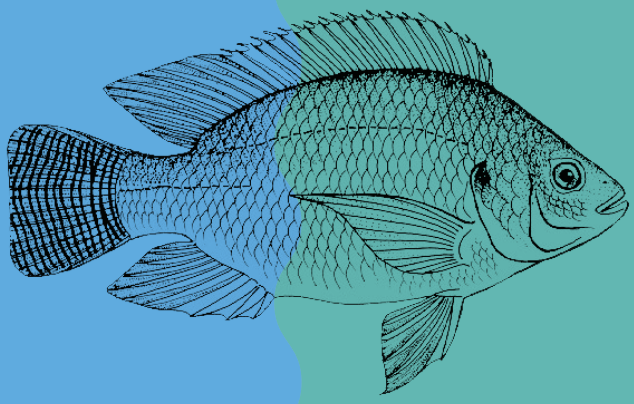
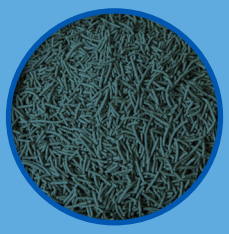
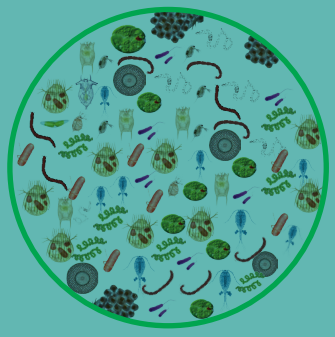
Feeding fish or pond...?







Kazi Ahmed Kabir

Propositions

1. The optimal nutrient composition of pond feed is different from feed for fish only. (this thesis)
2. Quantification of natural food production is not important to formulate pond feed. (this thesis)
3. The future of rice-fish systems will be determined by the availability of freshwater in tropical deltas.
4. We cannot keep habitat corridors for coastal biodiversity.
5. Scientific innovations have made the world more unstable.
6. Balancing between passion and reality is almost impossible.

Propositions belonging to the thesis, entitled

Feeding fish or pond . . . ?

Kazi Ahmed Kabir

Wageningen, 18 June 2019

Feeding fish or pond . . .?

Kazi Ahmed Kabir

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This research was conducted under the auspices of the Graduate School, Wageningen Institute of Animal Sciences (WIAS).

Feeding fish or pond . . .?

Kazi Ahmed Kabir

Thesis

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To my parents

Abstract

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Wageningen University and Research, The Netherlands.

Globally pond aquaculture is the predominant fish/shrimp farming system. Roughly 70% of the farmed fish and shrimps are produced in semi-intensive ponds where feed is a major nutrient supplier but where also natural food contributes to the productivity. Current knowledge on fish nutrition is based on studies where natural food is absent or available at a minimum level. When a complete feed, formulated on the basis of the above nutritional studies, is applied as supplementary feed in a pond where also natural food is available, much of the nutrients are not retained in the fish and the efficiency goes down. The aim of this research was to generate knowledge on how dietary macro-nutrients (with a focus on dietary carbohydrates) determine fish growth directly and indirectly (via natural food) in ponds. In a pond, the carbon to nitrogen (C:N) ratio in the system determines the productivity of natural food. The hypothesis underlying the current PhD research was that lowering the scarce and expensive macronutrients (e.g. protein and fat) in the feed would be compensated by an increased contribution of natural food as a result of a higher C:N input into the system, and thus not leading to lower fish yields. We conducted four experiments to test this hypothesis and have observed that lowering the dietary protein to energy ratio in the feed increased fish production at the pond level, mainly because of an indirect effect of the diet by enhancing the natural food in the pond. The type of non-protein dietary energy (lipid or carbohydrate) did not affect fish performance, not directly as feed nor indirectly via influencing the natural food web of the pond. However, the type of dietary non-starch polysaccharides (NSP) influenced natural food production and ultimately fish growth. Fish performance was better with slowly degradable NSPs in the feed. In all our studies we have noticed that feeding level increased fish production but also increased FCR. We have also observed that fish production increased with increasing stocking density (within the tested levels). Culture intensity (feeding level and stocking density) did not interact with the influence of the dietary macro-nutrient composition (i.e. P:E ratio) on pond productivity which means that enhancement of the natural food web through diets is possible even with increasing culture intensity. So, in ponds with a functional natural food web, the optimal macronutrient composition of supplementary feeds for tilapia differs from the optimal composition as recommended by NRC (1993, 2011) for tilapia. This may be related to the fact that the NRC recommendations were developed without the presence of natural food, and that the effect of the latter may be related to a possible enhancement of its production due to the extra nutrient input in the ecosystem via the waste of the fish (fertilizing effect). However, when monitoring the food web in the ponds, we have barely noticed differences among the treatments. This calls for improved methods to quantify the contribution of natural food to the fish production if this needs to be included in the feed formulation. A paradigm shift, however, is needed in fish nutrition studies, specially for the species produced in ponds under semi-intensive condition, to evaluate the performance of macro-nutrients on the system instead of on the fish for efficiency, and sustainability.

Contents

Chapter 1

General Introduction

1.1 Growth and challenges of aquaculture

Fish play an important role in meeting the current and future food demands within the global food system. Fish, from aquaculture and fisheries combined, provide at least 15% of the animal protein demand of 4.3 billion people (Lynch, 2017) and contributes 3.11% of global GDP (FAO, 2019). Fish demand increases with population growth. Supply of fish from capture fishery has been static for the last decades, meaning that current increases in fish supplies are coming from aquaculture. Consumption of fish from aquaculture as part of global supply grew from 6% to 53% in the last 50 years.

Aquaculture is the fastest growing food sector and in 2016 had a total annual production of 80 million metric ton, valuing ~180 billion USD (FAO, 2018). Out of the aquaculture production, 78% are finfish and crustaceans, which are mainly produced in ponds (FAO, 2018). In addition to genetic improvement of major aquaculture species, improvement of husbandry conditions and advancement of fish health management contributed to this growth, the major factor driving this increase was the conversion of 70% of the area of non-fed ponds into fed ponds (Tacon and Metian, 2015). This created an enormous demand for fish feed. As a result, fish feed production doubled from 30 million metric tonne in 2008 to 60 million metric tonnes in 2018. Aquaculture will continue to grow and by 2050 fish production needs to double if it wants to provide the required animal protein to the growing population (World Bank, 2013). This projected growth of the aquaculture sector will largely depend on increased supply of fish feed.

Fish feed is formulated based on the nutritional requirement of the target species. The efficiency with which feed is converted into fish biomass is a key determinant for the economic profitability of aquaculture. Fishmeal and fish oil are considered as ideal ingredients to provide the required essential amino acids and fatty acids for the fish. However, the global supplies of fishmeal and oil are limited and are not suffice to meet the growing demands by the growing aqua feed industry (Fig. 1). In addition, the growing scarcity of fishmeal and oil on the market will surge prices and make feeds expensive. Supplying fish feed at an affordable cost without compromising efficiency is an important consideration for future economic viability. Apart from economic viability, environmental sustainability is also a growing concern. Waste produced from aquaculture is often criticized from an environmental point of view. Aquaculture waste usually originates from feed. Therefore, more efficient feed with a lower footprint is also required. In conclusion, sustaining growth of aquaculture requires a sustainable growth of fish feed production, which has three major challenges, *e.g.*, fishmeal scarcity, price and environmental suitability.

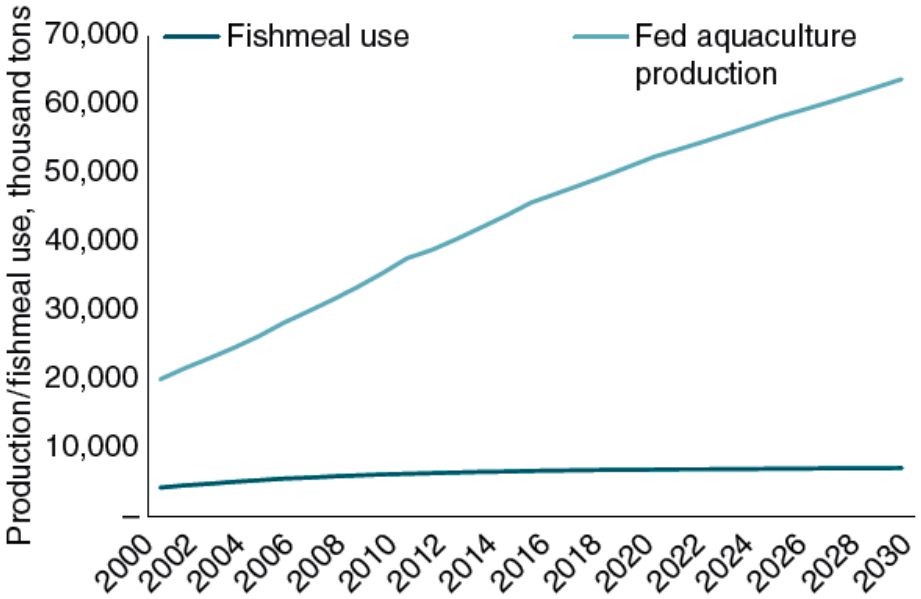

Fig.1. Projected fed aquaculture production and fishmeal use in global aquaculture (Copied from World Bank 2013)

1.2 Approaches for a sustainable growth of aquaculture

There are several ways to continue the growth of aquaculture. One way is expansion of marine aquaculture as part of blue economic growth. Compared to land, ocean areas are vast and underutilised. Another line of development is recirculating aquaculture systems (RAS) which allow very intensive production at an efficient resource use. A third way is intensification of pond aquaculture by using high quality feed, aeration and continuous/frequent water exchange. The latter approach received a lot of attention among the producers of whiteleg shrimp (*Litopenaeus vannamei*) and striped catfish (*Pangasianodon hypophthalmus*). Fulfilling demand for more fish through horizontal expansion of the culture area; or, through production of fish fillet by tissue culture can be considered as well. Yet we believe that an ecological intensification of pond culture, taking care of the potential of the pond ecosystem, can also contribute to double fish production.

