, a low percentage of similarity was calculated between microbiota of larvae gut from salt water and system water (10.5±7.31%). In the same day, a high percentage similarity was calculated between fresh water fish gut DGGE profiles and system water (37.6±7.39%) (**Table XII**).

Miyazaki *et al*. (1998) measured the drinking ratio (water ingestion) of Mozambique tilapia in fresh and salt water. Results indicated that tilapia from salt water had a higher drinking rate and started ingesting water sooner when compared with fresh water tilapia. In the present experiment, over time, there was an increase of similarity between fish gut from salt water and system water, which resulted from the proliferation of bacterial communities common to both groups.

Almost every day, 10-20% of water was removed (recommended water exchange in Avnimelech, 2009 was 10%) from the saltwater treatment and replaced with ozonated salt water to control nitrite and maintain good water quality. This might have affected the establishment of the bacterial communities in the saltwater

treatment. Other explanation for the low similarity values between fish gut and water is the carbon/nitrogen (C:N) ratio (**Table VI**). Heterotrophic bacteria utilize carbohydrate sugars, starch and cellulose as a food source to produce energy for growth and reproduction (Avnimelech, 1999). In a culture system, these energy sources originate from fish waste and non-eaten feed (Azim *et al.,* 2008). An optimum C:N ratio in active suspension ponds is very important, especially for the water quality. If the carbon is a limiting factor, unwanted compounds like ammonia and nitrite start to dangerously accumulate, (**Table VI**) compromising the growth of heterotrophic bacteria (Avnimelech, 1999; Avnimelech, 2006). If the system is carbon limited, it is natural that no relevant effect of the system water was observed in the gut of larvae from both fresh and salt water (Avnimelech, 1999). In salt water treatment, the poor C:N ratio and frequent water exchange could have contributed to a destabilization of the bacterial communities in the system water. The C:N average ratio observed in the present study was 2.9:1 in salt water and 1.4:1 in fresh water,(**Table VI**). Crab *et al.* (2010) considered 10:1 to be an optimal C:N ratio in fish ponds.

Several authors suggested the addition of an extra source of carbon to improve bacterial growth (Avnimelech, 1999; Schneider *et al*., 2006; Azin and Little 2008; Crab *et al*., 2010). In the present study, no carbon was added since there was the possibility of manipulating the bacterial communities in the water.

Feed microbial communities are one of the major initial colonizers of fish gut (Ringo *et al.,* 1995; Grisez *et al*., 1997; Ringø and Gatesoupe, 1998). With the first feed, the bacterial load in fish larvae increases (Verner-Jeffreys *et al.,* 2003; Reid *et al.,* 2009). This statement is in line with the results obtained in this study, (**Table XIII**) since an increase in similarity between feed and fish larvae was registered, after first feeding, in both fresh and salt water treatments. Feed has less effect on saltwater guts because the similarity became stable in the middle of the experiment (Day 14). Nonetheless, feed has a higher similarity over time when compared with system water, which can represent a higher effect of feed in fish gut at the beginning of intestinal colonization.

For the fresh water treatment, feed similarity was highest on sampling point

4, the same point where the minimum similarity between system water and fish gut was observed. There was, however, no evidence that the effect of feed would prevail over water, in larvae micro flora, over time. Water and feed profiles are related and can influence each other (**Table XIV**). The major factor that manipulates the similarity between water and feed of fresh and saltwater treatments is the capacity of the bacteria present in feed to tolerate NaCl concentrations (Sakata, 1980). Nevertheless these facts do not explain the differences between the effect of feed in fresh and saltwater treatments.

Salinity affects the dominance of gastrointestinal bacterial communities in fresh and salt water adult fish, as was reported by Sakata *et al.* (1980) in Nile Tilapia and by Ringo *et al*. (1995) in salmonids. Differences between the gut flora of larvae and adult fish (Sugita *et al.* 1988) and the two months time frame necessary for the establishment of gut microbiota in Nile tilapia (Sugita *et al.,* 1982 *in* Sugita *et al.* 1988), might explain the fluctuation in similarities between fresh and saltwater treatments **(Table XI).** Nevertheless, to be certain of any salinity effect, some profiles should be sequenced to identify the groups of bacteria present in the guts of the different treatments.

6.1.3 Effect of Feed Sterilization

It is of general knowledge that feed irradiation is an effective method of controlling bacterial populations, especially pathogens (Ogbato, 1988). With no known exception, the present results are in line with this statement that feed sterilization affects larval gut microbial communities over time (**Table XI**). There is little research regarding the effect of feed sterilization in fish micro flora, and the one that exists strictly concerns live feed, such as *rotifers* (Munro *et al.,* 1999) and *artemia* (Gimenez *et al.,* 2006). For obvious reasons, comparing live feed to artificial feed would not be correct.

Despite the main result of feed sterilization affecting gut microbiota, variations of similarity were observed over time within non-sterile and sterile feed treatments (**Table XI**). According to the obtained results, sterile feed fed fish exhibited significant variance in similarity during the experiment. Days 7 and 28 were

those with the less pronounced similarity in fish gut, representing a differential response of larvae to sterile feed. Non-sterile feed had a higher effect on fish gut in the early sampling points (day 0 and 7) compared with the latest (similarity stayed the same). As the gut is considered sterile (Ringo *et al.,* 1995) the introduction of feed has an enormous effect on the gut micro flora (Verner-Jeffreys, 2003; Reid *et al.,* 2009).

Water DGGE profiles are becoming more similar with fish DGGE profiles fed with non-sterile feed (**Table XII**) mostly due to the eventual non-eaten pellets full of bacteria and due to fish waste (Sakata *et al.,* 1980). Some bacterial species are well adapted to aquatic environments so, with space and nutrients, the populations compete and the best adapted will proliferate, (Ringo and Olsen, 1999) making profiles more similar. As sterile feed is free of bacteria, the similarity between water and fish gut seemed to stabilize after day 28 (**Table XII**). A lower similarity of water with larvae fed with non-sterile feed was expected, so it is likely that the entire bacterial load came from the communities established in the system water.

System water has an equal effect on fish gut in both non-sterile and sterile treatments during the first 2 sampling points (**Table XII**). It is possible that, because only two weeks had passed since the beginning of the experiment, the bacterial load from the feed was not enough to distinguish the effect of feed sterilization (**Table XIV**).

6.2 Growth

6.2.1 Effect of Salinity

Contrary to expected, growth was not affected by salinity (**Table VII and VIII**); Chervinski, (1982 (b)) had the same results for *Oreochromis mossambicus* and *Oreochromis aureus.* On the other hand, Linkongme *et al.* (1996) found significant growth at different salinities, at the same temperature, in *Oreochromis niloticus*.

One explanation for the result obtained by this study, is the evidence that in tilapia culture it is often seen the cross breeding between species, hybrids, such as red tilapia. *O. niloticus* x *O. mossambicus*. The tilapia larvae used in this study were the offspring of Nile tilapia with a salinity tolerant ancestor. The combinations of a

high growth performance characteristic of Nile tilapia and the salinity tolerance of Mozambique tilapia (Kamal and Mair, 2005), can explain equal growth rates in different salinities in this experiment.

The proximate analysis results (**Table IX**) showed a significant difference between dry matter content in fresh and salt water fish, meaning that fish from different systems had a significant weight difference (Appendix 7). Other data that supports these results is the amount of feed given. Significant differences were observed between fresh and saltwater fish. Feed limitation does not permit total satisfaction and growth might be below the maximum potential (Bowen, 1982). This did not seem to be the case due to the similar growth and FCR. Possible explanations for this outcome are the over estimation of fish performance in the first two weeks of the experiment (more feed was given) and/or feed spillage.

FCR was equal in all treatments (**Table IX**). A good FCR, as the one seen in this study (FCR >1), means that all fish were in good physiological condition and there were no apparent stressing agents (poor water quality) (Hepher, 1982). Other reason that can affect FCR is feed size. The pellets used in the experiment had a diameter of 0.5 mm. According to Bowen (1982), small sized pellets can improve the enzymatic-substrate interactions and decrease the resistance to peristaltic mixing, resulting in better incorporation of protein in fish biomass.

6.2.2 Effect of Feed Sterilization

Despite significant differences in growth between fish fed with non-sterile feed and fish fed with sterile feed not being found, the values of FCR and feed intake varied (**Table VII** and **VIII**).

The reason why feed intake is different between fish fed with non-sterile feed and fish fed with sterile feed can be due to feed being less attractive or even have an unpleasant taste. Armstrong *et al.* (1994) and Farkas (1998) stated that high gamma irradiation modifies the smell and taste of feed.

