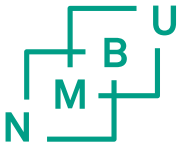

U
B
M
N

# Does feeding frequency affect utilization of added amino acids in Nile tilapia?

Master Thesis (30 credits)

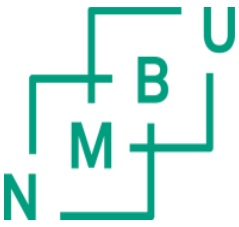
Biswas Bajgai and Rezaul Hoque

Department of Animal and Aquacultural Sciences

Faculty of Veterinarian Medicine and Biosciences

Norwegian University of Life Sciences

Ås, 2014



## Abstract

Nile tilapia (*Oreochromis niloticus*) is one of the major farmed fish species, with main production in Asia, South and Central America that can tolerate a wide range of environmental stress and easily adapt with low quality of feed ingredients. The aims of the experiments were to determine effects of feeding frequency on utilization of protein and energy in Nile tilapia, to quantify differences in excretion of ammonia and ammonium in Nile tilapia fed the same daily ration, distributed over 2 and 4 meals a day. The aims were derived from a hypothesis that frequent feeding facilitates higher overlap in absorption of crystalline dietary amino acid supplement and protein-bound amino acids, since digestion of protein requires time and thereby may improve utilization of dietary protein. Significant differences were indicated for $P<0.05$.

The fish were kept in indoors rearing tank (70×50×50 cm) with equal water flow (180 l $h^{-1}$) supplied with freshwater from a recirculation system. Each tank contained 30 tilapias (mean weight ± SEM; 721 ± 0.1 g) with an average weight of individual fish 24 g. All tanks had 24 h $d^{-1}$ light. The water temperature and dissolved oxygen were recorded on a daily basis. The average water temperature was 27.5 °C with 7.5 mg $l^{-1}$ dissolved oxygen and pH 7 during the experimental period. The fish were fed for 46 days

One plant ingredient based diet was prepared with 31% crude protein, 37% starch and 6 % fat in the dry matter. There were two different feeding regimes. One was only twice in a day (at 10:00 and 20:00) and the other was four times per day (at 08:00, 12:00, 16:00 and 20:00). Each meal lasted for 70 min when the fish were fed 2 meals $d^{-1}$, and 35 min for 4 meals $d^{-1}$. In three reference tanks feeding was twice per day to satiation level, and the fish in the remaining tanks were pair-fed 90 % of the dry matter consumed by the fish fed to appetite the previous day. No significant differences were found for growth performance, body composition, or feed conversion ratio when the fish received 90% of appetite level in 2 or 4 meals. However, liver weight in percent of whole body weight was significantly higher for 2 than 4 meals. Feeding the fish to satiation in 2 meals resulted in higher feed intake, and lower protein and energy retentions than feeding 90% of satiation in 2 meals.

Ammonium ($NH_4^+$), ammonia ($NH_3$), total ammonia nitrogen (TAN) and nitrite ($NO_2^-$) were measured one hour before the last meal (19:00) and every 2 hours after last meal ( 22:00;

00:00; 02:00; 04:00; 06:00) over night at days of 14, 26 and 39. During this measurement water samples were collected from the inlet and outlet of tanks. At the end of the experiment (day 45) nitrogenous catabolites were allowed to accumulate in a stagnant system (water flow closed), following the same sampling intervals as described for the run-through sampling approach. From the flow-through system, significant differences were observed between feeding frequency, time interval and their interaction in all parameters (TAN, ammonium, ammonia) at days 14, 26 and 39. Similarly, significant differences were seen for nitrite measurement at different period of time. However, no significant difference was seen for feeding frequency and the interaction between feeding frequency and measurement at different period.

In the flow-through system, the highest TAN was found at 4 hours after feeding in 2 meals (90% and appetitive) while for 4 meals (90%) it was at 2 hours after feeding during all the sampling days. The maximum TAN at 588 µg $l^{-1}$ water was measured at day 39 in 2 meals (appetitive), 4 h after feeding. The lowest value at 89 µg $l^{-1}$ was measured at day 14 in 4 meals (90%), 10 h after last feeding in the day. TAN was consistently highest for to 2 meals (appetitive) and lowest for 4 meals (90%). The TAN excretion started immediately after feeding, then reached peak value, and thereafter gradually declined to the based level. Nitrite accumulation did not follow any particular pattern in the flow-through system, bur tended to reach peak value 2 hours after feeding.

The accuracy of the results obtained from the stagnant system was high, and metabolic nitrogen excretion (with or without values for nitrite) over time was described ($R^2$=0.98-0.99) by 3$^{rd}$ degree polynomial patterns. Maximum nitrogen excretion, with nitrite was maximum at 4.59, 4.61 and 2.88 h after feeding in 2 meals (appetitive), 2 meals (90%) and 4 meals (90%) respectively. The estimates without nitrite included showed maxima at 4.59, 3.64 and 4.02 h after feeding in 2 meals (appetitive), 2 meals (90%) and 4 meals (90%) successively. $NO_2^-$, which was not a catabolite increased linearly over time. A probable reason for the observed increase in nitrite may be oxidation of TAN from metabolism and/or microbial oxidation of nitrogen in intestinal contents and faeces.

The nitrogen excretion rate over gills (not including excretion of urea via the kidneys) 4 h after last feeding was the highest (150 µmoles (kg body weight (BW)* h)$^{-1}$) for 2 meals (90%) and the lowest (113µmoles (kg BW * h)$^{-1}$) for 4 meals (90%). Whereas for 2 meals (appetitive) showed very close value 145 µmoles (kg body weight * h)$^{-1}$ to 2 meals (90%).

Meanwhile, 2 meals (appetitive) reached at peak (149 µmoles (kg BW * h)$^{-1}$) at 6 h after last feeding and the lowest (88 µmoles (kg BW * h)$^{-1}$) for 4 meals (90%). Therefore, it was seen in all the observations that the maximum level of nitrogen excretion was found in 2 meals (appetitive) and lowest in 4 meals (90%).

In conclusion, there were no significant differences in weight gain, feed utilization, body composition, protein and energy retention between 2 and 4 meals restricted. However, nitrogen excretion peak values were significantly higher in 2 meals (90%) than 4 meals (90%), indicating that the 4 meals (90%) feeding regime facilitated better water quality and thereby a better environment for the fish.

## Acknowledgements

The Experimental work of this thesis would not have been possible without close association with many people who always raised their hand for us when we needed them.

Firstly, we would like to state our heartfelt gratitude to our supervisor, Professor Trond Storebakken for his constant support, patience, motivation, enthusiasm and immense knowledge during the path of our thesis. His guidance helped us in all the time of research and writing of this thesis. Moreover, we would like to acknowledge Mr. Bjørn Reidar Hansen and Mr. Frank Sundby for their helps and constant practical advice during the period of our experiment in laboratory. Similarly, we are grateful to all of our respected teachers of the institute of Animal and Aquacultural Sciences for their guide and excellent cooperation during our whole study periods. Lastly but not the least, we thank our family and friends for supporting us spiritually.

We are a team and could not triumph without either of us. Therefore, we also want to say thanks to each other.

Thank you all.

Biswas Bajgai (Master in Feed Manufacturing Technology)

Rezaul Hoque (Master in Aquaculture)

Norwegian University of Life Sciences (NMBU)

Ås, 30 October 2014

## List of abbreviations

| | | | |
|---|---|---|---|
| % | Precent | mBW | Metabolic body weight |
| µg | Mircrogram | mg | Milligram |
| µmoles | Micromoles | min | Minute |
| AA | Amino acid | MS | Microsoft |
| ANOVA | Analysis of variance | $NaHCO_3$ | Sodium bicarbonate |
| ATP | Adenosine triphosphate | Nex. | Nitrogen excretion |
| BW | Body weight | $NH_3$ | Ammonia |
| $CO_2$ | Carbon dioxide | $NH_4^+$ | Ammonium |
| COOH | Carboxylic acid | $NH_4$-N | Ammonium-nitrogen |
| DM | Dry matter | NMBU | Norwegian university life sciences |
| FAO | Food and Agriculture Organization | $NO_2^-$ | Nitrite |
| FCR | Feed conversion ratio | $NO_2$-N | Nitrite-nitrogen |
| FI | Feed intake | $NO_3^-$ | Nitrate |
| Fig. | Figure | $^OC$ | Degree Celsius |
| GIFT | Genetically improved farmed tilapias | pH | Scale of acidity |
| h | Hour | SAS | Statistical analysis system |
| HCl | Hydrochloric acid | SBM | Soybean meal |
| ICP | Inductively coupled plasma | SEM | Standard error of the mean |
| kg | Kilogram | t | Time |
| kJ | Kilo Joule | TAN | Total ammonia nitrogen |
| l | Litre | WG | Weight gain |

**Table of contents** **Page**

**List of figures** Page

**List of tables** Page

# 1. Introduction

## 1.1 Tilapia and its importance

There are about 100 species of fish named tilapia but the Nile tilapia (*Oreochromis niloticus*) (Linnaeus, 1758), the Mozambique tilapia (*Oreochromis mossambicus*), and the Blue tilapia (*Oreochromis aureus*) are widely farmed. Among these three, Nile tilapia is the most commonly farmed and widely spread species (Pullin et al., 1997), due to its fast growth rate, higher fecundity and better flesh quality. The Nile tilapia, *Oreochromis niloticus* (Linnaues, 1758) is one of the most important freshwater fish worldwide (Coimbra et al., 2005), belongs to cichlidae family. It is native to North Africa and Middle East (Boyd, 2004). It can be cultured in many tropical and subtropical countries of the world (Lin et al., 2008). Its farming has expanding quickly during last decade and is predicted to grow in coming years (Tveteras and Nystøyl, 2011). Tilapias are the second largest fishes after carps in production, in world aquaculture (FAO, 2012). Tilapia is produced in more than 100 countries surpassing the other farmed fish (Fitzsimmons et al., 2011; EI-Sayed, 2006). The total worldwide production of farmed tilapia has increased from 3,83,654 metric tons i.e. 4.5% of total farmed fish production to 34,97,391 metric tons or 8.9% of total farmed fish produced between the years 1990 to 2010 (FAO, 2012). China is the largest producer of tilapia with 1.2 million tons that occupy about 40% of total tilapia production in the world (FAO, 2010). Tilapia is an omnivorous fish and can digest high fibre content feed. Tilapia can eat phytoplankton, periphyton, aquatic plants, invertebrates, benthic fauna, detritus, bacterial films, fish and fish eggs (Fitzsimmons and Watanabe 2010; FAO, 2012). The tilapia, therefore, can grow in lower protein and higher carbohydrate level in feed as compared to carnivorous farmed species (El-Sayed 2006). However, some factors including fish size, age, dietary protein source, energy content, water quality and culture conditions affect the protein requirement for the tilapia. Protein requirement decreases with increasing fish size and age. The protein requirement for tilapia ranges from 20- 50% (Nguyen, et al., 2009; El-Saidy and Gaber, 2005; Abdel-Tawwab et al., 2010; NRC., 1993; El-Sayed and Teshima, 1992; El-Sayed et al., 2003). Carbohydrate is one of the major dietary components; it does not only supply necessary energy but also have a protein-sparing effect in fish (Habib et al., 1994). Tilapia can utilize the high amount of starch (22-46%) efficiently (Wang et al., 2005). The lipid requirement for the tilapia is from 5-12% considering the linoleic (n-6) series fatty acids (18:2n-6 or 20:4n-6) because it

can enhance the growth better than the n-3 series (18:3n-3, 20:5n-3 or 22:6n-3) (Lim et al., 2011).

Tilapia can tolerate a wider range of environmental conditions—including factors such as salinity, dissolved oxygen, temperature, pH, and ammonia levels. Tilapia can endure low dissolved oxygen concentration about 0.1 mg $l^{-1}$ (Magid and Babiker, 1975) but the optimum growth is obtained at 3 mg $l^{-1}$ (Ross, 2000). Temperature plays a major metabolic role in fish. The optimal growth temperature for growth is 22-29°C and growth decreases with lowering temperature (Teichert-Coddington et al., 1997). The growth of tilapia is poor below 20° C and it cannot survive in temperatures below 10° C. Likewise, it cannot stand the temperatures as high as 42° C (Morgan, 1972; Mires, 1995). Tilapia can tolerate pH range of 3.7 to 11, but pH 7 to 9 is the ideal range for achieving the best growth (Ross, 2000). Ammonia is toxic to tilapia at concentrations of 7.1 mg $l^{-1}$ as unionized ammonia (Redner and Stickney, 1979; El-Sherif et al., 2008) and reduces feed intake and growth at concentrations as low as 0.1 mg $l^{-1}$ (El- Sherif et al., 2008). Optimum concentrations are estimated to be below 0.05 mg $l^{-1}$ (El-Sherif et al., 2008).

## 1.2 Some important plant protein sources used in tilapia diets

The major plant ingredients used in the fish feeds are pulses and protein concentrate meals (peas, lupins), oilseed meals (soybean, sunflower, rapeseed and soybean protein concentrates) and cereals and cereal by-products (wheat, maize, rice, barley, sorghum, oats, rye, millet, wheat gluten, dry distiller's grains with soluble, rice bran) (Tacon et al., 2011; Gatlin et al., 2007). The plant ingredients contain more indigestible organic matter like insoluble carbohydrate and fiber that may have harmful effects (Naylor et al., 2009; Gatlin et al., 2007). Soybean is the most commonly used feed ingredient for tilapia. Soybean meal is the product of soybean after removal of oil from soybean. It is a major alternative plant protein sources globally (Hertrampf and Piedad-Pascual, 2000). Soybean meal with Solvent extraction of the oil has 44% crude protein and 48% crude protein of soybean hulls and without the hulls respectively (NRC, 1993). It has high protein content, and comes with relatively well-balanced amino acid profiles, a reasonable price and steady supply (EI-Sayed, 1999). The most commonly used products are toasted soybean meals, dehulled soybean meal, non-dehulled soybean meals and ungrounded soybean cakes all around the world. All these

products are used depending on availability in the particular locality. Soybean meal is highly palatable to the warm water fish (Lowell, 1998). Methionine is the limiting amino acid in soybean meal (SBM). Soybean meal could replace 25% and 30% of fish meal protein without any effect on growth performance in tilapia (Jackson et al. 1982) and feed intake in seabream (Kissil et al., 2000). Incorporation of soy protein concentrate in carp larvae diets up to 40% did not affect the growth (Escaffre et al., 1997).

Corn gluten is another feed ingredient for tilapia. Corn gluten meal is a by-product of the wet-milling of maize that produces starch or ethanol. Corn gluten meal is mainly used in wet or dried form as feed for fish. Corn gluten meal is rich in protein ranging from 62.2-72.5 %, with high digestible energy content and low fiber, and well balance amino acid profile. It has also no anti-nutritional factors. But it is deficient in lysine contents (Pereira and Oliva-Teles, 2003). Some studies report that partial replacement of dietary fish meal with corn gluten meal (12-26% of the diets) has led to sufficient results of growth rates and feed employment in diets for the rainbow trout *(Oncorhynchus mykiss)* (Robaina et al., 1995). There was not any adverse effect in growth or feed efficiency when fish meal replaced by corn gluten meal at levels up to 20% in sea bass juveniles (Alliot et al., 1979).

## 1.3 Utilization of protein

Protein is the most essential nutrient for maintaining life and promotion of growth for fishes. Amino Acids are the building units of proteins, which are organic compounds with an amino group at one end and a carboxyl group at the other. Proteins are polymers of amino acids linked together by peptide bonds. There are about 300 amino acids occurring in nature but only 20 of them enter in proteins synthesis. Each amino acid has 4 different groups attached to α-carbon (the C-atom next to COOH). These 4 groups are amino group, COOH, hydrogen atom and side chain (R). In fish, the dietary proteins are hydrolyzed to free from amino acids, dipeptides and tripeptides in the intestinal lumen. These dipeptides and tripeptides are present in high concentrations, which are absorbed either directly or after hydrolysis, to amino acids (Bakke et al., 2010; Verri et al., 2010). Peptides are absorbed more rapidly than absorption of free amino acids. They are hydrolyzed within the enterocyte. Only free amino acids are absorbed and transported to blood and mainly diffusion and $Na^+$ independent carriers transport them. Amino acids diffuse across the basolateral membrane (Enterocytes → portal blood → liver → tissues). Moreover, liver is the major site of amino acid metabolism in the

body and the major site of urea synthesis. The liver is also the major site of amino acid degradation, and partially oxidizes most amino acids, converting the carbon skeleton to glucose, ketone bodies, or $CO_2$. In the liver the primary catabolism of amino acids take place in the tricarboxylic acid cycle to generate energy in the form of adenosine triphosphate (ATP). In the tricarboxylic acid cycle, the mechanism for degradation of amino acids is trans-deamination, in which the amino group is transferred to α-ketoglutarate to form glutamate. Other metabolic pathways for amino acid catabolism in the liver are gluconeogenesis, with glucogenic amino acids being the main source of carbon to form glucose, and lipogenesis, with amino acids being the preferred carbon source for lipid synthesis. Ammonia is the main end product of amino acid catabolism in fish.