Currently ~70% of finfish and ~60% of shrimps are produced in semi-intensive ponds. Production in these ponds can be further intensified rationally. Also, the 30% of non-fed ponds which has not yet been converted to fed ponds can be included under this approach. Several studies indicated that natural food contributes roughly 50% to the fish growth in outdoor pond aquaculture (Anderson et al., 1987; Asaduzzaman et al., 2010; Focken et al., 1998; Pucher and Focken, 2017). Analysis of the applied feeds and fish yield statistics also support such a

conclusion. For example, in 2012, 35 million metric tonne of feed were used for carp and tilapia for a combined total fish production of ±40 million metric ton (FAO, 2016; Tacon and Metian, 2015). These data imply that the FCR is very low, which is an indication that the natural food contributes to the fish growth. If we compare the N input (1.1 tg.N.yr^{-1}) and output (1.33 tg.N.yr^{-1}) in Chinese aquaculture, which contributes 80% of global yield, more N was harvested in fish than applied with the feed N (Luo et al., 2018). This indicates that pond based fed aquaculture is very efficient in terms of nutrient input and output. Observed nutrient (N) use efficiency was thus above 100%, which at fish level is not possible. This strongly indicates that fish farmed in ponds can harvest nutrients from the environment via the natural food. If this potential to extract nutrients could be realized to a higher degree, pond aquaculture will be able to grow more fish with less input while keeping environmental impacts disproportional small. Hence, this approach of ecological intensification can be a solution to the expansion of aquaculture production needed by 2050.

1.3 Concept of feeding the pond

Supplementary feeding contributes directly to fish growth by the intake, digestion and absorption of dietary nutrients by the fish and indirectly via stimulating the natural food, which also contributes to fish growth. Supplementary feeds stimulate the natural food in the pond mainly by the feed energy (carbon) that becomes available from faecal waste. The goal is to take advantage of in situ reuse of uneaten feed, faecal and metabolic waste through the microbial and planktonic food web present in the pond (Fig. 2). Thus, it well fits the concept of circular food production (or circular economy) turning waste into resources. Due to the presence of microbial biodegradation processes in the pond, non-conventional (low quality) feed ingredients may have a better utilization potential, as possible waste will still be a useful resource to stimulate the food web. Hence, low quality ingredients may still be very suitable for these supplementary pond feeds. Utilization of feed driven waste by the planktonic, benthic and microbial food web, and uptake of those by fish grazing will minimize accumulation of waste at the pond bottom, keeping the environment suitable to accommodate more fish in the system (Wahab et al., 2003). In other words, it might increase the carrying capacity of the pond. Moreover, if local ingredients or by products can be utilized well via the ecological process of the pond, the footprint for feed production as well as for fish production will be minimized.

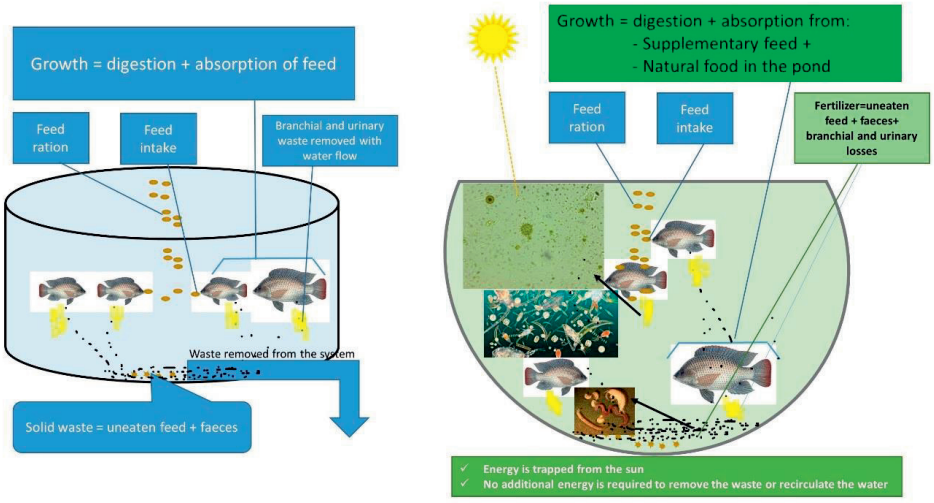

Fig. 2. Conventional feeding approach in absence of natural food (A) versus role of feed in pond culture (B)

The application of this concept requires better understanding on how feed works in a pond system and what the key processes are that will lead diet driven waste to stimulate natural food in the pond. This knowledge is scarce.

As feed constitutes a major expenditure for the farm, fish nutrition studies focused on making efficient feed. To do this, the dietary requirements of the major aquaculture species have been estimated. Research focused on (1) maximizing feed efficiency by altering concentrations of macro and micro nutrients (Adeoyea, et al., 2016; Thongprajukaew, et al., 2015; Long, et al., 2015; Hassaan, et al., 2015; Huang, et al., 2015) (2) improving feed management (Castro, *et al.*, 2014; Bhujel, *et al.*, 2007; Tsadik and Bart, 2007; Chiua, *et al.*, 2013; Cho, *et al.*, 2001), (3) finding alternative sources to expensive and resource scarce ingredients (Davies *et al.*, 2011; Koch *et al.*, 2016; Cavalheiro *et al.*, 2007; Köprücü and Özdemir, 2005; Watanabe, 2002; Allan *et al.*, 2000; Boonyaratpalin *et al.*, 1998; Carter and Hauler, 2000; Fontainhas-Fernandes *et al.*, 1999; Kikuchi, 2007; Mambrini *et al.*, 1999; McGoogan and Gatlin, 1997) and (4) minimizing waste production resulting from feeding (Cho and Bureau, 2001; Crab *et al.*, 2007).

With the current trend of using plant based ingredients in fish feed, studies on essential amino acids (Colt, 2018; Mo *et al.*, 2019; Rito *et al.*, 2019) and essential fatty acids (Carvalho *et al.*, 2019; Yıldız *et al.*, 2018), minimizing anti-nutrient effects by adding enzymes (Maas *et al.*, 2018), processing of non-starch polysaccharides (NSP) and determination of their inclusion level (Amirkolaie *et al.*, 2006) to optimize feed efficiency is getting more priority. Estimation

of optimal macro nutrients ratio, *i.e.*. protein to energy ratio (Haidar *et al.*, 2018; Koch *et al.*, 2017), carbohydrate to lipid ratio (Ali and Al-Asgah, 2001; Wang *et al.*, 2014; Xie *et al.*, 2017) and energy utilization processes by fish in general (Schrama *et al.*, 2018) have also been studied to some extent.

However, all the above research has been done in absence of natural food while application is predominantly for fish culture in ponds with a natural food web. If feed would be formulated not only considering the fish, but also its effect on the food web, the accumulation of feed waste might be reduced, while more natural food might be produced and contribute to fish production. Therefore, when applied in ponds, the outcome may be quite different from what is expected from the research.

High dietary crude protein is often desired for fish growth maximization. However, in a pond, high protein diets do not necessarily produce higher fish yields than low protein diets. High protein diets may cause higher total ammonia nitrogen (TAN) and NO_2–N concentrations in the water column (Hari *et al.*, 2004) and in the sediment (Li and Lovell, 1992). This may reduce fish yields (Wahab *et al.*, 2003). By immobilizing harmful inorganic N-species resulting from protein catabolism into microbial protein (algae or microbial biomass) or by converting them into NO_3-N through nitrification, concentrations of TAN and NO_2–N can be kept below threshold levels, creating room to increase production. By raising the C:N ratio of the feed input to 15–20, in-situ immobilization of N and nitrification will be enhanced (Asaduzzaman *et al.*, 2010; Asaduzzaman *et al.*, 2008). Change in the dietary macronutrient composition, *e.g.*, crude protein, fat, starch and non-starch polysaccharides (NSP) can influence the C:N ratio of the nutrient input and thereby the microbial food web in ponds. Altering the carbohydrate content especially the NSP content will also alter the digestible protein:digestible energy ratio (DP/DE) in aquafeeds and the C:N ratio in the different types of metabolic wastes. In addition, the faster nutrients are immobilized, the fewer nutrients accumulate in the sediment (Verdegem, 2013). The latter is preferred as bottom accumulation tends to stimulate denitrification, causing loss of valuable nitrogen (Jackson *et al.*, 2003; Martin *et al.*, 1998; Thakur and Lin, 2003).

The above two research streams, fish nutrition and pond fertilization, were rarely combined to reach a sustainable solution for pond diets. Few studies have been carried out on the optimal feed composition for pond aquaculture, and when done, the effect on enhancement of natural food remained untouched. So, there is limited knowledge available on how dietary nutrients really work in a pond to stimulate fish growth, directly via the nutritional pathways and indirectly as a stimulant to the natural food production.

1.4 Selection of tilapia as experimental fish

Tilapia is the second largest aquaculture fish group, produced in over 100 countries. Tilapia farming is projected to at a rate of over 5% per year for the next 5 years (Technavio, 2018), which is just after the white leg shrimp (projected growth 5.7%). It is consumed by all social levels, including the poor. In addition to local consumption, the fish is also traded globally. Biologically the fish is omnivorous and therefore, has higher potential to harvest natural food from the pond. The fish also has a high range of tolerance to environmental changes (*e.g.* temperature, dissolved oxygen, pH, salinity *etc.*). It can be produced both in inland freshwater and in brackish water ponds and has a high capacity for adaptation to the impacts of climate change and salinity intrusion. It is also suitable for both monoculture and polyculture systems. Annually 12 million metric tonnes of tilapia feed is produced globally which is 20% of the total fish feed manufactured (Tacon and Metian, 2015). Plenty of research on diet formulation of tilapia, ingredient diversification and energy evaluation has been done (NRC, 1993, 2013). However, an ideal recommendation for a diet, suitable for pond culture is missing.

