

# Effect of dietary protein level on fish growth, economic and ecological performance of three types of feed for Nile tilapia (*Oreochromis Niloticus*)

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## Abstract

With a growing world population, food production needs to increase as well. Part of the human diet is fish, and with world fisheries declining, the aquaculture industry is increasing. Nile tilapia is a major aquaculture species, especially for poorer people in tropical countries. In Africa, the main form of aquaculture are smallholder tilapia farms, which could benefit enormously from well-formulated supplemental feed. This study compared three diets (complete, nutritious, and supplemental), varying in dietary protein:energy levels, on fish performance, economic performance, and ecological performance. Fifteen ponds were built, lined with plastic, and fertilised with sugar molasses and cow dung. Each diet was tested in five replicate ponds. All ponds, regardless of treatment, received the same amount of feed, and the feeding ration was set at 12g/kg0.8/d. Dissolved oxygen (DO), pH, and temperature were measured twice a day, in the morning and afternoon, both times right before feeding and were kept at optimum levels for fish production. Aeration was provided during the night to avoid low DO levels in the morning. The experiment lasted eight weeks, and was conducted in Zambia. The complete diet (33% protein) had the best fish performance, with the lowest FCR (0.9), the nutritious diet (22% protein) ranked second with a FCR of 1.1, and the supplemental diet (18% protein) ranked last with a FCR 1.2 ( $p=0.00$ ). Protein efficiency decreased with increasing dietary protein level ( $p=0.00$ ). Because of the respective FCRs, the complete diet also scored best in terms of economic and ecological performance per kg of fish produced, while on its own, the diet was the most expensive and had the highest greenhouse gas emissions. The difference in economic performance was not significant between the complete and supplemental diets, meaning that the supplemental diet would be preferable for smallholder farmers because of its lower costs and improved affordability. The supplemental diet had the highest greenhouse gas emissions per kg of fish produced. The results regarding fish performance found in this study are contrary to results found in previous studies comparing supplemental pond feed varying in dietary protein:energy levels, which is most likely due to the turbidity levels experienced in this study. With decreasing protein:energy levels, turbidity increased over time, and high turbidity levels have been found to hamper fish performance. Higher turbidity levels are probably caused by fish behaviour, with decreasing dietary protein levels, the fish are more likely to compensate, and forage in the sediments, causing the soil particles to disperse higher in the water column and create higher turbidity levels. For future research, turbidity issues should be avoided.

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## Abbreviations

AA: amino acids  
ANF: anti-nutritional factors  
AP: acidification potential  
ASF: animal source Food  
C-feed: complete pond feed  
CL: crude lipid  
CP: crude protein  
DC: digestibility coefficient  
DE: digestible energy  
DHC: direct human consumption  
DM: dry matter  
DO: dissolved oxygen  
DP: digestible protein  
EEA: essential amino acids  
EFA: essential fatty acids  
EP: eutrophication potential  
FAO: Food and Agriculture Organization  
FCR: feed conversion ratio  
FI: feed intake  
FIC: feed intake capacity  
GHG: greenhouse gas  
GWP: global warming potential  
HDP: human digestible protein  
HLPE: high-level panel of experts on Food Security and Nutrition  
IPCC: Intergovernmental Panel on Climate Change  
IUU: illegal, unreported and unregulated (fishing practices)  
LCA: life-cycle analysis  
LCF: low- opportunity cost feed  
LU: land use  
MSY: maximum sustainable yield  
MT: metric tonnes  
N-feed: nutritious pond feed  
NRC: national research council  
NSP: non-starch polysaccharides  
PER: protein efficiency ratio  
RAS: recirculating aquaculture system  
SDG: Sustainable Development Goals  
S-feed: supplemental pond feed  
SME: small- to medium scale enterprises  
SSA: Sub-Saharan Africa  
Temp.: temperature  
WUR: Wageningen University and Research  
WW: wet weight

# 1. Introduction

An increasing food demand is one of the consequences of an increasing global population, and fish is providing 4.5 billion people with at least 15% of their animal protein demand (Van Zanten et al, 2018; Bhujel, 2014; Paukert et al, 2017; Béné et al, 2015). Fish is essential to food security because of the high nutritional value and because fish production and capture are a source of income for millions of people (Bhujel, 2014; Béné et al, 2015). Production from capture fisheries has declined since 2016 and aquaculture now contributes around 50% of the global seafood supply (figure 1; FAO, 2018). Finfish production constitutes the largest part of total aquaculture production, and is expected to increase over the coming decades (FAO, 2018). The FAO even states that the contribution of the aquaculture sector to fighting poverty is increasing and that the sector is essential in meeting their goal of zero hunger and malnutrition (FAO, 2018).

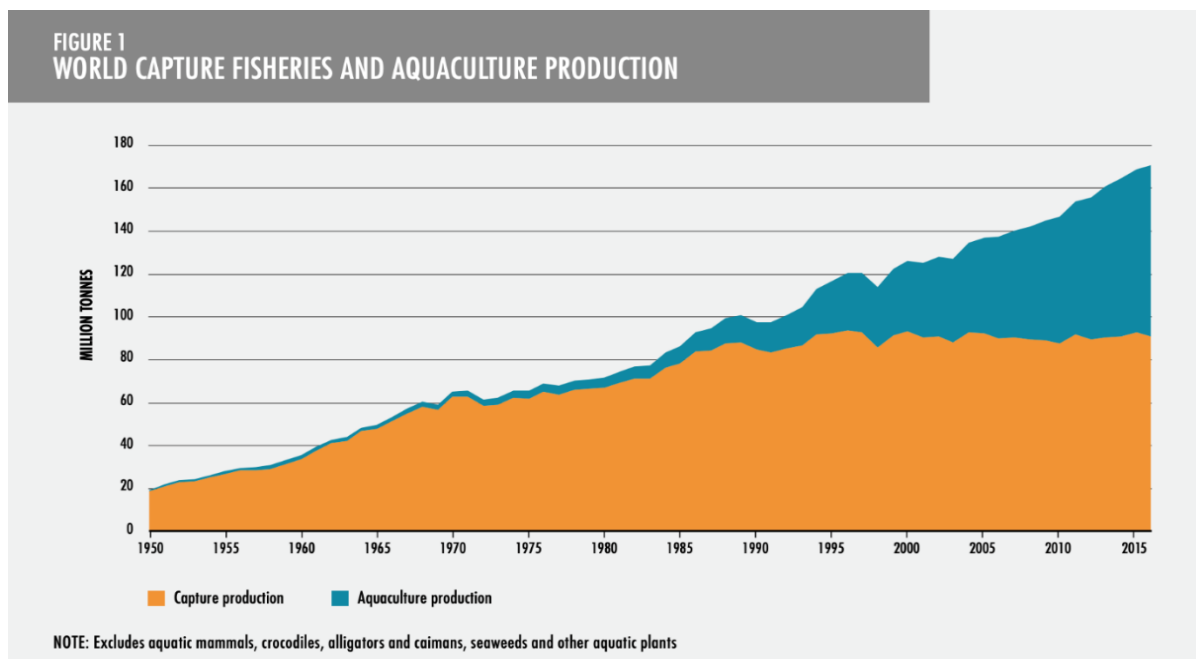


Figure 1: Visualisation of fish production from capture fisheries (orange) and aquaculture (blue) from 1950-2016. Source: FAO, 2018.

The expansion of aquaculture means that the aquafeed industry needs to grow accordingly, which is already shown by duplication in aquafeed production from 30 million MT to 60 million MT in the last decade, alongside the rapid growth of the aquaculture sector (World Bank, 2013). Formulation of fish feed is done based on various aspects, including type of production system, the target species, the economic value of the target species, the feeding habits of the target species, availability of ingredients, and financial resources (Tacon & Metian, 2015; Kabir, 2019).

The feed-conversion-ratio (FCR) of fish is lower than that of livestock, and provides opportunities for sustainable growth (El-Sayed, 2014; Mottet et al, 2017). Feed produced for production animals is a large part of the CO<sub>2</sub>- footprint of animal-source-food (ASF) and often raw materials are used which compete directly with human consumption (DHC) (El-Sayed, 2014; Mottet et al, 2017; Van Zanten et al, 2018; Van Hal et al, 2019). The latter requires land to grow crops used in aquafeeds, which is considered a central factor in climate change, as it is related to other types of environmental issues (e.g. eutrophication, acidification, biodiversity loss, deforestation) (Van Zanten et al, 2018; Van Hal et al, 2019; Clark & Tilman, 2017). Moreover, bad water quality in wastewater from aquaculture often originates from feed, and the use of fishmeal- and oil is often perceived as unsustainable when they are sourced from (overfished) marine stocks (World Bank, 2013; FAO, 2018; El-Sayed, 2014; El-Sayed, 1999; Frankic & Hershner, 2003; Kabir, 2019). Their limited availability will inevitably lead to an increasing shortage, and consequently a rise in prices, making feeds more expensive (Kabir, 2019), while feed is one of the major expenditures at farm level (Yuan et al, 2017).

Nile tilapia (*Oreochromis niloticus*), an omnivorous fish, has been the second most-farmed fish species group worldwide since 2005 and is a very important source of protein for human consumption (Studer, 2018; Bhujel, 2014). In 2016 Nile tilapia production reached 8% of the total aquaculture production, and it is mostly produced in tropical countries (FAO, 2018; Inocap, 2013). Nile tilapia production has seen a rapid growth during the last decades, which can be explained by their tolerance for low water quality, their high survival and growth, and their ability to eat a variety of feedstuffs and natural-occurring algae and plankton, abating the costs of buying commercially produced fish feed (Bhujel, 2014). However, feeding does result in faster growth, and replacing fishmeal and fish-oil in Nile tilapia diets with cheaper and more sustainable (plant-based) protein sources is a priority to make the sector more sustainable (El-Sayed, 1999; Köprücü & Özdemir, 2005; Kasiga & Lochmann, 2014; Fontainhas-Fernandes, 1999; Valdez-González et al, 2017).

The diet of Nile tilapia consists of a wide variety of ingredients and feeds, as they are omnivorous in early life stages and herbivorous in later life stages (Bhujel, 2014; FAO, 2005-2019; El-Sayed, 2006). This means that they feed on a low trophic level and natural food sources including algae, benthic invertebrates (i.e. organisms that live on the bottom), aquatic macrophytes (i.e. aquatic plants), phyto- and zooplankton, detritus, decomposing organic matter and other fish larvae (Bhujel, 2014; FAO, 2005-2019; FAO, 2019a; Cocker & Green, 2015; EL-Sayed, 2006; Shaheen, 2013). Because of their ability to efficiently harvest plankton from water, they're considered filter feeders (Bhujel, 2014; Shaheen, 2013).



In different production systems, the diet of Nile tilapia varies considerably (El-Sayed, 2006; FAO, 2019a; FAO, 2019b). In extensive systems (i.e. systems lacking any input other than the fish), the diet consists of natural foods present in the system (Bhujel, 2014; El-Sayed, 2006; Shaheen, 2013). Semi-intensive systems (i.e. systems with few inputs, such as fertilizer) make use of the same natural foods, which production is enhanced by pond fertilization (Bhujel, 2014; Milstein et al, 2008; FAO, 2019b; FAO, 2019c). Fertilization is done by supplying the pond with nitrogen (N), carbon (C) and phosphorus (P) (El-Sayed, 2006). The ratio between these elements is important for the nutrient composition of phytoplankton, which is 50% C, 10% N and 1% P (Bhujel, 2014). Both organic (e.g. animal manure) and inorganic fertilizers are used, as well as periphyton culture (El-Sayed, 2006; Milstein et al, 2008). Sometimes, semi-intensive systems also use supplemental feeding leading to a significant increase in crop yields (Bhujel, 2014; FAO, 2019b; FAO, 2019c). When supplemental feeding is provided, the natural food-web still accounts for roughly 30-50% of the fish growth (Bhujel, 2014; Anderson et al, 1987; Asaduzzaman et al, 2010; Pucher & Focken, 2017).

Using supplemental feed in pond systems contributes directly (i.e. direct supply of nutrients) as well as indirectly to fish growth by stimulating the natural food web (Kabir et al, 2019). This is done via supplying energy (carbon) and protein (nitrogen) through the fish faeces, which enhances the microbial and planktonic food web (Kabir, 2019). Kabir et al (2019) investigated the effect of dietary protein: energy ratio on fish (Nile tilapia) and pond performance, and showed that with a higher carbon (C) to nitrogen (N) ratio, fish production at pond level increased. However, the evidence for this was only provided from Bangladesh and it needs to be tested at a larger geographical scale, with a focus on areas where aquaculture is an important source of enterprise, or expected to become one.

Developing countries, and Africa in particular, struggle with the aquaculture value chain and Africa is unable to meet its own consumption demands (Kleih et al, 2013). Local feed supply is one of the issues causing the under-performance, as well as the lack of support for the small and medium-scale enterprises (SME) (Kleih et al, 2013). SME aquaculture in Africa mainly consists of (earthen) ponds with fertilisation and supplemental food supply (European Commission, 2017). The need for optimisation of fish feed formulation, feeding strategies, and performance enhancement is therefore high in Africa.

## 1.1 Study objective, research questions and hypothesis

The main objective of this thesis is to compare fish production and pond performance receiving three different feeds, varying in protein: energy ratio: Complete pond feed (C-Feed), Nutritious pond feed (N-Feed) and Supplemental pond feed (S-Feed), with a C:N ratio of 8.7, 11.2 and 14.1, respectively. The specific research questions of this study are as follows:

- 1) *How is fish (Nile tilapia) production affected by lowering the dietary protein level?*
- 2) *How is pond water quality affected by lowering the dietary protein level?*
- 3) *Which feed has the most favourable economic performance?*
- 4) *Which feed has the most favourable ecological performance?*

The hypothesis is that with a decrease in dietary protein level (thus an increase in dietary C:N ratio), the metabolic wastes will stimulate more natural food production, resulting in a higher contributing of natural food to total fish production and lower production costs for SME farmers, as well as a lower environmental footprint.

Comparing the three feeds on fish production, the main study objective, consisted of feeding twice daily, which wasn't a lot of work. Therefore, the feeds are also compared on ecological performance, considering greenhouse gas emissions and circularity.

## 2. Background

### 2.1 Fish production

It is almost common knowledge nowadays that capture fisheries have exceeded sustainable fishing levels and more and more fish stocks are depleted (FAO, 2018; Béné et al, 2015; Bhujel, 2014; Allison, 2011; Pauly et al, 2005) (Figure 2). Besides the allowed overfishing practices, the fishery sector also has to deal with the illegal, unreported and unregulated (IUU) fishing practices, which not only worsens the issue of wild stock depletion, it also complicates managing and recovering the wild stocks (FAO, 2018). The aquaculture sector, on the other hand, is still increasing faster than any other sector in food production, - making it the fastest growing sector related to food production- and now contributes around 50% of fish consumed by humans (FAO, 2018). Moreover, 37 countries produced more farmed fish than wild-caught fish in 2016 and these countries cover almost half of the global human population (FAO, 2018). The growth in the sector can be explained by a series of technological innovations, that still continue to provide opportunities for sustainable growth (HLPE, 2014). Nile tilapia production reached 4.2 million tonnes worldwide in 2016, which is 8% of the global fish production (FAO, 2018). Three carp-species produced more, representing 11%, 10% and 8% of the total fish production (FAO, 2018).

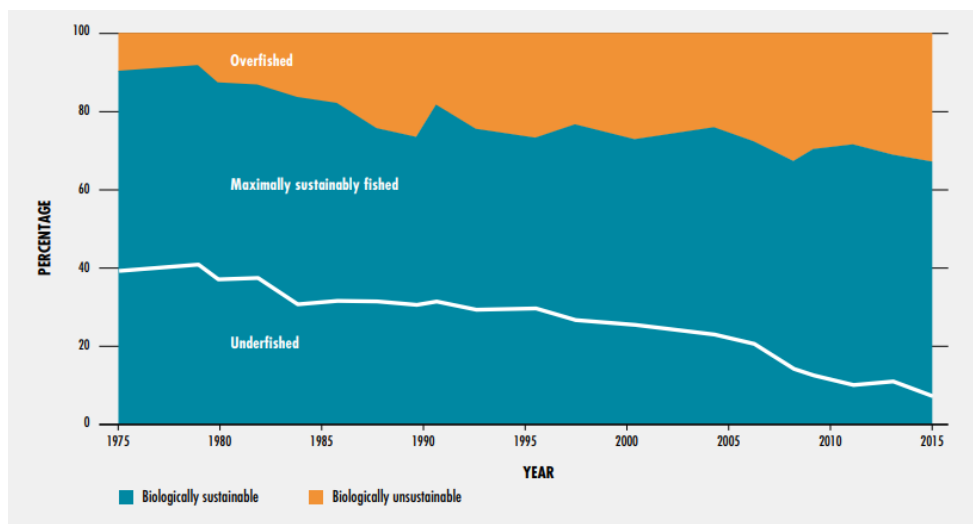


Figure 2. Global trends in the state of the world's marine fish stocks, from 1974 to 2015. The trends show the portion of fish stocks that are underfished, maximally sustainably fished or overfished as a percentage of the total fish stocks. Source: FAO, 2018.

### *2.1.1 Nile tilapia production*

Nile tilapia originates from Africa, and it is the second-most important group of cultured finfish, carp-species being the first most important group (Cocker & Green, 2015; FAO, 2005-2019). Presently, China is the largest producer of Nile tilapia, followed by Egypt (FAO, 2005-2019; Shalaan et al, 2018). In (sub)tropical countries, Nile tilapia production is most economical, as their temperatures are favourable for growth and no energy for heating is required, reducing the costs of production (FAO, 2005-2019).

Nile tilapia is known to survive in unfavourable environmental conditions, such as poor water quality, and it takes only six to twelve months to grow Nile tilapia to a marketable size (Belton et al, 2009; Bhujel, 2014; Cocker & Green, 2015). Therefore, Nile tilapia is a species of interest for fish farmers (Belton et al, 2009; Bhujel, 2014; Cocker & Green, 2015). Besides their ability to grow in adverse environmental conditions, there are numerous reasons explaining the popularity of Nile tilapia culture (Belton et al, 2009; Bhujel, 2014; Cocker & Green, 2015): no hormonal treatment is needed to induce breeding; they have a high survival and growth rate; their flavour is appreciated by a large group of consumers; Nile tilapia is well-established in international markets; they are able to breed and grow in a wide range of environments, increasing possibilities of various production systems and; their diet is variable and they are able to eat a variety of feedstuffs and natural-occurring algae and plankton, abating the costs of buying commercial fish feed (Bhujel, 2014; Cocker & Green, 2015).