## 1.4 Use of crystalline amino acids as dietary supplements

The most common and complete fish feed ingredient fishmeal was substituted by the plant protein sources. These plant protein sources are similar protein content but these sources do not have complete amino acids profile like fish meal. Consequently it needs great consideration when feed is prepared (Wilson et al. 1981). Dietary protein is actually required for amino acids. Amino acids are the building blocks of protein and amino acids are the products of protein hydrolysis. Animals require amino acids for maintenance and growth and naturally occurring proteins are the primary source of amino acids. So, fish requires amino acids for maintenance and growth. The level of protein needed by fish varies with the species and the amino acid composition of the protein fed. There are over 20 amino acids in body protein which are physiologically essential. Fish is unable to synthesize 10 of these amino acids that are called essential amino acids (Ketola, 1982; Ravindran and Bryden, 1999). Therefore, these amino acids must be supplied in the diet. These essential amino acids are arginine, lysine, histidine, threonine, valine, leucine, isoleucine, methionine, phenylalanine and tryptophan (Wilson, 1989). Amino acid is very important and we should make sure when feed is formulating with different ingredients.

Amino acids can supply in the form of intact and purified, as well as crystalline form as dietary supplements. Intact amino acids are those amino acids available in feed ingredients. Most of the plant-based ingredients lack some essential amino acids. Inclusion of artificially made purified amino acids in crystalline form in the least-cost plant protein ingredients based diets are the best way to satisfy those lacking the amino acid profile.

Crystalline amino acids have been successfully used to terrestrial livestock's feed to optimize dietary amino acids profile (Lewis and Bayley, 1995). It has also been used in aquaculture especially when plant protein sources are used in the fish diets because these sources are cheap, also due to the deficiencies of some amino acids (Li and Robinson 1998; Shiau 1998; Alam et al. 2002). Essential amino acids in intact protein are utilized more efficiently and effectively than those amino acids which are provided in crystalline form. Crystalline amino acids are absorbed and catabolized more rapidly than amino acids from intact proteins may be possible reason. Amino acid concentration in blood plasma increased immediately in the fishes fed with crystalline amino acids in comparison to those fed with intact proteins when some researchers conducted amino acids trails in fish (Yamada et al. 1981; Murai et al. 1987; Schuhmacher et al. 1995). It is necessary to balance the supply of free amino acids to blood plasma and muscle tissues for optimum growth and feed utilization in fish. Large portion of amino acids from the diets are deaminated and used in energy and only 30-40 % is retention in the body (Cowey, 1994). Yamada et al. (1981) concluded that rainbow trout fed with crystalline amino acids showed the inferior growth rate and feed conversion ratio due to the rapid uptake of amino acids from the gut into the peripheral blood that lead to deamination of the excess amino acids and also high rates of nitrogen excretion. Mixing many different plant protein sources and supply the minimum quantity of adequate amino acids can fulfill adequate amino acids.

Lysine is essential amino acid for fish, which are found highly in carcass of fish (Wilson and Cowey, 1985). It is usually limited in plant protein sources such as corn meal, sesame meal, zein, gluten or corn-based proteins and casein (Ball et al. 2007, Espe et al. 2007; Mai et al. 2006; Small and Soares 2000). It is the first-limiting essential amino acid when some plant protein sources are used for feed production (Hauler and Carter, 2001). Lysine is the precursor for carnitine, which is required for the transport of long-chained fatty acids from the cytosol into mitochondria for β-oxidation and plays important role in energy production (Harpaz, 2005; Horne et al. 1971; Walton et al. 1984). In addition, lysine also affects collagen synthesis, as its hydroxylation product, which is necessary for formation of the intermolecular crosslinks in collagen (Eyre, 1980; Piez and Likins, 1957). Furthermore, lysine has highly reactive Ɛ-amino group; it is very sensitive to heat damage and non-enzymatic glycolysis reactions, resulting in the production of Maillard reaction products (Moughan and Rutherfurd, 1996). In general, the lysine requirement for Nile tilapia needs 5.0-5.7% (Santiago and Lovell,

1988). Methionine is another an essential amino acid and one of the most limiting amino acids in many fish diets containing plant protein sources such as soybean meal, peanut meal, copra meal, leucaena leaf meal or cassava leaf meal (Coloso, et al., 1999). Supplementing commercially available methionine to soybean meal has been shown to improve growth of many fish species (Cai and Burtle, 1996).

## 1.5 Ammonia and its consequences

There are several nitrogenous wastes products in the water environment such as ammonia ($NH_3$), nitrite and nitrate in fish tank. Amongst them, ammonia is more toxic than the other two. Between the lateral two, nitrite is more toxic than nitrate. Ammonia is the nitrogenous end- product of amino acid and protein oxidation (Smith and Rumsey, 1976). It could be a great obstacle for fish growth and can cause mortality. Contrarily, Nitrite is the nitrogenous intermediate formed out of nitrate during the nitrification of ammonia by bacteria. Similarly nitrite could be a lethal factor for recirculating system. It also plays a role in interrupting the normal structure of blood hemoglobin and converting haeme group into nitrite-bund forms. As a result, it adversely affects the oxygen-carrying capacity of hemoglobin (Jensen 1990; Margiocco et al., 1983).

Ammonia exists in equilibrium between the two forms in soluble: the soluble ammonia gas ($NH_3$) and ammonium ion ($NH_4^+$), together known as total ammonia. Mainly it will exist in ionic form ($NH4^+$) and is converted to $NH_3$ when the temperature and pH is increased (Wood 1993). Total ammonia nitrogen (TAN= $NH_3$ and $NH_4^+$) is converted to nitrite ($NO_2^-$) by *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira* and *Nitrosolobus* bacteria. Under normal conditions, nitrites are quickly converted to non-toxic nitrate ($NO_3^-$) by naturally occurring bacteria like *Nitrobacter*, *Nitrococcus*, *Nitrospira* and *Nitrospina* (Parker, 2002; Timmons et al., 2002, Chen et al., 2006; Emparanza, 2009).

Likewise, fish excretion is the main source of ammonia (Cheng, 2004). The excretion rate is directly related to the feeding rate and the protein level in the feed. The remaining portions of the nitrogen that used after the body building energy and excreted through the gills are ammonia. It is the dominant product of protein metabolism in fish (Handy and Poxton, 1993)

and at least 80% of nitrogenous metabolic waste excreted through fish gills is ammonia. Urea generally represents only about 10- 15 % (Kaushik and Cowey, 1991). Urea usually excretes through kidney. Ammonia is toxic even at low concentrations, particularly in $NH_3$ (unionized ammonia) form (Chew et al., 2006; Felipo and Butterworth, 2002; Ip et al., 2004; Wicks and Randall, 2002) and it is a toxic compound which can adversely affect fish health. The main internal source of ammonia in fish is through the catabolism of proteins (amino acids) and most of ammonia is produced in liver during the transamination of amino acids followed by deamination of glutamate (Wicks and Randall, 2002).

Ammonia is mainly excreted through gills (Wilkie, 2002). There is a direct relation between protein intake and ammonia excretion in fish (Rychly, 1980; Beamish and Thomas, 1984; Li and Lovell, 1992; Ballestrazzi et al., 1994; Chakraborty and Chakraborty, 1998). The rate of excretion increases after feeding, and is the highest during the four hours after feeding. Excretion continues for one to four days. After that it declines to the original baseline levels (Altinok and Grizzle, 2004; Jobling, 1981; Dosdat et al., 1996; Peres and Oliva-Teles, 2006). Ammonia excretion rate varies upon fish species, fish size, protein quality, energy level, feed quality, water quality, water temperature and pH (Kaushik and Cowey, 1991; Chakraborty and Chakraborty, 1998; Fu-Guang et al., 2009; Kieffer and Wakefield, 2009; Green and Hardy, 2008; Peres and Oliva-Teles, 2006) and feeding schedule (Gelineau et al., 1998; Wicks et al., 2002; Zakes et al., 2006). Diets formulated with poor amino acids profile also influence the excretion of more ammonia (Bureau, 2004).

The nature and degree of toxicity depends on many factors, including the chemical form of ammonia, the pH and temperature of the water, the length of exposure, and the life stage of the exposed fish. Ammonia exists in $NH_3$ (unionized) and $NH_4^+$ (ionized) form at dissolved water. $NH_4^+$ is very difficult to enter into the fish body through gills and less bioavailable as compared to $NH_3$. $NH_3$ can easily enter from water to fish body and change into $NH_4^+$ and causes cellular damage US EPA (1989). At pH 7-8, $NH_4^+$ (ionized) is the primary from of total body ammonia in fish, which is responsible for toxic effect in the fish body. But $NH_3$ affects the whole aquatic plants and animals. In higher temperature and pH values, $NH_3$ is more toxic (US EPA, 1999). According to US EPA (2009), the ratio of $NH_3$ to $NH_4^+$ (ionized) increased 10 times each unit rise in pH and about 2 times for each 100C rise in temperature

from 0-30$^0$C. Length of exposure is one of the important factors in ammonia toxicity. Lower concentration of ammonia may not inversely affect the fish in short period exposure time but fish could be adversely affected under longer exposure. Ammonia toxicity susceptibility varies with fish life stage. Susceptibility decreases as fish develop from fry to juveniles and increases from juvenile to adults (Thurston et al. 1983). Ammonia enters a fish through it gills epithelium in fresh water. It occurs through passive diffusion in solution (Randall and Tsui 2002; Eddy, 2005). Ammonia is the end product of metabolism and it also excreted across the gill epithelium (Randall and Wright, 1987; Wright, 1995; Randall and Tsui, 2002). When excretion of ammonia is lower than entering through gill is higher, it can accumulate in the fish body. Ammonia can be very toxic to the fish due to high concentration inside the body. It causes loss of equilibrium, hyper excitability, increased breathing, cardiac output, and oxygen uptake, and in extreme cases, it also affects the central nervous system causing convulsions and death (US EPA, 1989). If the concentration of ammonia is lower, it can cause a reduction in hatching success, reduction in growth rate and morphological development, and pathologic changes in tissues of gills, livers, and kidneys (US EPA, 1989).

## 1.6 Objectives of the study

The first objective of the experiment was to find out effects of feeding frequency on utilization of protein and energy in Nile tilapia.

The second objective was to quantify differences in excretion of ammonia and ammonium in Nile tilapia fed the same daily ration, distributed over 2 and 4 meals a day.

## 2. Materials and methods

## 2.1 Fish and rearing unit

The trial was conducted at Fish Nutrition Laboratory (Norwegian University of life sciences) during the period from 12th April to 29th May 2014. The experimental fish were hatched at the same laboratory and fed on a commercial diet (Aller Aqua, Denmark), until the individual body weight was approximately 24 g. The brood stock was GIFT tilapia (Eknath et al., 1993) originated from the 12th generation of selection for rapid growth by Genomar AS (Oslo, Norway). The indoor rearing tanks (70×50×50 cm) had freshwater recirculation systems with a water level of 50 cm in each tank. Each tank was stocked with 30 fish (mean weight ± S.E.M; 721 ± 0.1 g). The tanks were subjected to a photo regime 24 h light during the whole

**Table 1.** Chemical composition of ingredients used in feed (g $kg^{-1}$)*****

| Chemical composition | Soybean meal | Corn Gluten Meal | Potato Starch |
|---|---|---|---|
| DM | 888 | 895 | 904 |
| Protein | 424 | 579 | 12 |
| Fat | 30 | 41 | 3 |
| Starch | 20 | 191 | 806 |
| Ash | 95 | 11 | 2 |
| Arginine | 33 | 18 | 1 |
| Histidine | 12 | 12 | 0.3 |
| Isoleucine | 21 | 24 | 1 |
| Leucine | 33 | 95 | 1 |
| Valine | 21 | 27 | 1 |
| Lysine | 27 | 10 | 1 |
| Methionine | 6 | 13 | 0.3 |
| Phenylalanine | 22 | 36 | 1 |
| Threonine | 17 | 20 | 1 |
| Tryptophan | 6 | 3 | 0.2 |

*Fôrtabell, 2008 from NMBU.

Table 2. Formulation and chemical composition of the experimental diet.

| Ingredients, g $kg^{-1}$ | |
|---|---|
| Soybean meal[a] | 350 |
| Corn gluten meal[b] | 200 |
| Potato starch[c] | 355 |
| Threonine[d] | 0.6 |
| Methionine[e] | 4.6 |
| Phenylalanine[f] | 0.4 |
| Taurine[g] | 1.35 |
| Lysine[h] | 3.20 |
| Mono calcium phosphate[i] | 10 |
| Rapeseed oil[j] | 45 |
| Premix[k] | 10 |
| $Y_2O_3$[l] | 0.08 |
| Vit-C 35%[m] | 0.10 |
| Sodium alginate[n] | 20 |
| Feed composition, $kg^{-1}$ | |
| Dry matter, g | 949 |
| Crude protein, g | 292 |
| Starch, g | 396 |
| Lipid, g | 44 |
| Ash, g | 43 |
| Energy, MJ | 20 |

[a]Soybean meal, Denosoy, Denofa, Fredrikstad, Norway. [b]Maize gluten, Cargill 13864. [c]Gelatinized potato starch, Culinar, LYGel F60. [e]Adisseo Brasil Nutricao Animal Ltda, Sao Paulo, Brazil. [g]Taurine-JP8, Qianjiang Yongan Pharmaceutical Co., Ltd., Hubei, China. [j]Food grade Eldorado, Oslo, Norway. [k]Contents per Kg: Vitamin A 2500.0 IU; Vitamin $D_3$ 2400.0 IU; Vitamin E 0.2 IU; Vitamin $K_3$ 40.0 mg; Thiamine 15.0 mg; Riboflavin 25.0 mg; d-Ca-Pantothenate 40.0 mg; Niacin 150.0 mg; Biotin 3.0 mg; Cyanocobalamine 20.0 g; Folic acid 5.0 mg; Pyridoxine 15.0 mg; Vitamin C: 0.098 g (Stay-C 35, ascorbic acid phosphate, DSM Nutritional Products, Basel, Switzerland); Cu: 12.0 mg; Zn: 90.0 mg; Mn: 35.0 mg; I: 2.0 mg; Se: 0.2 mg; Cd = 3.0 g; Pb = 28.0 g; total Ca: 0.915 g; total K 1.38 g; total Na 0.001 g; total Cl 1.252 g; Trouw Nutrition, LA Putten, The Netherlands. [l]Metal Rare Earth Limited, Jiaxing, China. [m]Stay-C 35, ascorbic acid phosphate, DSM Nutritional Products, Basel, Switzerland.

experimental period. The water temperature and the dissolved oxygen were recorded on a daily basis- average temperature was 27.5 °C with 7.5 mg $l^{-1}$ dissolved oxygen during the experimental period. Dissolved oxygen was measured daily online by oxygen meter (Oxyguard Commander, DO probe, Farum, Denmark). Also around equal water flow was kept in all tanks, which was 180 litres per hour in an average.

## 2.2 Experimental diet

Only one complete plant based diet was prepared for the experiment. The feed ingredients were soybean meal, corn gluten, potato starch, rapeseed oil, some crystalline essential amino acids (threonine, methionine, phenylamine and lysine), mono calcium phosphate, taurine, premix, yttrium oxide ($Y_2O_3$), vitamin C (35%) and sodium alginate. The diet was formulated with 31% crude protein, 7 % fat and 37% starch. The composition and formulation of the diets are shown in Tables 1 and 2 respectively.

## 2.3 Feed Preparation

Feed was prepared in the feed lab at NMBU and each time 5 kg (total 30 kg) of feed was prepared. Preparation of the diets began with grinding the soybean meal and maize gluten meal by using 1mm screen (Retsch Gmbh Retsch-Allee 1-5, 42781, Haan, Germany). All the ingredients were correctly weighed. After all dry ingredients had been weighed; ingredients were mixed properly and carefully. Small amount ingredients were mixed with small portion of large quantity, again mixed with another small portion of large quantity. After that uniform mixing of all the large and small quantity was possible. The spiral dough mixer (Moretti Forni Grain, Italy) was used to mix more uniformly about 15 min. During the mixing time, cold water for 40% of total feed weight and all the amount of rapeseed oil were mixed by pouring slowly and gently. All the feed dough was transfer to a pasta extruder (P55DV, Italgy, Carasco, Italy) and properly conditioned and mixed than cut in pellet at 2 mm size by using the pellet cutter at the edge of the craft opening. The process was repeated thrice. Likewise, the temperature of the pasta machine was $54^0$C. The prepared pellets were transferred on the dryer at $54^0$C in five hours. Finally, the prepared pellets were cooled and packed air tight and stored at $-20^0$C. Feed moisture was measured before storing the feed that was 5%. Each time 100 g of sample diet was taken for chemical composition analysis.

## 2.4 Experimental layout

There were two different feeding regimes. One was feeding only twice daily at 10:00 and 20:00 where 70 min were allocated per feeding. The other was four times a day at 8:00, 12:00, 16:00 and 20:00 where 35 minutes were allocated per feeding. Each treatment had three tanks. Feeding was done by electrically driven band feeding machine. Tilapias in 3 tanks were fed 2 meals a day in excess of appetite. Dietary dry matter intake was assessed as the difference between daily rations fed and the amounts collected from the water outlet during and immediately after every meal. Daily collections of uneaten feed were pooled by tank and dried at 105$^{o}$C overnight. Fish in the remaining 6 tanks were pair-fed 90% of the average previous day dry matter intake of the fish fed in excess. This amount was given to fish in 3 tanks divided into 2 meals a day, while fish in the remaining 3 tanks were fed 4 meals.