1.5 Aim and outline of the research

Dietary requirements for fish are normally determined in absence of natural food, while the majority of fish (incl. tilapia) are cultured in ponds, in which the natural food also contributes to fish growth. The relative contribution of the natural food to fish growth is dependent on the amount of supplementary feed given (Fig. 3). In semi-intensive systems, both supplementary feed as well as natural food contribute to fish growth. The supplementary feed can enhance fish growth directly (*i.e.* by digestion/absorption of dietary nutrients) and indirectly by stimulating the natural food of the pond thereby increasing the uptake of nutrients from the food web. This indirect effect is related to the type of waste (*e.g.* faeces) produced by the fish, more particularly the amount and type of carbon coming available for the food web. It is hypothesised that the optimal macro-nutrient composition for fish in semi-intensive pond systems is different from that of intensive culture systems where there is minimal or no contribution of the natural food.

In other words, the central hypothesis of this research is that “requirements” of macro-nutrients via supplementary feed in pond aquaculture are different from the known optimum levels as indicated in NRC (1993 and 2011) due to the presence of natural food. Since the relative role of natural food and supplementary feed in pond aquaculture changes with increasing feeding level and culture intensity (Fig. 3) the macro-nutrient “requirements” of a pond feed might change.

The aim of this research is to generate knowledge on how dietary macro-nutrients with a focus on dietary carbohydrates determine fish growth directly and indirectly (via food web) in

ponds. In the current study tilapia is used as model species. The following aspects were assessed:

- is the optimal dietary protein to energy ratio of pond feeds different from the current recommendation of NRC (1993 and 2011)?
- Does the type of carbon source (non-protein energy type; type of non-starch polysaccharides) influence pond productivity?
- Does the culture intensity (feeding level, stocking density) interacts with the response to diets differing in macro-nutrient composition?

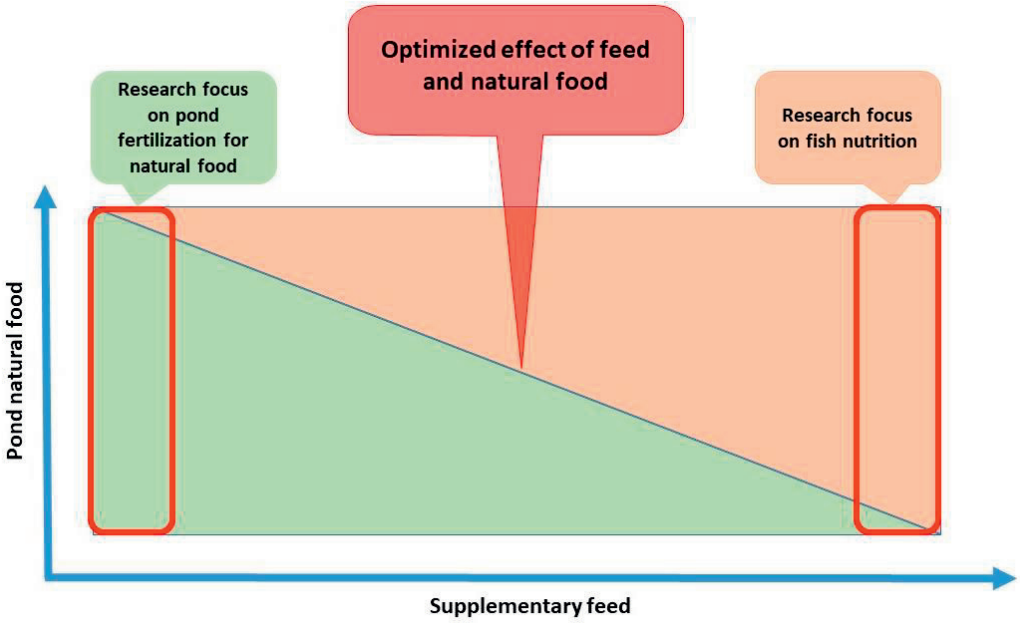

Fig. 3. Relative change in the focus of natural food and supplementary feed on fish production

To test the hypothesis related to macronutrients, first 3 experiments were conducted in experimental ponds. In each experiment two contrasting diets were formulated. One based on the NRC requirements and the other diet based on the observations of a pond fertilization approach. We tested the diets under “No” “Low” and “High” feeding levels, in a compartmentalized pond setting (Fig. 4) to estimate the effects of feeding level on fish performance as well as on enhancement of natural food. The ponds were divided into three equal compartments in a way that the dissolved nutrients can pass between the compartments by the fish and the feed cannot due to the small mesh size of the net used for partitioning (Fig. 4). The bottom of the pond compartments were separated by a concrete wall to avoid passing of the sediment nutrients as well as the benthos growing on the pond bottom.

As the dissolved nutrients were well mixed in the water column, we considered that the contribution of natural food from the water column was equal in all compartments. While sediment deposition is more likely different by feeding level and thus the impact of natural food from pond bottom is more likely different between feeding levels. The aim of assigning a single diet in each pond with three feeding levels nested in it in a split plot design was to test the effect of diet and feeding level on the specific parts of the natural food in the pond.

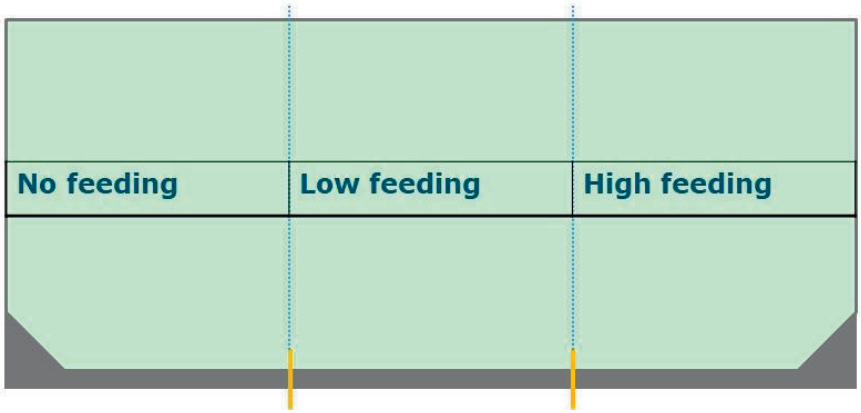

Fig. 4. Sketch of the pond compartments. The dotted line indicates the net and frame, solid bar indicates the concrete wall and the grey bottom indicates the mud layer of the pond bottom

The composition of macro-nutrients was tested in three steps:

1. Finding optimum protein (nitrogen) to energy (carbon) ratio for pond diet
2. Exploring the effect of types of carbon on pond diet. This has been tested further by types of carbon as energy source (*i.e.*. carbohydrate to lipid ratio) on fish growth; and also, by types of fibre (*i.e.*. types of non-starch polysaccharides) on faecal waste production and natural food stimulation
3. Finally, we tested how the composition of macro nutrients interact with changing culture intensity (stocking density and feeding level) at small fish farms.

In **chapter 2** we tested the effect of two diets, contrasting in dietary protein to energy ratio, under three feeding levels on fish performance and on enhancing the natural food web in the pond. Enhancement effect of the diet on natural food was monitored by sampling major elements of the natural food in the pond water and bottom soil at the start, middle and end of the experiment.

In **chapter 3** we studied how changing the dietary non-protein energy from lipid to carbohydrate affected fish performance (*e.g.*, growth, survival, feed conversion ratio *etc.*), fish

body composition (*i.e.* nutrient composition in the fish body), and apparent digestibility co-efficient (ADC) of macronutrients, at three feeding levels.

In **chapter 4** we determined the effect of dietary non-starch polysaccharides (NSP) on fish performance, body composition and ADC of macro-nutrients. We have also estimated the amount and composition of faeces produced due to feeding and their impact on natural food in the pond. The dietary effect on enhancement of natural food was measured by sampling a set of major indicators over time. The contribution of natural food to fish growth was assessed by fish stomach content and also by analysing organic matter composition via direct (as an effect of feed) and indirect (via enhancement of natural food) on $\Delta^{13}C$ and $\Delta^{15}N$ in the feed, fish, plankton and periphyton from the pond.

In **chapter 5** we tested the interaction of dietary macro nutrient composition with feeding level (two feeding levels) and stocking density (two stocking density) with a focus on practical application. We observed fish growth, fish body composition and pond nutrient dynamics. We have also presented a preliminary economic analysis to indicate profitability.

In the final chapter (**chapter 6**) the main outcome of the studies of this thesis was summarized and discussed in the context of feeding the pond and its role in sustaining aquaculture growth, and stake in the concept of circular food production system.