FCR variance can be related to inferior digestibility of the feed, or the fact that in general the sterile feed is poorer in terms of protein, fat and energy when compared with non-sterile feed (**Table X**). It is important to say that gamma

irradiation has a negative effect on oxidant protection of feeds, because it destroys the vitamin E that is a natural anti-oxidant of lipids in fish (Urbain, 1978; Armstrong *et al.,* 1994; Farkas, 1998).

7. Conclusions

The current study aimed at analyzing the effect of salinity and feed sterilization in interactions between water and gut microbial communities in Nile tilapia (*Oreochromis niloticus)* larvae. Growth performance was evaluated in relation to salinity and feed sterilization. From the present work the following conclusions can be drawn:

- Regarding the influence of factor salinity in the microbial communities in fish gut, the distinction of microbial communities in different salinities is not clear in this study. Salinity presents a random effect in the different sampling points meaning that the microbiota of larvae gut is not completely established.
- Sterile feed interferes with the dominance of the microbial communities in fish gut. With feed sterilization, bacteria from other environments, such as water, are in advantage to growth and proliferate.
- Surrounding environment, such as system water and diet affects bacterial populations over time, however feed has a stronger influence on the bacterial communities when compared with water.
- System water and feed bacterial communities are related and influence each other. Over time can be observed a clear significant increase of similarity between the microbial communities in both system water and feed.
- Fish growth is not affected by salinity or feed sterilization.

Suggestions for improvement of experiment set-up:

The establishment of gut microbiota is achieved 60 days after hatching (according to literature), before that it can observed, variations among bacteria dominance. Would be interesting to increase the length of the experiment to enhance the knowledge of the effect of salinity in microbial communities dominance in larvae.

Sequencing is definitely an important analysis to make, in order to identify and relate the dominant bacterial populations of fish gut, water and feed profiles.

Different molecular techniques, such as RNA, would turn this study more

accurate. Also increasing the number of individuals analyzed would contribute to reduce the statistical error.

Different diets can also influence gut microbiotas, for that reason future studies of gut biota under different diets is also recommended.

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Appendices

Appendix 1. Protocols for Dry Matter Content

Procedure ID: 67 Owner: fcf

Dry matter determination

1. Introduction

Determination of the loss in mass on drying under specified conditions.

2. Materials and equipment

Analytical balance (precision 0.1 mg).
Dry matter container with lid.
Oven with aircirculation.
Desiccator with silicagel.

3. Protocol

Depending on the sample: air dry sample in duplicate and fresh sample in triplicate.
Number all the dry matter containers.(number the container and the lid)
Put them in the oven at 103 C (the lid is placed besides the container)for at least 1 hour and maxium for 3 hours.
Put the lid on the container and put them in the desiccator.(max 8 containers per desiccator)
Close the desiccator (the valve) after 30 seconds.
Weigh the containers(container and lid together)after 60 minutes.
Depending on the sample: airdry sample 3-5 gram and fresh sample at least 10 gram
Put into the container ...gram sample and weigh the container(= container,lid and sample)accurately.
Put fresh sample first overnight in the oven at 70 C.
Put the container into the oven at 103C.
The lid is placed besides the container.
Put the lid on the container after 4 hours and put them back into the desiccator.
Close the desiccator after 30 seconds.
Weigh the containers after 60 minutes.
Put the containers back in the oven at 103 C (the lid besides the container)for 2 hours and put them back in the desiccator.
Close the desiccator after 30 seconds.
Weigh the containers after 60 minutes.
If the difference between the first and the second dryingperiod is more than 0.1 % of the sample-weight,then repeat the 2 hours drying.

4. Waste

5. Calculations

Gd - Ge
-------- X1000 = Dm (g/kg)
Gs - ge

Gd = the lowest weight of the container after drying
Ge = the weight of the empty container
Gs = the weight of the container and the sample

6. Precision

The difference between the duplicates: no more than 2 g/kg absolute.
The difference by a triplicate: The difference between each individual sample and the average is no more than 1 g/kg.

7. Remarks

If the results between the second and the third drying-period is more than 0.1 % of the sample-weight, you should take new samples and dry under vacuum at 80 C.

8. References

ISO-standard 6496
NEN-method 3332

9. Web reference

http://

10. Author

tino leffering

Chemicals

Chemical	code	CAS number	R-sentence	S-sentence

Last update

18-02-2003 14:52:26

Appendix 2. Protocols for Ash Determination

Procedure ID: 58 Owner: fcf

Ash determination

1. Introduction

Decomposition of organic matter of a test portion by incineration and weighing of the remaining ash.

2. Materials and equipment

Analytical balance (precision 0.1 mg).
Porcelain crucible.
Muffle furnace (550 C).
Desiccator with silicagel.

Reagents.

Ammonium chloride solution: Dissolve 200 g ammoniumchloride in 1 L distilled water.

3. Protocol

Depending on the amount of the sample will be chosen for small or large crucible.
Number the crucibles with a pencil.
Put the crucibles in order in the muffle furnace for at least 1 hour at 550 C.
Put them after 1 hour into the desiccator (max 8 crucibles per desiccator).
Close the desiccator after 30 seconds.
Open the desiccator after 60 minutes and weigh the crucibles.
Weigh 3 till 5 gram sample into the crucibles.
(fresh sample if possible 10 gram; in case of fresh sample first follow the dry matter determination)
Put the crucibles in a furnace (the start temp. should be lower than 200 C)for 3 hours at 550 C.
Put the crucibles in a desiccator, close the desiccator after 30 seconds.
Open the desiccator very carefully after 60 minutes and weigh the crucibles.
After weighing put the crucibles back in the furnace for 1 hour.
After 1 hour put the crucibles into the desiccator and close after 30 seconds.
Let them cool down for 60 minutes,open the desiccator carefully and weigh again.
If the difference between the first and the second ashing-period is more than 0.1 % of the sample-weight, then repeat the 1 hour ashing-period.

4. Waste

5. Calculations

Gd - Ge
------- x 1000 = Ash (g/kg)
Gs - Ge

Gd = the lowest weight of the crucible after ashing
Ge = the weight of the empty crucible
Gs = the weight of the crucible and sample

6. Precision

The difference between the duplicates shall not exceed:
2 g/kg (absolute value) for ash contents lower than 40 g/kg
5 % of mean value for ash contents from 40 till 60 g/kg
3 g/kg (absolute value) for ash contents from 60 till 100 g/kg
3 % of mean value for ash contents from 100 till 200 g/kg
6 g/kg (absolute value) for ash contents of 200 g/kg or more.

7. Remarks

When the ash contains carbonaceous particles that do not disappear on longer ashing, sometimes it is helpfull to add a few drops of water or a few drops of a solution of ammonium chloride (200 g/L), then add 4 or 5 ml of water, dry the sample again (1 hour at 103 C) and ash again for 1 hour.
If sample contains high levels of NaCl or carbonates, then use crucible with a lid.

8. References

ISO 5984 (1978)
NEN 3323 (1969)

9. Web reference

http://

10. Author

tino leffering

Last update

18-02-2003 14:11:16

Chemicals

Chemical	code	CAS number	R-sentence	S-sentence
ammonium chloride		12125-02-9	R22 Harmful if swallowed R36 Irritating to eyes	S22 Do not breathe dust

Appendix 3. Protocols for Crude Fat Content

Procedure ID: 58 Owner: fcf

Ash determination

1. Introduction

Decomposition of organic matter of a test portion by incineration and weighing of the remaining ash.

2. Materials and equipment

Analytical balance (precision 0.1 mg).
Porcelain crucible.
Muffle furnace (550 C).
Desiccator with silicagel.

Reagents.

Ammonium chloride solution: Dissolve 200 g ammoniumchloride in 1 L distilled water.

3. Protocol

Depending on the amount of the sample will be chosen for small or large crucible.
Number the crucibles with a pencil.
Put the crucibles in order in the muffle furnace for at least 1 hour at 550 C.
Put them after 1 hour into the desiccator (max 8 crucibles per desiccator).
Close the desiccator after 30 seconds.
Open the desiccator after 60 minutes and weigh the crucibles.
Weigh 3 till 5 gram sample into the crucibles.
(fresh sample if possible 10 gram; in case of fresh sample first follow the dry matter determination)
Put the crucibles in a furnace (the start temp. should be lower than 200 C)for 3 hours at 550 C.
Put the crucibles in a desiccator, close the desiccator after 30 seconds.
Open the desiccator very carefully after 60 minutes and weigh the crucibles.
After weighing put the crucibles back in the furnace for 1 hour.
After 1 hour put the crucibles into the desiccator and close after 30 seconds.
Let them cool down for 60 minutes,open the desiccator carefully and weigh again.
If the difference between the first and the second ashing-period is more than 0.1 %

of the sample-weight, then repeat the 1 hour ashing-period.