### *2.2 Nile tilapia nutrition*

Nile tilapia are natural filter feeders, which consume mainly zooplankton in earlier life stages and phytoplankton in later life stages (Bhujel, 2014; FAO, 2005-2019; El-Sayed, 2006; Abdel-Tawwab, 2011). They're also tolerant towards relatively high levels of carbohydrates and fibre, which is beneficial for feed formulation, as (animal) protein is usually more expensive than plant protein (El-Sayed, 2006; Sarker et al, 2016; Maas et al, 2020). Regardless, high-quality (well-balanced) feed supports high growth rates and crop yields in intensive culture systems (i.e. systems with high use of inputs such as feed and fertilizer, as well as high water exchange rates) (Bhujel, 2014; El-Sayed, 2006; NRC, 2011). Even in semi-intensive systems, a well formulated feed contributes significantly to fish growth through nutrient supply and natural food web (Kabir et al, 2019; Asaduzzaman et al, 2008). The possible natural food contribution to tilapia production has not yet been included in the NRCs recommendations for pond feeds (Kabir et al, 2019). Regardless of production system, energy and protein are deemed the most essential macronutrients for fish growth and survival (El-Sayed, 2006; Craig et al, 2017). Furthermore, these are also

the most important nutrients for building a natural food web in pond systems, as carbon (energy) is the building block for all organic matter and nitrogen (protein) is a primary component for plant growth (Kabir et al, 2019; Boyd & Tucker, 1998).

### *2.2.1 Protein and energy*

All processes in the body are driven by energy, such as anabolic and catabolic reactions, and muscle contractions (Halver & Hardy, 2003). Energy can be derived from a number of sources, but generally carbohydrates are the cheapest source (El-Sayed, 2006; NRC, 2011; Bhujel, 2014). Lipids and proteins are catabolized as energy when carbohydrates are insufficiently present to meet energy demands (Hardy, 2010; NRC, 2011; Halver & Hardy, 2003). Generally, carbohydrates and lipids are used as a source for digestible energy, while proteins are used as a source for muscle protein growth (Kaushik & Seiliez, 2010; El-Sayed, 2006; Haidar et al, 2018).

Feed should therefore not only be sufficient in energy, but also in protein (Bureau & Encarçao, 2006; Carter & Hauler, 2011; El-Sayed, 2006). More importantly, the ratio between these two should be well to ensure proper protein utilization, and is commonly referred to as the digestible protein to digestible energy ratio (DP:DE)(Haidar et al, 2018; Kabir et al, 2019; Carter & Hauler, 2011; Bureau & Encarçao, 2006; El-Sayed, 2006). Although for common aquaculture species this ratio is in the range of 18 to 23 g/MJ, for tilapia it might be as low as 16 g/MJ if part of the protein or energy requirement is provided by the system (Carter & Hauler, 2011; Haidar et al, 2018; El-Sayed, 2006). With increasing size and age, protein requirements for tilapia decrease, and the optimal DP:DE ratio decreases as well (El-Sayed, 2006; Bhujel, 2014).

### *2.2.2 Plant-based ingredients and anti-nutritional-factors*

Protein in aquafeeds is usually supplied by fishmeal because of its favourable amino acid composition, however, this is considered to be unsustainable due to depletion of marine fish stocks and limited availability (Al-Thobaiti et al, 2018; El-Sayed, 1999; Köprücü & Özdemir, 2005; Kasiga & Lochmann, 2014; Fontainhas-Fernandes, 1999; Valdez-González et al, 2017; Tacon & Metian, 2015; FAO, 2018; Cocker & Green, 2015; Yacout et al, 2016). 20 Million tonnes capture fish were reduced to fishmeal and oil in 2016, which could also be directly consumed by people and is another reason why fishmeal use is considered unsustainable (FAO, 2018; Tacon & Metian, 2015; Cocker & Green, 2015; Froese et al, 2018). About 17% of the total aquafeed used in the aquaculture sector is used by tilapia species (FAO, 2018). Fishmeal and fish oil is included in the diet of tilapia at a level of 2-4%, leading to their rank 6<sup>th</sup> in terms of fishmeal usage, after marine shrimp, marine fish, salmon, freshwater crustaceans, and fed carp (FAO, 2018; Coker & Green, 2015).

A lot of research has explored the extent to which expensive and unsustainable fishmeal can be replaced by cheaper and more sustainable plant proteins in aquafeeds (Al-Thobaiti et al, 2018; El-Sayed, 1999; Köprücü & Özdemir, 2005; Kasiga & Lochmann, 2014; Fontainhas-Fernandes, 1999; Valdez-Gonzaléz et al, 2017; Maas et al, 2018). One of the challenges that arises with increasing the level of plant ingredients in aquafeed (as is the case when the DP:DE ratio is decreased) are the anti-nutritional-factors (ANF) (Glencross et al, 2007; Valdez- Gonzalez et al, 2017; FAO, 2019b; Ng & Romano, 2013; El-Sayed, 2006; Ogunji et al, 2008; Schrama, 2018; Hardy, 2010). Antinutritional compounds lead to less digestibility of the nutrients in fish and consequently, reduced growth (Valdez-Gonzalez et al, 2017; Glencross et al, 2007; Schrama, 2018). Other effects of ANF involve thyroid dysfunction, damage to intestinal mucosa, and reduced mineral availability (Schrama, 2018). Examples of ANF include, but are not limited to: protease inhibitors, tannins, glucosinolates, phytates, saponins, and non-starch polysaccharides (NSP) (Schrama, 2018).

#### Protease inhibitors

Protease inhibitors interfere with specific enzymes (e.g. trypsin and chymotrypsin), disabling their ability to break down proteins (Schrama, 2018). Protease inhibitors are found in, amongst others, soybean meal (SBM), which is a popular fishmeal substitute because of high protein content and essential amino acid (EAA) availability (El-Sayed, 2006; Ng & Romano, 2013; Valdez-Gonzalez, 2017). Protease inhibitors cause reduced protein utilization, leading to reduced growth (El-Sayed, 2006; Schrama, 2018). However, this can be prevented by heating the ingredients before using (El-Sayed, 2006; Schrama, 2018). Furthermore, it should be noted that tilapia seem relatively tolerant to protease inhibitors (Schrama, 2018; El-Sayed, 2006).

#### Tannins

Tannins are phenolic compounds, which are found in pea seed meal, rapeseed meal, and mustard oil cake (Schrama, 2018; Ng & Romano, 2013; Valdez-Gonzalez, 2017). Tannins bind to minerals, starch, enzymes or proteins, reducing nutrient digestibility, and causing growth disruption (Schrama, 2018). Heat treatment and dehulling of seeds may reduce the negative effects of tannins (Schrama, 2018; Valdez-Gonzalez, 2017).

#### Glucosinolates

Glucosinolates are mainly present in mustard oil cake and rapeseed meal, and are toxic after hydrolysis by thioglucosidase enzymes resulting in cell damage (Schrama, 2018). They cause thyroid dysfunction, and liver and kidney damage, leading to disrupted metabolism and growth (Schrama, 2018; Ng & Romano, 2013) The severity of the toxic effects depends on overall inclusion of glucosinolates in the diet and it can be treated with heat and water extraction (Schrama, 2018; El-Sayed, 2006; FAO, 2019b).

### Phytates

Phytate (or phytic acid) is the main form of phosphorus (P) storage in plant and is poorly available for growth, as opposed to P in fishmeal, which is highly available for growth (Francis et al, 2001; Lall and Lewis-McCrea, 2007). Fish are unable to break down phytate, leading to damaged intestinal epithelia, reduced protein and mineral availability, recessed nutrient absorption and finally reduced growth (Schrama, 2018; Valdez-Gonzalez et al, 2017; El-Sayed, 2006). Heat-treatment, fermentation, milling and supplementing dietary phytase may reduce the negative effects of phytic acid (Schrama, 2018; El-Sayed, 2006; Maas et al, 2018).

### Saponins

Saponins are glucosides (i.e. glycoside derived from glucose) that are often found in plants (Schrama, 2018; FAO, 2019b; Glencross et al, 2007). Saponins damage biological membranes (e.g. intestinal epithelium) which increases the intestinal permeability, and inhibits nutrient transport, causing reducing growth (Schrama, 2018). Saponins are extremely soluble in water, so extracting water may reduce the negative effects (Schrama, 2018).

### Non-starch polysaccharides

Non-starch polysaccharides (NSPs) are major ANF present in plant ingredients and often remain undigested in monogastric animals because they lack enzymes to break NSPs down (Maas et al, 2019). In fish the enzymes necessary for the break down of NSPs are not- to very limitedly present in the gastrointestinal tract, which is why NSPs are considered to be of very little nutritional value (Sinha et al, 2011; Maas et al, 2019). Presence of NSPs in fish diets interferes with nutrient (e.g. fat and protein) utilization, and reduces fish performance (Sinha et al, 2011; Maas et al, 2020). Though NSPs are considered to be indigestible, several studies found that Nile tilapia can digest up to 24% of the NSP fraction (Leenhouwers et al, 2007; Maas et al, 2020).

Adequate treatment and processing of ingredients will reduce or even annihilate the effects of most ANF, increasing the suitability of utilizing more plant-based ingredients in Nile tilapia feeds, and lowering the DP:DE ratio (Schrama, 2018; Valdez-Gonzalez, 2017). Lowering the DP:DE ratio also reduces feed costs and environmental footprint (Tacon & Metian, 2015; Kabir et al, 2019).

## 2.3 Pressure on resources due to (aqua)feed

The increasing global food demand requires, among others, land and water, making them essential resources that need to be treated with caution as they are under pressure, because of a growing world population and environmental changes (Schneider et al, 2011; Clark & Tilman, 2017; Tilman et al, 2011; FAO, 2017; Van Zanten et al, 2018). About 40%

of Earth's land surface is used for agriculture as well as over 70% of freshwater usage (Molden, 2013), a quarter of the greenhouse gas emissions, acidification and eutrophication of terrestrial and aquatic ecosystems, deforestation, and it results in biodiversity loss (Clark & Tilman, 2017; Van Zanten et al, 2018).

### *2.3.1 Land use*

Consequently, the upscaling of food production leads to critique on the current consumption pattern, and in particular the Animal-Source-Food (ASF) production and consumption (Van Hal et al, 2019; Yacout et al, 2016). High environmental impact associated with ASF-production is one of the sources for these critiques and land use is regarded to be the most important environmental factor (Aleksandrowicz et al, 2016; Bajzeli et al, 2014; Hällstrom et al, 2015; Schneider et al, 2011). Land use is at the centre of other environmental issues, such as greenhouse gas emissions, acidification and eutrophication, biodiversity loss, and deforestation (Van Zanten et al, 2018). About 40% of land utilized by agriculture is used for feed production (i.e. food for production animals), which leads to feed-food competition, and it increases with the increase in ASF consumption (Godfray et al, 2010; Foley et al, 2011; Van Zanten et al, 2018; Wilkinson & Lee, 2018). Feed-food competition describes that utilizing land for crops for direct human consumption (DHC) is more efficient than utilizing land for feed crops (Foley et al, 2011; Godfray et al, 2010). In a conventional system, pigs and poultry (monogastric animals) consume around 2kg of human-edible protein to produce 1kg of human-edible protein, meaning they consume more protein than they produce (Mottet et al, 2017). For fish, this ratio is generally lower (El-Sayed, 2014; Mottet et al, 2017). The greater part of land used for Nile tilapia culture is caused by feed production, which has the largest environmental impacts in Nile tilapia culture (Yacout et al, 2016; Mungkung et al, 2013). Formulating aquafeed based on by-products or crop wastes, reduces the land use as the land used for the crop production is allocated to the primary crop (Van Zanten et al, 2018).

### *2.3.2 Water pollution*

Fish farming is associated with water pollution because of effluent streams from farms (Cocker & Green, 2015; El-Sayed, 2006; FAO, 2018; Shaheen, 2013; Verdegem, 2018; Bosma & Verdegem, 2011). Pollution is caused by fertilisers, antibiotics, pesticides, hormones and feed (Shaheen, 2013; Verdegem, 2018; El-Sayed, 2006; Bosma & Verdegem, 2011), which may accumulate in the environment and cause detrimental ecological effects by disturbing the functioning of the ecosystem (Bosma & Verdegem, 2011; El-Sayed, 2006). Furthermore, these accumulated pollutants may pose a threat to other organisms as well as human consumption of these organisms (Bosma & Verdegem,



2011; El-Sayed, 2006). Less inputs and effluents reduce pollution (El-Sayed, 2006; Verdegem, 2018; Yacout et al, 2016; Shaheen, 2013).

### *2.3.3 Acidification*

Acidification occurs when soil, marine water or freshwaters decrease in pH, because of built-up of hydrogen cations, the uptake of carbon dioxide (CO<sub>2</sub>) and soil-leaching of SO<sub>x</sub> and NO<sub>x</sub>, respectively (Psenner, 1994; Caldeira & Wicket, 2003; Robson, 2012). Acidification potential (AP) is measured in sulphur dioxide (SO<sub>2</sub>) equivalents (Yacout et al, 2016; Mungkung et al, 2013; Pelletier & Tyedmers, 2010). Nitrate leaching is a source of acidification, and this occurs in agriculture as well as in aquaculture (Gazey, 2018; Yacout et al, 2016). Yacout et al (2016) found that for Nile tilapia, the acidification potential is higher in semi-intensive pond systems than in intensive tank systems, which they explained by the relative difference in energy used for aeration and higher water use in the semi- intensive systems (Yacout et al, 2016). In semi- intensive systems more water is used per kg of fish produced, while less energy is needed for aeration, and nutrient efficiency is better (Yacout et al, 2016; Mungkung et al, 2013; Pelletier & Tyedmers, 2010). Higher inputs for maintaining water quality in semi-intensive pond systems leads to a higher AP, while in intensive systems feed usage is the main contributor to AP (Yacout et al, 2016; Pelletier & Tyedmers, 2010). Overall, in aquaculture, feed production is the main contributor to AP (Yacout et al, 2016; Mungkung et al, 2013; Pelletier & Tyedmers, 2010).

### *2.3.4 Eutrophication*

Excessive enrichment of water bodies with nutrients leads to eutrophication (Chislock et al, 2013). Eutrophication is often caused by fertilizers (containing phosphate or nitrate) in an aquatic system and is associated with aquaculture, because ponds are often fertilized on purpose (Chislock et al, 2013). Eutrophication potential (EP) associated with Nile tilapia culture gets higher with higher nutrient loading (Yacout et al, 2016). Feed input in each system is a major contributor to eutrophication potential, as is faeces, and more animals leads to more feeding and more faeces, which is why intensive systems generally have a higher EP than semi-intensive or extensive systems (Yacout et al, 2016; Pelletier & Tyedmers, 2010). The FCR in pond systems is usually lower than in tank or cage systems because of the natural food web present, which also leads to a lower nutrient loading and EP (Pelletier & Tyedmers, 2010). However, when ponds are intensely used (i.e. high stocking densities and inputs), the nutrient loading in pond effluent water can get quite high as well, leading to a higher acidification and eutrophication risk (Bosma & Verdegem, 2011).

### 2.3.5 Greenhouse gas emissions

Global warming is mainly affected by greenhouse gas (GHG) emissions, and carbon dioxide (CO<sub>2</sub>), methane (CH<sub>4</sub>) and nitrous oxide (N<sub>2</sub>O) are the main anthropogenic greenhouse gasses (Hu *et al.*, 2013; IPCC, 2014; Olivier *et al.*, 2017). All of these are emitted by aquaculture (Hu *et al.*, 2013; IPCC, 2014; Olivier *et al.*, 2017). The impact of these gasses is expressed in Global Warming Potential (GWP), and it describes how much heat each gas traps in the atmosphere, relative to CO<sub>2</sub> (IPCC, 2013). Hence, the GWP of CO<sub>2</sub> is 1 CO<sub>2</sub>-equivalent, while the GWP of CH<sub>4</sub> is 28 CO<sub>2</sub>-eq., and that of N<sub>2</sub>O is 298 CO<sub>2</sub>-eq. (IPCC,2013).

Aquaculture is estimated to be responsible for 5% of total emissions from agriculture, and this is expected to increase due to the expansion of the aquaculture sector (Waite *et al.*, 2014; Hasan & Soto, 2017; FAO, 2018). CO<sub>2</sub> is primarily emitted by combustion of fossil fuels, while CH<sub>4</sub> emission is caused by fossil fuel extraction and refining, as well as anaerobic mud layers (Van den Burg *et al.*, 2012; Vellinga *et al.*, 2013). Intensive pond culture is mainly responsible for these emissions (Van den Burg *et al.*, 2012). Utilisation of fish meal and fish oil also results in higher GHG emissions because of fossil fuel combustion in fisheries (Rasenberget *et al.*, 2013; Hasan & Soto, 2017; FAO, 2018).

Fertiliser production and input, as well incomplete denitrification and anammox processes are the sources of N<sub>2</sub>O emissions in aquaculture (Van den Burg *et al.*, 2012). The largest part of aquaculture emissions is due to production of crops and animal protein for aquafeed (Ewoukem *et al.*, 2013; Yacout *et al.*, 2016; Robb *et al.*, 2017). Most ingredients used in aquafeed are by-products, such as wheat bran, in which case emissions from processing to get the by-products consumable and transportation from farm to factory to farm should be allocated to the specific ingredients, while inputs such as fertiliser and machinery should be allocated to the main crop product. When main crops are used, such as wheat, all inputs and emissions should be taken into account. Furthermore, the FCR is of high importance, as this describes how much feed is needed to produce one kg of fish, and feed is the main source of GHG emissions (Yacout *et al.*, 2016; Rasenberget *et al.*, 2013; Hasan & Soto, 2017).

Despite the increase in GHG emissions, fish is a more efficient source of protein for human consumption than terrestrial livestock, as cultured fish has a lower environmental footprint than any other animal production system (Nijdam *et al.*, 2012; Bené *et al.*, 2015; MacLeod, 2017). On the one hand, Nile tilapia are considered to be one of the most efficient converters of feed (El-Sayed, 2006; Bené *et al.*, 2015; GAA, 2019), while on the other hand, Fry *et al.* (2018) found that tilapia have a low fillet yield, and therefore a rather low caloric retention (figure 3). The protein retention of tilapia was found to be higher than the caloric retention (Fry *et al.*, 2018).