Chapter 2

Effect of dietary protein to energy ratio on performance of Nile tilapia and food web enhancement in semi-intensive pond aquaculture

This chapter has been published as:
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Abstract

When fish have only access to formulated feed, the optimal dietary protein to energy ratio (P:E) for tilapia ranges between 18 and 23 g.MJ^{-1}. In pond culture, where natural foods complement administrated feed, increasing the carbon:nitrogen (C:N) ratio stimulates the natural food productivity. This study assessed if lowering the dietary P:E ratio (and thus increasing the C:N ratio of the feed input in the pond) below the optimal P:E ratio affects fish productivity, food web dynamics and nitrogen balances in semi-intensively managed tilapia ponds. Twelve ponds, each divided into three equally-sized compartments, were assigned to test the effect of two diets, which differed in P:E ratio (19 *vs* 14 g.MJ^{-1}). Three feeding levels (no, "low" and "high") were nested in each pond in a split plot design. Initial fish biomass was 1166 (±16) g.compartment^{-1} and the experiment lasted 60 days. Decreasing P:E ratio enhanced tilapia production and specific growth rate (P<0.05; 1195 *vs*. 986 g.compartment^{-1} and 1.76 *vs* 1.55 %.d^{-1}). Body composition of tilapia was unaffected by diet and feeding level. Despite the difference in performance, final fat content was 5% of body weight and unaffected by treatments. Averaged over both diets, survival and feed conversion ratio increased with increasing feeding level (P<0.001). Diet composition did not alter measured water quality, and abundance and diversity related parameters of the food web. The total amount of N accumulated in tilapia.pond^{-1} was higher with the low P:E ratio diet (*i.e.*, low protein diet). The data on N gain and N balance at the pond level suggest that the food web productivity was stimulated by reducing the dietary P:E ratio below the reported optimal levels in the literature. It is hypothesized that the optimal dietary P:E ratio is dependent on the culture intensity (extensive, semi-extensive or intensive pond culture).

2.1 Introduction

Nutritional studies that formed the basis of National Research Council (NRC) recommendation for dietary requirements in fish (NRC 1993; 2011), were mostly done in absence of the natural food. In outdoor ponds, which is the most commonly used farming system in Asia (FAO, 2016), fish have access to natural foods besides the formulated feed (Porchas-cornejo *et al.*, 2012; Pucher and Focken, 2017; Rahman *et al.*, 2008; Roy *et al.*, 2012). Therefore, in ponds, not all nutrients required by the culture species need to be provided in the formulated diet, as is the case with cage or flow-through tanks (Verdegem, 2013).

The protein to energy (P:E) ratio is one of the important determinants for quality feed formulation. This P:E ratio is often expressed in term of digestible protein over digestible energy (DP:DE). The optimum DP:DE ratio for common aquaculture species ranges between 18-23 $g.MJ^{-1}$ (Fernandes *et al.*, 2016; Helland *et al.*, 2010; Lanari *et al.*, 1995; Lanari and Agaro, 2002, NRC, 1993). Since in most formulated diets, protein and energy digestibility are similar, the optimal range in DP:DE is similar to the P:E ratio. Recently, Haidar *et al.* (2018) suggested that the optimal P:E ratio for tilapia is probably below 16 $g.MJ^{-1}$ based on the observation that fish performance linearly increased with decreasing P:E ratio. Haidar *et al.* (2018) did not test diets with a P:E ratio below 16 $g.MJ^{-1}$, but recommended further experiments to determine the optimal P:E ratio.

By increasing the carbon or energy availability in the pond, production can be increased. The latter is mostly done by supplying carbohydrates (not via the fish feed), raising the C:N ratio of nutrient inputs to 15-20 (Asaduzzaman *et al.*, 2010a, 2008, Hari *et al.*, 2006, 2004). When the C:N ratio of the nutrient input raises above 10, heterotrophic bacteria become dominant (Boyd, 1996; Lancelot and Billen, 1985), contributing substantial amounts of bacterial biomass to the food web. Organic, but also inorganic nitrogen are taken up by heterotrophic bacteria, thus keeping ammonia and nitrite levels in the pond low (Avnimelech, 1999; Hari *et al.*, 2006, 2004)). Heterotrophic bacteria, are a protein source, stimulating the food web and the production of fish grazing on natural foods. Asaduzzaman (2008) found a higher concentration of natural foods in the water column and the benthos, as well as a higher fish production in ponds with a C:N ratio of 15 or higher. The P:E ratio of the combination of diet and carbohydrate input (C:N ratio 20) used by Asaduzzaman (2008) was 9.5 $g.MJ^{-1}$, assuming protein, fat and carbohydrate contain respectively 23.6, 39.5 and 17.2 $kJ.g^{-1}$ energy (NRC, 2011). This P:E ratio is 50% of the NRC recommended P:E ratio of 18-23 $g.MJ^{-1}$.

In pond studies, the P:E ratio was adjusted by application of carbohydrate directly into pond and not via the formulated diet (Asaduzzaman *et al.*, 2010b, 2008; Dauda *et al.*, 2018; Hari *et al.*, 2006, 2004; Kidd *et al.*, 2011; Xu *et al.*, 2018). In this approach feeding and carbohydrate

addition are often not synchronized, for instance when feeding 3 times per day, while applying the carbohydrate once in the morning (Asaduzzaman *et al.*, 2010a). In addition, the carbohydrates and metabolic wastes resulting from feeding are not equally distributed in the pond. If the P:E ratio is changed by feed formulation, application of carbohydrates separately from the formulated feed is no longer necessary. Other advantages include, homogenous spreading by the fish of faeces and branchiary and urinary wastes over the pond area and introduction of an organic carbon source already exposed to bacteria in the fish gut, thus facilitating decomposition in the pond. However, in the past, P:E ratio optimization in ponds has never been tested by changing the diet composition.

In this study, two experimental diets were formulated with different P:E ratio. The low P:E diet was compared to a regular P:E ratio tilapia diet. The effects of lowering the P:E ratio in the diet on fish production and nitrogen retention and on natural food availability in the pond were assessed. In addition, the effect of feeding level was also investigated. We hypothesised that when fish receive diets with an equal energy content, fish eating the low protein diet will benefit from a more productive food web, allowing them to compensate the lack of protein in the diet by a higher availability of natural foods for fish in the pond.

2.2 Methods

Two different diets with a contrast in P:E ratio were tested on Nile tilapia *(Oreochromis niloticus)* in 12 outdoor ponds for 60 days. Ponds were divided into three compartments and three different feeding levels were assigned within each diet by pond in a split plot design.

2.2.1 Diets

Two diets, which differed in P:E ratio, were prepared. Diets were made by steam pelleting. Pellet size was 2.5 mm. The first diet ("High P:E") was formulated to have a P:E ratio of 19 $g.MJ^{-1}$, which is within the recommended range for tilapia by NRC (1993). This "High P:E" diet had a C:N ratio of 8.8 $g.g^{-1}$. The second diet ("Low P:E") was formulated to have an increased C:N ratio (11.8 $g.g^{-1}$). This was achieved by adding wheat and rice bran in the "Low P:E" diet while reducing the amount of ingredient providing protein into the diet. Both diets were identical in energy content. This was confirmed by the chemical analysis of the feed (Table 1).

2.2.2 Fish, rearing and housing facilities

All male, juvenile Nile tilapia *(O. niloticus)*, 14^{th} generation WorldFish GIFT strain were collected from Genetic Hatchery, a GIFT Nile tilapia Multiplication Center in Bangladesh, for this experiment.

Table 1. Ingredients and analysed chemical composition of the experimental Nile tilapia *(Oreochromis niloticus)* diets differing in P:E ratio.

		Diets	
Ingredients (%)		"High P:E" ratio	"Low P:E" ratio
Maize		19	19
Soybean meal		12	6
Wheat bran			15
Wheat flour		20	20
Rice bran			12
Sunflower meal		12	6
Rapeseed meal		12	6
Meat & bone meal		15	8
Fish meal		5	3
Fish oil		2	2
Vitamin & Mineral premix[a]		1	1
Mono calcium phosphate (MCP)		0.7	0.8
DL Methionine		0.3	0.2
Diamol		1	1
Chemical composition			
Dry matter (DM),	($g.kg^{-1}$)	908	910
Crude Protein (CP)	($g.kg^{-1}$ DM)	313	244
Fat	($g.kg^{-1}$ DM)	55	59
Ash	($g.kg^{-1}$ DM)	113	86
Phosphorus	($g.kg^{-1}$ DM)	15	11
Carbohydrate[b]	($g.kg^{-1}$ DM)	519	611
Gross energy	($kj.g^{-1}$ DM)	19	19
P:E ratio	($g.MJ^{-1}$)	19	14
C:N ratio[c]	($g.g^{-1}$ DM)	8.8	11.8

[a] commercial product made by ACI Godrej Agrovet Private Limited.

[b]This is calculated value where Carbohydrate= 1000-CP-FAT-ASh

[c]This is calculated C:N ratio considering 16% N content in the protein and 47, 70 and 50% C content in protein, fat and carbohydrate respectively (Waal and Boersma, 2012).

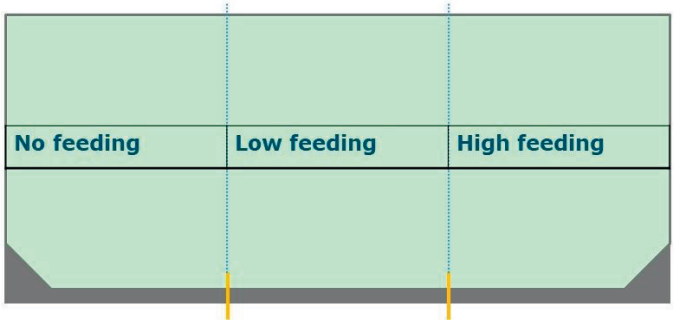


Fig. 1. Sketch of the pond compartments. The dotted line indicates the net & frame, solid bar indicates the concrete block and the grey bottom indicates the mud layer of the pond bottom

Twelve outdoor ponds, each thirty square meters, in a field experimental station were used for this experiment. Each pond was divided into three equal compartments (Fig. 1). Water column of the pond was divided by bamboo-frame fitted with 1mm mesh sized nets that allow well mixing of nutrients and dissolved solids within the compartments but prevents passing of the pellets and fish between the compartments. At the bottom, the compartments were separated by concrete block of 105 cm height, of which 75 cm below the soil and 30 cm extended above the pond bottom to prevent the exchange of uneaten feed and benthos between the compartments.