The dry matter intake was confirmed by the same routine as for the fish fed ad libitum. From the value from initial fish weight and expected FCR, daily feed intake percentage per fish and daily weight gain were estimated in an MS EXCEL sheet. After end of the experiment, the final weight of the fish per tank was measured. Then, the FCR estimate was adjusted so that final weight gain was identical to the values obtained by weighing the fish. Based on the assumption that FCR was the same throughout the experiment, daily average fish weights were estimated. These estimates were used to calculate daily dry matter intake in percent of body weight.

## 2.5 Sampling Procedures

Before starting the feeding trail, all the fish were starved for 24 h. All fish were netted with minimum disturbance. It was anesthetized by tricanine methanesulfonate (MS-222, 0.1 g $l^{-1}$ water, buffered with $NaHCO_3$, 0.1 g $l^{-1}$ water, Western Chemical Inc. Washington USA). All fish were weighed individually and separated under and oversized fish. Thirty homogenously sized fish were stocked in each tank. At the same time, five fish were randomly taken from the tank where experimental fish were taken and kept in freezer at -20$^{o}$C for initial whole body composition analysis and calculation of retention. At day 28 of the feeding trial, fish in each tank were again weighed for calculating for growth and feed utilization. That sampling was done after 24 h starvation. At the same time, five fish were randomly taken from each of three replicate tanks for body proximate composition analysis (Procedure described in 2.6).

After sampling, the remaining fish were readjusted and distributed into the same tanks. It was adopted with their respective diets.

Blood sampling was done at day 41. Blood was collected at 2, 4, 6, 8 and 10 h after the last meal. Three individual fish from each tank each time were taken out for blood sampling. So, total 15 fish in five times. Analysis of blood is not included in this thesis.

At the same time after blood sampled, feces were collected at same time as mentioned above. Ammonia concentration was measured at days 14, 26, 39 and 45 (Procedure described in Figure 2.7). Remaining seven fishes were taken from each tank and weighed the fish and liver individually. Liver weight in percentage was calculated. Seven fish were sampled from each tank and that was done at day 46 after ammonia measurement.

## 2.6 Chemical composition of diet, fish bodies and feces

Diets and tilapia body composition was done by proximate analysis. The sampled fish were removed from the - $20^0$C freezer and half thawed prior to analysis and weigh in (Sartorius AG, Göttingen, Germany). After that, the fish were homogenized in a grinder, and ground fish was freeze-dried for a week. Then the samples were reground with of $CO_2$ ice finer size. Dry matter content of fish was determined as weight loss after drying the samples at $105^o$C (until constant weight) for 20 h in an oven (ISO, 1983). Crude proteins (Kjeldahl N×6.25) were determined by Kjeltec auto 1035/1038 system (Tecator, Sweden). Starch was analyzed as glucose after starch hydrolysis with a heat tolerant amylo-glucosidase in accordance with the procedure of (McCleary et al., 1994). Hydrochloric acid (HCl) hydrolysis followed by diethyl ether extraction (Commission dir.98/64/EC) method was used to determine crude fat. Ash content was determined by heating at $500^o$C in muffle furnace. Yttrium oxide ($Y_2O_3$) was quantified in diet and freeze-dried feces by ICP analysis. Fecal nitrogen was analyzed by the Dumas method.

## 2.7 Ammonia and nitrite measurements

Ammonium ($NH_4$-N) and nitrite ($NO_2$-N) concentration in water was measured one hour before (19:00) the last meal and every 2 h after last meal (22:00, 00:00, 02:00, 04:00 and 06:00). Ammonia concentration was measured at day 14, 26, 39 and 45 of experiments. At days 14, 26 and 39 of the experiments, ammonium ($NH_4$-N) and nitrite ($NO_2$-N) were measured from inlet and outlet water of each tank. For actual value, subtract the value of outlet and inlet of each tank each time of measurement. After blood sampling, the remaining seven fishes in each tank was adjusted. Fishes were continuously maintained on their respective diets for 3 days. At day 45, ammonium ($NH_4$-N) and nitrite ($NO_2$-N) were measured by closing the inlet and outlet and water sample was taken from inside tank. The tanks were covered by black polythene to prevent from evaporation. Spectroquant® NOVA 60 was used to measure Ammonium ($NH_4$-N) and nitrites ($NO_2$-N). About 50 ml water was sampled per tank per measurement. The detail procedures to Ammonium ($NH_4$-N) and nitrites ($NO_2$-N) measurements are described in appendixes 1 and 2. Later, $NH_4^+$ and $NO_2^-$ were calculated on the basis of conversion Table 3. After that, ammonia ($NH_3$) was calculated based on the pH and temperature of the water. The observed average water temperature was $27.5^0$C and pH was 7. Therefore, the $NH_4^+$ and $NH_3$ are 99.33% and 0.67% respectively in total 100% of total ammonia nitrogen (for details see appendix 3).

**Table 3 Conversion table of $NH_4^+$, $NH_3$ and $NO_2^-$**

| |
|---|
| $NH_3 = NH_3\text{-}N * 1.22$ |
| $NH_4^+ = NH_4\text{-}N * 1.29$ |
| $NO_2^- = NO_2\text{-}N * 3.28$ |

In the stagnant system at days 45, the total ammonia nitrogen (TAN), ammonium ($NH_4^+$), ammonia ($NH_3$) and nitrite ($NO^-_2$) were measured in 2 hours interval after feeding. According to the Periodic Table, the atomic mass of nitrogen is 14.01, hydrogen is 1.01 and oxygen is 16.00. So, total mass of $NH_4^+$ is 14.01 + 4*(1.01) = 18.05. Likewise, $NH_3$ is 17.04 and $NO^-_2$ is 46.01. That means one mole of $NH_4^+$, $NH_3$ and $NO^-_2$ are considered as 18.05, 17.04 and 46.01 grams successively. After that, the entire milligram was changed to micromoles. Then, metabolic nitrogen excretion was calculated. For calculating the nitrogen excretion, change in

nitrogen excretion per time of sampling should be calculated. We used the formula that is shown below:

$\Delta N = (N*V)\ T_{n+2} - (N*V)\ T_n$

where,

N is moles of $NH_4^+$, $NH_3$, TAN and $NO_2^-$

V = water volume in l

$T_n$ = hours past feeding, n = 0, 1, 2, ..10.

Calculations were made for ΔTAN without ($\Delta NH_4^+ + \Delta NH_3$) or with $\Delta NO_2^-$. Finally, the sum of those values was divided by body weight (BW, kg) or metabolic weight (mBW, $kg^{0.8}$). The use of mBW did not give any increase in $R^2$ in the regression analysis (to be described later) compared to BW, so BW is used in the presentation of results. Thus two alternative values for metabolic nitrogen excretion over the gills are presented in µmoles (kg BW * h)$^{-1}$:

1. Metabolic nitrogen excretion including $\Delta NO_2^-$ = ($\Delta NH_4^+ + \Delta NH_3 + \Delta NO_2^-$) * ((BW (kg) * time interval of measurement (h)))$^{-1}$
2. Metabolic nitrogen excretion excluding $\Delta NO_2^-$ = ($\Delta NH_4^+ + \Delta NH_3$) * ((BW (kg) * time interval of measurement (h)))$^{-1}$

## 2.8 Fish growth performance, feed and protein utilization

$$\text{Feed conversion ratio (FCR)} = \text{Dietary dry matter intake (g)/weight gain (g)}$$

$$\text{Protein Retention (\%)} = 100 * \frac{(\text{Final protein content in fish (g)} - \text{initial protein content in fish(g)})}{\text{protein intake by fish (g)}}$$

$$\text{Energy Retention (\%)} = 100 * \frac{(\text{Final energy content in fish (kJ)} - \text{initial energy content in fish(kJ)})}{\text{energy intake by fish (kJ)}}$$

$$\text{Liver weight percent} = 100 * \left(\frac{\text{liver weight of fish (g)}}{\text{body weight of fish (g)}}\right)$$

## 2.9 Digestibility measurement

At day 41, we took 15 fishes from each tank and dissected and collected feces from the last 10 cm of intestines by gentle squeezing. Crude protein and yttrium oxide contents were measured from the feces (pooled by tank), and the same measurement was also done in feed.

$$\text{Apparent digestibility} = 100 - 100 * \left(\frac{\text{nutrient in faeces (\%)}}{\text{nutrient in feed (\%)}} * \frac{\text{Yttrium in feed (\%)}}{\text{Yttrium in faeces (\%)}}\right)$$

## 2.10 Statistical analysis

One-way and two-way analysis of variance was employed to analyze the results statistically. Significant ($P<0.05$) differences were ranked in SAS by P-diff under Least-square means, and are indicated by different superscript letters $^{a, b}$. Trends are indicated for $0.10 < P \leq 0.05$. Data concerning nitrogen excretion in the stagnant system and daily feed intake were subject to linear, 2$^{nd}$ or 3rd degree polynomial regression analysis in MS EXCEL. The model chosen was based on highest $R^2$ values.

# 3. Results

## 3.1 Daily feed intake in percent of estimated body weight

Daily feed intake in percent of estimated daily body weight during the four weeks of feeding is presented in Fig 1. The regression of weight gain on body weight (range of body weights after the fish has adapted to the experimental feeding regimes) is presented in Fig 2. The relationship between feed intake (FI, g DM $fish^{-1}$) and weight gain (WG, g $fish^{-1}$) was best described by linear regressions, which are presented in Table 4.

Table 4. Regression analysis of feed intake (FI, g DM $fish^{-1}$) on weight gain (WG, g $fish^{-1}$).

| Feeding regime | $R^2$, linear | Regression, FI = |
|---|---|---|
| Two meals, appetitive | 0.84 | - 0.049 * WG + 6.47 |
| Two meals, 90% | 0.77 | - 0.049 * WG + 6.09 |
| Four meals, 90% | 0.90 | - 0.05 * WG + 6.08 |

## 3.2 Growth performance and feed utilization

In the experiment, only one fish died, in 2 meals (appetitive). Thus, survival rate was not significantly different among treatments (P=0.42). The cause of death was that the fish was trapped in the outlet.

The initial mean weights of the groups were not significantly different (P=0.67) (Table 5). There was a tendency (P=0.06) that appetitive feeding resulted in higher final weight and weight gain than the two restricted feeding regimes. Feed intake was higher (P=0.0001) and FCR tended (P=0.07) to be higher for the fish fed appetitive than for the ones with a 10% restriction in daily ration.

## 3.3 Proximate composition of fish

The chemical composition of Nile tilapia is also presented in Table 5. There were no significant differences in whole body dry matter, crude protein, lipid, or energy contents of the fish, while ash tended (P=0.09) to differ, with the lowest value for the fish fed ad libitum.

## 3.4 Protein and energy retention

Protein and energy retention are presented in Table 5. Both retention of protein and energy were significantly lower in fish fed ad libitum than in the tilapias subject to feed restriction. Among the fish fed 90%, no significant differences were seen.

## 3.5 Protein digestibility

Protein digestibility is presented at Table 5 and There were significant differences (P=0.04) among all treatment. Protein digestibility was lower in 4 meals (90%) as compared to others.

## 3.6 Liver weight

Liver in percent of whole body weight is also presented in Table 5. The ratio on liver weight to body weight was similar value from 2 meals (appetitive) and 2 meals (90%) but 4 meals (90%) was lowest value. The liver weigh, in percentage of body weight, was significantly lower (P=0.04) in the fish fed 4 meals, compared to the tilapias fed 2 meals.

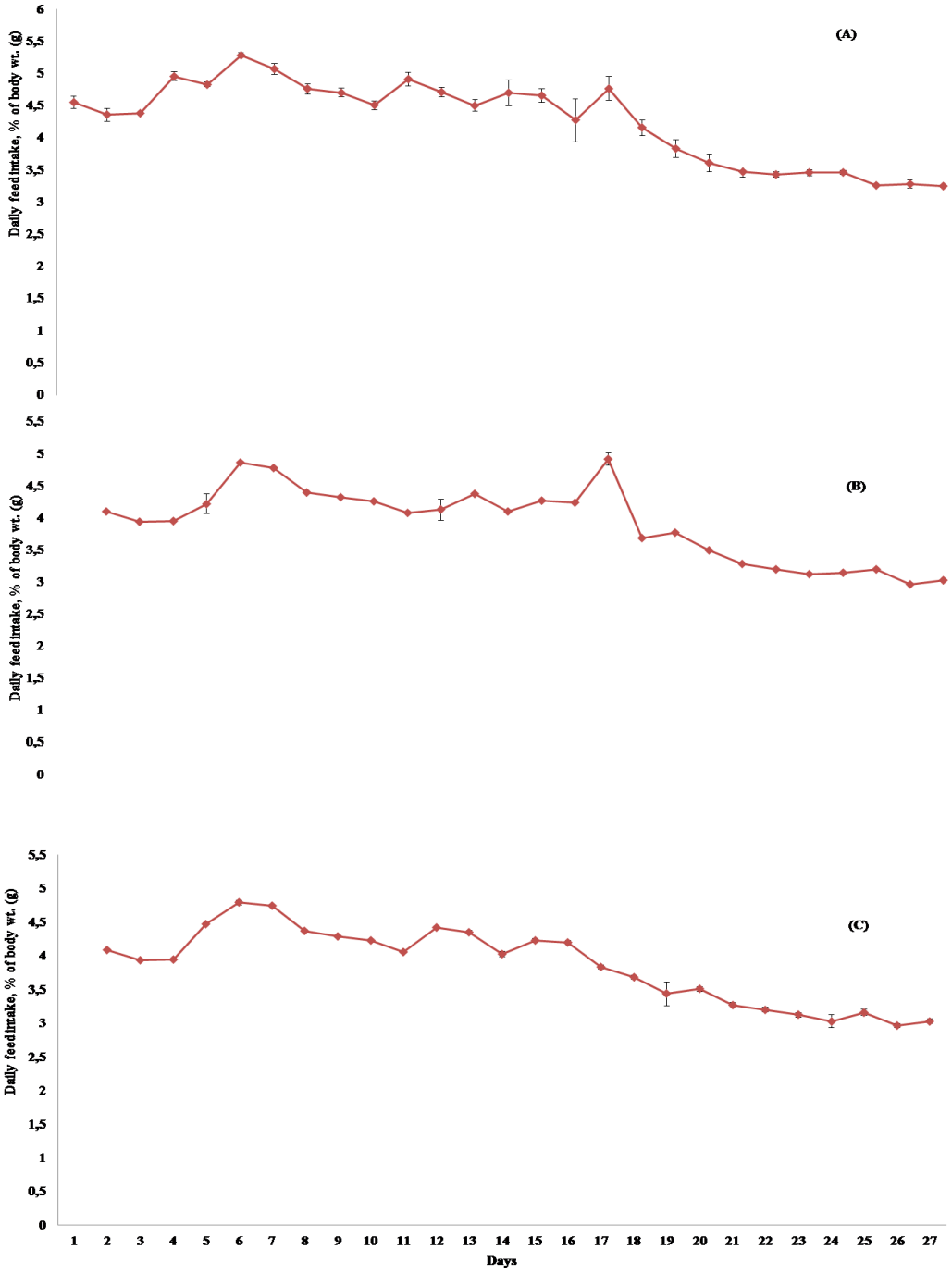



Figure 1. Daily feed intake of fish (A) 2 meals (appetitive), (B) 2 meals (90%) and (C) 4 meals (90%) during 4 weeks of experimental period which are presented in percentage of estimated body weight (mean ± s.e.m. of 3 tanks).