4. Waste

5. Calculations

Gd - Ge
------- x 1000 = Ash (g/kg)
Gs - Ge

Gd = the lowest weight of the crucible after ashing
Ge = the weight of the empty crucible
Gs = the weight of the crucible and sample

6. Precision

The difference between the duplicates shall not exceed:
2 g/kg (absolute value) for ash contents lower than 40 g/kg
5 % of mean value for ash contents from 40 till 60 g/kg
3 g/kg (absolute value) for ash contents from 60 till 100 g/kg
3 % of mean value for ash contents from 100 till 200 g/kg
6 g/kg (absolute value) for ash contents of 200 g/kg or more.

7. Remarks

When the ash contains carbonaceous particles that do not disappear on longer ashing, sometimes it is helpfull to add a few drops of water or a few drops of a solution of ammonium chloride (200 g/L), then add 4 or 5 ml of water, dry the sample again (1 hour at 103 C) and ash again for 1 hour.
If sample contains high levels of NaCl or carbonates, then use crucible with a lid.

8. References

ISO 5984 (1978)
NEN 3323 (1969)

9. Web reference

http://

10. Author

tino leffering

Last update

18-02-2003 14:11:16

Chemicals

Chemical	code	CAS number	R-sentence	S-sentence
ammonium chloride		12125-02-9	R22 Harmful if swallowed R36 Irritating to eyes	S22 Do not breathe dust

Appendix 4. Protocols for Crude Protein Determination

General:

Always ask one of the technicians which method has to be used.

The factor that influences the selection of a macro- or micro kjeldahl-method to determine organic nitrogen is: concentration and/or sample size.

Sample	Macro weight (mg)	Micro weight (mg)
Feed dry	300	50 - 100
Raw material dry	300 – 600	100
Fish fresh	1000	
Fish dry	300	
Digesta fresh	1500	
Digesta dry	500	50 - 100
Feaces fresh	1500	
Feaces dry	500	50 - 100
Sludge > 10 % DM	1500	
Sludge dry	500	50 - 100
Sludge < 10 % DM	10000	
Water		30000 – 50000
Acetanilide	200	40

1: Storage of samples.

The most reliable results are obtained on fresh samples. If an immediate analysis is not possible, preserve samples for kjeldahl digestion by acidifying to pH 1.5 to 2.0 with concentrated or 25 % sulfuric acid (H_2SO_4).

2: Interferences.

Nitrate: During kjeldahl digestion, nitrate in excess of 10 mg/l can oxidize a portion of ammonia released from the digested organic nitrogen, producing N_2O and resulting in negative values. This means for water samples with high nitrate-values, diluted the samples before sampling.

Inorganic salts and solids: The acid and salt content of the kjeldahl digestion reagent is intended to produce a digestion temperature of about 380 ^{0}C. If the sample contains a very large quantity of salt or inorganic solids that dissolve during digestion, the temperature may rise above 400 ^{0}C, at which point pyrolytic loss of nitrogen begins to occur.

Organic matter: During kjeldahl digestion, H_2SO_4 oxidizes organic matter to CO_2 and H_2O. If a large amount of organic matter is present, a large amount of sulfuric acid will be comsumed, the ratio of salt to acid will increase, and the digestion temperature will increase.

3. Principle.

In the presence of sulfuric acid (H_2SO_4), potassium sulfate (K_2SO_4) and a catalyst like copper sulfate ($CuSO_4$), amino nitrogen of many organic materials is converted to ammonium salts. Free ammonia is also converted to ammonium. The reaction mixture is then made alkaline, distilled and the ammonia is collected in boric acid and titrated with sulfuric acid.. The nitrogen content is calculated and by multiplication with the international protein factor, the crude protein content is obtained.

4. Chemicals.

- Kjeltabs CK: 3.5 g potassium sulfate (K_2SO_4) and 0.4 g copper sulfate ($CuSO_4$), (Boom 6205333).
- Sulfuric acid 95-97 %: H_2SO_4 (Fluka 84720).
- Sodium hydroxide: NaOH (Boom 50430)
- Boric acid: H_3BO_3 (Merck 1.00165)
- Methyl red sodium salt indicator: $C_{15}H_{14}N_3NaO_2$ (Merck 1.06078)
- Bromocresol green indicator: $C_{21}H_{14}Br_4O_5S$ (Merck 1.08121)
- Ammonium sulfate: $(NH_4)_2SO_4$ (Merck 1.01217)
- Acetanilide: C_8H_9NO (Fluka 00401)
- Titration acid: 0.1000 N H_2SO_4 (Merck 1.09074).
- Tritation acid: 0.0200 N H_2SO_4: dilute 0.1000 N 5x.

5. Reagents.

- Sodium hydroxide solution 50 %: Dissolve 5 kg NaOH in 9 l demi-water, cool to room temperature.
- Boric acid 1 %: Dissolve 50 g H_3BO_3 in 3 l demi-water, add 35 ml methyl red solution (1g/l methanol) and 50 ml bromocresol green solution (1g/l methanol) and dilute with demi-water to 5 l.

- Ammonium sulfate: Dissolve 8.2588 g ammonium sulfate (previously dried for 2 hours at 103 ^{0}C) in 500 ml demi-water and dilute to exact 1000 ml with demi-water. (1 ml solution = 1.25 ml 0.1000N H_2SO_4).

6. Materials en equipment.

- Polyethylene bags
- Analytical balance
- Plastic bucket (10 l) to prepare 50 % NaOH solution
- Destruction unit
- Set of 20 destruction tubes in a rack
- Erlenmeyer flask 5 l to prepare boric acid 1 % solution
- Dispensor for concentrated sulfuric acid
- Dispensor for demi-water
- Vortex
- Stirer
- Destillation unit

7. Method.

The execution of the analyses is to be done completely in laboratory A0.053 and the connecting weighing room A0.047.

Ask one of the technicians: before starting.

Pick up a set of 20 destruction tubes in a rack. (Tube 1, 2, and 3 is used as blank, tube 4 and 5 is used as control.)

Open de computer file. (Fill in rack-number and samples).

Place a polyethylene bag in a glass beaker of 25 ml. Bring in this bag about ….g (see general) of the sample and/or acetanilide to be analyzed and weigh accurately, using the magic key or print scrn-key.

Bring 2 catalyst pills (**take care: there are 2 types of catalyst)** into the bag with the sample, which will be brought on the bottom of a 250 ml destruction tube.

For all now following acts you are obliged to wear safety glasses and laboratory coat.

Add with a dispensor 22.5 ml concentrated sulfuric acid. Take care that some of the sulfuric acid comes into the plastic bags. The tubes are now ready for destruction.

Destruction: Asked one of the technicians which method has to be used and how to start the destruction unit. After the destruction, do not cool the tubes for a long time, because some tubes could crystallize. Add 30 ml demi-water with a dispensor and mix with a vortex.

When the solution is clear, give another 30 ml demi-water and mix again.

Distillation: The distillation will be done, by one of the technicians. The results will be printed automaticly.

8. Calculations.

$$Cp\ (g/kg) = \frac{(V1-V2) \times T \times 14.008 \times F}{G}$$

V1 = ml sulfuric acid used for titration

V2 = ml sulfuric acid used for the blank

T = titer sulfuric acid (macro = 0.1000 N, micro = 0.0200 N)

F = International protein factor, generally 6.25. (6.38 for milk)

G = weight of the sample in grams.

9. Waste.

The waste of the kjeldahl-analyses is collected in jerrycans on the assigned trolley with drip-container in room A0.053.

The wast of the destruction is collected in a special vessel.

10. Precision.

Macro: The difference between duplicates shall not exceed:

2 g/kg (absolute value) for CP-contents less then 200 g/kg.

1 % of the mean value for CP-contents of 200 – 400 g/kg.

4 g/kg (absolute value) for CP-contents higher then 400 g/kg.

Micro: 5 % of the mean value.

11. Literature.

This procedure is derived from ISO-standard 5983 and APHA: Standard Methode, Examination of water and wastewater 20th edition 1998, page 4-123.

Appendix 5. Protocols for Determination of Gross Energy

1. Scope and field of application

Determination of gross energy content.

The method is applicable for combustible materials such as, feeding stuffs, compound feed ingredients and products of animal origin, such as faeces and urine. Benzoic acid must be burned in the form of pellets. Oven-dry powder from wood, chips, hay, straw etc. burn in an explosive manner!! They must be moistened first and folded completely in a special (plastic) PEF-bag!! Readily combustible liquids with a low vapor pressure must not come in direct contact with the cotton thread.