2.2.3 Experimental procedure

Ponds were dried by pumping out the water. Two hundred fifty g $CaCO_3$ was applied at the bottom soil of each pond compartment (PC) before water filling. After water filling, 40g Dolomite ($CaMg(CO_3)_2$) was spread over the water surface of each compartment. Ten g urea (CH_4N_2O) and 20g Triple Super Phosphate (TSP), [$Ca(H_2PO_4)_2.H_2O$], per pond compartment (Rakocy and Mcginty, 1989) were applied 1 week after liming. Fish were stocked in the ponds 5 days after fertilization.

Fish were fed twice a day at 8.00 and 16.00 hours. “High” and “low” feeding levels started with 2 & 1% of fish biomass. The feed ration was gradually reduced, reaching 1 & 0.5% at the high and low feeding level compartments, respectively, at the end of the experiment. Feed rations were adjusted based on body weight sampling conducted at day 30 and 45. The amount of feed given of each diet was based on the measured DM content of the diet, thereby ensuring equal amount of feed given of both diets within each feeding level. Current experiment was mimicked with semi-intensive production. The nutrient input to the ponds was kept low to maintain water quality and assure good conditions for mineralization and food web development. So, in this experiment, the “high feeding” level was set at ~50% of the feed input normally applied in semi-intensive ponds in Bangladesh.

Duration of the experiment was 60 days, which was divided into two phases. Phase 1 (Day 1-30) was the time to build the natural food web in the ponds. During this period no measurements were taken. Phase 2 (Day 31-60) was the monitoring period.

2.2.3.1 Water quality monitoring

From day 31 dissolved oxygen (DO), pH, total dissolved solid (TDS), transparency, temperature and salinity of each pond were measured daily at 6.00, 9.00, 10.00, 12.00, 14.00 and 14.30 hours; by using Lutron dissolved oxygen meter model PDO-519, Hanna instruments pocket tester HI98128-phep5, Lutron conductivity meter model PCD-431, Secchi disc, Hanna digital thermometer model HI98501 and Atago refractometer model MASTER-S28M instruments.

NH_4^+, NO_2 and NO_3 were measured only at day 31, 45 and 60 by colourimetric, acc. to Neßler, with colour card and sliding comparator: 108025 | Nitrite Test, 111117 | Ammonium Test, 110022 | Nitrite Test; Merck KGaA, Darmstadt, Germany.

2.2.3.2 Sampling and analysing plankton

Phytoplankton and zooplankton samples were collected at day 31, 45 and 60. Samples were collected between 9.00-11.00 hrs from 3 points, equally spaced on a diagonal line in each pond compartment. At each point 15 L water was passed through the 45 µm mesh plankton net, thus pulling together 45 L of water from each compartment were sampled.

The concentrated samples were preserved in small plastic bottles with 5% buffered formalin. Diversity (group.L^{-1}) and abundance (ind.L^{-1}) estimations of plankton were done using a Sedgewick– Rafter (S-R) cell containing 1000 1-mm^3 cells. A 1 ml sample was put in the S-R cell and was left 15 min undisturbed to allow plankton to settle. The plankton in 10 randomly selected cells were identified where possible up to genus level and counted under a binocular microscope (LABOMED America.inc; Lx 300). Plankton were identified using keys by Ward and Whipple (1959), Prescott (1962), Belcher and Swale (1976), and Bellinger (1992). Plankton abundance was calculated using the following formula:

N = (P×C×100)/V. Here, N is the number of plankton cells or units per litter of original water; P, the number of planktons counted in 10 fields; C, the volume of final concentrate of the sample (ml); V= the volume of the pond water sample in litter.

2.2.3.3 Sampling and analysing benthos

The benthic macroinvertebrate samples were also collected on day 31, 45 and 60 with an Ekman grab (area: 225 cm^2). In each pond compartment, bottom mud samples were collected from three different locations, which were then combined into a composite sample. Benthic macroinvertebrates were collected after filtering sediments through 4 different mesh sieve and preserved in a plastic vial containing a 10% buffered formalin. Identification keys used for benthic macroinvertebrates were Brinkhurst (1971), and Pinder and Reiss (1983). Benthic macroinvertebrates density was calculated using the formula,

N = Y ×10,000/3A. Here, N=the number of benthic organisms per square meter; Y=total number of benthic organisms counted in 3 samples; A= Area of the Ekman dredge.

2.2.3.4 Sampling and analysing bacteria

In order to isolate and quantify the bacterial population, samples from both water and soil sediments were collected at day 31, 45 and 60. All samples were collected from three different locations of each pond compartment in sterile containers (15 ml tube, Falcon, USA), mixed homogenously before transported back to the Limnological Laboratory of the Environmental Science Discipline of Khulna University, Bangladesh. 1 ml water sample was transferred with

a sterile pipette to a test tube containing 9 ml of phosphate buffered saline (PBS) and the tube was shaken thoroughly, while 5 g of each sediment and water samples were weighed and transferred to a sterile conical flask and made up to 50 ml with PBS and the contents were mixed thoroughly to prepare a stock solution. Serial dilution of up to 10−6 for water and 10−8 for sediment were prepared with PBS. Volumes (0.1ml) of each dilution were spread over the surface of duplicate plates of tryptone soya agar (TSA; Difco, Detroit, MI, USA) with incubation at 30 °C for 24–48 h. Plates with 30– 300 colony forming units (CFU) were counted with a Leica Quebec Dark field Colony Counter (Leica, Inc., Buffalo, NY, USA) and expressed as CFU/ ml.

2.2.3.5 Sampling and analysing Chlorophyll a

Water samples were collected from specific locations (Fig. 1) in each compartment of the ponds from the mixed water column. Water samples from three parts of the pond were well mixed and kept into 500 ml bottles. The samples were transferred to the lab within an hour for analysis. There we filtered 250 ml of water through Whatman GF/C filter paper. We then torn the filter paper into 5-6 pieces and inserted them into a 50 ml centrifuge tube. Thereafter, we added 20 ml of methanol into each tube to cover the filter paper pieces in it, shook well and vortex until the filter paper was broken up. Kept them in the freezer overnight. Centrifuged at 3200 rpm for 10 minutes. Poured off the supernatant into a 1 cm cuvette and Measured the extinction at both 665 nm and 750 nm (zero with methanol). Chlorophyll a was calculated as Chl-a ($ug.L^{-1}$) = ((Abs[665nm]-Abs[750nm]) x A x Vm)/ Vf x L. Here, A = absorbance coefficient of chlorophyll-a in methanol (12.63); Vm = volume of methanol used for extinction (ml); Vf = litres of water filtered; and L = path length of cuvette.

2.2.3.6 Sampling and analysing proximate composition of fish and feed

Initial body composition was determined in 25 fish, which were randomly selected at the start of the experiment. For final body composition, 5 fish were randomly selected per compartment at the end of the experiment. Fish, which were used for body composition analysis, were euthanized by an overdose of a phenoxy-ethanol solution (1.0ml. L^{-1}) and stored at −20°C. Before chemical analysis, the sampled fish were cut into small pieces, homogenised by grinding in a mincing machine twice through a 4·5 mm screen grinder and subsequently oven-dried. Chemical analyses were done in triplicate. Dry matter (DM) was determined gravimetrically after drying at 103°C for 4 and 24 h (h) until constant weight, respectively, for feed and fish samples (ISO 6496, 1983). Crude ash was determined after incineration at 550°C for 4 h (ISO 5984, 1978). Crude protein (CP) was determined by the Kjeldahl method (ISO 5983, 1979) and calculated by multiplying the measured N content by 6·25. Fat was quantified by petroleum–diethyl ether extraction (ISO 6492, 1999). Before fat analysis, feed samples were hydrolysed by boiling for 1 h with 3 M-HCl. Dietary energy content

was measured by direct combustion in an adiabatic bomb calorimeter (IKA-C-7000; IKA analysentechnik, Weitersheim, Germany).

2.2.3 Analytical procedures and calculations

2.2.3.1 Performance

Biomass gain (g) was calculated as the difference between the biomass stocked and biomass harvested per compartment. The specific growth rate (SGR) was calculated as $SGR=((\ln(IndBW_{60})-\ln(IndBW_0))/60)\times100$; where $IndBW_{60}$ and $IndBW_0$ means individual body weight at day 60 and day 0. Feed conversion ratio (FCR) was calculated per compartment using the feed given and weight gain. The survival of fish per compartment was calculated as $(N_f/N_i)\times100$, where N_f is the final number of fish and N_i the initial number at PC level.

2.2.3.2 Nitrogen(N) retention

N Gain in fish was calculated by the difference between N_f and N_i. N Feed was calculated by total feed input per compartment multiplying the N content in feed; N balance was calculated by deducting N gain in fish from total N input from both the feed based on proximate composition and fertilizer (Urea, 46% N). N retained from Food Web was calculated by deducting N retention from feed from the total N gain in fish.

2.2.3.3 Sensitivity test to calculate the relative contribution of feed and food web to fish growth

In this study we didn't measure the apparent digestibility. So, the calculation of N retention was based on apparent digestibility coefficient (ADC) 90% for N (Azevedo *et al.*, 2004; Kaushik *et al.*, 1995) and N retention efficiency (RE) of 40% (Azevedo *et al.*, 2004). We assumed same ADC and RE for the N coming from food web. We then made a range of assumptions on the ADC (80%,85%,90% & 95%) and RE (30%, 35%, 40% & 45%) of N to evaluate how sensitive the contribution of formulated feed and natural foods to production is to changes in these parameters.