The retention rates Fry et al (2018) calculated were done with the following equations, and based on data found in Appendix I:

**Equation (1). Protein retention of selected aquatic and terrestrial animals.**

$$\begin{aligned} \text{Protein retention} &= (\text{g protein in edible portion}) / (\text{g protein in feed}) \\ &= (\text{edible portion}) (\text{g protein per 100 g of edible portion}) / (\text{FCR}) (\text{g protein per 100 g feed}) \end{aligned}$$

**Equation (2). Calorie retention of selected aquatic and terrestrial animals.**

$$\begin{aligned} \text{Calorie retention} &= (\text{calories in edible portion}) / (\text{calories in feed}) \\ &= (\text{edible portion}) (\text{calories per 100g edible portion}) / (\text{FCR}) (\text{calories per 100 g feed}) \end{aligned}$$

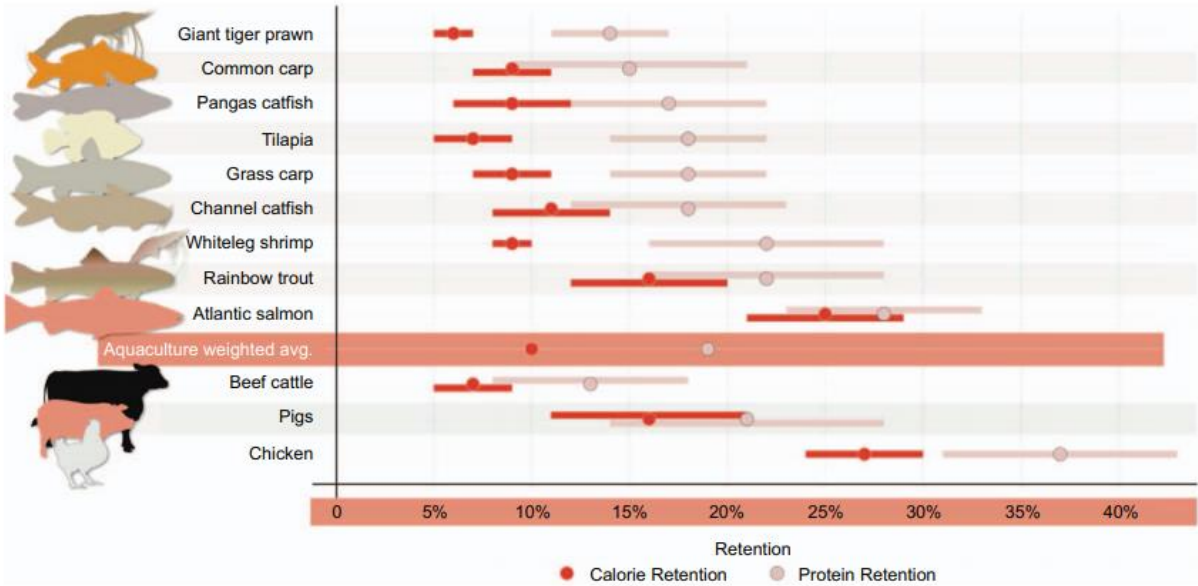


Figure 3. Feed conversion efficiency of fish compared to chickens, pigs and cattle. Dots represent the average score and the bars represent standard deviation. Source: Fry et al, 2018. Underlying data can be found in Appendix I.

**2.4 Aquaculture in Africa**

The rapid growth seen in the aquaculture sector the last decades is mainly due to smallholders and small-and-medium size enterprises (SMEs) (European Commission, 2017). Asia is currently the largest producer in the aquaculture sector, accounting for 92% of all production, while the Americas and Africa only produce 3% and 2%, respectively (European Commission, 2017). Around 1.74 million tonnes of aquaculture produce is produced in Africa annually, mainly Egypt (1.1 million tonnes), Nigeria (313,000 tonnes), and Uganda (111,000 tonnes) (European Commission, 2017). Nile tilapia accounts for almost half of the total aquaculture produce in Africa (43.6%), with African catfish (11.9%) and common carp (10.5%) in second and third place for most important freshwater species (European Commission, 2017).

Most of the tilapia and catfish are reared semi-intensely, with the use of supplemental feed (European Commission, 2017). There is motivation to develop aquaculture in Sub-Saharan-Africa (SSA), in the form of small-scale cage systems in large lakes, as well as fish farming integration into family agriculture systems (European Commission, 2017; Van Duijn et al, 2018). Increasing production seen in Asia can be explained by improvement in husbandry skills, as well as better feeding practices and production technologies (European Commission, 2017). However, the commercialisation of small-scale aquaculture in Africa is inhibited by several factors, including the limited government support, socioeconomic circumstances and the poor availability of inputs, such as feed (European Commission, 2017; Kleih et al, 2013). WorldFish (2010) found that productivity in Africa has not seen the same growth as in Asia, and it is falling behind as human population and food demands increase (WorldFish, 2010; Kleih et al, 2013).

Generally, aquaculture production systems are classified based on productivity levels, and can vary from backyard subsistence level fish farming to industrial-scale production in recirculation systems (European Commission, 2017). In the context of rural development, however, a classification based on ownership, labour, management, and markets is more appropriate (figure 4) (Edwards, 2013).

	Commercial		↔		Subsistence-oriented	
	Industrial aquaculture	Small to medium enterprise (SME) aquaculture	Small-scale commercial aquaculture	Subsistence aquaculture		
<b>FAO typology</b>	Large-scale commercial	SME enterprises	Small-scale aquaculture enterprises			
<b>Production systems</b>	Tanks (flow/re-circulated), cages, pond arrays	Tanks (flow), ponds, cages	Mainly ponds, lagoons, tanks, small cages/pens	Ponds (rain-filled)		
<b>Labour</b>	Salaried employees	Mixed, presence of permanent employees	Mainly family members. Activities are integrated into other small-holder farming activities			
<b>Capital</b>	Shared ownership	Family or family groups	Family ownership only			
<b>Management</b>	Financial management with on-farm technical support	Mainly family members, with some professional assistance	Mainly family, possibly with some professional assistance	Family only		
<b>Market type</b>	100% sales, including export	Mainly sales, both local and regional	Mixed sales and subsistence	Fully subsistence, little or no sales		
<b>Legal status</b>	Operated as a limited company	Limited company or association, independent or none	Sole trader/farmer, or none	Little or no legal status as operators		
<b>Access rights to land and water</b>	Legal concession for use	Land owned by the operator or family, or rented	Access to land through customary or family rights			

Figure 4. Classification of aquaculture production systems in terms of management, ownership, labour and markets. Source: European Commission, 2017.

Yalelo Zambia is an example of industrial-scale fish farming in Africa, with salaried employees, shared ownership, financial management, and 100% sales, while most fish farming activities in Africa consist of smaller scale operations. SME aquaculture can contribute significantly to local fish production, and they tend to be highly entrepreneurial and innovative (European Commission, 2017). Small-scale pond farmers who use Best-Management-Practices (BMPs) are found to have the largest potential to impact poverty, through stimulating economic growth (Kassam & Dorward, 2017).

Usually, juveniles are stocked at high densities, ponds are fertilised to enhance natural food web production, supplemental feed is used, and water quality is regulated (European Commission, 2017). With proper management and the right composition of supplemental feed, the production of these enterprises can successfully increase (European Commission, 2017), and an increase in supplemental feed production would be necessary to accommodate the demand as well (as seen in industrial aquaculture). Supplemental feed production might overtake industrial aquafeed production, because of the volume and increase in SME aquaculture farms. Small-scale and subsistence farmers are generally characterized by the integration of aquaculture into other farming activities, making aquaculture a secondary income and task (European Commission, 2017; Van Duijn et al, 2018). Earthen pond systems are usually the common type of production system for subsistence level farming, small-scale farming, and SME (European Commission, 2017; Van Duijn et al, 2018; Kassam & Dorward, 2017).

## 2.5 Pond systems

Since the majority of fish farming in Africa is done in pond systems, it's key to understand the dynamics of these systems. Pond systems not only serve to produce fish, there is also a natural food web present, consisting of benthic organisms, phyto- and zooplankton, and bacteria (Bosma & Verdegem, 2011; Cunha et al, 2019). Essentially, managing pond systems properly means balancing catabolism (i.e. decomposition) and anabolism (i.e. production) processes (Bosma & Verdegem, 2011; Kabir et al, 2019). Decomposition of faeces and uneaten feed consumes oxygen, while potentially dangerous nitrogen compounds are released (Bosma & Verdegem, 2011). Although high protein diets are usually key for high growth rates, in pond systems this may lead to high accumulation of Total Ammonia Nitrogen (TAN), and nitrite (NO<sub>2</sub>) concentrations in the water (Hari et al, 2004). On one hand, this is undesired as it may hamper fish yields and even lead to mass mortality (Wahab et al, 2003; Bosma & Verdegem, 2011; Magondu et al, 2013). While on the other hand, providing nutrients to pond water may stimulate the natural food web (Kabir et al, 2019; Asaduzzaman et al, 2008; Bosma & Verdegem, 2011; Cunha et al,

2019). Frequent water exchange and providing aeration (i.e. adding oxygen) are two ways of lowering the toxic nitrogen compound levels (Brune & Drapcho, 1991).

Aeration also helps increase production capacity, as oxygen availability is the main constraint in pond production (Bosma & Verdegem, 2011). Oxygen is necessary to decompose feed and faeces, as well as respiration of fish and algae (Bosma & Verdegem, 2011). During the day (i.e. when the sun is shining) phytoplankton provide oxygen as a by-product of photosynthesis (Wurts & Durborow, 1992). Part of this primary production is available as fish feed (Bosma & Verdegem, 2011; Kabir et al, 2019; Cunha et al, 2019). Stimulating growth of heterotrophic bacteria and plankton increases productivity at pond level, because nutrients from waste are converted into potential feed for fish (Kabir et al, 2019; Magondu et al, 2013; Asaduzzaman et al, 2008; Kumar et al, 2013; Cunha et al, 2019). Increasing carbon in the ponds stimulates growth of heterotrophic bacteria, and the contribution of the natural food web to fish growth (Kabir et al, 2019; Asaduzzaman et al, 2008). Increasing carbon can be done via direct application of a carbon source to the water, such as molasses, or via increasing the carbon in the feed (Kabir et al 2019).

Fish growth in fed pond systems is therefore due not only to the provided feed, but also because of the natural food web present (Kabir et al, 2019; Bosma & Verdegem, 2011; Asaduzzaman et al, 2008). Low quality feed ingredients, such as food waste and agricultural by- products, can therefore be upcycled as feed for fish and nutrient providers for plankton and bacteria (figure 5) (Kabir et al, 2019).

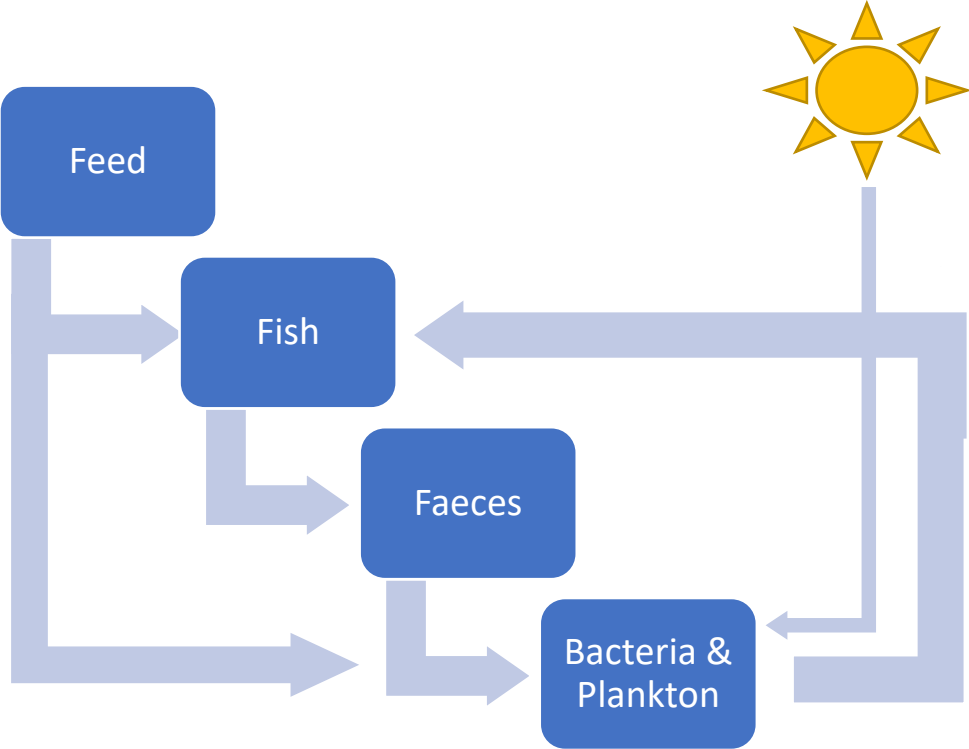


Figure 5. Pond system and natural food web dynamics.

## 3. Materials and methods

### 3.1 Diets

Three diets (complete, nutritious, and supplemental) were prepared for this experiment, which varied in protein: energy (P:E) ratio (table 1). The feed was prepared by extrusion after steam preconditioning, with a pellet size of 2 mm. The complete pond feed contained the highest P:E ratio (20.9 g/MJ), and the lowest carbon:nitrogen (C:N) ratio (8.7 g/g). Following the complete pond feed is the nutritious pond feed, with a protein: energy of 16.1 MJ/g and a C:N ratio of 11.2 g/g. Lastly, the supplemental pond feed with the lowest P:E level (14.1 MJ/g) and the highest C:N ratio (14.1 g/g). The increase in C:N ratio was achieved by increasing the level of wheat bran, and decreasing the level of animal protein. The main ingredients in all diets are blood products, wheat products, maize, soya oil and soya (table 1).

### 3.2 Experimental procedure

All male fingerling Nile tilapia (*Oreochromis niloticus*) were collected from the Yalelo hatchery, house strain Yalelo. Fifteen small (8 m<sup>2</sup>), outdoor ponds were constructed for this experiment. Each pond was covered with plastic liner and 20 cm soil. The soil in each pond was topped with 200 grams lime, and filled with water after 24h. 72h After liming, organic fertilizer (1 kg cow dung) was bagged and put in each pond. During fertilization, diluted sugar molasses were added as well, at a rate of 900 grams/pond. Fish (average bodyweight 2.82±0.29 grams) were stocked in the ponds one week after fertilization.

Fish were fed twice daily, between 7 and 8 a.m. (40% of total ration) and between 14 and 15 p.m. (60% of total ration). All ponds were fed with an equal amount of feed regardless of treatment and expected FCR (appendix II). The total feeding ration per day was set at 12g/kg<sup>0.8</sup> assuming an FCR of 1.1, set for the nutritious feed treatment. The other treatments were fed the same amount. The feeding rate started at 4.3% of fish biomass, and gradually declined to 2.4%, 3% and 3.2% of fish biomass, with a corresponding FCR of 0.8, 1.1, and 1.2, for the complete, nutritious and supplemental diet, respectively. Feed rations were not adjusted throughout the experiment. There were five replicate ponds per diet.

The total duration of the experiment was 56 days, divided into two phases. The first phase (day 1- 28) was meant to build the natural food web, and the second phase (day 28- 56) was the monitoring phase. During the first phase a few parameters were measured daily: dissolved oxygen (DO), temperature, pH, secchi disc transparency, and conductivity.



**Table 1. Ingredient composition in % inclusion and proximate nutrient composition of experimental feeds in dry matter (DM) content.**

<b>DM table</b>	<b>C-feed</b>	<b>N- feed</b>	<b>S- feed</b>
<i>Nutrients in g/kg DM</i>			
Dry matter	0.9	0.9	0.9
Crude protein	366.67	249	201
Crude fat	55.56	52	49
Crude fibre	55.56	56	76
Crude ash	68.89	86	57
Starch		341	335
NSP		225	302
Ca		18.2	3
Phosphorus	10.8	14.8	11
IP		5.4	4.7
DE (MJ)	14.4	12.4	11.4
DP		199.6	161.4
DP/DE (mg/kj)	20.9	16.1	14.1
lysine		14	11.2
methionine		6	4.9
<i>AA (g/kg CP)</i>			
lysine		56	56
methionine		24	24
threonine		35	35
tryptophan		11	12
C:N ratio feed g/g	8.7	11.2	14.1
C:N ratio faeces		29.9	46.8
C:N ratio pond		4.1	6.4
<b>Ingredient composition</b>	<b>C-feed</b>	<b>N-feed</b>	<b>S-feed</b>
Maize	20	38.1	32.35
Meat and bone meal	10.62	10	
Soya bean meal		20	15
Wheat bran	22.82	30	50
Soya oil	1	1	1
Mineral premix	0.5	0.5	0.5
Monocalcium phosphate			0.8
DL Methionine	0.41	0.2	0.15
L-Lysine HCl	0.42	0.2	0.2
Soya dehulled	29.23		
Fishmeal	3		
Poultry meal	4		
Returned raw materials	8		
<b>Total</b>	<b>100</b>	<b>100</b>	<b>100</b>

C-feed= complete feed (33% protein), N-feed= nutritious feed (22% protein, S-feed=supplemental feed (18% protein).



### 3.3 Area description

This experiment was carried out at the Yalelo production site, in Siavonga, Zambia. Siavonga is located near Lake Kariba, which is the production site for Yalelo cages. Their hatchery is based inland, which is the site where this experiment took place. The bottom was a sandy soil, and the experiment was conducted partly during the dry season (corresponding to winter), and partly during the wet season (corresponding to summer). The first phase of the experiment took place in the dry season, and phase two took place in the wet season. Water from the lake was used to fill the experimental ponds.

### 3.4 Water quality monitoring

Throughout the entire experiment DO, temperature, and pH were measured twice daily, at 7 a.m. and 14 p.m. DO and temperature were measured with an OxyGuard Handy Polaris, and pH was measured with a Hanna instruments pHep<sup>+</sup>, HI98108. Conductivity was measured every morning, with a TDS & EC meter (hold), brand XSMeterHouse. Ammonia was measured weekly by API ammonia test kit and colour card. In phase two, nitrate and nitrite were tested with Tetra tests and colour cards. Alkalinity was measured in week 7, with eXact iDip 570 freshwater aquarium kit. Turbidity was measured in week 6, 7 and 8 with Hanna instruments HI 98713 Turbidimeter. Transparency was measured every morning with a secchi disc. On day 55, a 24 hour observation of water quality was done, where DO, temperature and pH were measured every two hours. This was done at day 55 to look at the widest possible range of these parameters, as the nutrient loading increased over time, resulting in higher variations during the day.