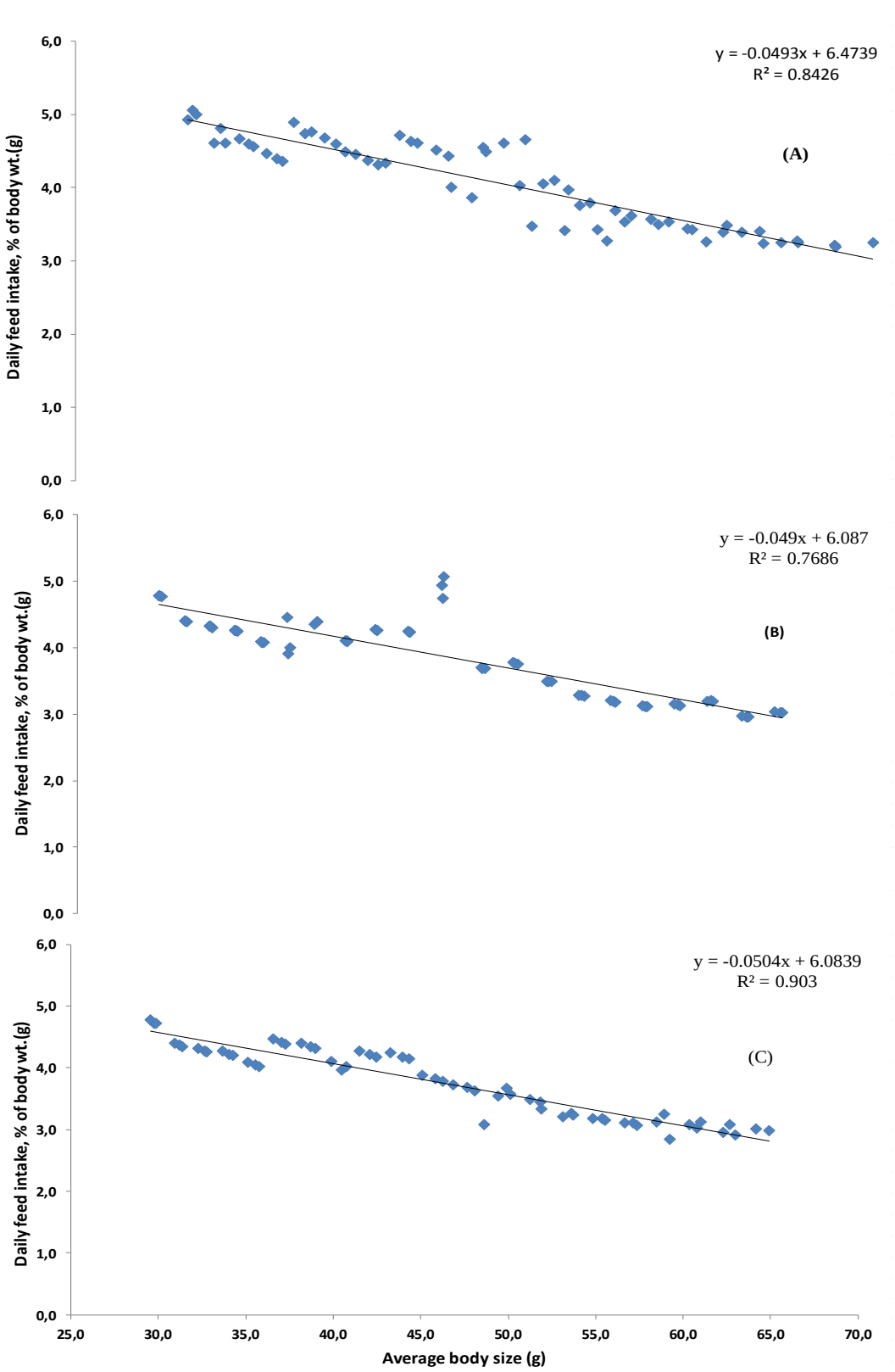



Figure 2. Daily feed intake of the tilapias following the period of adaptation (A) 2 meals (appetitive), (B) 2 meals (90%) and (C) 4 meals (90%) with respect to average body size at $27.5^0$C best fitted at linear regression pattern during 4 weeks of experimental period (mean of 3 tanks).

Table 5. Start weight, final weight, weight gain, feed intake (FI), whole body composition, feed conversion energy and protein retentions, protein digestibility and Liver weight[1].

| | Start sample | 2 meals appetite | 2 meal 90% | 4 meal 90% | Pooled s.e.m. | P<F |
|---|---|---|---|---|---|---|
| Survival rate, % | - | 99.9 | 100 | 100 | 0.11 | 0.42 |
| Fish$^{-1}$ | | | | | | |
| Start weight, g | - | 24.0 | 24.0 | 24.0 | 0.01 | 0.67 |
| Final weight, g | - | 70.7 | 66.9 | 66.9 | 2.4 | 0.06 |
| Weight gain, g | - | 46.7 | 42.9 | 42.9 | 1.8 | 0.06 |
| Feed intake, g | - | 49.1$^{a}$ | 42.3$^{b}$ | 41.8$^{b}$ | 0.47 | <0.0001 |
| Body composition, Kg$^{-1}$ | | | | | | |
| Dry matter, g | 297 | 325 | 325 | 322 | 40 | 0.52 |
| Crude protein, g | 166 | 133 | 135 | 136 | 23 | 0.22 |
| Lipid, g | 93 | 116 | 114 | 121 | 48 | 0.27 |
| Ash, g | 41 | 25 | 27 | 26 | 6 | 0.09 |
| Energy, MJ | 79 | 83 | 82 | 83 | 12 | 0.34 |
| Feed utilization | | | | | | |
| FCR | - | 1.06 | 0.99 | 0.97 | 0.04 | 0.07 |
| Retention , Kg$^{-1}$ | | | | | | |
| Protein, g | - | 405$^{a}$ | 450$^{b}$ | 449$^{b}$ | 15 | 0.02 |
| Energy, g | - | 410$^{a}$ | 438$^{b}$ | 432$^{b}$ | 12 | 0.02 |
| Protein digestibility, % | - | 98.3$^{b}$ | 98.5$^{b}$ | 97.6$^{a}$ | 0.46 | 0.01 |
| Liver weight,% | - | 4.7$^{b}$ | 4.3$^{b}$ | 3.6$^{a}$ | 0.4 | 0.04 |

[1]The values are given as mean of 3 tanks of fish. Different superscript letters$^{a,b}$ indicate significant (P<0.05) differences, ranked by P-diff in the LS means procedure in SAS (1991)

## 3.7 Ammonia and Nitrite measurement in flow through system

Pre and postprandial TAN, ammonium, ammonia and nitrite excretion rates µg l$^{-1}$ of water in some particular days and hours are presented in Figs. 3, 4, and 5 constantly. Unlike, postprandial TAN, ammonium, ammonia and nitrite excretion rates µg l$^{-1}$ of water is presented in Fig. 6 which was accumulation in tank during 10 hours of analysis. The same patterns of change in 2 meals (appetitive), 2 meals (90%) and 4 meals (90%) were observed at days 14 and 39. According to the Figs 3 and 5, we could see the level of TAN, ammonium

and ammonia was higher at one hour (19:00) earlier than two hours (22:00) later of last feeding, while it was opposite at days 26 which is presented in Fig. 4. However, the highest rate of production with a rapid increased was after 4 hours ( 00:00) of last meal, which was the same for all of the observation days. Similarly, TAN, ammonium and ammonia excretion were speedily decreased till 10 hours (06:00) in all the observed days. Unlike, at days 14 and 39, nitrite production was increased 8 hours (04:00) after last feeding and reached to the peck. Whereas, it was not the same at day 26. Based on two-way ANOVA there were significance difference ($P<0.05$) between feeding frequency, measurement time and their interaction in all parameters (TAN, ammonium, ammonia) at days 14, 26 and 39. Likewise, there was significantly different ($P<0.05$) among nitrite measurement at different period. However, there were not significantly different ($P>0.05$) among feeding frequency and their interaction (feeding frequency and measurement at different period).

### 3.7.1 Measurement at day 14

Production of TAN, Ammonium and Ammonia were observed at 2, 4, 6, 8 and 10 hours after last feeding, which showed the highest value in 2 meals (appetitive) and lowest in 4 meals (90%) successively. It was medium in 2 meals (90%) during the whole observed time compare to others. The rate was increased speedily, which was the highest after 4 hours (00:00) of feeding then it was gradually decreased and reached at lowest value after 10 hours (06:00). At the same time the level of nitrite was also observed. Similar pattern was found for all meals, which was increased rapidly after 2 hours (22:00) of feeding and reached to maximum level after 8 hours (04:00). Then it was decreased slowly and went to the lowest level at 10:00.

### 3.7.2 Measurement at day 26

During all the observed time the highest level of TAN, ammonium and ammonia was after 4 hours (00:00) of last feeding for all sorts of meals and 2 meals (appetitive) reached the maximum level. Contrary, the 2 meals (90%) and 4 meals (90%) were observed almost the

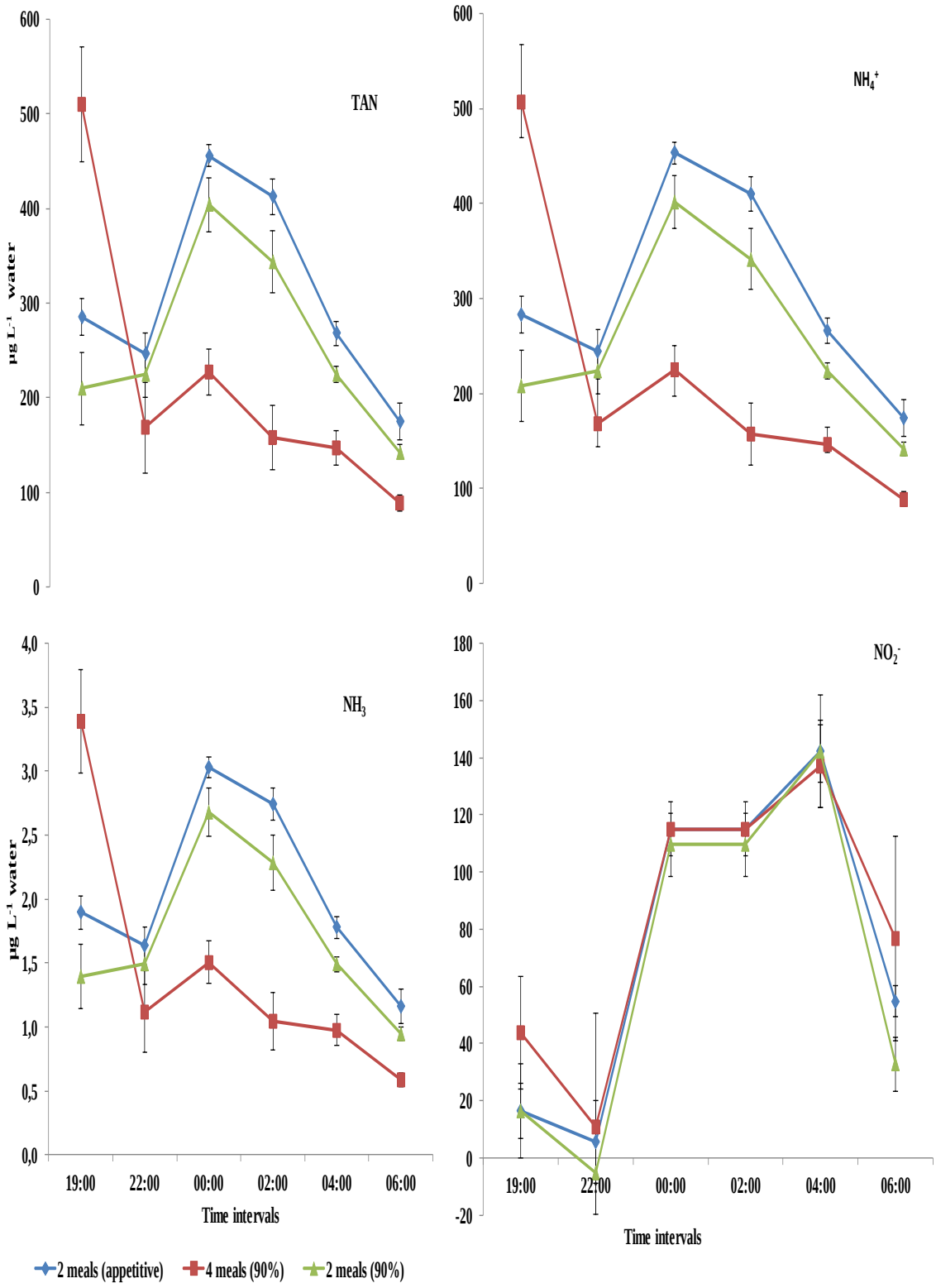



Figure 3. Measurement of TAN, $NH_4^+$, $NH_3$ and $NO_2^-$ from inlet and outlet in different time interval. (hrs.) at 2 meals (appetitive), 2 meals (90%) and 4 meals (90%) at day 14. (Mean of 3 tanks ± s.e.m).

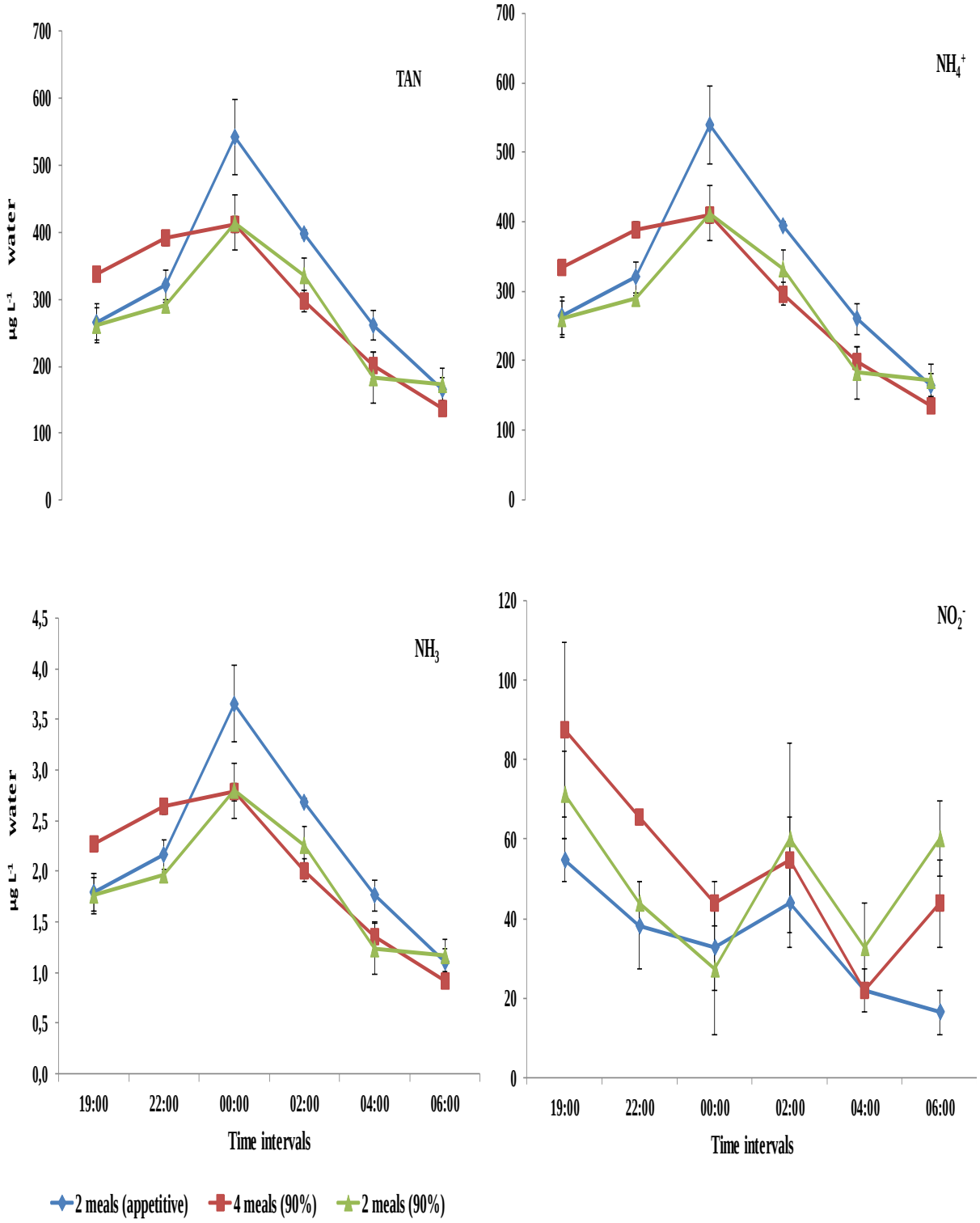



Figure 4. Measurement of TAN, $NH_4^+$, $NH_3$ and $NO_2^-$ from inlet and outlet in different time interval. (hrs.) at 2 meals (appetitive), 2 meals (90%) and 4 meals (90%) at day 26. (Mean of 3 tanks ± s.e.m).

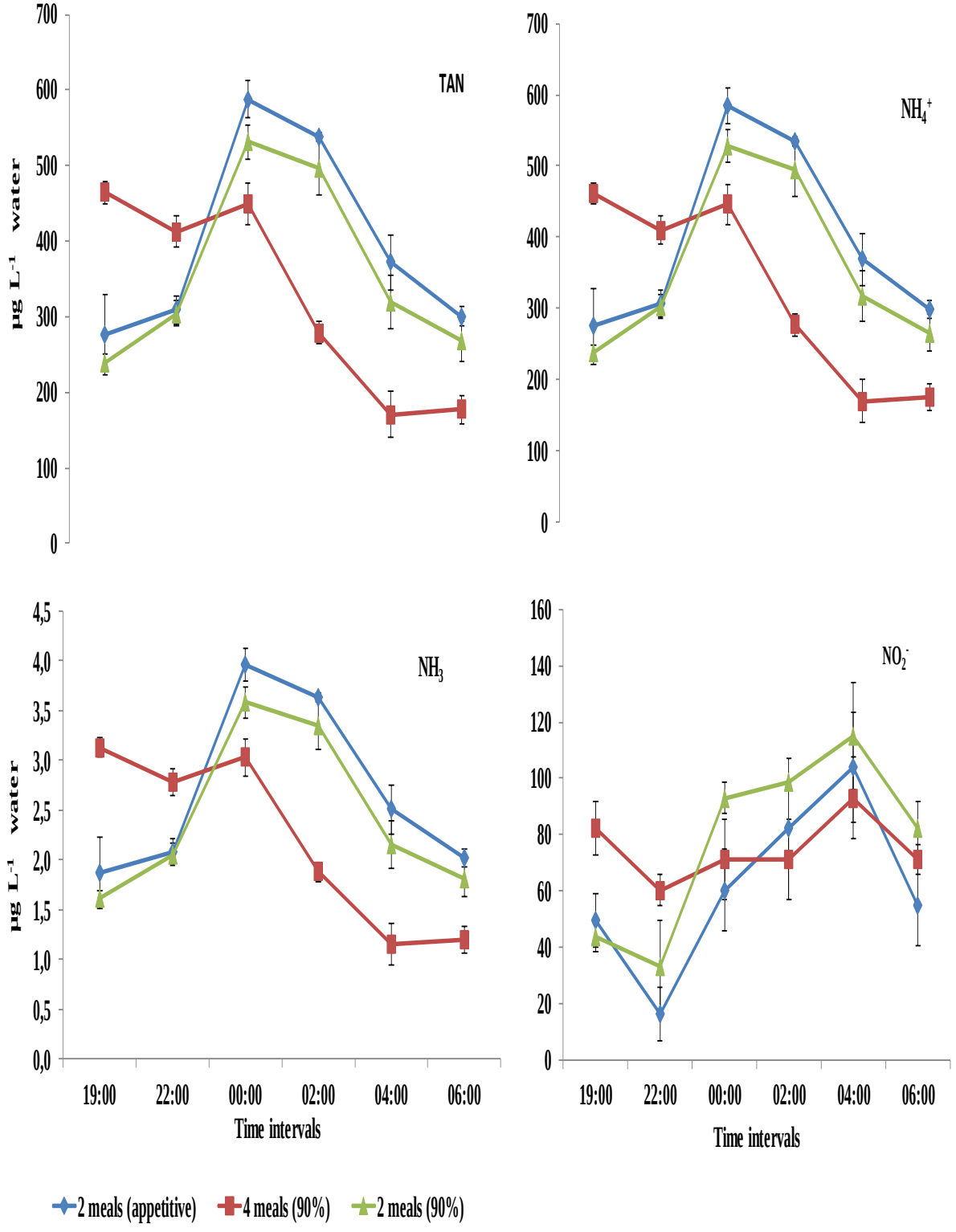



Figure 5. Measurement of TAN, $NH_4^+$, $NH_3$ and $NO_2^-$ from inlet and outlet in different time interval. (hrs.) at 2 meals (appetitive), 2 meals (90%) and 4 meals (90%) at day 39. (Mean of 3 tanks ± s.e.m).