2.2.3.4 Statistical analysis

The data were analysed using the IBM SPSS software package version 23. All were analysed for the effect of diet, feeding level and their interaction by two-way repeated measure ANOVA using the procedure general linear model (GLM). When significant interaction found multiple comparisons of means using Tukey's multiple range test were performed.

2.3 Result

2.3.1 Fish performance

Fish performance at low P:E diet at all feeding levels was better compared to the other diet (Table 2). Biomass gain at low P:E diet was 1195 g.compartment^{-1} compared to 985 g.compartment^{-1}, which is more than 20% increase. Both diet (P≤0.05) and feeding level (P≤0.001) influenced growth (biomass gain per compartment) but there was no interaction effect between them. Even in non-fed compartments there was a diet effect and fish growth with the low P:E diet was 134% higher than the high P:E diet. Survival was not different between diets (P>0.05) and increased with increasing feeding level (P≤0.001). FCR ranged between 0.5 to 1.6 and increased with increasing feeding level (P≤0.001). Difference in FCR between diets at high feeding level was higher than that at low feeding level. SGR was also higher in low P:E diet and was significantly influenced by diet (P≤0.05) and feeding level (P≤0.001).

2.3.2 Body composition

Initial body composition for dry matter (DM), crude protein (CP), crude fat (CFat) and ash were respectively 274, 157, 54, and 39 g.kg^{-1}. There was no effect of diet and feeding level or their interaction on final fish body composition (Table 3).

Table 3. Effect of dietary protein to energy ratio (P:E) and feeding level on final body composition of Nile tilapia *(Oreochromis niloticus)*.

g.kg^{-1}	"Low P:E" diet	"High P:E" diet	Standard Error	P values		
				D	F	D*F
Dry matter	283	287	4.5	0.5	0.6	0.7
Crude protein	167	167	1.4	0.9	0.7	0.4
Crude Fat	52	54	1.6	0.4	0.6	0.7
Ash	39	42	1.2	0.1	0.3	0.9

*D=Diet and F=Feeding Level, D*F=Diet and Feeding level interactions

2.3.3 Feed nitrogen (N) input and output

At compartment level, feed N input was higher with the high P:E diet in fed compartments, as was intended. However, the N gain in fish per compartment was higher (P≤0.05) with the low P:E diet. The N gain increased with increasing feeding level (Table 4). At compartment level N balance was also influenced by the diet (P≤0.001) and the feeding level (P≤0.001).

Table 2. Effect of dietary protein to energy ratio (P:E) and feeding level on performance of Nile tilapia *(Oreochromis niloticus)*.

Per compartment	Units	**"Low P:E" diet**									**"High P/:E" diet**									**P values**		
		NF			**LF**			**HF**			**NF**			**LF**			**HF**			**D**	**F**	**D*F**
Biomass stocked	g	1193	±	12	1179	±	21	1175	±	16	1155	±	12	1155	±	21	1141	±	16	**0.04**	0.6	0.9
Biomass harvested	g	1490	±	46	2409	±	133	3233	±	117	1282	±	46	2317	±	133	2805	±	117	**0.006**	**0.000**	0.35
Biomass gain	g	297	±	37	1230	±	131	2058	±	125	127	±	37	1162	±	131	1665	±	125	**0.014**	**0.000**	0.37
Survival	%	52	±	4	69	±	6	86	±	5	57	±	4	72	±	6	84	±	5	0.64	**0.000**	0.73
Feed given	g	NA			860	±	20	2025	±	27	NA			880	±	20	2001	±	27	0.93	**0.000**	0.26
FCR	$g.g^{-1}$	NA			0.8	±	0.9	1.0	±	0.9	NA			0.8	±	0.9	1.3	±	0.9	0.09	**0.005**	0.25
SGR	$\%.d^{-1}$	1.5	±	0	1.8	±	0.07	2.0	±	0.1	1.1	±	0	1.7	±	0.07	1.8	±	0.06	**0.02**	**0.000**	0.27

*NF= No Feeding, LF="Low Feeding" and HF=High Feeding, D=Diet and F=Feeding Level, D*F=Diet and Feeding level interactions

2.3.4 Food web

There was no diet effect on the measured parameters of the food web (Table 5 and Supplementary Table 1). Abundance and diversity of phytoplankton, and total count of bacteria in soil and water column changed (P≤0.001) over time (Supplementary Table 1). Zooplankton diversity was influenced by feeding level (P≤0.05) and sampling time (P≤0.001). There was a trend towards significant effect of feeding level (P≤0.08) on abundance of zooplankton and time (P≤0.07) on chlorophyll a. Variation between samples taken at the same time within diet and feeding level, was very high, especially for abundance of phytoplankton, zooplankton and total counts of both soil and water bacteria (Supplementary Table 1).

2.3.5 Water quality

There was a clear effect of time (P<0.001) for all measured water quality parameters (Table 6). Dissolved oxygen concentration in the morning decreased over time from 4.25 $mg.L^{-1}$ at the beginning of the experiment to 3.6 $mg.L^{-1}$ at end. On the other hand, NO_2, NO_3 and NH_4^+ increased over time (Table 6). However, for all time points, the measured water quality parameters were within the optimal range for Nile tilapia. N accumulation with the high P:E diet was higher by arithmetic mean compared to the low P:E diet but the difference by diet was not significant (Table 6).

2.4 Discussion

2.4.1 Effect of dietary protein to energy ratio on growth and body composition

We investigated the effect of dietary P:E ratio on fish performance and on the food web contribution to fish growth. The low P:E ratio diet ($14g.MJ^{-1}$) performed better than the high P:E ratio diet ($19g.MJ^{-1}$). The P:E ratio of better performed diet ($14g.MJ^{-1}$) is lower than the recommended P:E ratios ($18\text{-}23g.MJ^{-1}$) for Nile tilapia (El-Sayed and Teshima, 1992; Kaushik *et al.*, 1995; NRC, 1993). The previously reported lowest P:E ratio was 16 $g.MJ^{-1}$ (Haidar *et al.*, 2018). However, in Haidar *et al.* (2018) recommended diet CP content was $382g.kg^{-1}$ and gross energy content was 23.5 $kj.g^{-1}$ which in the current study is 36% less for CP and 19% less for gross energy. Reducing the gross amount of CP and energy content in the diet might have practical implication on economic benefits. The reduced inclusion of CP in the diet has been complemented by the natural food of the pond. Several studies on different fish species cultured in the pond supported this outcome (Asaduzzaman *et al.*, 2010a, 2010b; Bombeo-Tuburan *et al.*, 1993; Jackson *et al.*, 2013; Porchas-cornejo *et al.*, 2012; Pucher and Focken, 2017; Rahman *et al.*, 2008).

Table 4. Effect of dietary protein to energy ratio (P:E) and feeding level on feed N input and gain in fish

Per compartment		"Low P:E" diet			"High P:E" diet			P values		
		NF	**LF**	**HF**	**NF**	**LF**	**HF**	**D**	**F**	**D*F**
N Feed	g	NA	30 ± 0.7	72 ± 1.1	NA	40 ± 0.7	91 ± 1.2	**0.00**	**0.00**	**0.00**
N gain Fish	g	10 ± 1.1	36 ± 3.9	56 ± 4.0	4.4 ± 1.1	32 ± 3.8	48 ± 4.0	**0.04**	**0.00**	0.70
N Balance	g	-10 ± 1.1	-5 ± 3.8	15 ± 4.7	-4.4 ± 1.1	7 ± 3.8	43 ± 4.7	**0.00**	**0.00**	0.25

* NF= No Feeding, LF=Low Feeding and HF=High Feeding, D=Diet and F=Feeding Level, D*F=Diet and Feeding level interactions

Table 5. Effect of dietary protein to energy ratio (P:E) on food web in the pond

		"Low P:E" diet			"High P:E" diet			P values
Abundance of Phytoplankton	ind.L^{-1}	71201.3	±	4302.082	83619.81	±	5934.276	0.121
Diversity of Phytoplankton	genus.L^{-1}	13.25926	±	0.544835	13.01852	±	0.598638	0.772
Abundance of Zooplankton	ind.L^{-1}	13453.7	±	878.8398	14361.11	±	1849.772	0.667
Diversity of Zooplankton	genus.L^{-1}	6.777778	±	0.353699	6.62963	±	0.145344	0.707
Abundance of Benthos	ind.m^{-2}	1244.4	±	470.6	1508.3	±	633.4	0.432
Diversity of Benthos	group.m^{-2}	2.777778	±	0.289742	3.111111	±	0.400617	0.515
Chlorophyll a	µg.L^{-1}	0.010389	±	0.000945	0.011778	±	0.000834	0.296
Total count of bacteria from water	CFU.100ml^{-1}	41818.52	±	4029.02	44981.48	±	5763.505	0.662
Total count of bacteria from soil	CFU.100ml^{-1}	95992.59	±	2349.662	95881.48	±	4005.571	0.981

Table 6. Effect of dietary protein to energy ratio (P:E) and Sampling time on pond water quality