1.1 Detection limit

The detection limit is 0.002 kJ/g

1.2 Determination limit

The determination limit is 0.010 kJ/g

1.3 Maximum energy input is 30.000 J (30 kJ)

2. Definition and Principle

The gross energy is the gross calorific value of the test sample measured at room temperature.
This is done by measuring the calorific value of the decomposition vessel (the bomb-vessel) at room temp. and the increasing in temp. of the decomposition vessel wall after complete burning of the sample with an over-pressure of 30 bar oxygen. With a C-value, from a Benzoic acid standard, with a guaranteed calorific value, according to the National Bureau of Standards, the specific calorific value for that sample is calculated.

The gross energy depends on the chemical composition of the samples.

3. Safety instructions

Operation of decomposition vessels is only permitted in combination with the IKA calorimeter C7000. The composition vessels must only be used for determination of the gross calorific value of solid and liquid combustible materials in accordance with DIN 51900, BS 1016 T5, ISO 1928, ASTM 5468 / 5865 / 4809.

When handling combustion samples, residues and auxiliary materials, the appropriate safety requirements must be maintained, for example: by corrosive, easily flammable, contaminated with bacteria and toxic materials. In addition toxic residues of combustion are possible in the form of gasses, ash or precipitates on the inner wall of the decomposition vessel.

Therefore wear personal safety equipment, such as lab-coat and fitting gloves and degas the vessel in the fume cupboard, trow away the cleaning paper after cleaning the decomposition vessel.

When working with oxygen, be aware of the danger warning: As a compressed gas, oxygen promotes combustion, supports combustion intensively and may react violently with combustible substances**. Do not use any oil or grease!!**

The condition of the seals must be checked for functionality and no leaks (see "Leak test").

4. Precision

4.1 Repeatability

The difference between duplicates shall not exceed 2% of the average value.
4.2 Reproducibility is +/- 0.1% to DIN 51900 (See page 45 in the manual 0695 IKA)

4.3 Precision is 0.0001 kJ/g

5. Materials

5.1 Reagents

The water should be demineralised (specific resistance ≥ 0.1 MΩ*cm, specific conductivity ≤ 10 µS/cm), or water of comparable quality (Demi-water).

Reagents (ascorbic acid tablets 50 pieces C723, order number 3243000) can be stored at room temperature for five years and reagent solutions can be stored for one year if not otherwise stated.

5.1.1 PEF bags weighed on a 0.1 mg Analytical balance, cotton threats 50 J/piece (500 pieces C710.4, order number L04L1312K46), ignition wire 30 J/piece (500 pieces C710.3 order number), O-ring Viton 51414 (Eriks 4x1.5 mm 100 pieces order number 10024019), O-ring Vion51414 green (Eriks 46.04x3.53 mm 100 pieces order number 10025097).

5.2 Equipment:

5.2.1 Analytical balance (0.1mg).

5.2.2 Oxygen gas, 30 bar minimum pressure, Linde gas

5.2.3 IKA C7000 Calorimeter

5.2.4 Cooler JulaboFC 600

5.2.5 IKA C7002 Cooling system

5.2.6 Stainless steel crucibles C4

5.2.7 Venting handle C7010.8

5.2.8 Oxygen filling station C48

5.2.9 Decomposition vessel C7010

5.2.10 Digital Ohm meter for checking the vessel resistance

5.2.11 Tweezers

6. Procedure

6.1 Preparation

Worksheets are generated by the LIMS. A worksheet contains at maximum 24 samples. Each worksheet contains at least one natural standard with a known gross energy content.
Before each sampling the sample is mixed carefully. Before starting the analyses of samples, for each bomb an amount of 2 PEF bag weighing 0.45 – 0.50 gram should be analysed 3 times for calibrating. The energy should be between 46.4 and 47.2 KJ/gram.

If the value is outside this range, the equipment should first be calibrated.

The first analysis result should not be used, this is meant to warm up the calorimeter.

After the samples 2 more times the calibration with PEF-bags is repeated.

6.2 Equipment settings:

6.2.1 Oxygen pressure should be 30 bars.

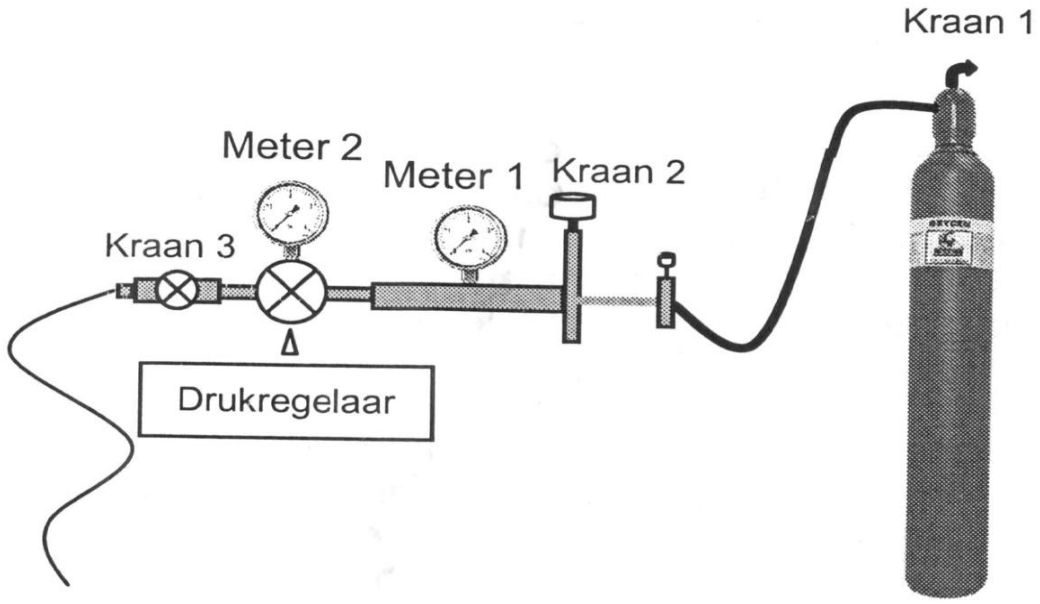
6.2.2 Cooler (5.2.4) should be at 4°C

6.3 Preparation of equipment:

6.3.1 Turn the cooler (5.2.4) on with the green switch on the backside (right and downside) and adjust it at 4°C
This should be done at least 15 minutes before the first analysis.

6.3.2 Open the oxygen cylinder in the laboratory (one turn).

6.3.3 Open the grey oxygen valve with the blue inner circle (two turns) in the laboratory



6.3.4 Check the oxygen pressure at the filling system, this should be 30 bars, adjust if necessary.

6.3.5 Turn on the calorimeter with the green switch on the front.

6.3.6 Turn on the cooling system (5.2.5) with the black switch on the backside (right and downside).

6.3.6 Check the serial numbers on the parts of the bomb (such as container and top ring), so they don't get mixed.

6.3.7 Before the first analysis the empty bomb with nut, but without cover has to be placed in the cooling system (5.2.5).

6.3.8 Fit the bomb together according to page 5 in the manual.

6.4 Determination of PEF value

6.4.1 Attach a metal ignition wire to both electrodes of the cover.

6.4.2 Attach a cotton thread to the middle of the ignition wire.

6.4.3 Place the metal crucible in the holder.

6.4.4 Hang the cotton thread in the steel crucible.

6.4.5 Weigh 2 PEF-bags on a glass disk and notice the weight, also in the computer-file

6.4.6 Place the bags in the crucible and make sure it is in contact with the cotton thread.

6.4.8 Place the cover in the bomb.
6.4.9 Close the bomb, don't screw it to tight.
Fill the bomb with oxygen up to 31 bars and continue at 6.6.
Repeat this procedure for at least 3 times with bags for each bomb used.

The average of 5 values found for each bomb, will be used in the calculation. (See 7). After the samples repeat 2 times for each bomb.

6.5 Determination of samples

6.5.1 Attach a metal ignition wire to both electrodes of the cover

6.5.2 Attach a cotton thread to the middle of the ignition wire

6.5.3 Place the metal crucible in the holder

6.5.4 Hang the cotton thread in the steel crucible

6.5.5 Weigh a PEF bag and notice the weight

6.5.6 Put approximately 0.5 g sample with an accuracy of 0.1 mg in the PEF bag and notice the weight. This amount can vary depending on the gross energy content (see 7).
Liquid samples should after weighing first been freeze dried before analysis.

6.5.7 Place a weighed sample in the crucible and make sure it is in contact with the cotton thread

6.5.8 Place the cover in the bomb
6.5.9 Close the bomb, don't screw it to tight.
6.5.10 Fill the bomb with oxygen up to 31 bars

6.6 Measurement

6.6.1 Open the cover of the calorimeter

6.6.2 Carefully place the bomb in the calorimeter, make sure it sits right and is not wobbly.

6.6.3 Leave the cover of the calorimeter open!
6.6.4 Check if the display shows "menu 1". If not then push FCT and 8 together.
6.6.5 Press the S1 key, below the "pre-par" indication on the display.
6.6.6 Check if the display shows "sample code".