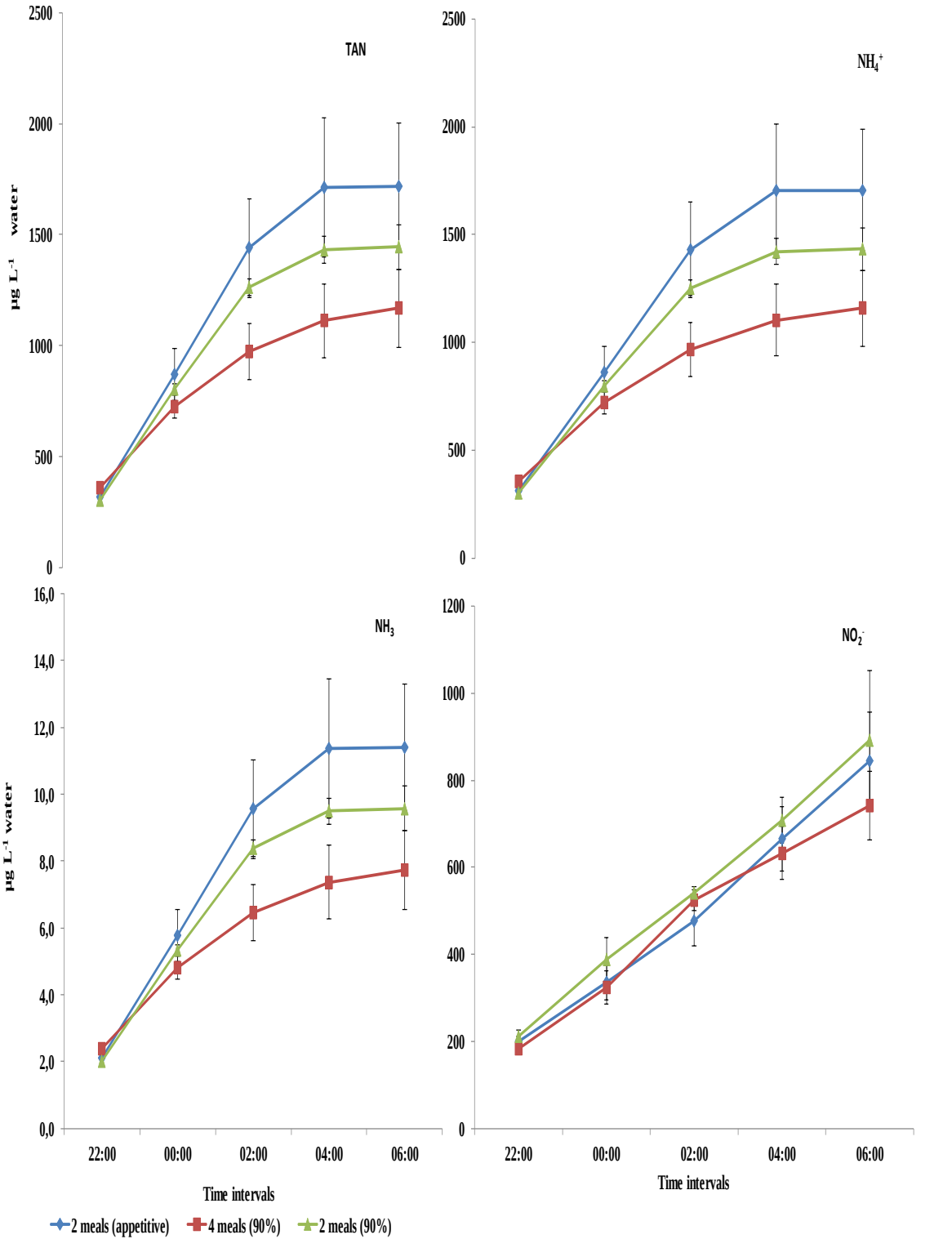



Figure 6. Measurement of TAN, $NH_4^+$, $NH_3$ and $NO_2^-$ from inlet and outlet in different time interval. (hrs.) at 2 meals (appetitive), 2 meals (90%) and 4 meals (90%) at day 45. (Mean of 3 tanks ± s.e.m).

same value that time. Then the rate of production for all meals decreased significantly and reached to the lowest level after 10 hours (06:00). On the other hand, maximum level of nitrite was measured after 2 hours (22:00) in 4 meals (90%), whereas it was after 6 hours (02:00) for 2 meals (appetitive) and 2 meals (90%). Then it decreased with some fluctuation and reached to the lowest level after 8 hours (04:00) in all meals.

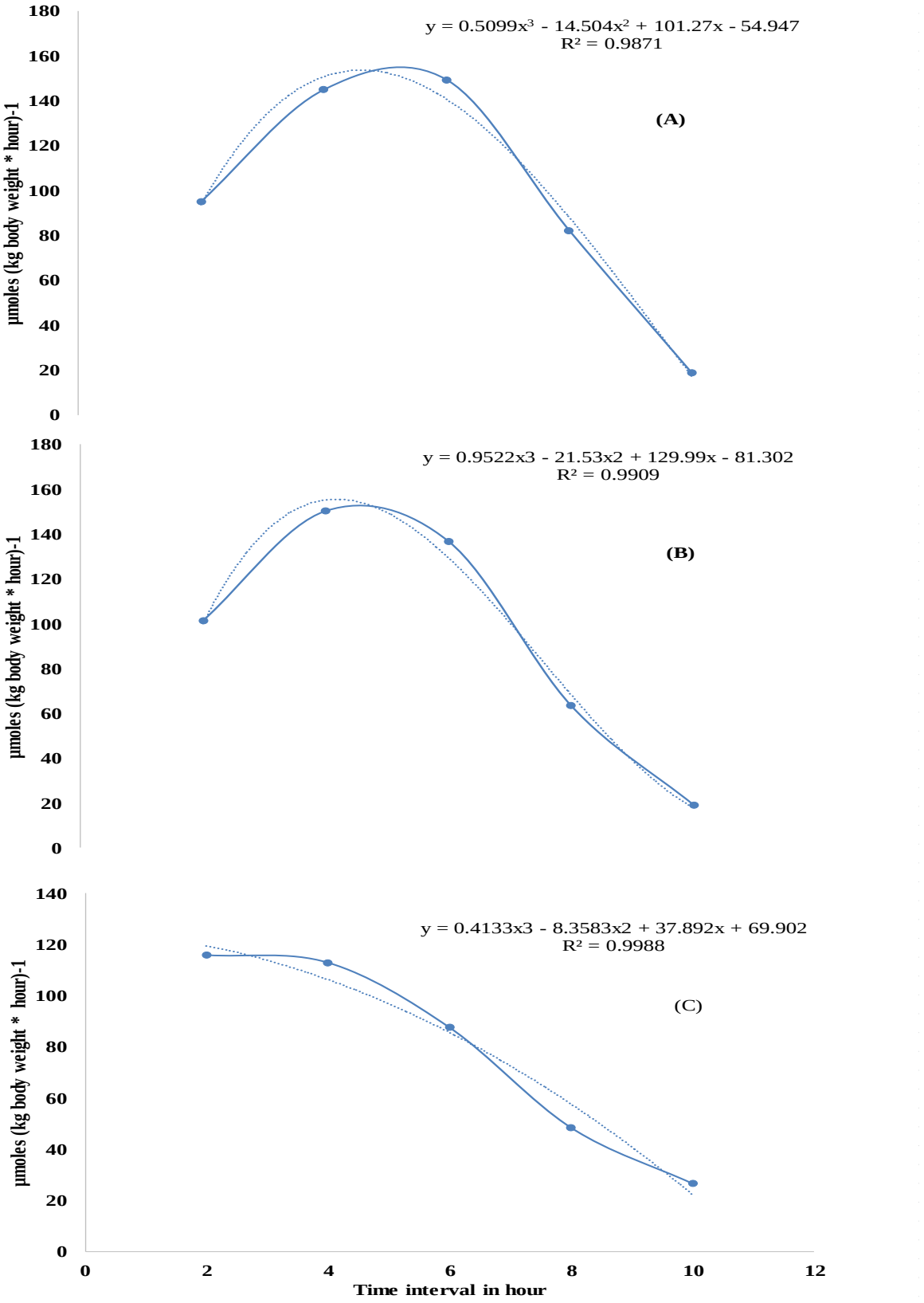
### 3.7.3 Measurement at day 39

The highest level of TAN, ammonium and ammonia were found after 4 hours (00:00) of last feeding for 2 meals (appetitive), 2 meals (90%) and 4 meals (90%) respectively. Then the rate of production decreased similarly in all group of meals and reached to the lowest 10 hours (06:00) after last feeding. However, the lowest level of nitrite were observed after 2 hours (22:00) of feeding, whereas the highest level were observed after 8 hours (04:00) in the 2 meals (appetitive), 2 meals (90%) and 4 meals (90%) consecutively.

## 3.8 Measurement at day 45 (stagnant system)

The accumulative TAN, ammonium, ammonia, and nitrite (µg $l^{-1}$) are presented in Fig. 6. After feeding, the production of TAN, ammonium and ammonia increased rapidly and reached to the peak level after 10 hours (06:00). The highest value was always for 2 meals (appetitive) and lowest for 4 meals (90%), while 2 meals (90%) gave intermediate values. The same ranking among feeding regimes was seen for nitrite. No time-dependent decrease in nitrite was observed.

Metabolic nitrogen excretions, with or without nitrite included are presented in Figs. 7 and 8. The relationship between nitrogen excretion (including $NO_2^-$, Nex, µmoles (kg body weight, hour)$^{-1}$ and time intervals (t, hour$^{-1}$) was best described by 3$^{rd}$ degree polynomial patterns are presented in Table 6. $R^2$ showed that the model was well fitted with the data. When we calculated the derivatives, we found the rate of change of meals with respect to time. It showed that maximum excretion was at 4.59, 4.61 and 2.88 hours in 2 meals (appetitive), 2 meals (90%)




Figures 7. Individual measurement of Metabolic nitrogen excretion (including $NO_2^-$) (($\Delta NH_4^+$ $+\Delta NH_3$ $+\Delta NO_2^-$) /fish weight * 2) from stagnant system in different time interval (hours) at 2 meals (appetitive) (A), 2 meals (90%) (B) and 4 meals (90%) (C) at day 45.

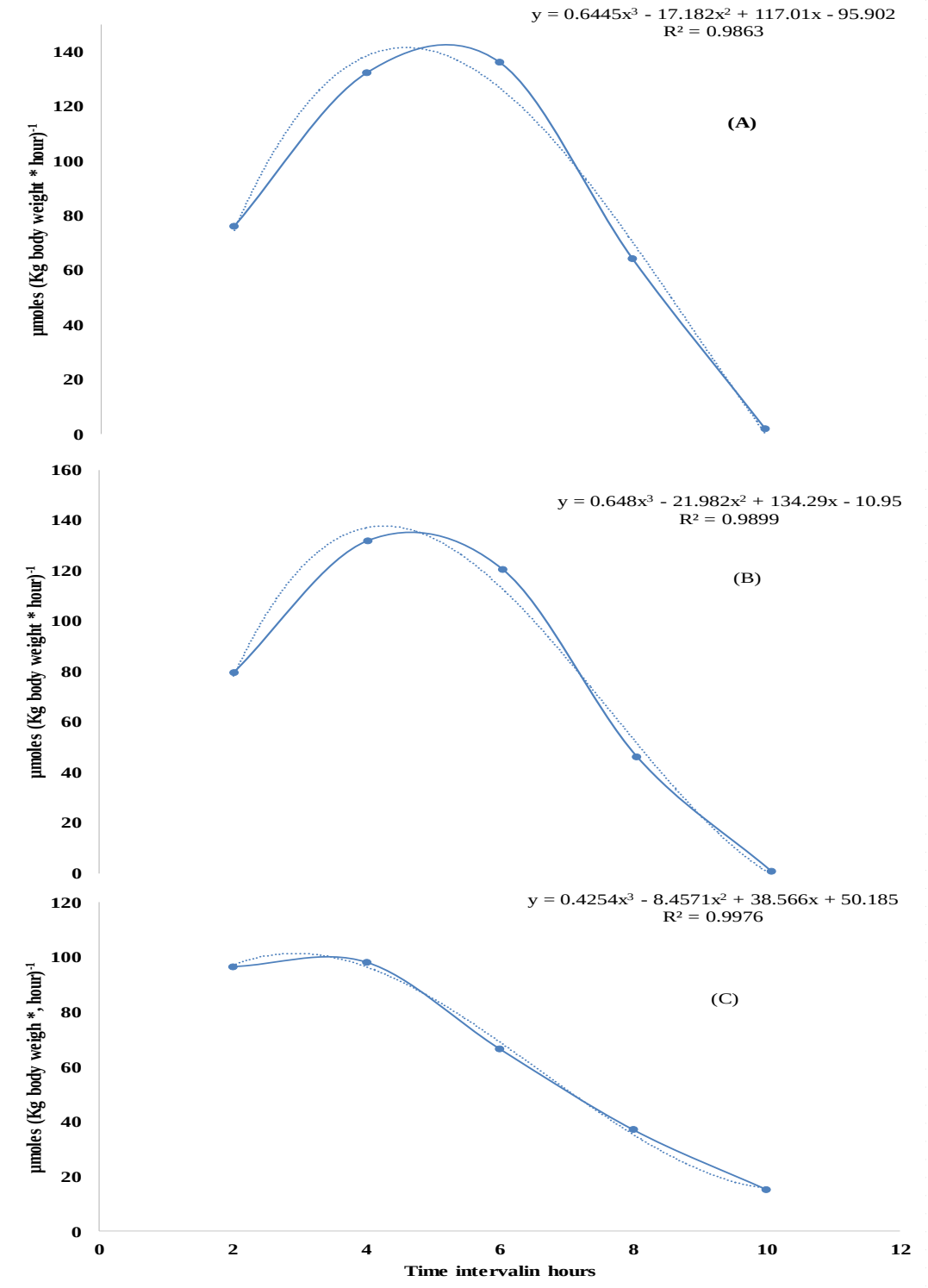



Figure 8. Individual measurement of Metabolic nitrogen excretion (excluding $NO_2^-$) (($\Delta NH_4^+$ + $\Delta NH_3$) /fish weight *2) from stagnant system in different time interval (hours) at 2 meals (appetitive) (A), 2 meals (90%) (B) and 4 meals (90%) (C) at day 45.

and 4 meals (90%) respectively. After that it was declining with increasing of time intervals. Though, Nitrogen excretion was reached at 2.88 hours after feeding in 4 meals (90%) and excretion rate was decreased very slowly

Table 6. Regression analysis of nitrogen excretion (including $NO_2^-$) on time intervals (t, $hour^{-1}$).

| Feeding regime | $R^2$, linear | $R^2$, 2nd degree | $R^2$, 3rd degree | Regression, Nex = |
|---|---|---|---|---|
| Two meals, appetitive | 0.40 | 0.97 | 0.99 | $0.51 * t^3 - 14.50 * t^2 + 101.3 * t - 55.0$ |
| Two meals, 90% | 0.54 | 0.92 | 0.99 | $0.95 * t^3 - 21.53 * t^2 + 130 * t - 81.3$ |
| Four meals, 90% | 0.94 | 0.98 | 0.99 | $0.41 * t^3 - 8.36 * t^2 + 37.9 * t + 69.9$ |

Again, the relationship between nitrogen excretion without $NO_2^-$, Nex, µmoles (kg body weight, $hour)^{-1}$ and time intervals (t, $hour^{-1}$) was best described by 3rd degree polynomial patterns are presented in Table 7. Here, $R^2$ indicated that the model was well fitted with the data. It was showed variation in meals. Once we calculated the derivatives, it showed that maximum excretion at 4.59, 3.64 and 4.02 hours in 2 meals (appetitive), 2 meals (90%) and 4 meals (90%) respectively. After that, it was declining with increasing of time intervals. When we observed the graphs (Fig. 7 and 8), Nitrogen excretion with nitrite (Fig 7) was showed more value with flat curves.