		"Low P:E" diet									"High P:E" diet									P values		
		T1			T2			T3			T1			T2			T3			D	T	D*T
Dissolved oxygen	$mg.L^{-1}$	4.25	±	0.01	3.66	±	0.01	3.6	±	0.01	4.2533	±	0.01	3.6517	±	0.01	3.6	±	0.01	0.72	**0.00**	0.75
pH		7.31	±	0.01	7.26	±	0.01	7.67	±	0.01	7.31	±	0.01	7.25	±	0.01	7.66	±	0.01	0.54	**0.00**	0.93
Temperature	˚C	30.5	±	0	31.2	±	0.04	31.4	±	0	30.497	±	0.05	31.15	±	0.04	31.4	±	0.04	0.64	**0.00**	0.80
Salinity	ppt	4.87	±	0.0	3.0	±	0.0	2.0	±	0.0	4.9	±	0.0	3.0	±	0.0	2.0	±	0.0	0.68	**0.00**	0.68
TDS	$mg.L^{-1}$	5056	±	7.1	4001	±	2.62	3337	±	5.9	1.0	±	5061	2.0	±	4003	3.0	±	3339	0.07	**0.00**	0.96
Transparency	inch	11.1	±	0.2	10.8	±	0.43	11.9	±	0.3	10.75	±	0.23	10.833	±	0.43	11.9	±	0.32	0.59	**0.01**	0.88
NO2	$mg.L^{-1}$	0.03	±	0	1.7	±	0.21	3.75	±	0.2	0.025	±	0	1.9	±	0.21	4.27	±	0.18	0.07	**0.00**	0.30
NO3	$mg.L^{-1}$	0	±	0	5	±	0	7.5	±	1	0	±	0	5	±	0	5.83	±	0.99	0.26	**0.00**	0.26
NH4+	$mg.L^{-1}$	0.5	±	0	1	±	0	1	±	0	0.5	±	0	1	±	0	1	±	0	0.40	**0.00**	0.10

*T1, 2 &3 = Sampling Day 31, 45 and 60, D=Diet, T=Sampling time, D*T= Diet and time interaction

The proximate composition of formulated feed affects fish body composition. By lowering the dietary P:E ratio (Haidar *et al.*, 2018), the fish fat content was 157 $g.kg^{-1}$ and by reducing the dietary CP content (Al Hafedh, 1999) the fish fat content was 116 $g.kg^{-1}$. These studies were done in absence of natural food. The low 5.2% fat content in fish at harvest in the current experiment suggests the fish obtained protein not only from the feed but also from natural foods present in the pond.

2.4.2 Contribution of food web to fish growth in a pond

To answer the above question, we calculated the amount of N coming from food web of the pond. We could not measure digestibility of nutrients in this experiment as it was conducted in ponds. So, we considered 90% protein digestibility (Azevedo *et al.*, 2004; Kaushik *et al.*, 1995) and 40% protein retention efficiency (RE) (Azevedo *et al.*, 2004) for both diets and natural food. Under these assumptions, the contribution of natural food is 64% for the low P:E diet compared to 45% for the high P:E diet (Fig. 2). As the above estimation was based on logical assumption but not on actual result, we did a sensitivity test by changing the ADC (80, 85, 90, and 95%) and RE (30,35,40,45 and 50%) for both diet and natural food. The outcome largely remained same (Supplementary Fig. 1). These estimated contributions, underpin the importance of natural food web to fish production in semi-intensive ponds. Previous studies (Anderson *et al.*, 1987; Burford *et al.*, 2002, 2004; Cam and Mariotti, 1991; Porchas-cornejo *et al.*, 2012) also confirmed that contribution of food web to fish growth can be between 40-68% in semi-intensive fed ponds based on the level of intensity and the type of species cultured. Findings of the current study remains within the previously reported results and we noticed that contribution of natural food decreased with an increasing feeding level (Fig. 3).

We further explored the composition of food web to understand the relative importance of different functional components, *i.e.*. the pelagic and benthic, part of it. We considered that tilapia production realized in the non-fed compartment is based on the pelagic food web. As the water column was well mixed and turbulent due to aeration, we assume the water column in each pond was quite uniform across compartments. When we measured the food web, we noticed presence of benthos in the non-fed compartments as well. However, we assumed that fish production in the non-fed compartment was gained only as an effect of the dissolved nutrients in the water column by the pelagic food web and their subsequent influence on other parts of the food web and fish.

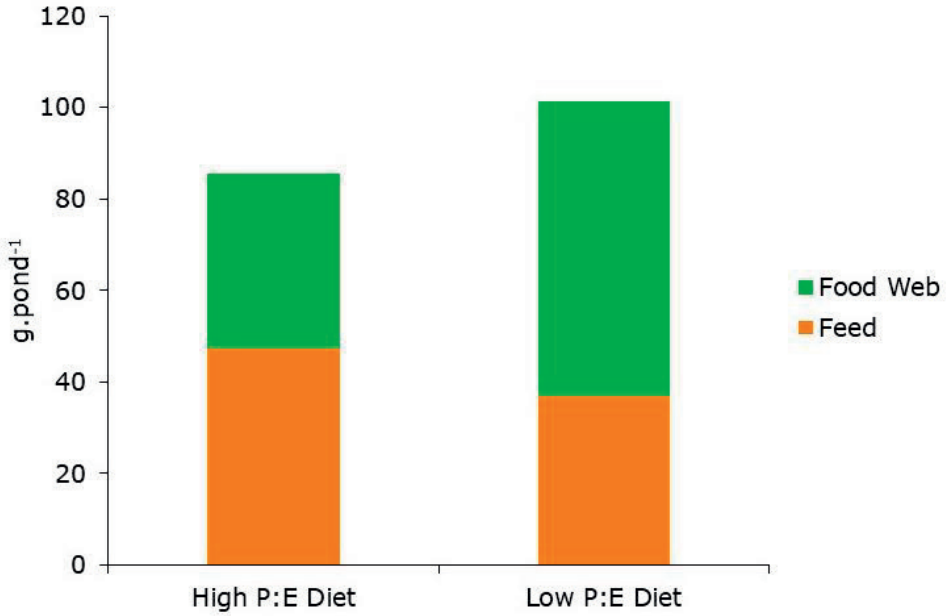


Fig. 2. Effect of dietary P:E ratio on the contribution of feed and food web to N gain in fish

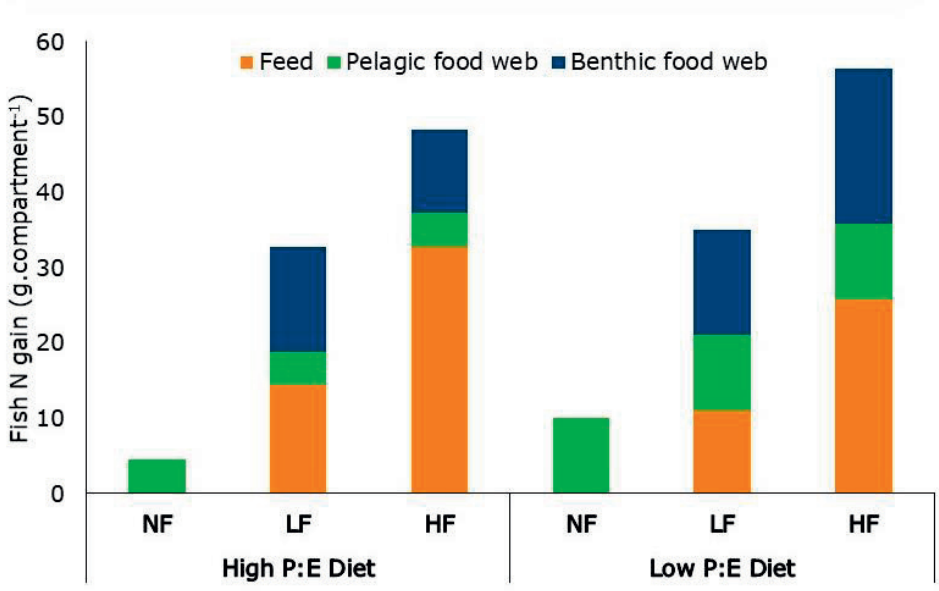


Fig. 3. Impact of dietary P:E ratio on contribution of fish N gain from feed, pelagic and benthic food web at different feeding levels (NF=no-feeding, LF=low feeding, and HF=high feeding).

The contribution of the benthic food web was estimated by deducting the contribution of feed and the pelagic food from the total achieved growth, in the remaining two fed compartments.

By this calculation, contribution of the N from the pelagic food web was 30% *vs* 16% between low and high P:E ratio diet, and for benthic food web it was 34% *vs* 29 % for the low and high P:E diet. At high feeding level contribution of the benthic food web was highest with the low P:E diet and lowest with the high P:E diet (Fig. 3). This is due to lack of adequate C in the waste of the pond fed by high P:E diet. So microorganisms cannot take up all the N in the waste. Wahab *et al.* (2003) reported a similar outcome of increased N accumulation and less growth in shrimp ponds. The striking thing here was that the effect of diet on the pelagic food web was different. In previous studies on pond food web enhancement by adding external carbon besides the diet prioritized the role of the bacterial and the benthic communities (Asaduzzaman *et al.*, 2010b, 2010a, 2008; Avnimelech, 1999; Xu *et al.*, 2018). The influence of lowering the dietary P:E ratio on both the pelagic and benthic food web in this study is probably due to making more carbon available for microbes to utilize available N in the pond. However, when we measured the food web we did not notice any difference between the diets (Supplementary Table 1). In extensive ponds, the food web follows a cyclic pattern of growth (Benincà *et al.*, 2015) but in a well fed pond it should ideally remain stable unless controlled by predation. So, the likely cause of no difference in the food web was grazing pressure of the fish.