### 3.5 Sampling and analysing of fish

Fish were stocked after being treated with a salt bath, to treat for potential parasites. At harvest, the ponds were drained, and the fish were scooped out with a dipnet. The fish were weighed per pond to determine total biomass, and consecutively counted to determine the survival rate. Afterwards, 500-520 gram samples were prepared for final body composition analysis. Since the fish were sourced from the same pond, initial body composition was assumed equal for all treatments. For final body composition, the samples taken at harvest were sent to the University of Zambia (UNZA) for proximate analysis (ash, lipids, protein and moisture). A description of the procedures done at UNZA can be found in Appendix III. Fish that were used for body composition were euthanized by asphyxiation and putting them on ice, and later stored at - 22 °C.

### 3.6 Analytical procedures and calculations

Gain in fish biomass was determined by subtracting the stocked biomass from the harvested biomass per pond. Individual biomass gain was calculated as the difference between the individual weight at harvest minus at stocking. The feed-conversion-ratio

(FCR) was calculated as the total feed fed per fish, divided by the individual biomass gain. The specific growth rate (SGR) was calculated as follows:  $SGR = \left( \frac{\ln(IndBW56) - \ln(IndBW0)}{56} \right) * 100$ , where *IndBW56* is the individual body weight on day 56, and *IndBW0* is the individual body weight on day 0. Survival percentage was calculated as (number of fish harvested/number of fish stocked) \*100. Lastly, protein efficiency ratio (PER) was calculated as total body weight gain (g) divided by total dietary protein input (g).

Results were tested for normality (Appendix IV) and consecutively analysed with one-way ANOVA to detect differences in means between treatment, with Tukey's multiple range tests as post-hoc analysis when significant differences ( $P < 0.05$ ) were found in the ANOVA. Data were analysed in Microsoft Excel and IBM SPSS software package version 23.

### 3.7 Economic and ecological performance

For (SME) farmers, economic performance is important to determine the costs per feed. To compare the economic performance of each feed, the costs per kg of feed was multiplied with the respective FCR, to determine the costs per kg of fish output . A similar calculation to determine the price per kg of protein in the fish was also done .

Greenhouse gas (GHG) emissions from processing and transportation were included in the calculations in case the ingredients were by-products. In case they were primary products (i.e. maize and soy oil), GHG emissions from crop production (including emissions from fertilizers and machine use) were also included. To compare the greenhouse gas emissions (in CO<sub>2</sub>-equivalent) related to each diet, data from FeedPrint was used. FeedPrint is a tool developed by the WUR to gather information and insights into the GHG emissions resulting from feed production and utilization (Vellinga et al, 2013). Each ingredient has a certain amount of CO<sub>2</sub>- emissions related to its usage, and therefore, the differences in emissions per diet are due to the differences in ingredient inclusion rates, and final CO<sub>2</sub>- emissions per diet were adjusted to match the inclusion rates in each diet.

## 4. Results

In this experiment, three diets (with five replicates) were assessed for fish performance, and several water quality parameters were analysed daily to maintain an optimal environment for fish growth.

### 4.1 Fish performance

Individual body weight (2.82 g), feed input per pond (3194 g) and survival (90.7 %) did not differ significantly ( $P=0.261$ ,  $P=0.298$ , and  $P=0.269$ , respectively) between treatments. Although not significant, the initial stocking biomass did show a trend with the nutritious treatment having the lowest initial biomass (528 g) and the complete treatment the highest initial biomass (602 g;  $P=0.082$ ; table 2; figure 1). Overall, the fish showed a faster growth rate with increasing dietary protein content, reflected by a higher SGR (%/day) and lower FCR (-). Alternatively, protein efficiency decreased with increasing dietary protein content.

Total biomass harvested per pond (g), final individual body weight (g), and individual body weight gain (g) differed significantly ( $P=0.000$ ,  $P=0.001$ , and  $P=0.001$ , respectively) between the complete treatment, and the nutritious and supplemental treatments (table 2, figures 6 and 7). The latter two did not differ significantly from each other for final individual body weight ( $P=0.140$ ; figure 7), but did show trends for total biomass harvested ( $P=0.091$ ; figure 6) and individual body weight gain ( $P=0.071$ ; figure 7).

The ANOVA analysis showed a significant difference in SGR (%/day) between treatments, and Tukey's test showed that the supplemental treatment differed significantly from the complete and nutritious treatments ( $P=0.012$ ), with no significant difference between the latter two ( $P=0.831$ ; table 2; figure 8). FCR (-) and PER (g weight gain/g protein fed) were significantly different between all treatments (both  $P=0.000$ ; table 2; figure 9 and 10).

The fish biomass harvested in the experiment concurs with 5290, 4240 and 3990 kg/ha for the complete, nutritious and supplemental feed, respectively.

**Table 2. Main results regarding growth per feed treatment.**

	Complete	Nutritious	Supplemental	P-value
<b>Initial biomass stocked (g)</b>	602±60	528±23	584±55	0.082
<b>Initial individual body weight (g)</b>	2.91±0.4	2.64±0.1	2.92±0.3	0.261
<b>Feed per pond (g)</b>	3194 ±0.2	3195±2	3194±0.3	0.298
<b>Protein input per pond (g)</b>	1053 <sup>a</sup> ±0.1	715 <sup>b</sup> ±0.5	578 <sup>c</sup> ±0.1	0.000
<b>Survival (%)</b>	92.8±4.8	87.9±2.7	91.5±6.3	0.269
<b>Total biomass harvested (g)</b>	4228 <sup>a</sup> ±97	3388 <sup>b</sup> ±147	3192 <sup>b</sup> ±149	0.000
<b>Final individual body weight (g)</b>	22.1 <sup>a</sup> ±1.8	19.3 <sup>b</sup> ±1.3	17.5 <sup>b</sup> ±1.0	0.001
<b>Individual body weight gain (g)</b>	19.1 <sup>a</sup> ±1.5	16.7 <sup>b</sup> ±1.3	14.6 <sup>b</sup> ±1.2	0.001
<b>SGR (%/day)</b>	3.62 <sup>a</sup> ±0.2	3.55 <sup>a</sup> ±0.2	3.20 <sup>b</sup> ±0.3	0.012
<b>FCR (-)</b>	0.87 <sup>c</sup> ±0.0	1.11 <sup>b</sup> ±0.1	1.22 <sup>a</sup> ±0.0	0.000
<b>PER (g weight gain/g protein fed)</b>	3.5 <sup>c</sup> ±0.1	4.1 <sup>b</sup> ±0.2	4.6 <sup>a</sup> ±0.2	0.000
<b>Protein content fish (%)</b>	9.15 <sup>a</sup> ±0.3	10.70 <sup>b</sup> ±0.5	10.11 <sup>b</sup> ±0.8	0.003
<b>Moisture content fish (%)</b>	75.34 <sup>a</sup> ±1.2	72.78 <sup>b</sup> ±0.9	73.75 <sup>ab</sup> ±1.0	0.008
<b>Ash content fish (%)</b>	4.67 <sup>a</sup> ±0.6	4.02 <sup>ab</sup> ±0.2	3.65 <sup>b</sup> ±0.8	0.056
<b>Fat content fish (%)</b>	9.16±1.7	10.11±1.7	7.61±1.5	0.084

<sup>a,b,c</sup> indicates differences between treatments. Treatments within the same row with a different letter are significantly different ( $P < 0.05$ ). When no superscripts are included, no significant difference was found ( $P > 0.05$ ). Complete = 33 % protein, nutritious = 22% protein and supplemental = 18% protein.

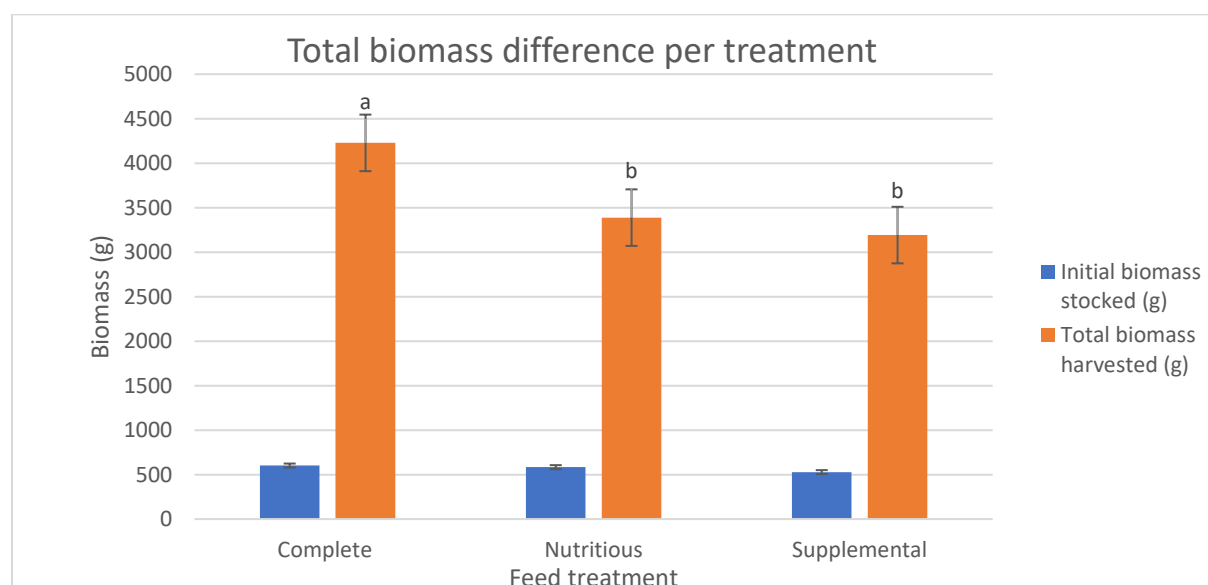


Figure 6. Initial biomass stocked in grams (blue bars,  $P=0.082$ ) and total harvested biomass in grams (orange bars,  $P=0.000$ ) per treatment per pond ( $n=5$ ). No significant difference between the nutritious and supplemental treatment was found for total biomass harvested ( $P=0.091$ ). Exact values are found in table 2. Complete = 33 % protein, nutritious = 22% protein and supplemental = 18% protein. Error bars show 5% confidence limits. <sup>a,b,c</sup> indicates differences between treatments. Treatments within the same bar colour with a different letter are significantly different ( $P < 0.05$ ). When no superscripts are included, no significant difference was found ( $P > 0.05$ ).

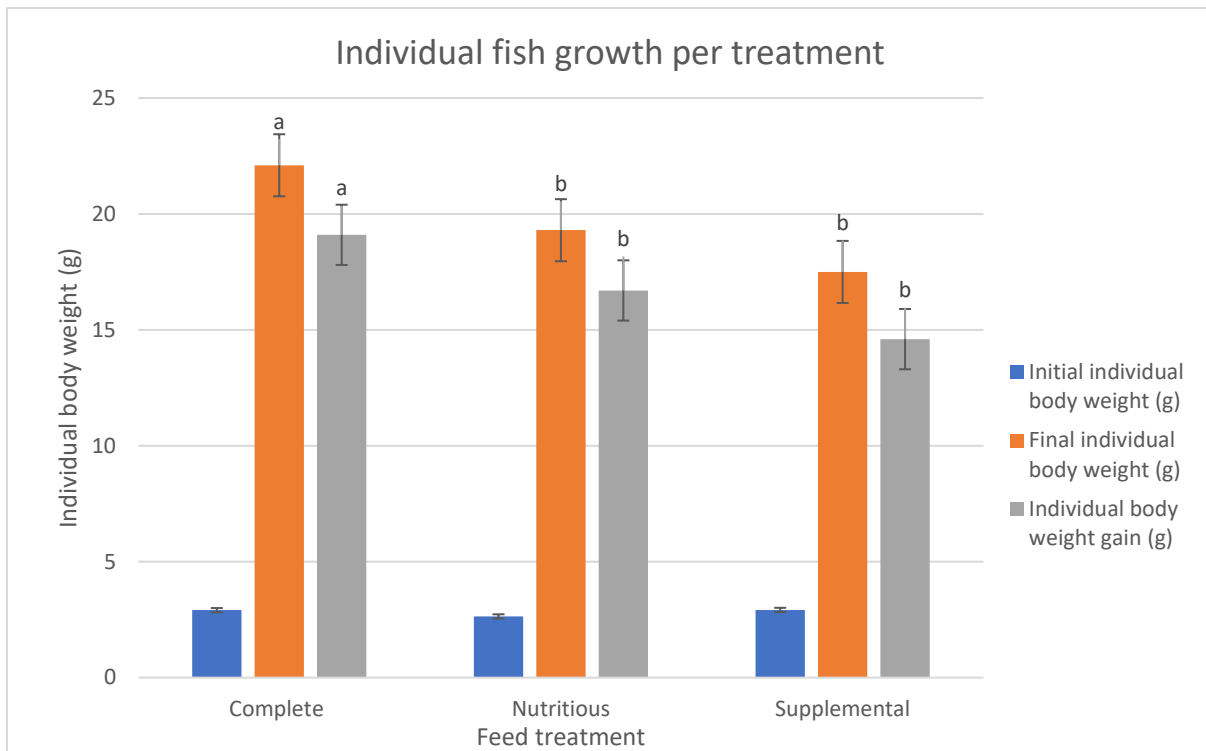


Figure 7. Initial individual body weight in grams (blue bars,  $P=0.261$ ), final individual body weight in grams (orange bars,  $P=0.001$ ) and individual body weight gain in grams (grey bars,  $P=0.001$ ) per treatment per pond ( $n=5$ ). No significant difference between the nutritious and supplemental treatment was found for final individual body weight ( $P=0.140$ ), and individual body weight gain ( $P=0.071$ ). No significant difference between all treatments was found for initial individual body weight ( $P=0.261$ ). Exact values are found in table 2. Complete = 33 % protein, nutritious = 22% protein and supplemental = 18% protein. Error bars show 5% confidence limits. a,b,c indicates differences between treatments. Treatments within the same bar colour with a different letter are significantly different ( $P < 0.05$ ). When no superscripts are included, no significant difference was found ( $P > 0.05$ ).

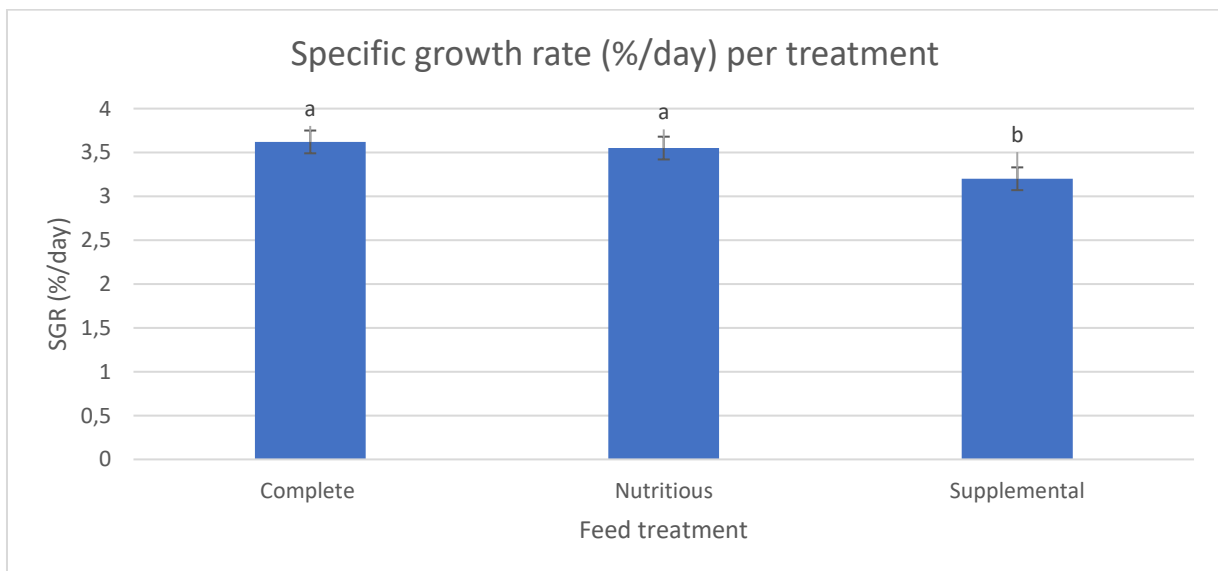


Figure 8. Specific growth rate (%/day) per feed treatment ( $n=5$ ). ANOVA showed significant differences between treatments ( $P=0.012$ ). No significant difference between the complete and nutritious treatments was found with post-hoc Tukey's multiple range tests ( $P=0.831$ ). Exact values are found in table 2. Complete = 33 % protein, nutritious = 22% protein and supplemental = 18% protein. Error bars show 5% confidence limits. a,b,c indicates differences between treatments. Treatments within the same bar colour with a different letter are significantly different ( $P < 0.05$ ). When no superscripts are included, no significant difference was found ( $P > 0.05$ ).