Table 7. Regression analysis of nitrogen excretion (including $NO_2^-$) on time intervals (t, $hour^{-1}$).

| Feeding regime | $R^2$, linear | $R^2$, 2nd degree | $R^2$, 3rd degree | Regression, Nex = |
|---|---|---|---|---|
| Two meals, appetitive | 0.38 | 0.95 | 0.99 | $0.64 * t^3 - 17.2 * t^2 + 117 * t - 95.9$ |
| Two meals, 90% | 0.50 | 0.92 | 0.98 | $0.65 * t^3 - 21.98 * t^2 + 134.3 * t - 10.95$ |
| Four meals, 90% | 0.94 | 0.97 | 0.99 | $0.43 * t^3 - 8.46 * t^2 + 38.6 * t + 50.19$ |

## 4. Discussions

Water quality was within adequate ranges for growth of Nile tilapia during the whole feeding trial. The average water temperature was 27.5$^0$C, pH (7), and dissolved oxygen (7.5 mg $l^{-1}$) of this experiment was optimum for tilapia production which was also described by Popma and Lovshin (1996). Bergheim (2007) also conducted the experiment on ten g to 1 kg Nile tilapia and best growth was found at 26-28$^0$C and pH 6.5 to 7. The time interval between the feeding frequencies is an important factor for feed intake, because it is related to stomach capacity, digestion rate and evacuation (Brett, 1971; Kono and Nose, 1971). Similarly, evacuation time is related to feeding sequence and fish size (Pandian, 1967; Noble, 1973; Fänge and Grove, 1979). Tilapia has small stomach and it can eat small portion of feed continuously (Moriarty, 1973). So tilapia needs feeding frequency for maximum growth and development. However, tilapia return in appetite following a satiation meal is approximately 4 hours (Riche et al., 2004).

The feed intake was found linear with weight gain. About 4.5% of estimated body weight was seen for 2 meals (appetitive) at initial, which was end with around 3.25%. Whereas, initially 2 meals (90%) and 4 meals (90%) were started approximately 4% of estimated body weight and end with about 3.0%. During the experiment of 4 weeks, at first week the daily feed intake was increased rapidly up to almost 0.5% of body weight, and then gradually decline with increasing body weight of fish in all groups. Fig. 2(A) presents satiation level with maximum feed intake. Thus, this is indicative of the feed intake potential of Generation 12 tilapia under the current experimental regime.

Two meals (appetitive) was the satiation level of feeding to fish and others two were about 90% of that. With respect to feed intake, there was a tendency (P=0.06) that appetitive feeding resulted in higher final weight and weight gain than the two restricted feeding regimes. As previous studies also explained that fish growth rate is directly proportional to feeding level (Zuanon et al., 2004) in a certain feeding point (Tesser and Sampaio, 2006). In this study FI rate was lower for the two and four meals (90%) and, however, growth was efficient and the FCR was lower.

Results of the present study indicate that feeding frequency did not significantly affect proximate composition of *O. niloticus*. The more efficient (lower) FCR was observed in this study. It tended (P=0.07) to be higher for the fish fed appetitive than for the ones with a 10%

restriction in daily feed allotment. But FCR, in this study, did not influence by feeding frequency. Some researcher like Webster et al. (1992) conducted research in channel catfish with one to four meals in a day and Wang et al. (1998) reared hybrid sunfish with 1 and 2 meals a day. Giberson and Litvak (2003) also reared Atlantic sturgeon and shortnose Sturgeon with four and eight meals and revealed that there were not significantly different on FCR. FCR did not influence by the frequency on those experiments. The current study is in agreement with those findings.

Protein and energy retention were significantly higher when meals were fed appetitive at 2 times a day than restricted feeding with 2 and 4 times a day. Riche et al. (2004) explained that the return of appetite succeeding a satiation level in Nile tilapia approximately 4 hour at 28$^{o}$C. The present study also followed four or more hours of feeding interval. There was also higher protein and energy retention observed in 2 meals (appetitive) because feeding interval and feed intake both were higher. Unlikely, there was no different 2 meals (90%) and 4 meals (90%) protein and energy retention, even there were difference in feeding frequencies. Therefore, feed intake rate could be important for nutrients retention.

In the present study, 4 meals (90%) had lower digestibility as compared to 2 meals (appetitive and 90%). Therefore, feeding frequency and rate could affect protein digestibility in tilapia. Liu and Liao (1999) explained that when the intervals between meals are shorter, the feed passes through digestive tract more quickly and causes less effective digestion. Moreover, Hudon and de La Noue (1984) did not find any difference in apparent digestibility of protein when feeding frequency was increased from two to six times per day in rainbow trout.

The liver is metabolic organ. So, the ratio of liver weight to body weight is a useful biomarker to detect the hazardous effects of the environmental stressors (Pait and Nelson, 2003). In the current study, the ratio of liver weight to body weight was observed the lowest value in 4 meals (90%) than others value from 2 meals (appetitive) and 2 meals (90%). So, comparing liver weight with body weight indicates that liver size was affected by the feeding frequency and level of feeding. 2 meals and 4 meals had taken equal amount of feed during experiment, but liver size was smaller in 4 meals. The reason behind the enlargement of liver should be more nutrients (lipid, glycogen) storing in 2 meals (appetitive and 90%). Likewise, liver deamination where excess amino acids converted into functional resources such as hydrogen, oxygen and carbon, one of the body's energy-production mechanisms could be deaminated for

these. Pyle et al. (2005) also explained that liver weight percentage as compared to fish body is associated with an indication of the status of energy reserve in an animal and metabolic activity.

The excretion values obtained in this experiment were lower than those observed in others study. The diets used in the present study was highly digestible (>96%) protein with a good crystalline essential amino acid balance. Hence, this could affect ammonia excretion. Ammonia excretion rate is not relative to the amount of feed ingested, but relative to the amount of protein ingested from the feed (Forsberg 1996; Pruszyński 2003). Protein amount in terms of AA balance, nutritional composition of ingredients, and their digestibility are the main factors affecting ammonia excretion in fish (Cho and Bureau, 2001; Green and Hardy, 2008; Peres and Oliva-Teles, 2006). Un-ionized ammonia concentration higher than 2000 µg $l^{-1}$ of water that flow in the tank causes mass mortality of tilapia in a few days (Lim and Webster, 2006). If tilapias are exposed to un-ionized ammonia concentration above 1000 µg $l^{-1}$ over a prolonged period of time, particularly fry and juveniles may die when the dissolved oxygen (DO) is low (Lim and Webster, 2006). Un-ionized ammonia begins to depress appetite of tilapia when the concentration in water is as low as 80 µg $l^{-1}$ (Popma and Masser, 1999).

After the feeding, the rate of ammonia excretion increases rapidly in response to feed intake (Jobling, 1981; Ballestrazzi et al., 1994; Handy and Poxton, 1993). Similarly, ammonia excretion rate of Nile tilapia was increased rapidly around 4-5 hours after feeding and maintained the highest and then excretion rate was decreased in this study. This is supported by previous studies (Altinok and Grizzle, 2004; Jobling, 1981., Dosdat et al., 1996; Peres and Oliva-Teles, 2006). Ten hours after last feeding, ammonia excretion rate was almost the same as one hour before last feeding for 2 meals. This is because of the same time interval between first and last meal. In case of 4 meals, the first measurement before one hour of last feeding was 3 hours later of third meals. That is why, $NH_3$, TAN, $NH_4^+$ measurements were the highest.

The majority of nitrogen from catabolism of amino acids is excreted through the gills as $NH_3$ and $NH_4^+$, while the kidney as urea excretes a small proportion. This experiment focus was only on excretion through gills, because the fish laboratory at NMBU did not have the facilities to analyse urea. Indigestible nitrogen is excreted by the intestine, and may be

oxidized by heterotrophic intestinal bacteria such as *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosolobus,* and *Nitrosovibrio* (Watson et al. 1981). The rate of bacterial activity in freshwater is limited by soluble phosphate, and the amount of autotropic bacteria growing in the water body of a freshwater recirculating aquaculture system is limited. As phosphorus levels increase, the amount of algae increases too. Therefore, phosphate generally limits the growth of freshwater autotropic bacteria (*Nitrobacter, Nitrospina, Nitrococcus,* and *Nitrospira*) and there is little activity of autotropic bacteria to utilize the produced nitrite (Watson et al. 1981). So, nitrite is mainly diluted and accumulated in water.

In this study, the excretion rate of ammonia after feeding increased, reached at peak value, and thereafter decreased. Nevertheless, there was consistent increase in nitrite over the whole sampling time. As the tilapias are expected to have an apparent digestibility of phosphorus at 40-50% from the current feed formulation (Storebakken personal communication), the rest passes undigested through the intestine. The undigested phosphorus provokes low solubility in water. However, due to microbial activities in intestine and feces the insoluble phosphorus may have been converted to soluble phosphorus and, which may have cause increasing $NO_2^-$. Thus, it may be that some of the nitrite originates from oxidation in the intestine. Another explanation may be that nitrogen is oxidized by bacterial activity in floating and sedimented feces, both "packed" in a mucous "bag" when released from the gut of the tilapia. Oxidative activity in feces may both have resulted in oxidation of nitrogen in feces, and eventually oxidation of ammonia and ammonium from metabolism, since phosphate does not limit bacterial growth in feces. The design of this experiment, however, does not permit the identification of the sources of the nitrite in the water.

The nitrogen excretion rates (including or excluding nitrite) showed an increasing trend after last feeding and gradually decreased after a certain time. The highest value was seen for 4 meals (90%) and the lowest for 2 meals (Appetitive) after 2 hours of last feeding. The values were 116 and 95 µmoles (kg body weight * hour)$^{-1}$ (including nitrite); 96 and 76 µmoles (kg body weight * hour)$^{-1}$ (excluding nitrite). While for 2 meals (90%) it was 101 µmoles (kg body weight * hour)$^{-1}$ (including nitrite) and 80 µmoles (kg body weight * hour)$^{-1}$ (excluding nitrite). Similarly, after 4 hours of last feeding, it was found maximum for 2 meals (90%) and the lowest for 4 meals (90%). The values were 150 and 113 µmoles (kg body weight * hour)$^{-1}$ (including nitrite); 132 and 98 µmoles (kg body weight * hour)$^{-1}$ (excluding nitrite), whereas it was 145 µmoles (kg body weight * hour)$^{-1}$ (including nitrite) for 2 meals (appetitive).

However, the value (excluding nitrite) (132 µmoles (kg body weight * hour)-1) was the same for 2 meals appetitive and 2 meals 90%). On the other hand, after 6 hours of last feeding the peak value was seen in 2 meals (appetitive) and the lowest in 4 meals (90%) which were 149 and 88 µmoles (kg body weight * hour)$^{-1}$ (including nitrite); 136 and 66 µmoles (kg body weight * hour)$^{-1}$ (excluding nitrite). In the same time it was 137 µmoles (kg body weight * hour)$^{-1}$ (including nitrite) and 121 µmoles (kg body weight * hour)$^{-1}$ (excluding nitrite) for 2 meals (90%). Moreover, for 4 meals (90%) a decline trend was seen after 4 hours of last feeding whereas, for the 2 meals (appetitive and 90%) it was seen after 6 hours of last feeding. Likewise, the highest nitrogen excretion was found for 2 meals (appetitive) and the lowest for 4 meals (90%). The values were 82 and 48 µmoles (kg body weight * hour)$^{-1}$ (including nitrite); 64 and 37 µmoles (kg body weight * hour)$^{-1}$ (excluding nitrite), while it was 63 µmoles (kg body weight * hour)$^{-1}$ (including nitrite) and 46 µmoles (kg body weight * hour)$^{-1}$ (excluding nitrite) for 2 meals (90%). Furthermore, after 10 hours of last feeding for 2 meals (appetitive and 90%) showed the lowest value compare to 4 meals (90%), which were 18.7, 19.0 and 27 µmoles (kg body weight * hour)$^{-1}$ (including nitrite); 2, 1 and 15 µmoles (kg body weight * hour)$^{-1}$ (excluding nitrite) respectively. Hence, after all observations it was seen that 2 meals (appetitive) had the maximum level of nitrogen excretion and the lowest for 4 meals (90%).

Excretion of nitrogen from the fish is higher with less frequent feeding. Nitrogen influences the growth of microorganism in water. Excess nitrogen play role for faster growth of microorganism. Significant increases of microorganisms affect water quality, food resources and the oxygen level. It severely decreases the level of dissolved oxygen, leading to increase diseases and can causes death a large numbers of fish. Therefore, restricted feeding with more frequency can reduce the level of nitrogen excretion and compose a higher water quality. Good water quality gives better production that can be beneficial for the farmers.

Materials from this study are being followed up by blood plasma amino acid pattern analysis, and analysis of transcription for amino acid catabolic enzymes in the liver (MSc thesis of Elena Gusokova). The combination of the results from this thesis and the follow-up analysis will hopefully give answer to the question if frequent feeding of Nile tilapia reduces amino acid catabolism. This continuation of our research may also contribute to explaining the observed differences in liver weight.

## 5. Conclusions

Distributing the same daily feed allotments to tilapias in two or four meals a day did not result in significantly differences in weight gain, feed utilization, body composition, protein or energy retention. However, the rate of nitrogen excretion was lower when the fish were fed 4 than 2 meals. This may have positive consequences for the water quality and thereby the farm environment.

***References***


Abdel-Tawwab M., Ahmad M.H., Khattab Y.A.E. and Shalaby A.M.E., 2010. Effect of dietary protein level, initial body weight, and their interaction on the growth, feed utilization, and physiological alterations of Nile tilapia, *Oreochromis niloticus* (L.). *Aquaculture*, **298**, 267-274.

Alam, M.S., Teshima, S., Koshio, S. and Ishikawa, M. (2002) Arginine requirement of juvenile Japanese flounder *Paralichthys olivaceus* estimated by growth and biochemical parameters. *Aquaculture*, **205**, 127–140.

Alliot E., Pastoreaud A., Pelaez J. and Metailler R. (1979) Partial substitution of fishmeal with corn gluten meal products in diets for sea bass (*Dicentrurchus labrax*). Proceeding World Symposium on Finfish Nutrition and Fish feed Technology, Hamburg, 20-23 June, 1978, pp. 229-238. Altinok, I. and Grizzle, J.M. (2004) Excretion of ammonia and urea by phylogenetically diverse fish species in low salinities. *Aquaculture*, **238**, 499-507.

Bakke, A.M., Glover, C. and Krogdahl, Å. (2010). Feeding, digestion and absorption of nutrients. *Fish Physiology*, **30**, 57-110. In Grosell, M., Farrel, P. and Brauner, C.J. (Eds) The multifunctional gut of fish. Elsevier, Amsterdam.

Ball, R.O., Urschel, K.L. and Pencharz, P.B. (2007) Nutritional consequences of interspecies differences in arginine and lysine metabolism. *The Journal Nutrition*, **137**, 1626-1641.

Ballestrazzi, R., Lanari, D., D'agaro, E., and Mion, A. (1994) The effect of dietary protein level and source on growth, body composition, total ammonia and reactive phosphate excretion of growing sea bass (*Dicentrarchus labrax*, L.). *Aquaculture*, **127**,197-206.

Beamish, F.W.H. and Thomas, E. (1984) Effects of dietary protein and lipid on nitrogen losses in rainbow trout, *Salmo gairdneri*. *Aquaculture*, **41**, 359-371.

Bergheim A. (2007) Water quality criteria in recirculation systems for Tilapia. International Research Institute of Stavanger, Stavanger, Norway.

Boyd, E.C. (2004) Farm-Level Issues in Aquaculture Certification: Tilapia. Report commissioned by WWF-U.S. Auburn University, AL, USA.

Brett, J.R. (1971) Satiation time, appetite and maximum food intake of sockeye salmon (*Oncorhynchus nerka*), *Journal of the Fisheries Research Board of Canada,* **28**, 409-415.

Bureau, P.D. (2004) Metabolic wastes in fish: Endogenous and exogenous determinants. Fish Nutrition Research Laboratory, Department of Animal and Poultry Science, University of Guelph, Ontario, Canada.

Cai, Y. and Burtle G.J. (1996) Methionine requirement of channel catfish fed soybean meal-corn-based diets. *Journal of Animal Science,* **74**, 514–521.

Chakraborty, S.C. and Chakraborty, S. (1998) Effect of dietary protein level on excretion of ammonia in Indian major carp, *Labeo rohita* fingerlings. *Aquaculture Nutrition*, 4, 47 – 51.

Chen, S., Ling, J. and Blancheton, J.P. (2006) Nitrification kinetics of biofilm as affected by water quality factors. *Aquaculture Engineering*, **34**, 179–197.

Cheng, S., Lee, W., Shieh, L. and Cehn, J. (2004) Increased production and excretion of urea in the Kuruma shrimp (*Marsupenaeus japonicas*) exposed to combined environments of increased ammonia and nitrite. *Archives of Environmental Contamination and Toxicology*, **47**, 352-362.