6.6.7 Enter the sample code with “clear button change code”, and press RET (=return key).
6.6.8 Check if the display shows “user”.
6.6.9 If this is the first time: enter your name and press RET, the next times your name will already be in the display and you only need to press RET.
6.6.10 Check if the display shows “sample mass”.
6.6.11 Enter the weight of the PEF bag, and press RET (do not enter the total weight or the real sample weight!)
6.6.12 Check if the display shows “Q-ext” and “80” (value for ignition wire and cotton thread)
6.6.13 Press RET
6.6.14 Press the X key
6.6.15 Close the cover
6.6.16 Check if the sample code is correct
6.5.17 Press the RET key, and the measurement will start.

6.5.18 Wait for the beep signal

6.5.19 Put together another bomb

6.5.20 Press the ATN key to shut down the noise

6.5.21 Write down the caloric value on the display (GE display)

6.5.22 Open the cover

6.7 Cleaning the Bomb

6.7.1 Take the bomb out of the calorimeter

6.7.2 Place the bomb in the cooling system (5.2.5), it will close automatically

6.7.3 Wait until the cooling system is completed, the cooling system will beep

6.7.4 Take the bomb out of the cooling system and place it in the ventilated fume cupboard

6.7.5 Turn on the ventilation of the fume cupboard

6.7.6 De-gases the bomb with the venting handle (5.2.7) until no hiss is heard anymore

6.7.7 Take the bomb out of the fume cupboard

6.7.8 Open the bomb

6.7.9 Clean the cover and the shield inside with a piece of paper towel (a small space, so use your little finger) and electrodes from ignition wires and the inside of the bomb with paper towel

6.7.10 Clean the crucible with the tweezers if necessary and stamp it out.

After all the measurements: clean the cover with all the electrodes parts separated with a lot of Demi-water from the tap and dry them with the pressed-air pistol and the inside of the vessel with a wet paper towel.

7. Calculation

The contents of gross energy (GE) are calculated as follows (see remark 10.2):

GE (kJ/g) = ((GE display (kJ/g) – average PEF value (kJ/g)) * weight PEF bag (g)) / weight sample (g)

For an accurate measurement, the increase of temperature should lay between 10 and 16 degrees Kelvin. If the temperature increase lies outside this range, contact directly a laboratory technician.

8. Conservation

8.1 Shelf life

Store the samples in the PEF bag in desiccators before analysis.

9. Registration

The results of the worksheet are stored in the LIMS-database.

10. Remarks

10.1 The system corrects for differences in environmental temperature before analysis. If the difference is to larger, the display will mention “Pretest drift to high” and the bomb should first be placed in the cooling system (5.2.5).

10.2 Explanation of the calculation:
The system automatically gives the energy content (kJ/g) of the sample included the PEF bag based on the weight of the PEF bag (6.6.11). Mathematically, it can be described as:

GE display (kJ/g) = ($Energy_{sample}$(kJ) + $Energy_{PEF\ bag}$(kJ)) / weight PEF bag (g) (1)

Or:

$Energy_{sample}$(kJ) = GE display (kJ/g) * weight PEF bag (g) - $Energy_{PEF\ bag}$(kJ) (2)

The energy of the PEF bag is:

$$Energy_{PEF\ bag}(kJ) = PEF\ value\ (kJ/g) * weight\ PEF\ bag\ (g) \quad (3)$$

Combination of 2 and 3, gives:

$$Energy_{sample}(kJ) = (GE\ display\ (kJ/g) - PEF\ value\ (kJ/g)) * weight\ PEF\ bag\ (g) \quad (4)$$

The GE content of the sample (in kJ/g) is $Energy_{sample}(kJ)$ / weight sample (g), therefore dividing equation 4 by weight of sample gives:

$$GE\ (kJ/g) = (\ GE\ display\ (kJ/g) - PEF\ value\ (kJ/g)\ ) * weight\ PEF\ bag(g)/weight\ sample\ (g)$$

which is used in the calculation.

11. Literature

ISO standard 9831 (1998).

DIN 51900

IKA Werke

Neumagen 27

D-79219 Staufen

Appendix 6. Proximate Analysis of 42-Days Old Tilapia and feed

	dm g/kg	ash g/kg	ash g/kg dm	cp g/kg	cp g/kg dm	energy kJ/g	energy kJ/g dm	fat g/kg	fat g/kg dm
FN1	193.42	24.03	124.24	108.89	562.99	4.87	25.16	58.49	302.43
FN2	201.54	24.34	120.76	112.36	557.52	5.14	25.52	63.07	312.97
FN3	196.77	23.42	119.04	109.48	556.38	5.02	25.51	62.15	315.83
FS1	189.83	23.02	121.25	105.05	553.39	4.81	25.35	60.01	316.10
FS2	192.49	22.39	116.34	106.69	554.28	4.98	25.89	62.06	322.40
FS3	199.22	24.70	123.98	112.24	563.41	5.02	25.22	60.21	302.22
SN1	188.51	23.18	122.96	106.57	565.34	4.68	24.84	56.56	300.06
SN2	194.17	22.97	118.29	109.03	561.54	4.88	25.13	60.12	309.61
SN3	182.50	21.13	115.79	100.04	548.16	4.68	25.62	60.40	330.97
SS1	182.63	20.90	114.42	100.59	550.79	4.67	25.59	60.13	329.26
SS2	163.27	18.83	115.33	91.68	561.55	4.18	25.62	51.85	317.59
SS3	181.13	20.68	114.16	99.25	547.94	4.64	25.63	60.90	336.20
Non-Sterile Feed	868.18	99.08	114.12	537.10	618.65	20.24	23.31	160.11	184.42
Sterile Feed	876.46	99.71	113.77	540.79	617.02	20.31	23.17	160.41	183.02

Appendix 7. Fish growth performance data in four periods.

Feed*	Water**	Tank	Week	Initial individual weight	Final individual weight	SGR	Metabolic growth	Feed Intake	Metabolic ration
1	1	1	1	0.01	0.03	17.0	19.6	0.01	14.00
1	1	2	1	0.01	0.03	15.8	18.0	0.01	14.46
1	1	3	1	0.01	0.03	18.6	22.0	0.01	13.36
1	2	1	1	0.01	0.03	16.7	19.2	0.01	14.79
1	2	2	1	0.01	0.03	15.9	18.0	0.01	15.16
1	2	3	1	0.01	0.03	18.3	21.6	0.01	14.15
2	1	1	1	0.01	0.03	16.8	19.3	0.01	14.07
2	1	2	1	0.01	0.03	16.8	19.4	0.01	14.04
2	1	3	1	0.01	0.03	16.9	19.6	0.01	14.01
2	2	1	1	0.01	0.03	19.4	23.2	0.01	13.72
2	2	2	1	0.01	0.03	18.2	21.4	0.01	14.19
2	2	3	1	0.01	0.03	18.9	22.4	0.01	13.92
1	1	1	2	0.03	0.09	16.9	24.8	0.03	12.86
1	1	2	2	0.03	0.09	18.5	27.2	0.04	15.60
1	1	3	2	0.03	0.09	15.2	22.2	0.03	12.62
1	2	1	2	0.03	0.09	17.3	25.4	0.04	15.12
1	2	2	2	0.03	0.09	18.5	27.2	0.04	15.63
1	2	3	2	0.03	0.11	18.0	27.3	0.05	16.05
2	1	1	2	0.03	0.09	16.8	24.5	0.04	16.57
2	1	2	2	0.03	0.10	18.3	27.2	0.04	13.72
2	1	3	2	0.03	0.09	17.2	25.3	0.04	15.80
2	2	1	2	0.03	0.10	16.0	23.9	0.04	14.94
2	2	2	2	0.03	0.11	18.2	27.6	0.04	15.27
2	2	3	2	0.03	0.11	17.8	27.0	0.04	14.60
1	1	1	3	0.09	0.62	13.8	30.1	0.31	17.56
1	1	2	3	0.09	0.61	13.4	29.3	0.32	18.17
1	1	3	3	0.09	0.60	13.5	29.2	0.32	18.61
1	2	1	3	0.09	0.54	12.6	26.6	0.30	17.69
1	2	2	3	0.09	0.52	12.3	25.7	0.29	17.44
1	2	3	3	0.11	0.61	12.4	26.8	0.32	16.85
2	1	1	3	0.09	0.51	12.4	25.9	0.29	18.18
2	1	2	3	0.10	0.51	11.7	24.4	0.32	19.14
2	1	3	3	0.09	0.56	12.9	27.4	0.29	16.88
2	2	1	3	0.10	0.52	11.7	24.5	0.29	17.15
2	2	2	3	0.11	0.50	10.9	22.4	0.27	15.61
2	2	3	3	0.11	0.51	11.0	22.7	0.29	16.41
1	1	1	4	0.62	2.12	8.7	24.0	0.98	15.63
1	1	2	4	0.61	2.13	8.9	24.4	0.98	15.88
1	1	3	4	0.60	2.20	9.3	25.8	0.96	15.48
1	2	1	4	0.54	2.11	9.7	26.7	0.96	16.41
1	2	2	4	0.52	2.30	10.6	29.7	0.98	16.32
1	2	3	4	0.61	1.86	7.9	21.2	0.72	12.27