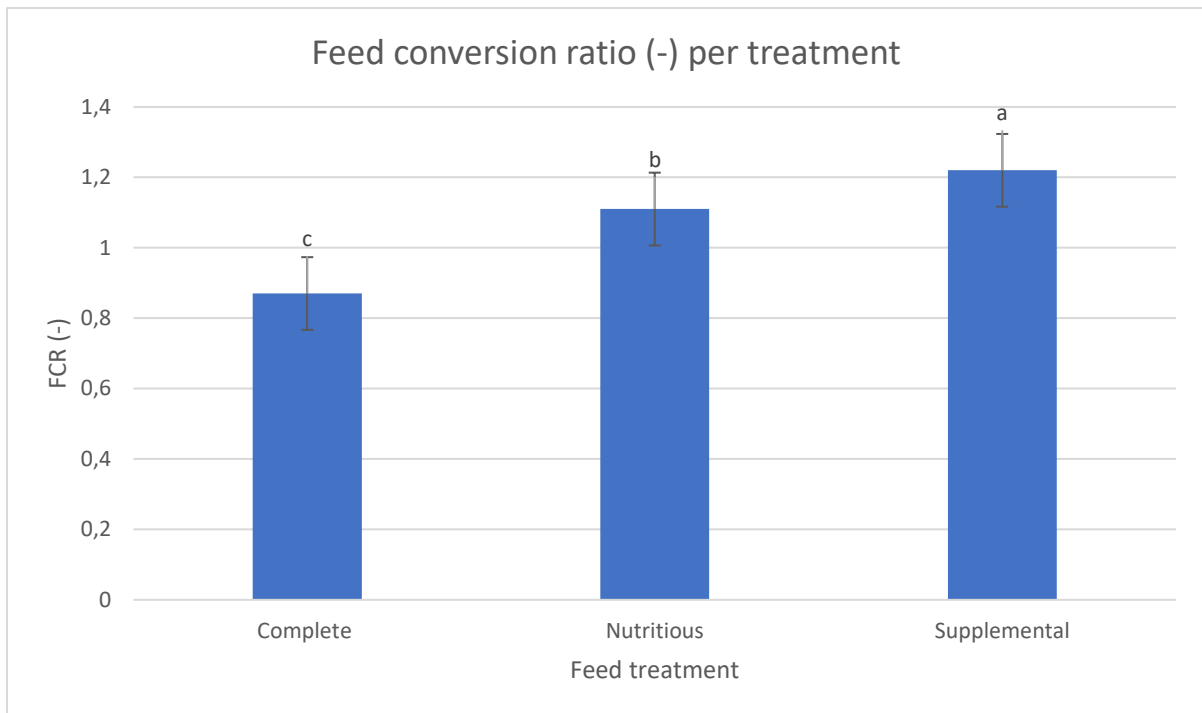


Figure 9. Feed conversion ratio (-) per feed treatment (n=5). All treatments differ significantly from each other (P=0.000). Exact values are found in table 2. Complete = 33 % protein, nutritious = 22% protein and supplemental = 18% protein. Error bars show 5% confidence limits. a,b,c indicates differences between treatments. Treatments within the same bar colour with a different letter are significantly different (P < 0.05). When no superscripts are included, no significant difference was found (P>0.05).

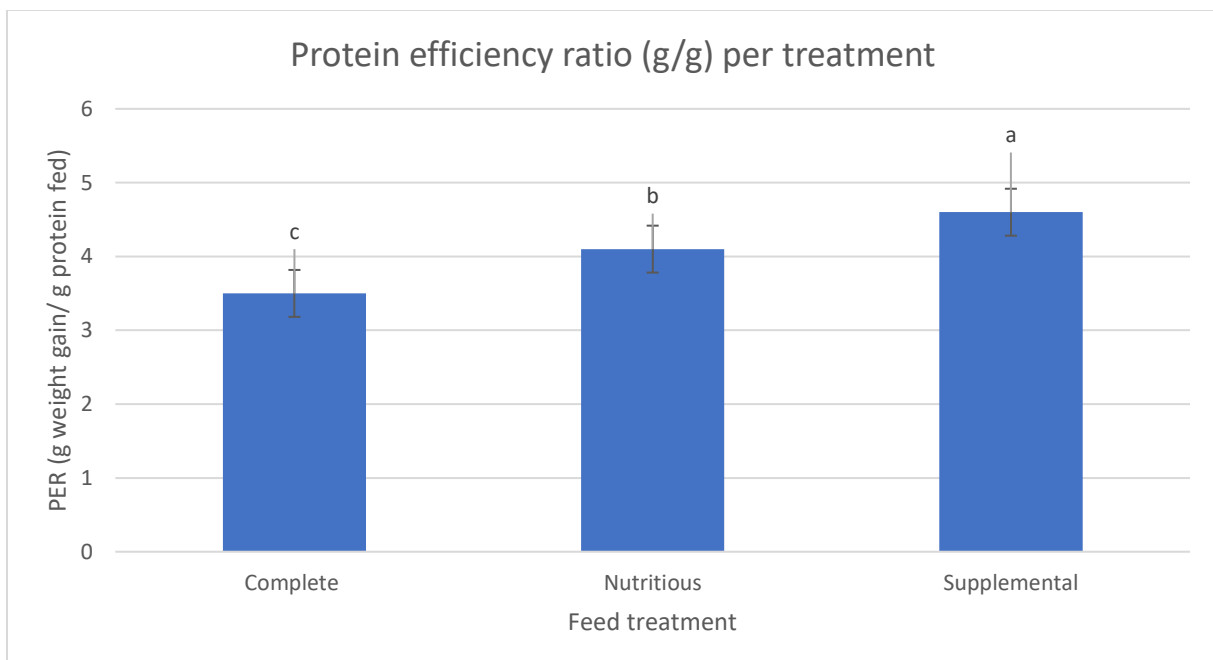


Figure 10. Protein efficiency ratio (g weight gain/ g protein fed) per feed treatment (n=5). All treatments differ significantly from each other (P=0.000). Exact values are found in table 2. Complete = 33 % protein, nutritious = 22% protein and supplemental = 18% protein. Error bars show 5% confidence limits. a,b,c indicates differences between treatments. Treatments within the same bar colour with a different letter are significantly different (P < 0.05). When no superscripts are included, no significant difference was found (P>0.05).

## 4.2 Body composition

Protein content (%) was lowest in the complete feed (9.15%), and highest in the nutritious feed treatment (10.70%;  $P=0.003$ ). The supplemental (10.11%) and nutritious treatment did not differ significantly from each other ( $P=0.261$ ; table 2; figure 11).

Moisture content (%) showed a similar but reverse curve, with the highest moisture content in the complete feed (75.34%), and the lowest in the nutritious feed treatment (72.78%;  $P=0.008$ ; table 2; figure 6). The supplemental feed treatment (73.75%) did not differ significantly from the nutritious feed treatment ( $P=0.344$ ), but did show a trend with the complete feed treatment ( $P=0.081$ ).

Ash content (%) decreased, although not significantly, with increasing dietary protein content ( $P=0.056$ ; table 2; figure 11). The nutritious feed treatment (4.02%) did not differ significantly from either one of the other two treatments ( $P=0.612$  for supplemental, and  $P=0.239$  for complete). The complete (4.67%) and supplemental (3.65%) treatments differed significantly from each other ( $P=0.049$ ).

Lastly, fat content was highest in the nutritious feed (10.11%) treatment, lowest in the supplemental feed (7.61%) treatment, and in between for the complete feed (9.16%) feed treatment. However, this was merely a trend and not significantly different ( $P=0.084$ ; table 2; figure 11).

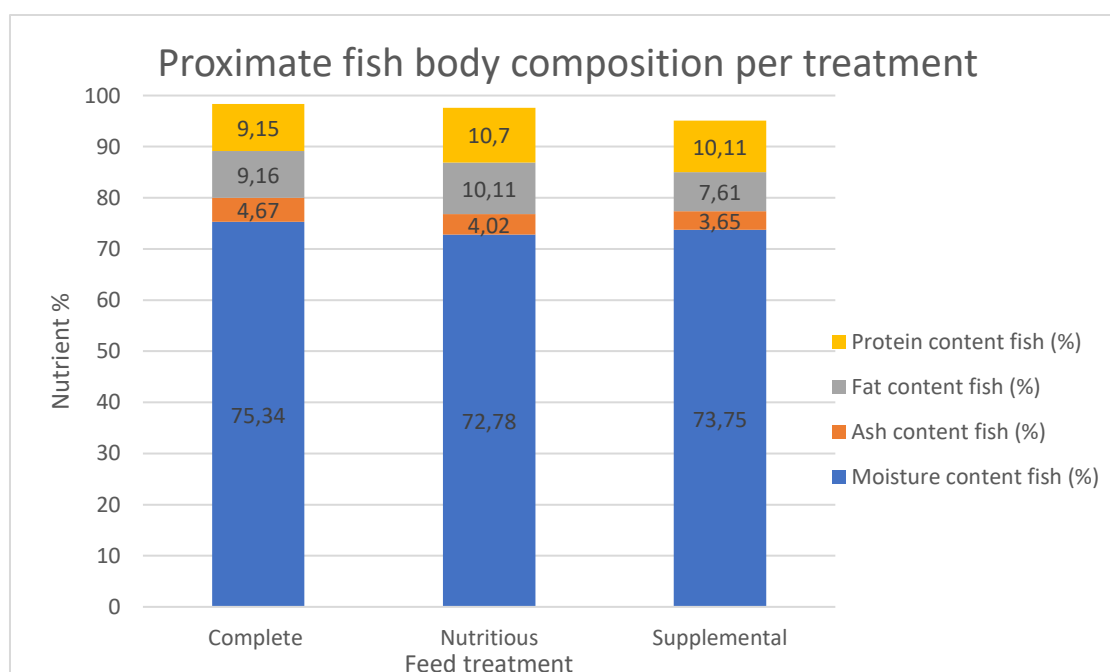


Figure 11. Proximate body composition per treatment ( $n=5$ ). Protein (yellow) and moisture (blue) content differ significantly between treatments ( $P=0.003$  and  $P=0.008$ , respectively), while ash (orange) and fat (grey) content merely showed a trend ( $P=0.056$  and  $P=0.084$ , respectively). Exact values are found in table 2. Complete feed = 33 % protein, nutritious feed = 22% protein and supplemental feed = 18% protein.

### 4.3 Water quality

Several water quality parameters were measured twice daily: dissolved oxygen (DO), pH and temperature. Morning DO (mg/L) decreased from 8.2 mg/L on average to 6.9 mg/L ( $P=0.000$ ), while afternoon DO increased from 7.5 mg/L to 11.8 mg/L on average ( $P=0.000$ ; figure 13). Morning pH (-) increased from 8.3 on average in week 1 to 8.5 on average in week 8 ( $P=0.023$ ), and afternoon pH increased from 8.8 on average in week 3 to 9.1 in week 8 ( $P=0.000$ ). Morning temperature increased from 27.9 degrees Celsius in week 1 to 28.6 degrees Celsius in week 8 ( $P=0.002$ ), and similarly afternoon temperature increased from 30.7 degrees Celsius in week 1 to 32.5 degrees Celsius in week 8 ( $P=0.000$ ). Overall, the aforementioned water quality parameters didn't differ significantly between treatments during most of the experiment (table 3).

Water quality parameters that weren't measured on a daily basis include ammonia (mg/L), nitrite (mg/L), nitrate (mg/L), alkalinity (ppm), total dissolved solids (TDS; ppm) and turbidity (formazin nephelometric units; FNU). Ammonia didn't reach toxic levels ( $>0.5$  mg/L) in any pond, with 0.07 mg/L on average in week 2 as well as in week 8. Nitrate and nitrite levels also remained below toxic levels, with 14.6 mg/L on average for nitrate and 25 mg/L maximum, and nitrite levels never surpassed 0.3 mg/L. Alkalinity didn't show significant differences, with 146.4 ppm on average for the complete feed, 137 for the nutritious feed, to 127 for the supplemental feed treatment ( $P=0.53$ ). Similarly, TDS didn't show significant differences with 121 ppm for the complete feed, 114 ppm for the nutritious feed and 104.4 ppm for the supplemental feed ( $P=0.12$ ).

Lastly, turbidity increased from 176.11 FNU on average on day 45, to 225 FNU on day 55 ( $P=0.040$ ). There was a significant difference between the complete feed (112 FNU) and the supplemental feed (287 FNU) on day 49 ( $P=0.043$ ), as well as on day 55 (114 FNU, and 333 FNU, respectively;  $P=0.002$ ). Overall, turbidity increased with time as well as decreasing dietary protein level, and there was no significant difference at all timepoints between nutritious and either of the other two feed treatments was found (figure 12).



**Table 3. Water quality parameters measured twice daily, at 08.00 a.m. and 14.00 p.m. respectively.**

		COMPLETE	NUTRITIOUS	SUPPLEMENTAL	P-value
WEEK 1	DO MO (mg/L)	8.08 <sup>a</sup>	8.09 <sup>a</sup>	8.43 <sup>b</sup>	0.010
	DO AF (mg/L)	7.70	7.30	7.58	0.407
	TEMP MO (°C)	27.96	28.05	27.85	0.445
	TEMP AF (°C)	30.93	30.55	30.66	0.118
	pH MO (-)	8.19	8.28	8.31	0.379
WEEK 2	DO MO (mg/L)	7.02	6.85	7.11	0.128
	DO AF (mg/L)	8.35	7.65	8.18	0.100
	TEMP MO (°C)	27.51	27.26	27.44	0.177
	TEMP AF (°C)	30.17	29.53	29.56	0.053
	pH MO (-)	8.00	8.00	8.00	-
WEEK 3	DO MO (mg/L)	6.77	6.96	6.91	0.408
	DO AF (mg/L)	10.03	9.84	10.17	0.658
	TEMP MO (°C)	28.15	27.86	27.84	0.045
	TEMP AF (°C)	32.28	31.25	31.50	0.059
	pH MO (-)	8.22	8.18	8.18	0.167
	pH AF (-)	8.72	8.74	8.78	0.462
WEEK 4	DO MO (mg/L)	6.83	6.90	6.93	0.648
	DO AF (mg/L)	10.79 <sup>a</sup>	9.27 <sup>b</sup>	10.11 <sup>ab</sup>	0.018
	TEMP MO (°C)	28.03 <sup>a</sup>	27.82 <sup>ab</sup>	27.69 <sup>b</sup>	0.010
	TEMP AF (°C)	32.58 <sup>a</sup>	32.43 <sup>a</sup>	32.04 <sup>b</sup>	0.007
	pH MO (-)	8.21	8.16	8.22	0.207
	pH AF (-)	8.71	8.65	8.66	0.206
WEEK 5	DO MO (mg/L)	6.62 <sup>a</sup>	6.38 <sup>b</sup>	6.73 <sup>a</sup>	0.003
	DO AF (mg/L)	11.36 <sup>a</sup>	10.38 <sup>b</sup>	10.64 <sup>ab</sup>	0.017
	TEMP MO (°C)	28.30 <sup>a</sup>	27.99 <sup>b</sup>	28.01 <sup>b</sup>	0.002
	TEMP AF (°C)	31.58 <sup>a</sup>	31.15 <sup>ab</sup>	31.06 <sup>b</sup>	0.028
	pH MO (-)	8.23	8.19	8.24	0.199
	pH AF (-)	8.64 <sup>a</sup>	8.56 <sup>b</sup>	8.62 <sup>a</sup>	0.007
WEEK 6	DO MO (mg/L)	6.08	6.18	6.43	0.082
	DO AF (mg/L)	10.09	8.80	10.24	0.082
	TEMP MO (°C)	27.33	27.38	26.03	0.423
	TEMP AF (°C)	30.27	30.32	30.36	0.819
	pH MO (-)	8.28	8.27	8.30	0.509
	pH AF (-)	8.50	8.39	8.44	0.455
WEEK 7	DO MO (mg/L)	5.97	5.99	6.20	0.280
	DO AF (mg/L)	14.00 <sup>a</sup>	10.62 <sup>b</sup>	12.19 <sup>c</sup>	0.000
	TEMP MO (°C)	27.95	27.83	27.79	0.13
	TEMP AF (°C)	32.71 <sup>a</sup>	32.49 <sup>ab</sup>	32.24 <sup>b</sup>	0.019
	pH MO (-)	8.40	8.31	8.37	0.167
	pH AF (-)	9.09 <sup>a</sup>	8.83 <sup>b</sup>	8.97 <sup>a</sup>	0.001
WEEK 8	DO MO (mg/L)	6.84	6.85	6.88	0.977
	DO AF (mg/L)	14.11 <sup>a</sup>	10.22 <sup>b</sup>	11.04 <sup>b</sup>	0.000
	TEMP MO (°C)	28.64	28.46	28.57	0.506
	TEMP AF (°C)	32.63	32.58	32.24	0.249
	pH MO (-)	8.46	8.46	8.50	0.104
	pH AF (-)	9.25 <sup>a</sup>	9.00 <sup>b</sup>	9.08 <sup>b</sup>	0.002

DO= dissolved oxygen, temp= temperature, MO= morning, AF= afternoon. <sup>a,b,c</sup> indicates differences between treatments. Treatments within the same row with the same letter are not significantly different (P > 0.05). Complete = 33 % protein, nutritious = 22% protein and supplemental = 18% protein

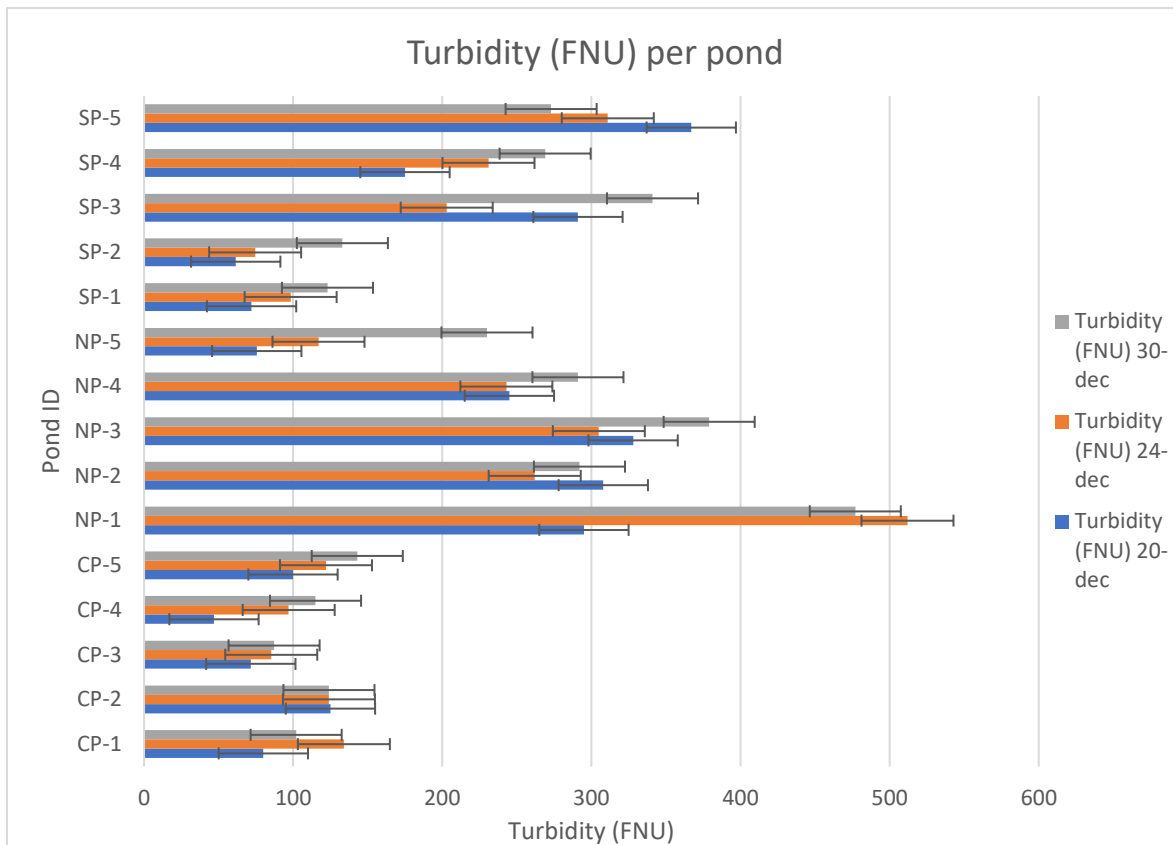


Figure 12. Turbidity values measured at three timepoints: day 45 (blue), 49 (orange), and 55 (grey) per pond (n=15). SP= supplemental ponds, NP= nutritious ponds, and CP= complete ponds. On day 49 and 55, complete feed and supplemental feed differed significantly from each other ( $P=0.043$  and  $P=0.002$ , respectively). No significant difference was found between the nutritious ponds and either one of the other two treatments. Complete = 33 % protein, nutritious = 22% protein and supplemental = 18% protein. Error bars show 5% confidence limits.