Chew, S.F., Wilson, J.M., Ip, Y.K., Randall, D.J. (2006) Nitrogenous excretion and defense against ammonia toxicity. In: Val, A.; Almeida-Val, V.; Randall, D.J. (Eds.). Fish physiology. The physiology of tropical fishes. New York: Academic Press, p. 307-395.

Cho, C.Y. and Bureau, D.P. (2001) A review of diet formulation strategies and feeding systems to reduce excretory and feed wastes in aquaculture. *Aquaculture Research*, **32**, 349-360.

Coimbra, A.M., Reis-Henriques, M.A. and Darras, V.M. (2005) Circulating thyroid hormone levels and iodothyronine deiodinase activities in Nile tilapia (*Oreochromis niloticus*) following dietary exposure to endosulfan and Arochlor 1254. *Comparative Biochemistry and Physiology*, **141(C)**, 8-14.

Coloso, R.M., Murillo-Gurrea D.P., Borlongan I.G. and Catacutan, M.R. (1999) Sulphur amino acid requirement of juvenile Asian Sea bass (*Lates calcarifer*). *Journal of Applied Ichthyology,* **15**, 54–58.

Cowey, C.B. (1994) Amino acid requirements of fish: a critical appraisal of present values. *Aquaculture*, **124**, 1–11.

Dosdat, A. Servais, F. Metáiller, R. Huelvan, C.M. and Desbruyeres, E. (1996) Comparison of nitrogenous losses in five teleost fish species. *Aquacultur*e, **141**,107-127.

Eddy, F.B. (2005) Ammonia in estuaries and effects on fish. *Journal of Fish Biology*, **67**, 1495-1513.

EI-Sayed, A.F.M. (1999) Alternative dietary protein sources for farmed tilapia, *Oreochromis Spp*. *Aquaculture*, **179**, 149-168.

Eknath, A.E., Tayamen, M.M., Palada-de Vera, M.S., Danting, J.C., Reyes, R.A., Dionisio, E. E., Capili, J.B., Bolivar, H.L., Abella, T.A., Circa, A.V., Bentsen, H.B., Gjerde, B., Gjedrem, T. and Pullin, R.S.V. (1993) Genetic improvement of farmed tilapias: the growth performance of eight strains of *Oreochromis niloticus* tested in different farm environments. *Aquaculture*, **111,** 171 – 188.

El-Saidy, D.M. S. and Gaber, M.M.A. (2005) Effect of dietary protein levels and feeding rates on growth performance, production traits and body composition of Nile tilapia, *Oreochromis niloticus* (L.) cultured in concrete tanks. *Aquaculture Research*, **36,** 163-171.

El-Sayed, A.M., Mansour, C.R. and Ezzat, A. A. (2003) Effects of dietary protein levels on spawning performance of Nile tilapia (*Oreochromis niloticus*) broodstock reared at different water salinities. *Aquaculture*, **220**, 619-632.

El-Sayed, A.-F.M. and Teshima, S. (1992) Protein and energy requirement of Nile tilapia, (*Oreochromis niloticus*) fry. *Aquaculture*, **103**, 55-63.

El-Sayed, A-F.M. (2006) Tilapia Culture. CABI Publishing, CAB International, Oxfordshire, UK. 277 pp.

El-sherif, M. S., Feky, E. and Amal, M. (2008) Effect of ammonia on Nile tilapia (*O. niloticus*) performance and some hematological and histological measures. Proceedings of the 8th International Symposium on tilapia in Aquaculture, October 12-14, 2008, Cairo, Egypt, pp: 513-530.

Emparanza, E. (2009) Problems affecting nitrification in commercial NOB with fixed-bed biofilters for salmonids in Chile. *Aquaculture Engineering*, **41**, 91-96.

Escaffre, A.M., Infante, J.L.Z., Cahu, C.L., Mambrini, M., Bergot, P. and Kushik, S.J. (1997) Nutritional value of soy protein concentrate for larvae of common carp (*Cyprinus carpio)* based on growth performance and digestive enzyme activities. *Aquaculture,* **153**, 63-80.

Espe, M., Lemme, A., Petri, A. and El-Mowafi, A. (2007) Assessment of lysine requirement for maximal protein accretion in Atlantic salmon using plant protein diets. *Aquaculture,* **263**, 168-178.

Eyre, D.R. (1980) Collagen - molecular diversity in the body's protein scaffold. *Science,* **207**, 1315-1322.

Fange, R. and Grove, D. (1979) Digestion. In. Hoar, W.S., Randall, D.J. and Brett, J.R. [eds.] Fish physiology, Bioenergetics and growth, **8**, 161-260. Academic Press, New York.

FAO (2010) The State of World Fisheries and Aquaculture. FAO, Rome.

FAO (2012) The State of World Fisheries and Aquaculture. FAO, Rome.

Felipo, V. and Butterworth, R.F. (2002) Neurobiology of ammonia. *Progress in Neurobiology,* **67**, 259-279.

Fitzsimmons, K. and Watanabe, W.O. (2010) Tilapia (Family: Cichlidae). In: Le Francois, N.R. Jobling, M., Carter, C., Blier, P.U. and Savoie, A. (Eds.) Finfish Aquaculture Diversification. CABI, Wallingford, UK, 374-396.

Fitzsimmons, K., Martinez-Garcia, R. and Pablo G-A. (2011) Why Tilapia is becoming the most Important Food Fish on the Planet. In: L. Liping and K. Fitzsimmons (Eds.) Proceedings of the 9th International Symposium on Tilapia in Aquaculture. Shanghai Ocean University, Shanghai, China, April 22-25.

Forsberg, O.I. (1996) Ammonia excretion rates from post-smolt Atlantic salmon, *Salmo salar* L., in land based farms. *Aquaculture Research,* **27**, 937-944.

Fôrtabellen (2008) http://statisk.umb.no/iha/fortabell/index.php

Fu-Guang, L., Shuenn-Der, Y. and Hon-Cheng, C. (2009) Effect of temperature, stocking density and fish size on the ammonia excretion in palmetto bass (*Morone saxatilis* x *M chrysops*). *Aquaculture Research*, **40**, 450-455.

Gatlin III, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E.,Hu, G., Krogdahl, Å., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., Souza, E., Stone, D., Wilson, R.P. and Wurtele, E. (2007). Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquaculture Research,* **38**, 551-579.

Gelineau, A., Medale, F. and Boujard, T. (1998) Effect of feeding time on postprandial nitrogen excretion and energy expenditure in rainbow trout. *Journal of Fish Biology*, **52**, 655-664.

Giberson A.V. and Litvak M.K. (2003) Effect of feeding frequency on growth, food conversion efficiency, and meal size of juvenile Atlantic sturgeon and Shortnose sturgeon. *North American Journal of Aquaculture*, **65**, 99-105.

Green, J.A. and Hardy R.W. (2008) The effects of dietary protein:energy ratio and amino acid pattern on nitrogen utilization and excretion of rainbow trout (*Oncorhynchus mykiss)* (Walbaum). *Journal of Fish Biology*, **73**, 663-682.

Habib, M.A.B., Hasan, M. R. and Akand, A.M. (1994) Dietary carbohydrate utilization by silver barb Puntius gonionotus. In DeSilva, S.S. (Ed.) Asian Fisheries Society Species Publication. *Asian Fisheries Society*, Manila, Phillippines, 57-62.

Handy, R.D. and Poxton, M.G. (1993) Nitrogen pollution in marine culture: toxicity and excretion of nitrogenous compounds by marine fish. *Revised Fish Biology*, **3**, 205 – 241.

Harpaz, S. (2005) *L*-carnitine and its attributed functions in fish culture and nutrition - a review. *Aquaculture,* **249**, 3-21.

Hauler, R.C. and Carter, C.G. (2001) Reevaluation of the quantitative dietary lysine requirements of fish. *Reviews in Fish Science,* **9**, 133-163.

Hertrampf, J.W. and Piedad-Pascual, F. (2000) Handbook on ingredients for aquaculture feeds. Kluwer Academic Publishers, 624.

Horne, D.W., Tanphaichitr, V. and Broquist, H.P. (1971) Role of lysine in carnitine biosynthesis in *Neurospora crassa*. *Journal of Biological Chemistry*, **246**, 4373-4375.

Hudon, B. and de La Noue, J. (1984) Influence of meal frequency on apparent nutrient digestibility in rainbow trout (*Salmo gairdneri*). *Bulletin Francais De La Peche Et De La Pisciculture*, **293**, 49–51.

Ip, Y.K.; Chew, S.F.; Wilson, J. M.; Randall, D.J. (2004) Defenses against ammonia toxicity in tropical air breathing fishes exposed to high concentrations of environmental ammonia: a review. *Journal of Comparative Physiology*, **174**, 565-575.

ISO, 6496. (1983) International Organization for Standardization. Animal Feeding Stuffs: Determination of Moisture Content, ISO, Geneve, Switzerland.

Jackson, A.J., Capper, B.S. and Matty A.J. (1982) Evaluation of some plant proteins in complete diets for the tilapia (*Sarotherodon mossambicus)*. *Aquaculture*, **27**, 97-109.

Jensen, F.B. (1990) Nitrite and red cell function in carp: control factors for nitrite entry, membrane potassium ion permeation, oxygen affinity and methaemoglobin formation. *The Journal of Experimental Biology*, **152**, 149–166.

Jobling, M. (1981) Some effects of temperature, feeding and body weight on nitrogenous excretion in young plaice *Pleuronectes platessa* L. *Journal of Fish Biology*, **18**, 87-96.

Kaushik, S.J. and Cowey, C.B. (1991) Dietary factors affecting nitrogen excretion by fish. In Cowey, C.B. and Cho, C.Y. (Eds.), Nutritional Strategies and Aquaculture Waste. Fish Nutrition Research Laboratory, University of Guelph, Guelph, Ontarto, pp. 3-19.

Ketola, H.D. (1982) Amino acid nutrition of fishes: Requirements and supplementation of diets. *Comparative Biochemistry and Physiology*, **73,** 17–24.

Kieffer, J.D. and Wakefield, A.M. (2009) Oxygen consumption, ammonia excretion and protein use in response to thermal changes in juvenile Atlantic salmon (*Salmo salar)*. *Journal of Fish Biology*, **74**, 591-603.

Kissil, G.W., Lupatsch, I., Higgs, D.A. and Hardy, R.W. (2000) Dietary substitution of soy and rapeseed protein concentrates for fishmeal, and their effects on growth and nutrient utilization in gilthead seabream *Saparus aurata* L. *Aquaculture Research*, **31**, 595-601.

Kono, H. and Nose, Y. (1971) Relationship between the amount of food taken and growth in fishes. 1. Frequency of feeding for a maximum daily ration, *Bulletin of the Japanese Society of Scientific Fisheries*, **37,** 169–173.

Lewis, A.J. and Bayley, H.S. (1995) Amino acid bioavailability. In: Ammerman, C. B., Baker, D. H. and Lewis, A. J. (Eds.), Bioavailability of Nutrients for Animals—Amino Acids, Minerals. Vitamins. Academic Press Inc, San Diego, CA, pp. 35–65.

Li, M. and Lovell, R.T. (1992) Comparison of satiate feeding and restricted feeding of channel catfish with various concentrations of dietary protein in production ponds. *Aquaculture*, **103**, 165 – 175.

Li, M.H. and Robinson, E.H. (1998) Effects of supplemental lysine and methionine in low protein diets on weight gain and body composition of young channel catfish *Ictalurus punctatus*. *Aquaculture*, **163**, 295–305.

Lim C., Oksoy M. and Klesius P. (2011) Lipid and fatty acid requirements of tilapia. *North American Journal of Aquaculture*, **73,** 188-193.

Lim, C.E. and Webster, C.D. (2006) Tilapia: Biology, Culture and Nutrition. Food Products Press, New York, USA, pp. 469-501.

Lin, Y.H. Lin, S.M. and Shiau, S.Y. (2008) Dietary manganese requirements of juvenile tilapia, *Oreochromis nilotis* x *O.aureus*. *Aquaculture*, **284**, 207-210.

Linnaeus, C. (1758) Systema naturae per regna tria naturae secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. Ed. 10, Tomus 1. L. Salvii, Stockholm, Sweden, 823 pp.

Liu F.G. and Liao C.I. (1999) Effect of feeding regimes on the food consumption, growth and body composition in hybrid striped bass (*Morone saxatilis* × *Morone chrysops*). *Fisheries Sciences*, **64**, 513–519.

Lowell, T. (1998) Nutrition and feeding of fish. *Aquaculture Series*, Springer, 267 pp.

Magid, A. and Babiker, M.M. (1975) Oxygen consumption and respiratory behaviour of three Nile fishes. *Hydrobiologia*, **46**, 359 – 367.

Mai, K.S., Zhang, L., Ai, Q.H., Duan, Q.Y., Zhang, C.X., Li, H.T., Wan, J.L. and Liufu, Z.G. (2006) Dietary lysine requirement of juvenile Japanese seabass, *Lateolabrax japonicus*. *Aquaculture*, **258**, 535-542.

Margiocco, C., Arrillo, A., Mensi, P. and Schenone, G. (1983) Nitrite bioaccumulation in *Salmo gairdneri* Rich. and hematological consequences. *Aquatic Toxicology*, **3**, 261-270.

McCleary B.V., Solah V. and Gibson T.S., (1994) Quantitative measurement of total starch in cereal flours and products. *Journal of Cereal Science*, **20**, 51-58.

Mires, D. (1995) Aquaculture and the aquatic environment: mutual impact and preventive management. *The Israeli Journal of Aquaculture – Bamidgeh*, **47**, 163-172.

Morgan P.R. (1972) Causes of mortality in the endemic tilapia of Lake Chilwa (Malawi). *Hydrobiologia*, **40**, 101-119.

Moriarty, D.J.W. (1973) The physiology of digestion of blue-green algae in the cichlid fish tilapia, *Oreochromis nilotica*. *Journal of Zoology*, **171**, 25-40.

Moughan, P.J. and Rutherfurd, S.M. (1996) A new method for determining digestible reactive lysine in foods. *Journal of Agriculture, Food and Chemistry*, **44**, 2202-2209.

Murai, T., Ogata, H., Hirasawa, Y., Akiyama, T. and Nose, T. (1987) Portal absorption and hepatic uptake of amino acids in rainbow trout force fed complete diets containing casein or crystalline amino acids. *Nippon Suisan Gakkaishi*, **53**:1847-1859.

Naylor, R.L., Hardy, R.W., Bureau, D.P., Chiu, A. and Elliott, M. (2009). Feeding aquaculture in an era of finite resources. *PNAS Direct Submission.* **106**, 15103-15110.

Nguyen T.N., Davis D.A. and Saoud I.P. (2009) Evaluation of Alternative Protein Sources to replace fishmeal in practical diets for juvenile tilapia, *Oreochromis* spp. *Journal of the World Aquaculture Society*, **40**,113-121.

Noble, R.L. (1973) Evacuation rates of young yellow perch, *Perca flavescens* (Mitchil). *Transactions of the American Fisheries Society*, **102**, *759-763.*

NRC (National Research Council) (1993) Nutrient requirement of fish, National Research Council. National Academy Press, Washington, DC, USA, 114.

Pait, A.S. and Nelson, J.O. (2003) Vitellogenesis in male Killifish, *Fundulus heteroclitus* induced by selected estrogenic compounds. *Aquatic Toxicology*, **64**, 331-342.

Pandian, T.J. (1967) Intake, digestion, absorption and conversion of food in the fishes *Megalops cyprinoides* and *Ophiocephalus striatus*, *Journal of Marine Biology*, **1**, 16-32.

Parker, R. (2002) Aquaculture science. Delmar, New York, 621 pp.

Pereira, T.G. and Oliva-Teles, A. (2003) Evaluation of corn gluten meal as a protein source in diets for gilthead sea bream (*Sparus aurata L.*) juveniles. *Aquaculture Research*, **34**, 1111-1117.

Peres, H. and Oliva-Teles, A. (2006) Effect of the dietary essential to nonessential amino acid ratio on growth, feed utilization and nitrogen metabolism of European sea bass (*Dicentrarchus labrax*). *Aquaculture*, **256**, 395-402.

Piez, K.A., Likins, R.C. (1957) The conversion of lysine to hydroxylysine and its relation to the biosynthesis of collagen in several tissues of the rat. *Journal of Biolology. Chemistry*, **229**, 101-109.

Popma, T. and Masser, M. (1999) Tilapia Life History and Biology. Southern Regional Aquaculture Center (SRAC). Publication Number 283.

Popma, T. J. and. Lovshin. L.L. (1996) Worldwide Prospects for Commercial Production of tilapia. *R* and *D* Series 41, International Center for Aquaculture and Aquatic Environments, Department of Fisheries and Allied Aquacultures, Auburn University, AL, USA.

Pruszyński, T. (2003) Effects of feeding on ammonium excretion and growth of the African catfish (*Clarias gariepinus*) fry. *Czech Journal of Animal Science*, **48**, 106–112.

Pullin, R.S.V and Lowe-McConnell, R.H. (1997) The Biology and Culture of Tilapias. ICLARM Conference Proceedings 7. International Center for Living Aquatic Resources Management, Manila, Philippines, pp. 58

Pyle, G.G., Rajotte, J.W. and Couture, P. (2005) Effects of industrial metals on wild fish populations along a metal contamination gradient. *Ecotoxicology and Environmental Safety*, **61**, 287–312.