2	1	1	4	0.51	1.73	8.8	23.1	0.83	15.67
2	1	2	4	0.51	1.71	8.6	22.6	0.78	14.72
2	1	3	4	0.56	2.31	10.1	28.3	1.18	19.13
2	2	1	4	0.52	1.76	8.7	23.0	0.92	17.05
2	2	2	4	0.50	1.65	8.6	22.5	0.91	17.74
2	2	3	4	0.51	2.02	9.8	26.7	0.96	17.00

*Feed: 1 Non-sterile; 2 Sterile
**Water: 1 Fresh; 2 Salt

Appendix 8. Measured values of inorganic carbon (IC), non-purgable organic carbon (NPOC), total carbon (TC), total amount of nitrogen (TAN-N), nitrite (NO_2^--N) + nitrate (NO_3^--N), total carbon (TC) and Carbon Nitrogen ratio in three different weeks in the four different systems: Fresh water non-sterile feed (FN) fresh water sterile feed (FS) salt water non-sterile feed (SN) and salt water sterile feed (SS).

	24-Jun				01-Jul				07-Jul			
	FN	FS	SN	SS	FN	FS	SN	SS	FN1	FS1	SN1	SS1
IC (mg/l)	13.6	14.2	19.1	13.2	14.1	13.8	18.7	18.2	9.2	8.0	17.0	16.3
NPOC (mg/l)	4.4	6.3	3.0	0.6	4.0	3.8	3.3	2.8	3.8	5.3	3.7	3.5
TC (mg/l)	17.9	19.4	23.0	15.9	18.1	18.5	22.7	22.6	14.2	14.3	22.4	21.2
$NH4^+$ (mg/l-N)	0.09	0.07	0.05	0.04	0.10	0.14	0.04	0.04	0.14	0.10	0.10	0.12
NO_3^- + NO_2^-(mg/l-N)	6.64	6.39	4.30	3.47	9.23	8.78	6.55	7.81	16.7	17.3	8.33	9.9
NT (mg/l)	7.6	7.4	4.8	3.8	10.3	9.7	7.1	8.3	17.7	19.6	9.3	10.8
Ratio C:N	2.4 : 1	2.6 : 1	4.8 : 1	4.2 : 1	1.8 : 1	1.9 : 1	3.2 : 1	2.7 : 1	0.8 : 1	0.7 : 1	2.4 : 1	2:1

Appendix 9. DGGE Protocol

Standard Operating Procedure
DGGE Version January 2010
G.H.J. Heilig, Laboratory of Microbiology, Wageningen University, Wageningen, The Netherlands

Equipment & Material
BioRad Econo pump (BioRad 731-8142)
Cellophane sheets (Sigma Z377600-1PAK)
20cm clamps (BioRad 165-1835)
Comb 25-well (BioRad 165-1862)
Gelbond (Lonza 54731)
Gel drying frame material
Glass plates
Gradient maker (Wageningen University Technical workshop)
Inner glass plate (BioRad 165-1823)
Magnetic stirrer (any)
Magnetic stirrer bar (any)
Outer glass plate (BioRad 165-1824)
Paper clamps
Power supply (BioRad 164-0302)
Scanner (BioRad 170-7980)
Shaker (any)
20cm spacers, 1mm (BioRad 165-1848)
2 Stainless steel trays

Chemicals
40% acrylamide:bisacrylamide (37.5:1) (BioRad 161-0148)
Ammonium persulphate (any)
EDTA (Sigma E5134-1KG)
Ethanol 96% (any)
Formaldehyde (Sigma F8775-500ML)
Formamide (Fluka 47680)
Glacial acetic acid (any)
Glycerol (Sigma G6279-1L)
NaOH (any)
Silver nitrate (Sigma 85228-50G)
Sodium borohydride (Aldrich S21173-264)
TEMED (Invitrogen 15524-010)
TRIS 7-9 (Sigma T1378)
Urea (Sigma 02493)

Solutions
50xTAE buffer
242 g TRIS base
57.1 ml glacial acetic acid

100 ml 0.5 M EDTA (pH 8.0)
Dem. H2O to 1L

0.5x TAE buffer
100 ml 50xTAE
Dem. H2O to 10L

100% denaturing PA
421.6 g urea
200 ml 40% acrylamide:bisacrylamide (37.5 : 1)
400 ml formamide
10 ml 50xTAE buffer
20 ml glycerol

Carefully heat to hand warm, add stirrer and dissolve. Adjust to final volume of 1 L with demi- H2O. Store in the dark at room temperature.

0% denaturing PA
200 ml 40% acrylamide:polyacrylamide (37.5 : 1)
10 ml 50xTAE buffer
20 ml glycerol
Dem. H2O to 1L. Store in the dark at room temperature.

Cairns' 8x fixation solution
800 ml 96 % ethanol
40 ml acetic acid
160 ml demi- H2O

Cairns' 1x fixation solution
Add 50 ml 8xCairns' fixing solution to 350 ml demi-water

Silver staining solution
0.4 g AgNO3 in 1x Cairns' fixing solution (200 ml)

Developer
A spatula tip of NaBH4 (approx. 10 mg)
250 ml 1.5 % NaOH solution
750 µl formaldehyde

Cairn's preservation solution
250 ml 96 % ethanol
100 ml glycerol
650 ml demi- H2O

Gel sandwich

Clean one outer and an inner glass plate with soap, dry them and clean them again with 96% ethanol.

Cut the gelbond to the size of the outer glass plate.

Add some water to the surface of the outer glass plate.

Place the gelbond hydrophobic side down on this glass plate (check this by adding a drop of water on the gelbond, it will roll of easily).

Fix the gelbond, without removing the paper sheet, with a roller or glass reaction tube, then take of the paper sheet.

Dry the gelbond carefully with some tissues.

Carefully align the gelond with the bottom part of the glass plate.

Clean a set of spacers with 96% ethanol, and place them on the gelbond.

Place the smaller glass plate on top.

Add the clamps to the sides of the sandwich, and place in the sandwich-holder. For proper distance between the spacers slide the plastic card between the glass plates.

Press the spacers down and fasten the screws on the clamps.

Place the sandwich on top of the rubber gasket and press down the handles.

Preparing the gel plug

Set a pipette to 1 ml and put on the tip ready to dispense the gel solution as soon as the TEMED and APS are added to the 'plug' solution.

Prepare the 'plug' solution as follows (volumes for one gel, or two):

1.5 ml (3 ml) 0% Denaturant

4.5 µl (9 µl) TEMED

15 µl (30 µl) 10% (w/v) Ammonium PerSulfate (APS)

Add 1.5 ml of the plug solution to the plate cassette, by pipetting the 1.5 ml volume in one go down the side of the spacer.

Repeat for the second gel.

Tilt the casting stand so the 'plug' solution runs level along the bottom taking care to avoid making any air bubbles.

Check that the stand is then still level.

Leave the plug for approx. 10 min to set (normally about the time it takes you to

make your gradient solutions, and rinse out the mixing chamber and tubing with water).

Cast the gradient gel and stacking gel as normal.

Preparing the gel

Prepare the gel solutions required, being high, low, and a stacking gel in 50ml Greiner tubes according to the mixing table in the flow hood.

Place on ice.

Rinse the gradient maker and tubes with demi-water, switch on the pump at running speed (19 ml/min) and drain the system.

Close the screw between the compartments of the gradient maker.

Dry the compartments with a tissue.

When the gel solution are cooled add 10% APS (only in high and low solutions).

Pour the high solution in the right compartment (closest to the drain), and the low in the left.

Add a magnetic stirring bar to the right compartment only.

Open the screw and immediately start the pump at 5 ml/min.

Start the stirrer use setting 400 rpm.

Place the needle between the glass plates.

The gradient maker should be emptied without tilting it.

Remove the needle when the gel is poured, switch off the pump and transfer to the erlenmeyer flask.

Rinse the compartments with demi-water and switch on the pump and drain the system.

Add the 10% APS to the stacking gel.

Close the screw between the compartments and add the stacking gel to the right compartment.