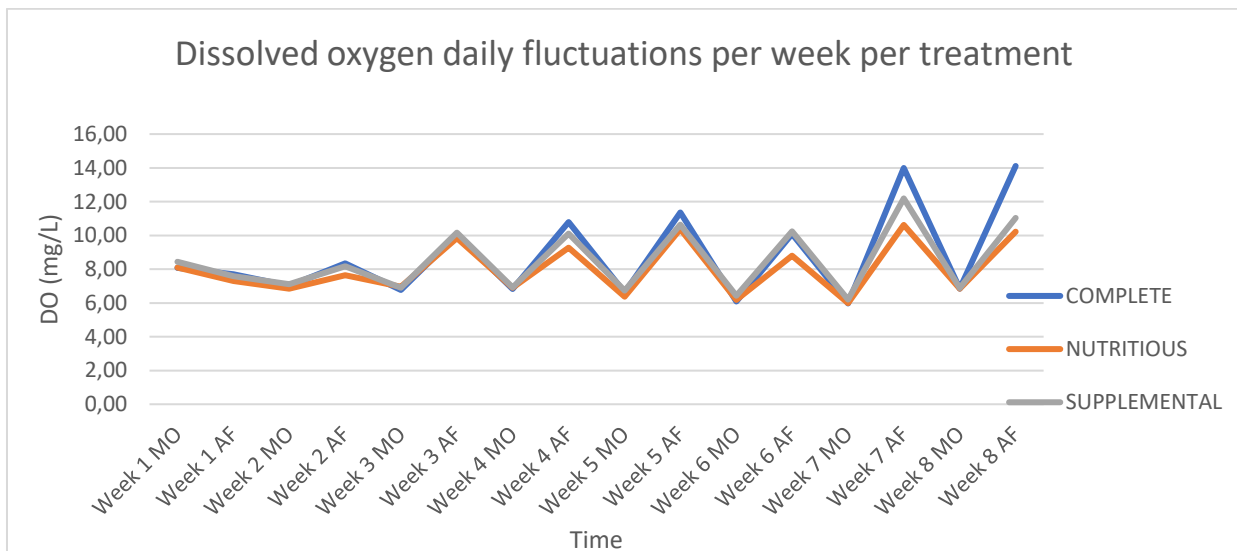


Figure 13. Dissolved oxygen fluctuations per treatment for the entire research duration. DO was measured twice daily, in the morning and afternoon. The complete diet (blue) shows the highest daily fluctuations near the end of the research, while nutritious (orange) and supplemental (grey) show lower fluctuations ( $P=0.000$ ). Overall, morning DO decreased over time and afternoon DO increased over time ( $P=0.000$ ). MO=morning, AF=afternoon. Complete = 33 % protein, nutritious = 22% protein and supplemental = 18% protein.

#### 4.3.1. 24 Hour observation of temperature, dissolved oxygen and pH

A 24 hour observation was done to monitor the fluctuations in temperature, DO and pH. Temperature was lowest at 06.00 a.m. and increased till 14.00 p.m (figure 14a). DO and pH followed similar trends (figure 14b and figure 14c, respectively). While temperatures in each pond didn't deviate much from each other, DO and pH did show differences. DO was highest in all complete ponds, and lowest in the supplemental ponds (figure 14b). pH showed the same trend (figure 14c).

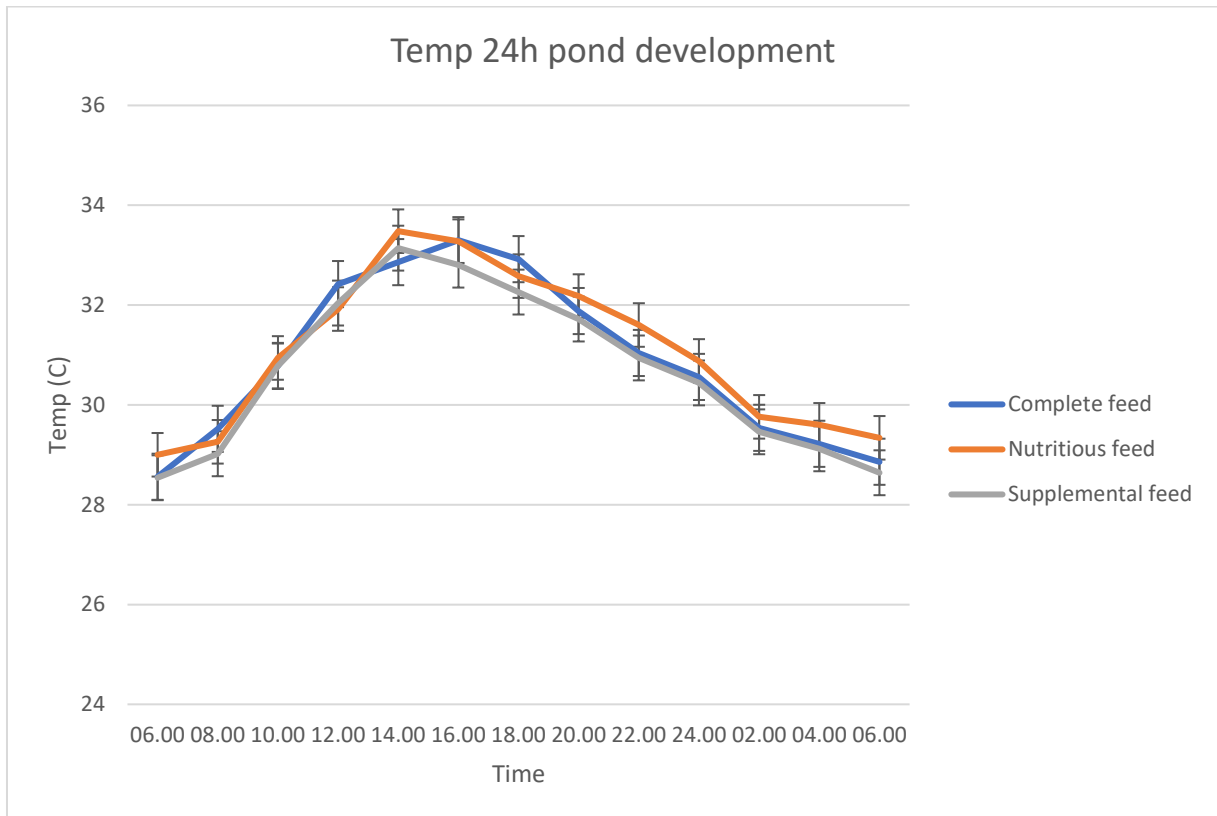


Figure 14a. A 24 hour development of temperature in every pond. CP=complete pond (33% dietary protein), SP= supplemental pond (18% dietary protein), NP= nutritious pond (22% dietary protein). Measurements were taken every 2 hours. CP1= complete pond 1, SP1= supplemental pond 1, NP= nutritious pond 1, and so forth. Measurements were taken at 55 of the experiment. Temperature in degrees Celsius is displayed on the Y-axis and timepoints are displayed on the X-axis. Error bars show 5% confidence limits.

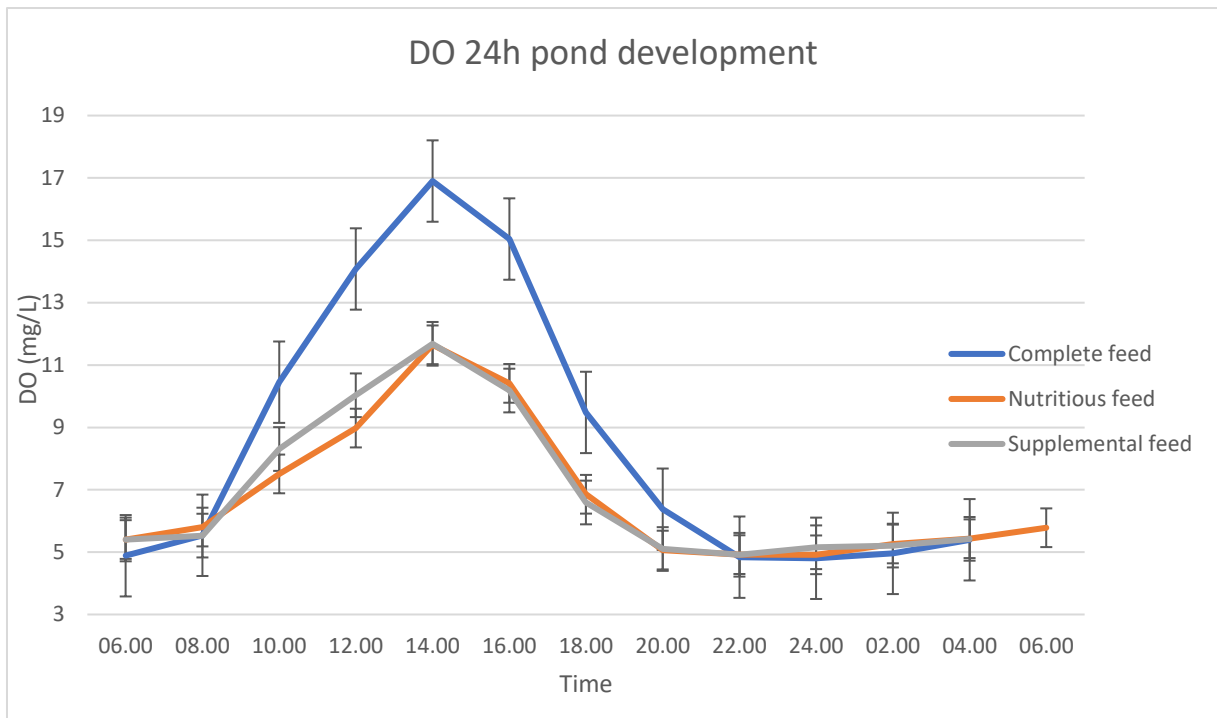


Figure 14b. A 24 hour development of dissolved oxygen (DO) in every pond. CP=complete pond (33% dietary protein), SP= supplemental pond (18% dietary protein), NP= nutritious pond (22% dietary protein). Measurements were taken every 2 hours. CP1= complete pond 1, SP1= supplemental pond 1, NP= nutritious pond 1, and so forth. Measurements were taken at 55 of the experiment. DO in mg/L is displayed on the Y-axis and timepoints are displayed on the X-axis. Error bars show 5% confidence limits.

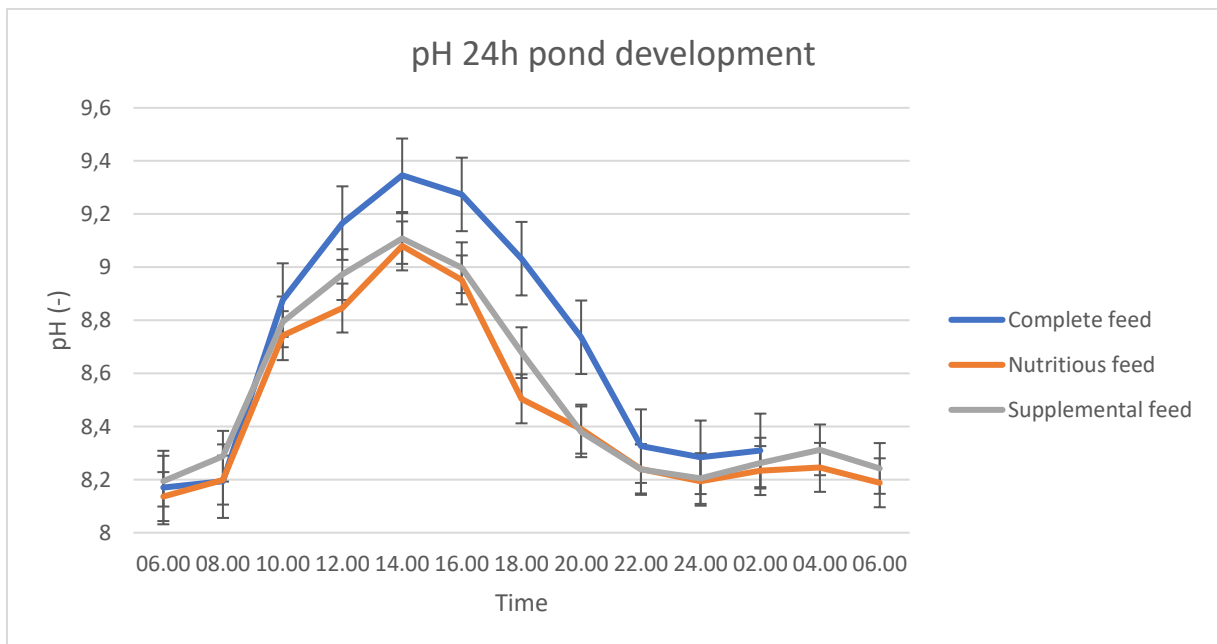


Figure 14c. A 24 hour development of pH in every pond. CP=complete pond (33% dietary protein), SP= supplemental pond (18% dietary protein), NP= nutritious pond (22% dietary protein). Measurements were taken every 2 hours. CP1= complete pond 1, SP1= supplemental pond 1, NP= nutritious pond 1, and so forth. Measurements were taken at 55 of the experiment. pH is displayed on the Y-axis and timepoints are displayed on the X-axis. Error bars show 5% confidence limits.

#### 4.4 Economic and ecological performance

There was a very small difference in economic performance between the complete and supplemental treatments (+0.01USD/kg for supplemental), and only a slight increase (+0.03USD/kg) in costs for the nutritious treatment compared to the previous two. Comparing the costs per kg of fish protein, results in a different outcome; The nutritious diet performs best with the lowest costs (5.90 USD/kg), and the complete diet performs worst with the highest costs (6.45 USD/kg) per kg of fish protein (table 4).

**Table 4. Economic performance of each diet.**

	Price/kg ZMW	FCR	Price/kg fish in ZMW	USD/kg fish produced	Protein content in fish (% ww)	USD/kg protein in fish
<b>Complete</b>	9.84	0.87	8.56	0.59	9.15	6.45
<b>Nutritious</b>	8.24	1.11	9.15	0.63	10.7	5.90
<b>Supplemental</b>	7.17	1.22	8.75	0.60	10.1	5.98

ZMW= Zambian Kwacha (currency), FCR= Feed- Conversion Ratio, USD= United States Dollars, ww= wet weight, kg= kilogram. Complete = 33 % protein, nutritious = 22% protein and supplemental = 18% protein

Similarly to economic performance, an estimation of ecological performance per diet was made. Each ingredient has a certain amount of CO<sub>2</sub>- emissions related to its usage, and FeedPrint was used to estimate the GHG emissions per ton ingredient (table 5). Final CO<sub>2</sub>- emissions per diet (table 6) were adjusted to match the inclusion rates in each diet. GHG emissions were reported in CO<sub>2</sub>- equivalents.

**Table 5. Carbon dioxide emissions per ton ingredient.**

Ingredient	CO <sub>2</sub> transport/ton	CO <sub>2</sub> processing/ton	CO <sub>2</sub> crop/ton	Total kg CO <sub>2</sub> /ton
<b>Maize</b>	63	0	421	484
<b>Wheat bran</b>	33	512	0	545
<b>Soya oil</b>	157	21	597	775
<b>Soybean meal</b>	134	123	0	257
<b>Soya dehulled</b>	132	520	4	656
<b>Meat and bone meal 45%</b>	28	485	0	513
<b>Mineral premix</b>	6	1087	0	1093
<b>Lysine</b>	6	6431	0	6437
<b>Methionine</b>	6	3044	0	3050
<b>Fish meal 63%</b>	181	1165	0	1346
<b>Poultry meal</b>	6	778	0	784

Additives such as lysine and methionine have the highest CO<sub>2</sub>- emissions per ton produced, followed by fish meal. The mineral premix, lysine and methionine are all included in similar rates in each diet, while fish meal is only included in the complete diet.

Per kg of feed, the nutritious diet scored best, with the lowest (0.483 kg CO<sub>2</sub>-eq/kg feed) GHG emissions per kg of feed, while the complete diet scored the worst, with the highest (0.592 kg CO<sub>2</sub>-eq/kg feed) GHG emissions per kg of feed (table 6). However, when taking their respective FCRs into account, the GHG emissions per kg of fish are lowest for the complete diet (0.515 kg CO<sub>2</sub>-eq/kg fish) and highest for the supplemental diet (0.608 kg CO<sub>2</sub>-eq/kg fish).