Randall, D.J. and Tsui, T.K N. (2002). Ammonia toxicity in fish. Marine pollution bulletin, **45**, 17-23.

Randall, D.J. and Wright, P.A. (1987) Ammonia distribution and excretion in fish. *Fish Physiology and Biochemistry*, **10**, 315–324.

Ravindran, V. and Bryden, W.L. (1999) Amino acid availability in poultry - in vitro and in vivo measurements. *Australian Journal Agriculture Research*, **50**, 889-908.

Redner , B.D. and. Stickney, R.R. (1979) Acclimation to ammonia by Tilapia aurea. *Transactions of the American Fisheries Society*, **108**, 383 – 388.

Riche, M., Haley, D.I., Oetker, M., Garbrecht, S. and Garling, D.L. (2004) Effect of feeding frequency on gastric evacuation and the return of appetite in tilapia *Oreochromis niloticus* (L.). *Aquaculture*, **234**, 657- 673.

Robaina, L., Izquierdo, M.S., Moyano, F.J., Socorro, J., Vergara, J.M., Montero, D. and FernCndea-Palacios, H. (1995) Soybean and lupin seed meals as protein sources in diets for gilthead seabream (*Sparus aurata*): Nutritional and histological implications. *Aquaculture*, **130**, 219-233.

Ross, L.G. (2000) Environmental physiology and energetics. In: Beveridge, M.C.M.; Mcandrew, B.J. (Eds.) Tilapias: biology and exploitation. Dordrecht: *Kluwer Academic Publishers*, 89-128.

Rychly, J. (1980) Nitrogen balance in trout. II. Nitrogen excretion and retention after feeding diets with varying protein and carbohydrate levels. *Aquaculture*, **20**, 343-350.

Santiago, C.B. and Lovell, R.T. (1988) Amino acid requirements for growth of Nile tilapia. *Journal of Nutrition*, **118**, 1540-1546.

Schuhmacher, A., Schon, J., Goldberg, M. and Gropp, J.M. (1995) Plasma amino acid levels in rainbow trout (*Oncorhynchus mykiss*). *Journal of Applied Ichthyology*, **11**, 309–316.

Shiau, S.Y. (1998) Nutrient requirements of penaeid shrimp. *Aquaculture*, **164**, 77–93.

Small, B.C. and Soares, J. H. (2000) Quantitative dietary lysine requirement of juvenile striped bass (*Morone saxatilis*). *Aquaculture Nutrition*, **6**, 207-212.

Smith, H. and Rumsey, G. 1976. Nutrient utilization by fish, In: First Int. Symp., Feed composition, animal nutrient requirements, and computerization of diets, Edited by: Fonnesbeck, P., Harris, L. and L. Kearl. Utah Agricultural Experiment Station, Utah State University, Logan, Utah, USA, 320 pp.

Tacon, A.G.J., Hasan, M.R. and Metian, M. (2011). *Demand and supply of feed ingredients for farmed fish and crustaceans: trends and prospects*: FAO Fisheries and Aquaculture Technical Paper No. 564, Rome, Italy.

Teichert-Coddington, D.R., Popma, T.J. and Lovshin, L.L. (1997) Atributes of tropical pond-cultured fish, 183-198. In: Enga H.S. and Boyd, C.E. (eds.), Dynamics of Pond Aquaculture. CRC Press, Boca Raton, Florida, USA.

Tesser, M.B. and Sampaio, L.A. (2006) Rearing of pejerrey (*Odontesthes argentinensis*) juveniles at different feeding rates. *Ciência Rural*, **36,** 1278-1282.

Thurston, R.V., Russo, R.C. and Phillips, G.R. (1983) Acute toxicity of ammonia to fathead minnows. *Transactions of the American Fisheries Society*, **112**, 705-711.

Timmons, M.B., Ebeling, J.M., Wheaton, F.W., Summerfelt, S.T. and Vinci, B.J. (2002) Recirculating Aquaculture Systems, 2nd Edition. Cayuga Aqua Ventures, Ithaca, NY 14850, USA. 800 p. NRAC Publication No. 01-002.

Tveteras, R. and Nystøyl, R. (2011) Fish Production Estimates and Trends 2011-2012. Global Outlook for Aquaculture Leadership Conference, November 6-9 2011 Santiago, Chile.

U.S. Environmental Protection Agency (EPA) (1989) Summary review of health effects associated with ammonia. Health issue assessment. EPA/600/89/052F.

U.S. Environmental Protection Agency (EPA) (2009) Draft 2009 update. Aquatic life ambient water quality. 941 criteria for ammonia- freshwater. Office of Water, EPA-822-D-09-001.

U.S. Environmental Protection Agency (EPA) (1999) Update of ambient water quality criteria for ammonia. EPA-822-R-99-014.

Verri, T., Romano, A., Barca, A., Kottra, G., Daniel, H. and Storelli, C. (2010) Transport of di- and tripeptides in teleost fish intestine. *Aquaculture Research*, **41**, 641-653.

Walton, M.J., Cowey, C.B. and Adron, J.W. (1984) The effect of dietary lysine levels on growth and metabolism of rainbow trout (*Salmo gairdneri*). *British Journal Nutrition*, **52**, 115-122.

Wang Y., Liu Y.-J., Tian L.-X., Du Z.-Y., Wang J.-T., Wang S. and Xiao W.P. (2005) Effects of dietary carbohydrate level on growth and body composition of juvenile tilapia, *Oreochromis niloticus*×*O. aureus*. *Aquaculture Research*, **36**, 1408-1413.

Wang, N., Hayward, R.S. and Noltie , D.B. (1998) Effect of feeding frequencyon food consumption, growth, size variation, and feeding pattern of age-0 hybrid sunfish**.** *Aquaculture*, **165**, *261-267.*

Watson, S.W., Valos, F.W. and Waterbury, J.B. (1981) The Family *Nitrobacteraceae*. In The Prokaryotes. Edited by M.P. Starr et al. Springer-Verlag, Berlin, Germay.

Webster, C.D., Tidwell, J.H., Goodgame, L. S., Yancey, D. H. and Mackey, L. (1992) Use of soybean meal and distillers grains with solubles as partial or total replacement of fishmeal in diets for channel catfish, *Ictalurus punctatus*. *Aquaculture*, **106**, 301-309.

Wicks, B.J. Joensen, R. Tang, Q. and Randall, D.J. (2002) Swimming and ammonia toxicity in salmonids: the effect of sub lethal ammonia exposure on the swimming performance of coho salmon and the acute toxicity of ammonia in swimming and resting rainbow trout. *Aquactic Toxicolology*, **59**, 55-69.

Wicks, B.J.; Randall, D.J. (2002) The effect of sub-lethal ammonia exposure on fed and unfed rainbow trout: the role of glutamine in regulation of ammonia. *Comparative Biochemistry and Physiology*, **132(C)**, 275-285.

Wilkie, M.P. (2002) Ammonia excretion and urea handling by the fish gills: present understanding and future research challenges. *Journal of Experimental Zoology*, **293**, 284-301.

Wilson, R.P. (1989) Amino acids and proteins. In: Fish Nutrition, 2nd edn. Ed: J. E. Halver, Academic Press, Inc., San Diego, CA, pp. 111-151.

Wilson, R.P., Robinson, E.H. and. Poe, W.E. (1981) Apparent and true availability of amino acids from common feed ingredients for channel catfish. *Journal of Nutrition,* **111**, 923-929.

Wilson, R.P. and Cowey, C.B. (1985). Amino-acid composition of whole-body Tissue of Rainbow-Trout and Atlantic salmon. *Aquaculture* **48**, 373-376.

Wright, P.A. (1995) Nitrogen excretion: three end products, many physiological roles. *Journal of Experimental Biology*, **281**, 273-281.

Yamada, S., Simpson, K.L., Tanaka, Y. and Katayama, T. (1981) Plasma amino acid changes in rainbow trout (*Salmo gairdneri*) force fed casein and a corresponding amino acid mixture. *Bulletin of the Japanese Society of Scientific Fisheries*, **47**, 1035 – 1040.

Zakes, Z. Demska-Zakes, K. Jarocki, P. and Stawecki, K. (2006) The effect of feeding on oxygen consumption and ammonia excretion of juvenile tench *Tinca tinca* (L.) reared in a water recirculating system. *Aquaculture International*, **14**, 127-140.

Zuanon, J.A.S., Asano, M. and Fernandes, J.B.K. (2004) Performance of Tricogaster (*Trichogaster trichopterus*) submitted to different feeding levels and stocking densities**.** *Revista Brasileira de Zootecnia*, **33**, 1639-1645.

## Appendix

### 1. Measurement of ammonium

Spectroquant®

# Ammonium

**14752**

Test

| | | | |
|---|---|---|---|
| **Measuring range:** | 0.05 – 3.00 mg/l $NH_4$-N | 0.06 – 3.86 mg/l $NH_4$ | 10-mm cell |
| | 0.03 – 1.50 mg/l $NH_4$-N | 0.04 – 1.93 mg/l $NH_4$ | 20-mm cell |
| | 0.010 – 0.500 mg/l $NH_4$-N | 0.013 – 0.644 mg/l $NH_4$ | 50-mm cell |
| | Expression of results also possible in mmol/l. | | |

Check the pH of the sample, specified range: pH 4 – 13.
If required, add dilute sodium hydroxide solution or sulfuric acid drop by drop to adjust the pH.

Pipette 5.0 ml of the sample into a test tube.

Add 0.60 ml of **$NH_4$-1** with pipette and mix.

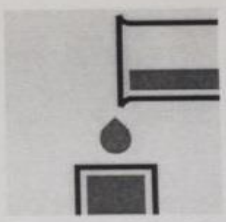
Add 1 level blue microspoon of **$NH_4$-2**.

Shake vigorously to dissolve the solid substance.

Reaction time:
5 minutes

Add 4 drops of **$NH_4$-3** and mix.

Reaction time:
5 minutes



Transfer the solution into a corresponding cell.

Select method with AutoSelector.

Place the cell into the cell compartment.

**Important:**

Very high ammonium concentrations in the sample produce turquoise-coloured solutions (measurement solution should be yellow-green to green) and false-low readings are yielded. In such cases the sample must be diluted (plausibility check).

To measure in the 50-mm cell, the sample volume and the volume of the reagents have to be doubled for each. Alternatively, the semi-microcell, Cat.No. 73502, can be used.

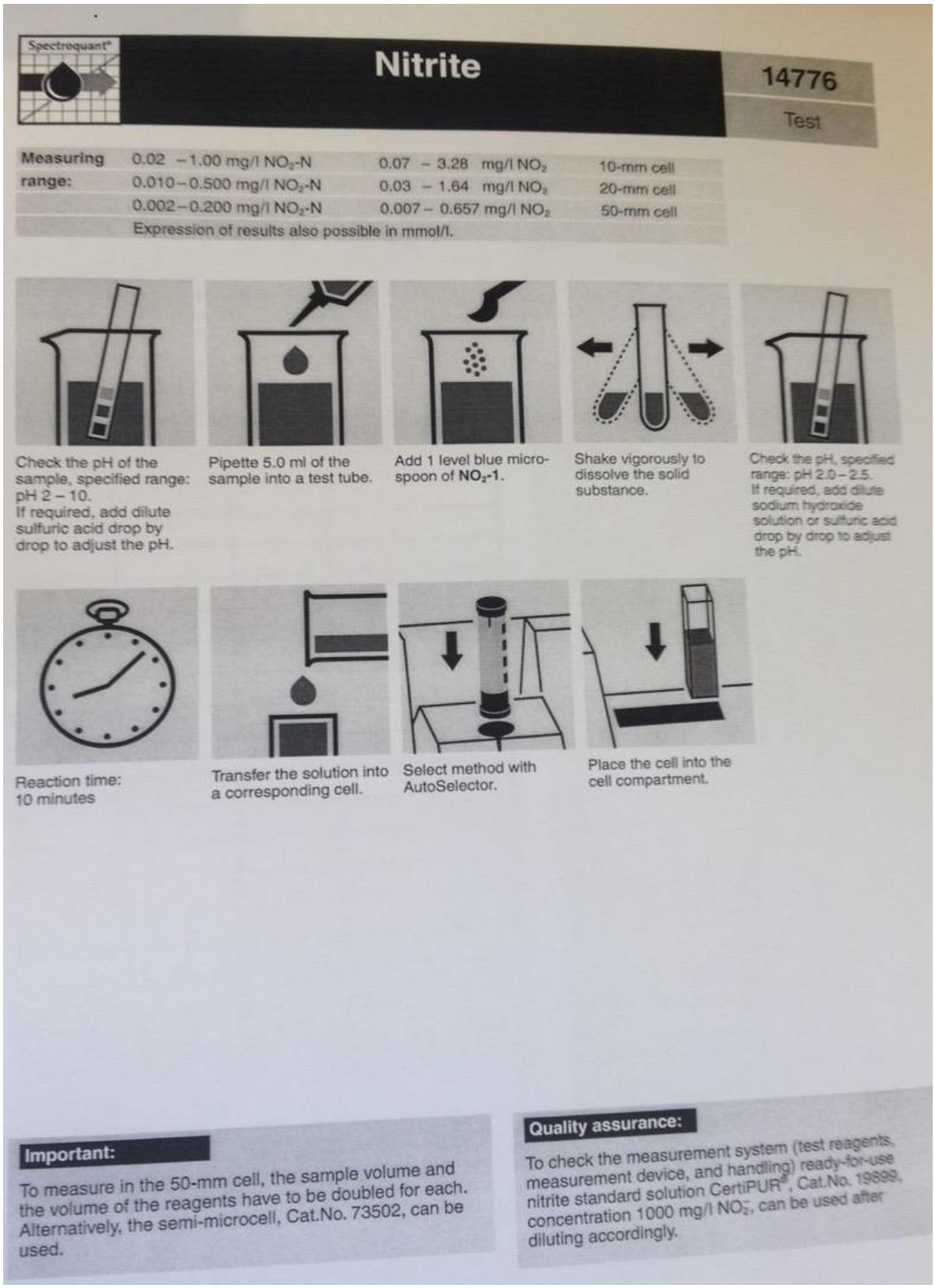
**Quality assurance:**

To check the measurement system (test reagents, measurement device, and handling) we recommended to use Spectroquant® CombiCheck 50, Cat.No. 14695.

Ready-for-use ammonium standard solution CertiPUR®, Cat.No. 19812, concentration 1000 mg/l $NH_4^+$, can also be used after diluting accordingly.

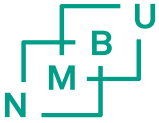
To check for sample-dependent effects the use of addition solutions (e.g. in CombiCheck 50) is highly recommended.

## 2. Measurement of nitrite



**3. Percent of un-ionized $NH_3$ in aqueous ammonia solutions for $27.5^0$C - $30^0$C and $p^H$ 6.2 – 7.8**

| | Temperature, $^0$C | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **pH** | **25.5** | **26.0** | **26.5** | **27.0** | **27.5** | **28.0** | **23.5** | **29.0** | **29.5** | **30.0** |
| 6.2 | .0933 | .0967 | .100 | .104 | .107 | .111 | .115 | .119 | .123 | .129 |
| 6.3 | .117 | .122 | .126 | .130 | .135 | .140 | .145 | .150 | .155 | .160 |
| 6.4 | .148 | .153 | .159 | .164 | .170 | .176 | .182 | .189 | .195 | .202 |
| 6.5 | .186 | .193 | .200 | .207 | .214 | .221 | .229 | .237 | .246 | .254 |
| 6.6 | .234 | .242 | .251 | .260 | .269 | .279 | .289 | .299 | .309 | .320 |
| 6.7 | .295 | .305 | .316 | .327 | .339 | .351 | .363 | .376 | .389 | .402 |
| 6.8 | .371 | .384 | .397 | .4ll | .426 | .441 | .456 | .472 | .489 | .506 |
| 6.9 | .466 | .483 | .500 | .517 | .536 | .554 | .574 | .594 | .615 | .636 |
| **7.0** | .586 | .607 | .628 | .651 | **.674** | .697 | .722 | .747 | .772 | .799 |
| 7.1 | .737 | .763 | .790 | .818 | .846 | .876 | .907 | .938 | .970 | 1.0O |
| 7.2 | .926 | .958 | .992 | 1.03 | 1.06 | 1.10 | 1.14 | 1.18 | 1.22 | 1.25 |
| 7.3 | 1.16 | 1.20 | 1.25 | 1.29 | 1.33 | 1.38 | 1.43 | 1.48 | l.53 | 1.58 |
| 7.4 | 1.46 | 1.51 | 1.56 | 1.62 | 1.67 | 1.73 | 1.79 | 1.85 | 1.92 | 1.98 |
| 7.5 | 1.83 | 1.89 | 1.96 | 2.03 | 2.10 | 2.17 | 2.25 | 2.32 | 2.40 | 2.48 |
| 7.6 | 2.29 | 2.37 | 2.46 | 2.54 | 2.63 | 2.72 | 2.81 | 2.91 | 3.01 | 3.11 |
| 7.7 | 2.87 | 2.97 | 3.07 | 3.18 | 3.29 | 3.40 | 3.5l | 3.63 | 2.75 | 3.88 |
| 7.8 | 3.59 | 3.71 | 3.84 | 3.97 | 4.10 | 4.24 | 4.38 | 4.53 | 4.68 | 4.84 |



Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no