Start the pump and set at running speed (19 ml/min) until the solution has reached the needle and a few drops have dripped into the erlenmeyer flask.

Stop the pump.

Place the needle between the glass plates and start the pump set at 3 ml/min.

When the stacking gel is poured place the comb that will form the slots carefully in the stacking gel. Avoid air-bubbles since the will appear as dents in all your bands.

Leave the gel for at least 1 hr.

Running a gel

Add freshly prepared 0.5xTAE buffer to the buffer tank and fill up to Fill.

Switch on the Dcode at least 90 minutes before electrophoresis, so that the buffer can heat up to 65ºC.

After one hour of polymersiation remove the comb carefully.

Rinse off non-polymerized gel in and above the slots with running buffer using a syringe and needle and click the sandwich in the sandwich-holder. (There should always be a sandwich at the other side to get a closed upper buffer compartment).

Switch off the Dcode, and take off the lid.

Take this super-sandwich and let is slide into the buffer tank.

Switch on the Dcode until the upper buffer compartment is filled with buffer.

Switch off the Dcode and take of the lid.

Add your samples (remember load mirror-wise).

Switch on the Dcode after returning the lid.

Lower the temperature of the DCode to 60ºC

Switch on the power supply for 10 min. at 200V and lower to 85V for 16 hrs.

DGGE Mixing Table

Gradient Comp.	0%	100%	Final Volume	TEMED	10% APS
%	ml	ml	ml	µl	µl
0	9	-	9	13	50
30	9.1	3.9	13	13	50
31	8.97	4.03	13	13	50
32	8.84	4.16	13	13	50
33	8.71	4.29	13	13	50
34	8.58	4.42	13	13	50
35	8.45	4.55	13	13	50
36	8.32	4.68	13	13	50
37	8.19	4.81	13	13	50
38	8.06	4.94	13	13	50
39	7.93	5.07	13	13	50
40	7.8	5.2	13	13	50
41	7.67	5.33	13	13	50
42	7.54	5.46	13	13	50
43	7.41	5.59	13	13	50
44	7.28	5.72	13	13	50
45	7.15	5.85	13	13	50
46	7.02	5.98	13	13	50
47	6.89	6.11	13	13	50
48	7.76	6.24	13	13	50
49	6.63	6.37	13	13	50
50	6.5	6.5	13	13	50
51	6.37	6.63	13	13	50
52	6.24	6.76	13	13	50
53	6.11	6.89	13	13	50
54	5.98	7.02	13	13	50
55	5.85	7.15	13	13	50
56	5.72	7.28	13	13	50
57	5.59	7.41	13	13	50
58	5.46	7.54	13	13	50
59	5.33	7.67	13	13	50
60	5.2	7.8	13	13	50

Staining a Gel

Prepare 1xCairn's fixation solution, silver staining solution and developer solution.

Put the gel in a stainless steel tray (keep one tray dedicated to silver staining and one

for developing gels).

Add 200 ml 1xCairn's fixation solution and rock the box for 5 min, remove the solution and store for later application.

Add 200 ml silver staining solution to the box and rock 10 min. Discard the solution (chemical waste!)

Rinse the box with demi-water (chemical waste!).

Add fresh demi-water and rock for 5 min.

Discard water.

Rinse the gel and gelbond with demi-water.

Place the gel in a stainless steel developer tray.

Add a small part of developer solution and rock a little, discard this part and repeat.

Add the remaining developer solution.

Rock until the gel has developed well.

Discard the developer solution.

Add the previously used 200 ml 1x Cairns' solution to the box and rock for 5 min.

Discard the fixation solution.

Add some demi-water and rock for 2 min.

Replace the demi-water with Cairns' preservation solution and rock for 7 min, add cellophane sheet to be soaked as well.

Place the gel on a glass plate (gelbond faced down).

Place the cellophane sheet on top the gel (avoid air bubbles, press the edges of the gel to the glass plate).

Use old spacers and paper clamps to frame the gel and cellophane sheet.

Dry the gel overnight at 55ºC.

Remove framing material and cut off excess gelbond.

Clean the gelbond side with 70% ethanol and a tissue.

Gel is ready for scanning.

<u>Scanner Manual</u>

Open PDquest

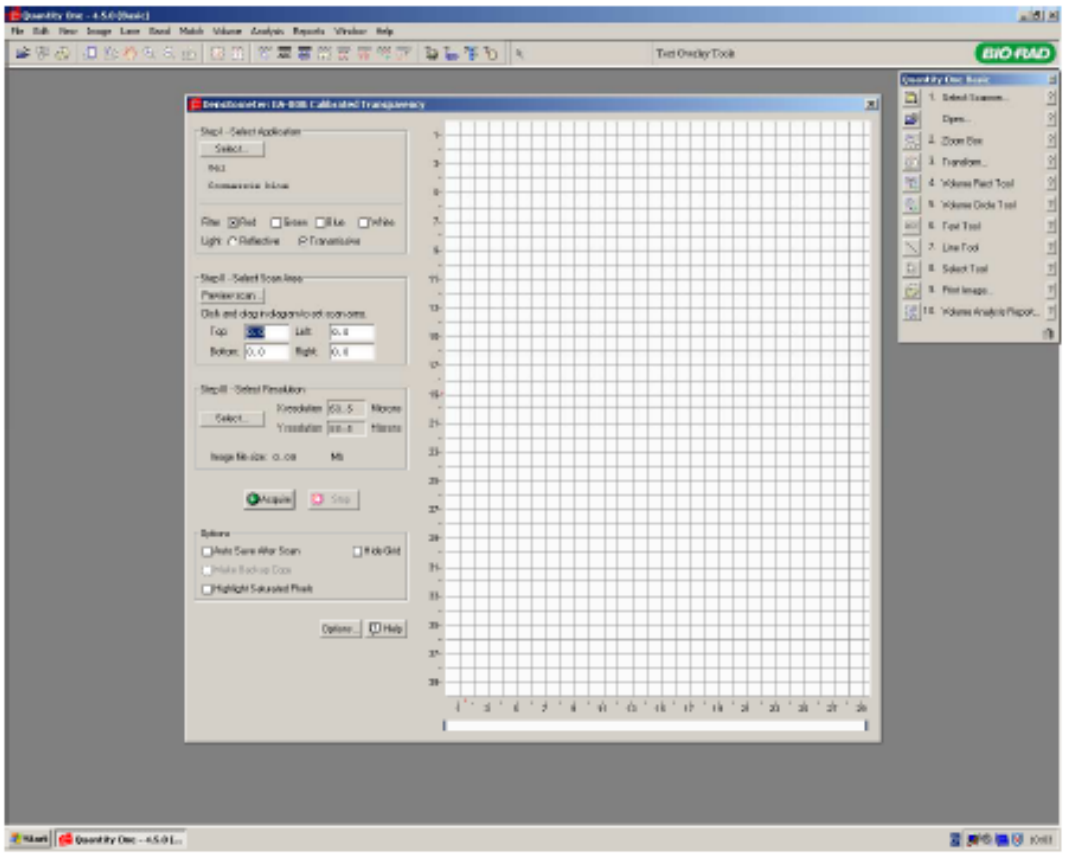
File

=>GS-800

Select

=>Gel

=>Coomassie blue (automatic selection filter red, light transmissive and gel resolution)



Preview Scan

Select the smallest possible gel area with +

Acquire

Image

=>Transform

=>adjust gamma

=>OK

Export to TIFF

Export view excluding overlay

Resolution

=>same as scan (400dpi)

=>Export =>Save

Appendix 10. Similarity tables of gut and water overtime

Calculated means of Pearson correlation similarities of fish gut overtime inside and outside of systems: fresh water non-sterile feed (FN), fresh water sterile feed (FS), salt water non-sterile feed (SN) and salt water sterile feed (SS).