**Table 6. Ecological performance of each diet.**

	Complete	Nutritious	Supplemental
<b>Total kg CO<sub>2</sub>-eq/kg feed</b>	0.592	0.483	0.498
<b>FCR</b>	0.87	1.11	1.22
<b>Total CO<sub>2</sub>-eq/kg fish</b>	0.515	0.536	0.608

FCR= feed conversion ratio. CO<sub>2</sub>-eq= CO<sub>2</sub>- equivalent. Complete = 33 % protein, nutritious = 22% protein and supplemental = 18% protein

## 5. Discussion

### 5.1 Fish performance

Overall, fish fed with the highest dietary protein content performed best in terms of growth rate and FCR, which is in line with the high protein recommendations provided by the NRC (1993), and other research (Hafedh, 1999). However, the relative differences in performance are smaller than the differences in protein fed: the diet with 33% protein content supplied 58% more protein than the diet with 18% protein content, while the FCR increased a mere 33%. Comparing the 22% protein content with the highest protein content resulted in smaller differences: the 22% protein diet supplied 38% less protein, and the FCR increased with 24%. The smallest differences in performance were found in comparing the 22% dietary protein content with the 18% dietary protein content: the protein fed was 21% lower for the lowest protein diet, and the FCR was 9% higher. This suggests that the protein in the lower protein diets is more efficiently converted into fish biomass, which is also reflected by the PER; the diet with the 18% dietary protein content had the highest PER, meaning that more growth in fish biomass is realised per gram of protein fed (table 2). These findings are all in line with previous studies, seeking to optimize dietary protein content for Nile tilapia reared in tanks, cages (Hafedh, 1999; Wang et al, 2017), and earthen ponds (Opiyo et al, 2014).

Interestingly, various previous studies found that in pond systems a lower protein: energy ratio is not only beneficial for the protein efficiency, but also for the growth rate and FCR (Kabir et al, 2019; Asaduzzaman et al, 2008). This is contrary to what was found in this study, and could have various explanations.

The first explanation could lie in the effects of high turbidity on fish physiology and behaviour (Collins et al, 2011; Sutherland & Meyer, 2007; Gray et al, 2016; Gray et al, 2014; Ardjosoediro & Ramnarine, 2002). With decreasing dietary protein level, turbidity increased more over time than for the higher dietary protein treatments (figure 12). Considering that all ponds had the same amount of soil, were the same depth, and had the same water levels, the turbidity difference is likely not due to any of these factors. This could be explained by the feeding behaviour resulting from the dietary protein differences; tilapia are known to be omnivorous, consuming plankton, insects, benthic fauna and detritus (Engdaw et al., 2013; Prabu, 2008; Tadesse, 1999; Getachew, 1993; Teferi et al., 2000; Tsegay et al., 2016; Parker et al, 1999; Abdel-Tawwab, 2011), and it is highly likely that the fish in this experiment compensated the protein deficiency by actively foraging in the bottom sediment, causing sediment particles to disperse higher into the water and consequently increasing the water turbidity. Several studies have shown that this is not uncommon (Persson, 1997; Lammens & Hoogenboezem, 1991; Zhang et al, 2016; Zhang et al, 2017). Moreover, previous studies found that when fed a low protein diet, Nile tilapia

compensate by consuming more of the natural food present in the water (Kabir et al, 2019), and Nile tilapia isn't the only species shown to compensate lower protein with higher utilization of the natural food web, this was also shown to occur in freshwater prawn ponds (Asaduzzaman et al, 2008).

Circling back to a possible explanation for the differences in results compared to Kabir et al (2019) and Asaduzzaman et al (2008): it has repeatedly been reported that high turbidity decreases growth and fitness in fish through gill damage and visual impairment (Collins et al, 2011; Sutherland & Meyer, 2007; Gray et al, 2016; Ardjosoediro & Ramnarine, 2002). Gill damage may cause respiratory issues, while visual impairment may decrease feed intake, both leading to a decreased growth rate (Sutherland & Meyer, 2007; Gray et al, 2016; Ardjosoediro & Ramnarine, 2002). Deeper ponds might have reduced the issues resulting from the high turbidity, as turbidity caused by suspended sediment is mainly reported in shallow waters (Zhang et al, 2017).

Another cause for lower growth rate of the low-protein feeds could be the method of fertilization. In this study, the ponds were fertilized by putting cow dung in a bag and punching holes in the bag, to slowly release the nutrients from the cow dung. However, since these were very small ponds (8m<sup>2</sup>), this was not the desired method of fertilization (FAO, 2020a; FAO, 2020b). Both the fertilizer source and the method of application could have resulted in different outcomes of this study. Cow dung has poorer nutritional properties than chicken or pig manure, and direct application of the manure to the water body might have given the natural food web a better kickstart as opposed to the slow nutrient release used in this study (FAO, 2020a; FAO, 2020b). However, the high DO in the afternoons suggest that there was photosynthesis and phytoplankton production (Wurts & Durborow, 1992).

## 5.2 Body composition

In terms of body composition, fish fed the nutritious diet (=middle protein), had the most favourable outcome: they had the lowest moisture content, and the highest protein and fat content (table 2). In general, fish fed with the highest protein diet had the lowest protein content and the highest moisture and ash content. The higher ash content and body weight of fish fed a higher protein diet suggest that they had a more skeletal growth, and less muscle growth, which is also supported by the lower protein content of the fish (Shearer, 1984; Reinitz & Hitzel, 1980; Wang et al, 2017). Inversely, the same trend can be seen for the lower protein diets, which contain less ash and more protein. Lipid content of the fish was not significantly affected by dietary protein. These findings are supported by e.g. Wang et al (2017), who found that with increasing dietary starch, body protein increased, while moisture and ash decreased, and lipid levels remained unaffected (Wang et al, 2017).



Though lipid content was not significantly affected by dietary protein, a trend was visible for lower body fat content of fish fed the supplemental diet ( $P=0.084$ ), with the largest difference between the nutritious and supplemental diets. This could have various explanations, including the potential burning of fat to meet the energy demand (Chowdhury & Bureau, 2009), a higher contribution of zoo- and phytoplankton in the diet (Abdel-Tawwab, 2011; Kabir et al, 2019; Napiórkowska-Krzebietke, 2017), both of which are lower in lipids during the summer than the formulated feed (Mitra et al, 2007; Abd El-Hady et al, 2016; Napiórkowska-Krzebietke, 2017), and a decreased digestibility for fat with increasing non-starch polysaccharides (NSP) in the diet, which was the case in this study (table 1) (Maas et al, 2020).

### 5.3 Water quality

Throughout the entire experiment, all water quality parameters measured remained within levels optimal for Nile tilapia production (El-Sayed, 2006; Makori et al, 2017; Towers, 2015; Shaheen, 2013; Cocker & Green, 2015). Temperature was the only exception, as this exceeded the upper optimal level of 29 °C frequently in the afternoons (table 3). Temperature wasn't affected by diet at all during the whole duration of the experiment, but pH and DO were affected by diet in week seven and eight (table 3). Because turbidity increased over time and with decreasing dietary protein (figure 12), sunlight was less able to penetrate the water column, and consequently photosynthesis activity by phytoplankton was lower in the lower protein diets. This was shown by afternoon DO values that were lower in the nutritious and supplemental ponds than in the complete ponds. In aquaculture, earthen ponds DO is the most crucial water quality parameter, as levels below 5mg/L may result in retarded fish growth (Boyd & Tucker, 1998). DO drops during the night, when no photosynthesis takes place and oxygen is used by fish and other aquatic organisms for respiration (Boyd & Tucker, 1998; Shoko et al, 2014). Because aeration was applied during the night in this study, DO levels were never too low in the morning.

As a result of the high DO values in the afternoons, pH also increased (Wurts & Durborow, 1992), and was significantly higher in the complete treatment ponds than in the others during the last two weeks. At the start of the experiment, the ponds were supplied with lime to improve and stabilize water quality, specifically pH (FAO, 2020c). That effect decreased with time as the nutrient loading increased with time, due to higher amounts of feed fed and fish growth, resulting in more faeces (Wurts & Durborow, 1992; Makori et al, 2017). The rise in afternoon pH is not only unfavourable because it deviates a lot from fish blood pH, it also causes a shift in the equilibrium between non-toxic ammonium ( $\text{NH}_4^+$ ) and toxic ammonia ( $\text{NH}_3$ ) (Wurts & Durborow, 1992).

Ammonia is not the only nitrogen-compound that poses a threat to fish production; nitrite ( $\text{NO}_2$ ) and nitrate ( $\text{NO}_3$ ), both results of ammonia oxidation, are harmful to aquatic

organisms as well (Shoko et al, 2014). Nitrogen build-up in outdoor earthen ponds is one of the major challenges faced by aquaculture nowadays, and is caused by nitrogen input via feeds and fertilizer (Li et al, 2009; Bosma & Verdegem, 2011; Magondu et al, 2013; Shoko et al, 2014). By keeping the feeding level low in this study, nitrogen input was minimized and toxic concentrations of ammonia were avoided.

#### 5.4 Economic and ecological performance

Small- and medium-sized enterprises (SMEs) in Africa contribute considerably to local fish production, and often make use of fertilizers to enhance natural food production in ponds, as well as supplemental feed to increase production levels (European Commission, 2017; Van Duijn et al, 2018). However, financial and technical issues still inhibit growth, and need to be overcome (Kleih et al, 2013). Considering that feed is often the major expenditure at farm level, the costs should remain low to improve affordability and stimulate growth of SMEs. In terms of economic performance, the supplemental feed is the most affordable, with the lowest costs per kg feed (table 4) and only 1 cent more per kg fish produced than the complete feed. The nutritious feed might be the second-cheapest option in cost-price per kg of feed, but the FCR counteracts this and leads to higher costs per kg of fish produced. While the complete feed is the most expensive option to buy, the FCR causes lower costs per kg of fish produced. Affordability for small-scale and SMEs increases by using the supplemental feed, and using supplemental feed results in higher productivity compared to no feeding (European Commission, 2017). Especially when the growth performance resembles the results of Kabir et al (2019) and Asaduzzaman et al (2008), the supplemental feed would become cheaper per kg of fish produced, and thus not only affordability would increase, but profitability as well. Growth of SMEs is crucial for impacting poverty, as Kassam & Dorward (2017) found that small-scale pond farmers who use best management practices (BMPs), have the best potential to impact poverty indirectly by generating economic growth, which is also important for the Sustainable Development Goals (SDGs) (Kassam & Dorward, 2017).

In 2015 seventeen SDGs were adopted by several countries with the intention of achieving specific targets over the next 15 years (UN, 2020). These goals cover hunger, poverty, gender equality, 'life below water', and economic growth (UN, 2020; European Commission, 2017). Aquaculture has the potential to contribute to these goals at various levels (i.e. regional and national) (European Commission, 2017). Because of the variation in aquaculture systems (figure 4), the contribution of each system to these goals varies as well (figure 15).

Aquaculture type	Sustainable Development Goal				
	SDG 1 No poverty	SDG 2 Zero hunger	SDG 5 Gender equality	SDG 8 Decent work and economic growth	SDGs 12, 13, 14 & 15 Environmental sustainability <sup>7</sup>
Subsistence aquaculture	✓	✓✓✓	✓✓✓	✓✓	✓✓✓
	Less family expenditure on food	Major household protein source	Equal opportunities at family level	Valued work, but limited impact	Low impact, integrated development
Small-scale commercial aquaculture	✓✓	✓✓✓	✓✓✓	✓✓	✓✓✓
	Generates some income	Sales at family and local levels	Equal opportunities at family level	Some local economic impact	Low impact, integrated development
SME aquaculture	✓✓✓	✓✓	✓✓	✓✓✓	✓✓
	Generates significant income	Sales at local level	Opportunities skewed towards males	Dynamic and progressive culture	Can have cumulative impacts
Industrial aquaculture	✓	✓	✓	✓✓	✓✓
	Efficient and increasingly automated	Most produce high value and exported	Opportunities skewed towards males	Long value chain, foreign income	Can have impacts

Scale: Blank no impact, ✓ some impact, ✓✓ considerable impact, ✓✓✓ major impact

Figure 15. Type of aquaculture system and their impact on the sustainable development goals. Source: European Commission, 2017.

Economic performance is not the only important parameter anymore, ecological performance of fish farming also needs to be considered in order for the sector to grow sustainably (Yacout et al, 2016; Clark & Tilman, 2017; Tacon & Metian, 2015). The feeds in this research differed slightly in GHG emissions (table 5), with the nutritious diet having the lowest emissions and the complete diet the highest. However, taking the FCR into account shifts this performance to the complete diet as the best scoring diet, with the lowest emissions per kg of fish produced (table 5).

The ecological performance of each feed in this research is estimated by using FeedPrint, a tool developed by the WUR. However, FeedPrint is designed for and based on European data (Vellinga et al, 2013), and not on data for crops produced in Africa. Aller Aqua Zambia, which produced the feeds in this research, imports gluten and animal protein from South-Africa and the rest of the raw materials are sourced from within Zambia (Greiling, 2020, pers. comm.). Considering that solely emissions related to processing of by-products and their transport are included in this research, the actual difference in emissions between crops grown in Europe and Africa will likely only deviate regarding transport. Leaving out emissions related to transport doesn't alter the original conclusion of emissions related to each feed; the nutritious diet still has the lowest emissions, and the complete diet the highest. Emissions for the complete diet will likely further increase, albeit a small amount,

when transport is included, because this diet has the highest inclusion of animal proteins, which are imported from South-Africa.

Maize and soy oil are primary products and therefore also emissions related to crop production were included in this research. In the case of maize, the actual emissions will likely be higher for maize grown in Africa, which is included as an ingredient in this research (Valin et al, 2013; FAO, 2017; Shiferaw et al, 2011; Reynolds et al, 2015). This is mainly due to the lower yields per ha and the strong link between deforestation and maize production in Sub-Saharan Africa (Valin et al, 2013; FAO, 2017). However, maize production in Sub-Saharan Africa is usually done with little to no inputs (such as fertilizer and pesticides), which are the major sources of GHG emissions related to crop production in other parts of the world (Valin et al, 2013; FAO, 2017; Reynolds et al, 2015).

Data on soy oil in FeedPrint is based on the largest source of soy oil in Europe, which are the Americas (Vellinga et al, 2013). However, soy oil is produced locally in Sub-Saharan Africa, decreasing emissions related to transport. Both in the Americas and in Sub-Saharan Africa, a strong relationship between deforestation and soy production is observed (Ordway et al, 2017), indicating that emissions related to crop production are similar. Emissions related to maize are most prevalent in the nutritious diet, followed by the supplemental diet and lastly the complete diet. Soy oil is included in each diet at the same level, and therefore the actual emissions related to soy oil inclusion won't differ between the diets.

Including primary products such as maize and soy oil in animal feed is not desired from a circular point of view (Van Zanten et al, 2018; Van Hal et al, 2019). In a circular model, only by-products, leftovers and grassland are used as animal feed (Van Zanten et al, 2018; Van Hal et al, 2019). Considering that Nile tilapia is an omnivorous fish, which is already fed with by-products and leftovers in extensive and semi-intensive systems, would make it an excellent candidate to incorporate in a circular agriculture model, decreasing land use and GHG emissions related to its production. Animals are limited in a circular model, due to the decrease in feed availability, and therefore animal proteins for feed are also more limited (Van Hal et al, 2019), meaning that a diet for Nile tilapia would likely consist of more crop by-products and lower dietary protein levels. This might mean a decrease in production, as seen in this study, but this is not necessarily the case, as several previous studies found that fish production in ponds wasn't altered by dietary protein level (Kabir et al, 2019; Asaduzzaman et al, 2008).

Fish meal production is related to higher CO<sub>2</sub>- emissions than the crops used in this study, and even though FeedPrint is based on European production, decreasing fish meal in fish diets is often seen as a good suggestion for decreasing the CO<sub>2</sub>- footprint of fish feed (Yacout et al, 2016; Mungkung et al, 2013; Tacon & Metian, 2015). Other than the CO<sub>2</sub>-

footprint, using fish meal is pressuring wild fish stocks, which is all the more reason to minimize or even eliminate fish meal from fish feed (Tacon & Metian, 2015). In the supplemental and nutritious diets, no fish meal is included which would thus be more sustainable options than the complete diet. The fish meal inclusion in the complete diet is rather low, which is also the case for tilapia diets in general (FAO, 2018; Cocker & Green, 2015). However, because of the high volumes of commercial tilapia feed, fish meal is still an important factor in determining the sustainability of tilapia feed (FAO, 2018; Cocker & Green, 2015; Tacon & Metian, 2015).

Algae, such as seaweed, as an alternative protein source in fish feeds also have a promising role for sustainable development (El-Sayed, 2006), and are considered a useful material in the circular economy (Van den Broek, 2017; O'Brien, 2018; European Seed, 2018; Van den Burg et al, 2016; Ministry of Agriculture, Nature and Food quality of the Netherlands, 2018). Algae are generally high in protein, with favourable amino acids compositions, and don't require a lot of land for cultivation (Siddik et al, 2015; El-Sayed, 2006; Mohammed et al, 2018;). Ghosh & Mitra (2015) even found that pond and prawn production increased when fishmeal was replaced with algae meal in the feed. Furthermore, including algal meal in aquafeeds has been shown to improve the immune system, increase protein content, and improve growth performance in Nile tilapia (Khalafalla & El-Hais, 2015; Al-Zayat, 2019; Belal et al, 2012). All of these aspects are highly relevant for fish farmers, as diseases and other pathogens pose a great risk to farm profitability (Ina-Salwany et al, 2019; Njiru & Okechi, 2019). Whether or not algal or seaweed inclusion is interesting for SME farmers is unknown in terms of costs, as no data was found comparing algae-based ingredient prices with animal protein prices.

## 6. Conclusions and recommendations

### 6.1 Conclusions

This research investigated the effect of lowering dietary protein: energy levels on fish growth, economic performance and ecological footprint. The complete feed (33% protein) had the highest protein levels, and best fish growth results with a FCR of 0.9. The supplemental feed had the lowest protein levels (18% protein), and worst fish growth with a FCR of 1.2. These results deviate from previous research done on dietary protein: energy levels in pond feed, as they have found that with lower dietary protein content, fish compensate by consuming more of the natural food web present in ponds. This difference is likely due to high turbidity levels experienced in the lower protein ponds.

In terms of economic performance the complete feed and supplemental feed didn't differ in costs per kg of fish produced (0.60 USD/kg fish), but they did differ in affordability: the supplemental feed is cheaper to buy than the complete feed. As for ecological performance, the complete feed is the least sustainable option, with the highest GHG emissions per kg of feed produced. However, when looking at the GHG emissions per kg of fish produced on the different feeds, the supplemental feed performed the worst, with the highest emissions.