		Sampling day				
		0	**7**	**14**	**28**	**42**
FN**	Similarity inside treatment*	82.0^{a,1} ±6.90	93.3^{a,1} ±3.65	87.2^{b,1} ±9.34	78.6^{a,1} ±7.76	79.1^{a,1} ±9.88
	Similarity outside treatment *	69.2^{b,2} ±13.80	66.5^{b,2} ±19.94	77.6^{c,2} ±17.99	39.9^{a,2} ±22.16	67.5^{b,2} ±13.02
FS**	Similarity inside treatment *	86.7^{bc,1} ±8.71	84.6^{b,1} ±11.46	92.0^{ac,1} ±2.48	75.2^{a,1} ±8.54	81.7^{b,1} ±7.99
	Similarity outside treatment *	69.5^{bc,2} ±15.01	65.2^{b,2} ±20.64	78.1^{d,2} ±17.81	46.0^{a,2} ±19.59	71.5^{c,2} ±11.30
SN**	Similarity inside treatment *	92.7^{b,1} ±3.15	79.0^{a,1} ±11.98	78.1^{a,1} ±12.10	80.3^{a,1} ±8.11	74.2^{a,1} ±14.28
	Similarity outside treatment *	73.1^{c,2} ±13.92	56.4^{b,2} ±17.35	59.7^{b,2} ±16.78	42.6^{a,2} ±18.21	68.6^{c,2} ±13.48
SS**	Similarity inside treatment *	69.5^{b,1}±23.01	27.2^{a,1} ±21.00	89.4^{c,1} ±7.17	60.1^{b,1} ±18.79	86.8^{c,1} ±5.67
	Similarity outside treatment *	64.2^{b,2} ±19.80	44.5^{a,2} ±16.86	79.1^{d,2} ±15.52	47.0^{a,2} ±18.34	70.3^{c,2} ±12.90

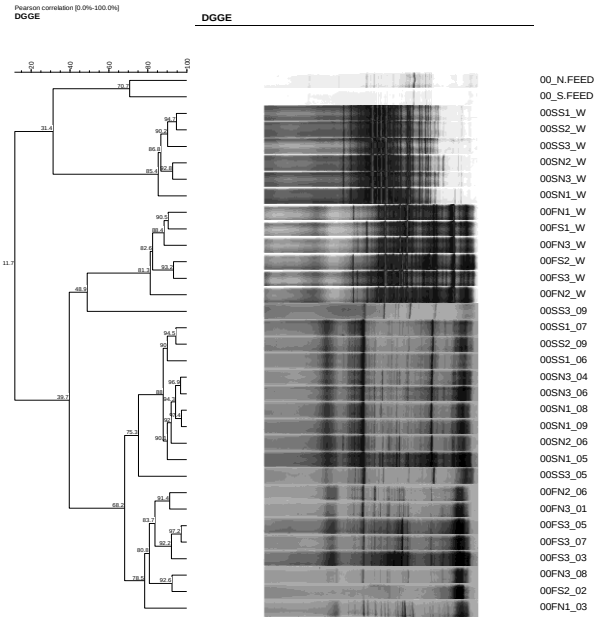
*Values are given as mean ± SD. Means followed by the same letter (**row**) are not significant (one way ANOVA (p>0.05)).
Means of similarity inside and outside group per individual system (FN, FS, SN, SS) followed by the same number (column**) are not significant (*T-test* (p>0.05)).

Calculated means of Pearson correlation similarities of water overtime inside and outside of systems: fresh water non-sterile feed (FN), fresh water sterile feed (FS), salt water non-sterile feed (SN) and salt water sterile feed (SS).

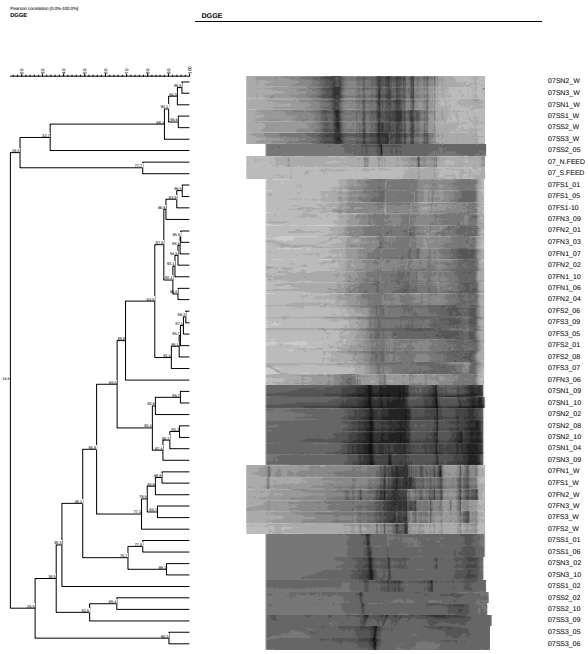
		Sampling day				
		0	**7**	**14**	**28**	**42**
FN**	Similarity inside treatment *	$84.3^{a,1}$ ±3.81	$78.6^{a,1}$ ±4.84	$83.8^{a,1}$ ±4.74	$81.3^{a,1}$ ±10.30	$92.2^{a,1}$ ±5.47
	Similarity outside treatment *	$48.7^{a,2}$ ±26.75	$50.5^{a,2}$ ±23.61	$51.8^{a,2}$ ±22.89	$59.7^{ab,1}$ ±21.20	$71.0^{b,1}$ ±18.52
FS**	Similarity inside treatment *	$82.2^{a,1}$ ±9.66	$79.1^{a,1}$ ±2.44	$76.2^{a,1}$ ±15.11	$85.4^{a,1}$ ±6.26	$88.5^{a,1}$ ±5.94
	Similarity outside treatment *	$49.8^{a,2}$ ±25.64	$52.1^{a,2}$ ±21.64	$46.9^{a,2}$ ±27.58	$68.3^{b,2}$ ±12.39	$70.0^{b,1}$ ±16.84
SN**	Similarity inside treatment *	$87.9^{a,1}$ ±4.35	$95^{ab,1}$ ±1.35	$80.3^{ab,1}$ ±4.86	91.5a^{b,1} ±1.03	$77.7^{b,1}$ ±9.85
	Similarity outside treatment *	$49.6^{ab,2}$ ±27.08	$52.1^{ab,2}$ ±27.60	$45.5^{a,2}$ ±25.98	$64.6^{b,2}$ ±19.05	$66.5^{a,1}$ ±15.64
SS**	Similarity inside treatment *	$87.9^{a,1}$ ±2.64	$90^{a,1}$ ±4.23	$86.6^{a,1}$ ±7.75	$82.9^{a,1}$ ±4.51	$90.0^{a,1}$ ±0.77
	Similarity outside treatment *	$49.8^{a,2}$ ±26.82	$56.0^{ab,2}$ ±24.99	$51.4^{ab,2}$ ±21.92	$65.4^{ab,1}$ ±18.30	$67.2^{b,2}$ ±11.19

Appendix 11. DGGE Results

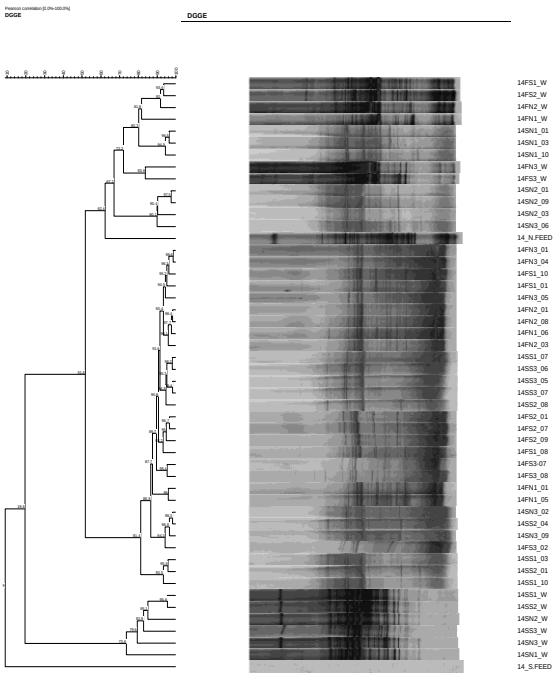
Day 0- Water, feed and fish gut



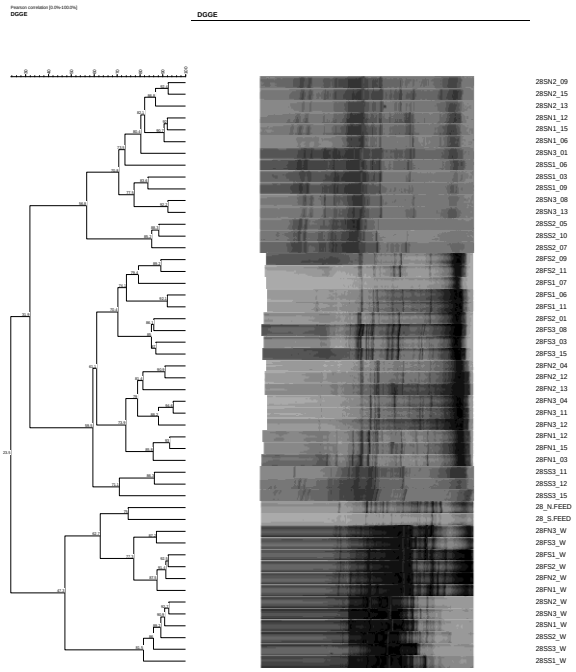
Day 7- Water, feed and fish gut



Day 14- Water, feed and fish gut



Day 28- Water, feed and fish gut



Day 42- Water, feed and fish gut