### 6.2 Recommendations

Because of turbidity levels, the FCR was higher for the lower protein feeds than expected based on previous research. In turn, the high FCR caused higher costs and emissions per kg of fish produced, while the supplemental feed has the lowest costs per kg of feed and second-to-lowest emissions. For future research, turbidity issues should be prevented (e.g. by deepening or enlarging the ponds) to assess the economic and ecological performance of the lower protein feeds, as they likely have better performances than seen in this research. Furthermore, feed made entirely from by-products and slaughter waste should be formulated and tested in the same way the three feeds were tested in this research, to explore the potential of feeding Nile tilapia in a circular food system. Algal meal or seaweeds could possibly be included in such a research. Lastly, the method of fertilization should be adjusted to the desired method of fertilization for the pond-types in use (i.e. small ponds – direct application) and the desired fertilizer source (i.e. pig or poultry manure instead of cow manure).

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## Appendix I Data retention rates Fry et al (2018)

**Table 1.** Data used to calculate protein and calorie retention for selected aquatic and terrestrial farmed animal species.

Species	FCR <sup>a</sup>	Edible portion of animal <sup>b</sup>	Feed content <sup>c</sup> (g or kcal per 100 g of feed)		Human nutrition <sup>d</sup> (g or kcal per 100 g serving)	
			Protein	Calories	Protein	Calories
Carp	1.5–2.0					
Common carp	–	0.36–0.54	17–45	175.8–554.2	18	109–127
Grass carp	–	0.36–0.54	25	326.0–345.5	17–18	112–127
Catfishes	1.2–2.2					
Channel catfish	–	0.35–0.63	28–32	345–390	15–17	117–119
Pangas catfish	–	0.35–0.63	26–32	339–388	15	97
Salmonids	–					
Atlantic salmon	1.2–1.5	0.58–0.88	35.5–44	372–554.5	20	208
Rainbow trout	1.0–2.0	0.40–0.82	40–47	383–454	20	141
Shrimps	1.2–2.4					
Giant tiger prawn	–	0.40	25–45	225–433	20	85
Whiteleg shrimp	–	0.62–0.65	25–45	277–417	20	85
Tilapias	1.4–2.4	0.37–0.45	20–32	216–404.4	20	96
Cattle	6.0–10	0.52–0.64	7–15.4	188–339	15–20	214–276
Chicken	1.7–2.0	0.70–0.78	18–23	320	18.6	215
Pigs	2.7–5.0	0.68–0.76	13.2–20.9	326.5–335.1	15–18.2	211–304

<sup>a</sup> Data sources: Tacon and Metian (2008) (aquatic species) [12]; Smil (2013) (livestock species) [13]; Shike (2013) (cattle) [14]; Zuidhof *et al* (2014) (chicken) [15]; Rabobank Research (2015) (pigs) [16].

<sup>b</sup> Data sources: see table S4.

<sup>c</sup> Data sources: see table S5.

<sup>d</sup> Data sources: USDA National Nutrient Database for Standard Reference [27]; Shauhua Zahn, Nanyang Technical University (personal communication); Seafood Health Facts [28]; USDA National Nutrient Database terms used for beef: ‘composite of trimmed retail cuts, separable lean and fat, trimmed to 1/8” fat, all grades, raw’ and ‘variety meats and by-products, mechanically separated beef, raw’; USDA National Nutrient Database term used for chicken: ‘meat and skin, raw’; USDA National Nutrient Database terms used for pork: ‘composite of trimmed leg, loin, shoulder, and spareribs, separable lean and fat, raw’ and ‘fresh, variety meats and by-products, mechanically separated, raw’.

Table taken directly from Fry et al (2018).



## Appendix II Feed intake all ponds

Table 1. Planned and executed feed intake all ponds, regardless of treatment. Feeding rate was set at 12 g/kg<sup>0.8</sup>/d. Each pond housed 200 fish (25/m<sup>2</sup>).

DAY	FEEDING RATE	FEEDING RATE	FEEDING RATE	FEEDING RATE
	MORNING	AFTERNOON		
	grams (60%) 08.00h	grams (40%) 15.00h	g fish <sup>-1</sup> d <sup>-1</sup>	% bw d <sup>-1</sup>
1	14.93	9.95	0.122	4.3%
2	15.39	10.26	0.125	4.3%
3	15.87	10.58	0.129	4.3%
4	16.35	10.90	0.132	4.2%
5	16.85	11.23	0.136	4.2%
6	17.36	11.57	0.140	4.2%
7	17.88	11.92	0.144	4.1%
8	18.41	12.27	0.148	4.1%
9	18.95	12.64	0.152	4.1%
10	19.51	13.01	0.156	4.1%
11	20.08	13.38	0.160	4.0%
12	20.65	13.77	0.164	4.0%
13	21.25	14.16	0.169	4.0%
14	21.85	14.57	0.173	3.9%
15	18.72	18.72	0.178	3.9%
16	19.25	19.25	0.182	3.9%
17	19.79	19.79	0.187	3.9%
18	20.33	20.33	0.192	3.8%
19	20.89	20.89	0.197	3.8%
20	21.46	21.46	0.202	3.8%
21	22.05	22.05	0.207	3.8%
22	18.11	27.17	0.212	3.8%
23	18.60	27.90	0.217	3.7%
24	19.09	28.64	0.223	3.7%
25	19.60	29.40	0.228	3.7%
26	20.11	30.17	0.234	3.7%
27	20.64	30.95	0.240	3.6%
28	21.17	31.76	0.245	3.6%
29	21.72	32.57	0.251	3.6%
30	22.27	33.41	0.257	3.6%
31	22.84	34.26	0.263	3.6%
32	23.42	35.12	0.270	3.5%
33	24.00	36.00	0.276	3.5%
34	24.60	36.90	0.283	3.5%
35	25.21	37.82	0.289	3.5%
36	25.83	38.75	0.296	3.5%
37	26.47	39.70	0.303	3.4%
38	27.11	40.67	0.309	3.4%
39	27.77	41.65	0.316	3.4%

<b>40</b>	28.44	42.66	0.324	3.4%
<b>41</b>	29.12	43.68	0.331	3.4%
<b>42</b>	29.81	44.71	0.338	3.3%
<b>43</b>	30.51	45.77	0.346	3.3%
<b>44</b>	31.23	46.85	0.353	3.3%
<b>45</b>	31.96	47.94	0.361	3.3%
<b>46</b>	32.71	49.06	0.369	3.3%
<b>47</b>	33.46	50.19	0.377	3.3%
<b>48</b>	34.23	51.34	0.385	3.2%
<b>49</b>	35.01	52.52	0.394	3.2%
<b>50</b>	35.81	53.71	0.402	3.2%
<b>51</b>	36.62	54.92	0.411	3.2%
<b>52</b>	37.44	56.16	0.419	3.2%
<b>53</b>	38.27	57.41	0.428	3.1%
<b>54</b>	39.13	58.69	0.437	3.1%
<b>55</b>	39.99	59.98	0.446	3.1%
<b>56</b>	40.87	61.30	0.455	3.1%

## Appendix III Procedures proximate analysis

The description of the procedures done at UNZA, kindly provided by mr. Ian Nachibanga, head of Food and Nutrition laboratory.

### MOISTURE METHOD PRINCIPLE

Evaporation method

The moisture content is determined by measuring the mass of a food sample before and after the water is removed by evaporation:

$$\% \text{ Moisture} = \frac{M_{\text{INITIAL}} - M_{\text{DRIED}}}{M_{\text{INITIAL}}} \times 100$$

Here,  $M_{\text{INITIAL}}$  and  $M_{\text{DRIED}}$  are the mass of the sample before and after drying, respectively. The basic principle of this technique is that water has a lower boiling point than the other major components within food, *e.g.*, lipids, proteins, carbohydrates and minerals. Sometimes a related parameter, known as the dry matter, is reported as a measure of the moisture content. The dry matter content is a measure of the amount of material remaining after all the water has been evaporated:

$$\% \text{ Dry Matter} = \frac{M_{\text{DRIED}}}{M_{\text{INITIAL}}} \times 100$$

Thus, %Dry matter = (100 - %Moisture). To obtain an accurate measurement of the moisture content or total solids of a food using evaporation methods it is necessary to remove all of the water molecules that were originally present in the feed sample, without changing the mass of the food matrix. This is often extremely difficult to achieve in practice because the high temperatures or long times required to remove all of the water molecules would lead to changes in the mass of the food matrix, *e.g.*, due to volatilization or chemical changes of some components. For this reason, the drying conditions used in evaporation methods are usually standardized in terms of temperature and time so as to obtain results that are as accurate and reproducible as possible given the practical constraints. Using a standard method of sample preparation and analysis helps to minimize sample-to-sample variations within and between laboratories.

### APPARATUS

*Aluminium dish*

*Oven set at 105°C*

### METHOD

- 1 weigh a clean dry Aluminium Dish and record its weight (W1). Do not remove from the balance.*
- 2 mix the sample thoroughly and add 2g of the sample to the Aluminium Dish*
- 3 record the new weight of that of the sample + Aluminium Dish (W2)*
- 4 transfer the Aluminium Dish with the weighed sample to the oven set at 105°C*
- 5 dry the sample for 4 hours*
- 6 after 4 hours cool the sample in the Dessiccator*
- 7 after cooling weigh the sample and record the weight (W3)*
- 8 transfer the sample to the oven set at 105°C*
- 9 further dry it for 1 hour*
- 10 after 1 hour, remove the sample from the oven and cool before reweighing*
- 11 note the weight (W4)*

***If the weight of W3 = W4, proceed with the calculation of moisture percentage. If W3 ≠ W4, record W4 and take the sample back to the oven for another 1 hour. After 1 hour remove the sample from the oven and cool. After cooling reweigh, till the weight is the same as the previous weight.***

**RESULTS AND CALCULATION**

Weight of aluminium dish	W1
Weight of aluminium dish + air dry sample	W2
Weight of air dry sample	W2 – W1
Weight of aluminium dish + oven dry sample	W3
Weight of oven dry sample	W3 – W1

$$\text{Moisture \%} = \frac{W2 - W3}{W2 - W1} \times 100$$

$$\text{Dry Matter \%} = 100 - \text{moisture \%}$$

----- end of moisture/dry matter analysis

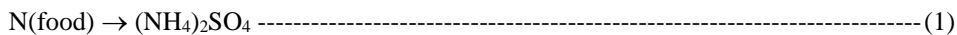
**PROTEIN DETERMINATION**

**Kjeldahl method**

The Kjeldahl method was developed in 1883 by a brewer called Johann Kjeldahl. A food is digested with a strong acid so that it releases nitrogen which can be determined by a suitable titration technique. The amount of protein present is then calculated from the nitrogen concentration of the food. The same basic approach is still used today, although a number of improvements have been made to speed up the process and to obtain more accurate measurements. It is usually considered to be *the* standard method of determining protein concentration. Because the Kjeldahl method does not measure the protein content directly a *conversion factor (F)* is needed to convert the measured nitrogen concentration to a protein concentration. A conversion factor of 6.25 (equivalent to 0.16 g nitrogen per gram of protein) is used for many applications, however, this is only an average value, and each protein has a different conversion factor depending on its amino-acid composition. The Kjeldahl method can conveniently be divided into three steps: digestion, neutralization and titration.

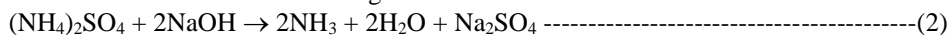
**Digestion Principle**

The food sample to be analyzed is weighed into a *digestion flask* and then digested by heating it in the presence of sulfuric acid (an oxidizing agent which digests the food), anhydrous sodium sulfate (to speed up the reaction by raising the boiling point) and a catalyst, such as copper, selenium, titanium, or mercury (to speed up the reaction). Digestion converts any nitrogen in the food (other than that which is in the form of nitrates or nitrites) into ammonia, and other organic matter to CO<sub>2</sub> and H<sub>2</sub>O. Ammonia gas is not liberated in an acid solution because the ammonia is in the form of the ammonium ion (NH<sub>4</sub><sup>+</sup>) which binds to the sulfate ion (SO<sub>4</sub><sup>2-</sup>) and thus remains in solution:

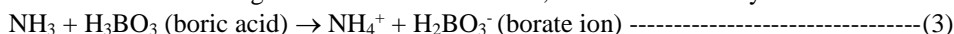


**Neutralization Principle**

After the digestion has been completed the digestion flask is connected to a *receiving flask* by a tube. The solution in the digestion flask is then made alkaline by addition of sodium hydroxide, which converts the ammonium sulfate into ammonia gas:

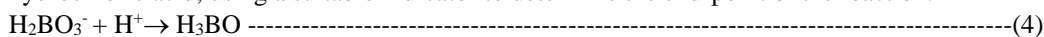


The ammonia gas that is formed is liberated from the solution and moves out of the digestion flask and into the receiving flask - which contains an excess of boric acid. The low pH of the solution in the receiving flask converts the ammonia gas into the ammonium ion, and simultaneously converts the boric acid to the borate ion:



**Titration**

The nitrogen content is then estimated by titration of the ammonium borate formed with standard sulfuric or hydrochloric acid, using a suitable indicator to determine the end-point of the reaction.



The concentration of hydrogen ions (in moles) required to reach the end-point is equivalent to the concentration of nitrogen that was in the original feed (Equation 3). The following equation can be used to determine the nitrogen concentration of a sample that weighs  $m$  grams using a  $x$ M HCl acid solution for the titration:

$$\% N = \frac{x \text{ moles}}{1000 \text{ cm}^3} \times \frac{(v_s - v_b) \text{ cm}^3}{m \text{ g}} \times \frac{14 \text{ g}}{\text{moles}} \times 100 \text{-----(5)}$$

Where  $V_s$  and  $V_b$  are the titration volumes of the sample and blank, and 14g is the molecular weight of nitrogen N. A blank sample is usually run at the same time as the material being analyzed to take into account any residual nitrogen which may be in the reagents used to carry out the analysis. Once the nitrogen content has been determined it is converted to a protein content using the appropriate conversion factor: %Protein = F× %N.

Advantages and Disadvantages

*Advantages.* The Kjeldahl method is widely used internationally and is still the standard method for comparison against all other methods. Its universality, high precision and good reproducibility have made it the major method for the estimation of protein in foods.

*Disadvantages.* It does not give a measure of the true protein, since all nitrogen in foods is not in the form of protein. Different proteins need different correction factors because they have different amino acid sequences. The use of concentrated sulphuric acid at high temperatures poses a considerable hazard, as does the use of some of the possible catalysts. The technique is time consuming to carry-out.

## APPARATUS

Kjeltec digestion unit

Kjeltec distillation unit

100ml measuring cylinder

Titration unit

## REAGENTS

0.1N sulphuric acid

Boric acid indicator

Concentrated sulphuric acid

Catalyst

## METHOD

### DIGESTION

- 1 Weigh 1g of sample into a kjeldahl flask
- 2 add catalyst (kjeltec) 12ml concentrated sulphuric acid
- 3 digest for 1 hour or till the mixture turns transparent green
- 4 after digestion switch off the digester to cool
- 5 remove the kjeldahl flask and add 75ml water
- 6 take the flask for distillation

### DISTILLATION

- 7 add 25ml boric acid indicator in a 100ml conical flask and place it on the receiver end of the Kjeltec distillation unit
- 8 add 50ml 40% sodium hydroxide and mount the flask on the distillation unit
- 9 distill for 5minutes or till 75ml of distillate is collected

### TITRATION

Titrate with 0.1N sulphuric acid

## RESULTS AND CALCULATION

Sample weight	1g
volume acid Titrated	$x$ ml

## CALCULATION

If exactly 1g was used apply this formula: Crude protein% = vol titrated x 0.875

Generally

$$\text{Crude Protein \%} = \frac{6.25 \times \text{Vol Acid} \times 1.4\text{mg}}{\text{sample mass (g)} \times 1000\text{mg}} \times 100$$

where 1.4 is mass of element nitrogen (14) x concentration of acid used in titration

*\*The relationship is that 1ml of 1N acid is equivalent to 14mg of nitrogen (or 1ml of 0.1N acid = 1.4mg nitrogen mass)*

----- end of crude protein analysis

## ETHER EXTRACT ANALYSIS (FAT ANALYSIS)

### PRINCIPLE

A dried, ground sample is extracted with diethyl ether which dissolves fats, oils, pigments and other fat soluble substances. The ether is then evaporated from the fat solution. The resulting residue is weighed and referred to as ether extract or crude fat. Both the ether and the samples must be free of moisture to avoid co-extraction of water-soluble components in the sample such as carbohydrates, urea, lactic acid, glycerol, etc. If water-soluble components are present in large amounts in the sample, they are washed out of the sample prior to drying. Low temperatures are used to evaporate the ether and remove residual moisture to prevent oxidation of the fat. Petroleum ether does not dissolve all of the plant lipid material, and therefore it cannot be substituted for diethyl ether.

### APPARATUS

Soxhlet apparatus unit

### REAGENTS

Diethyl ether solvent (*hexane, petroleum ether 40-60°C can as well be used*)

### METHOD

- 1 weigh the flask and record its weight (W1)
- 2 weigh 5g of the sample and transfer in a thimble. Plug the thimble with cotton wool to avoid particles of the sample be spilled in the flask by the extracting solvent
- 3 Transfer the thimble into the soxhlet
- 4 mount the soxhlet unit
- 5 add enough extracting solvent (150ml or more)
- 6 reflux for 6-8hours (*if the sample in the thimble is mixed with 1g sodium sulphate, it is possible to reflux for only 3 hours*)

- 7 remove as much solvent as possible from the flask after refluxing time is over
- 8 dry the flask in the oven set at 105°C until constant weight,
- 9 after cooling reweigh the flask (W2)

*(If doing a proximate analysis, the residue left in the thimble may be used to determine crude fiber)*

## RESULTS AND CALCULATION

Weight of sample	5g
Weight of empty flask	W1
Weight of flask + ether extract	W2
Weight of ether extract (oil)	W2 – W1

## CALCULATION

$$\% \text{ Crude fat (wet)} = \frac{W2 - W1}{\text{weight (g) of sample}} \times 100$$

Percent Crude Fat (Ether Extract), DM basis:

$$\% \text{ Crude fat (DM)} = \frac{W2 - W1}{\text{weight (g) of sample}} \times \text{DM}\%$$

## POSSIBLE ERRORS

The method of ether extract assumes ALL substances soluble in ether are fats This assumption is NOT TRUE. Plant pigments, wax which are also soluble in ether, but do NOT have the same nutritional values of fats However, this error is generally small.

----- *end of ether  
extract analysis*

# Appendix IV Q-Q plots for normality