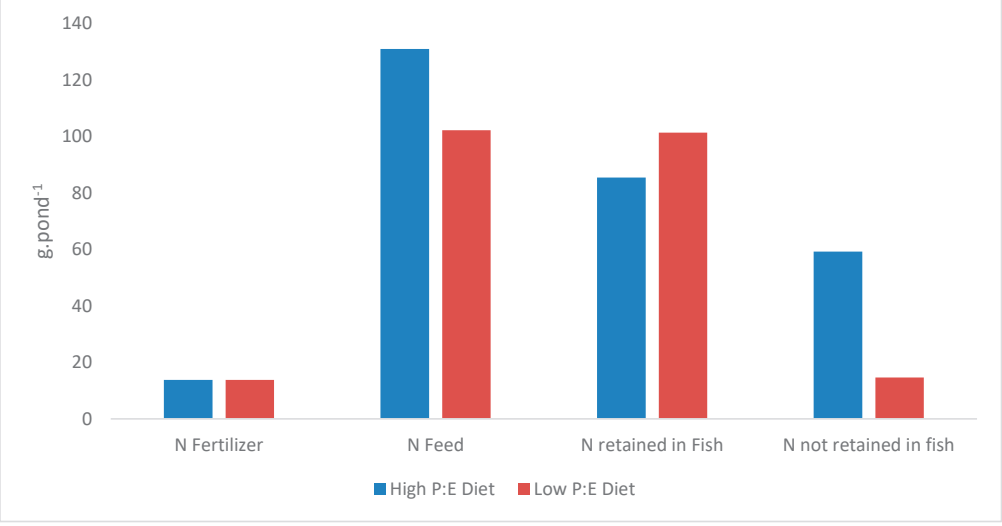


Fig. 4. Effect of dietary P:E ratio on N input, output and balance in the pond.

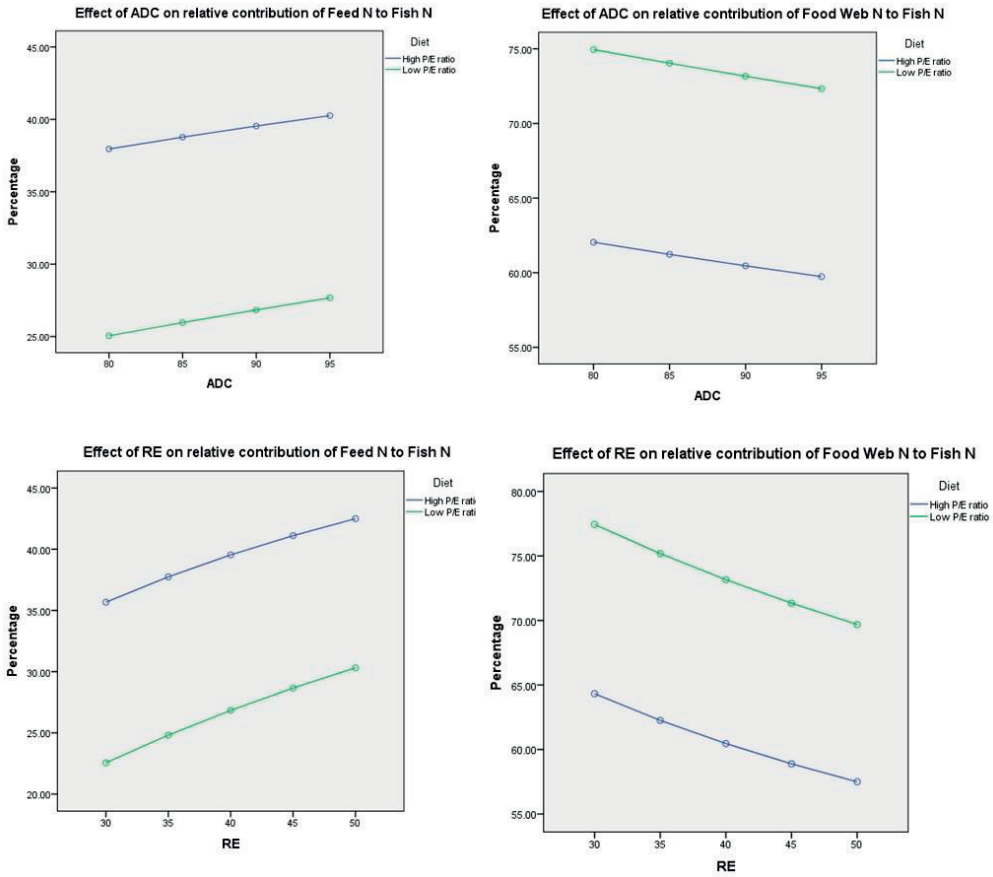
2.4.3 Varying Nitrogen retention efficiency with contrast dietary protein to energy level (or dietary protein level)

To estimate the effect of dietary P:E ratio on the fate of N in the system, we calculated total N input (g.pond^{-1}) including fertilizer and feed N, total N retained in the fish (g.fish^{-1}) and the non-retained N (g.pond^{-1}) in the pond. We observed N retention in fish (Fig. 4) is much higher

from the low P:E diet (87%) compared to the high P:E diet (59%). The amount of N that is not retained in fish can cause higher total ammonia nitrogen and NO_2–N concentrations at the water column (Hari *et al.*, 2004) and in the sediment (Li & Lovell, 1992). In this study, we did not see the inorganic N (NO_2, NO_3 and NH_4^+) accumulation in the water column. So, either this went to the pond bottom or returned to the atmosphere by denitrification. If the first case happens, high levels of ammonia could reduce the overall bottom productivity and fish yield (Wahab *et al.*, 2003) unless there is adequate C in the waste to maximize the use of additional N in the waste coming through high P:E diet. With the high P:E diet, the contribution of the benthic food web to fish production reduced with increasing feeing level. If the second case happens, it will return to atmosphere as N_2, and if denitrification becomes carbon limited there is a risk of N_2O volatilization, contributing to global warming (Hu *et al.*, 2012). In an ideal situation 98% of N input can be retained to fish biomass (Fan *et al.*, 2015). In current study the RE of N with the low P:E diet (87%) is close to the reported best level (Fan *et al.*, 2015).

2.5 Conclusion

Lowering the P:E ratio from 19 to 14 g.MJ^{-1} in formulated feeds applied in ponds improved tilapia production. The P:E ratio of 14 g.MJ^{-1} was much lower than the normally recommended P:E ratio of 18-23:E g.MJ^{-1}. Our analysis suggests that with the low P:E diet, fish consumed more natural foods to compensate for the lower nutrient input through the formulated feed. However, the higher contribution of natural foods to tilapia production was not shown by a higher abundance of plankton, benthos and soil and water microbiota. Hence, better parameters to explain natural food productivity and consumption by fish are needed. The latter will require additional research into the relation between the dietary P:E or C:N ratio, natural food productivity and the contribution of natural foods to total pond production.



Supplementary Fig. 1. Relative contribution of feed N and food web N to Fish N gain under different apparent digestibility coefficient(ADC) and retention efficiency (RF) of the food web N

Supplementary Table 1: Response of Food web in the pond over time in relation to the dietary ratio and feeding levels

			"Low P:E" diet			"High P:E" diet			P values						
		Time	NF	LF	HF	NF	LF	HF	D	F	D*F	T	T*D	T*F	T*D*F
Abundance of Phytoplankton	ind.L^{-1}	1	7.8E+04	6.3E+04	5.4E+04	8.7E+04	9.6E+04	6.8E+04	0.1	0.8	0.3	**0.00**	0.3	0.4	0.3
		2	6.6E+04	1.2E+05	9.2E+04	1.2E+05	9.4E+04	1.5E+05							
		3	4.4E+04	6.0E+04	6.2E+04	5.4E+04	4.0E+04	4.7E+04							
Diversity of Phytoplankton	group.L^{-1}	1	1.1E+01	1.1E+01	1.2E+01	1.1E+01	1.2E+01	1.1E+01	0.7	0.7	1.0	**0.00**	0.5	0.86	0.5
		2	1.5E+01	1.5E+01	1.5E+01	1.4E+01	1.6E+01	1.6E+01							
		3	1.3E+01	1.5E+01	1.4E+01	1.3E+01	1.3E+01	1.2E+01							
Abundance of Zooplankton	ind.L^{-1}	1	1.5E+04	1.2E+04	1.4E+04	1.5E+04	2.9E+04	1.3E+04	0.6	*0.08*	0.5	0.19	0.17	0.8	0.1
		2	9.4E+03	1.6E+04	1.3E+04	1.0E+04	1.2E+04	1.4E+04							
		3	8.5E+03	1.8E+04	1.6E+04	1.3E+04	1.3E+04	1.0E+04							
Diversity of Zooplankton	genus.L^{-1}	1	6.5E+00	5.3E+00	6.3E+00	5.3E+00	6.7E+00	5.7E+00	0.7	**0.02**	0.5	**0.00**	0.98	0.54	0.3
		2	6.0E+00	8.7E+00	8.3E+00	6.8E+00	8.3E+00	7.7E+00							
		3	5.0E+00	8.0E+00	6.8E+00	6.3E+00	6.5E+00	6.3E+00							
Abundance of Benthos	ind.m^{-2}	1	1.20E+03	7.75E+02	1.88E+03	1.55E+03	2.20E+03	1.20E+03	0.4	0.6	0.8	0.9	0.85	0.72	0.42
		2	1.35E+03	1.53E+03	1.00E+03	7.25E+02	1.73E+03	1.50E+03							
		3	1.13E+03	1.63E+03	7.25E+02	2.18E+03	1.58E+03	9.25E+02							
Diversity of Benthos	group.m^{-2}	1	2.5E+00	1.7E+00	3.7E+00	3.7E+00	3.2E+00	3.2E+00	0.4	0.7	0.8	0.35	0.74	0.19	0.59
		2	2.7E+00	3.3E+00	3.5E+00	2.2E+00	4.0E+00	3.8E+00							
		3	2.8E+00	3.2E+00	1.7E+00	2.5E+00	3.0E+00	2.5E+00							
Total count of bacteria from soil	CFU.100ml^{-1}	1	8.3E+04	8.6E+04	8.0E+04	9.2E+04	8.3E+04	8.4E+04	1.0	0.5	0.2	**0.00**	0.74	0.95	0.99
		2	1.0E+05	1.0E+05	1.0E+05	1.1E+05	9.1E+04	1.0E+05							
		3	1.0E+05	1.0E+05	1.0E+05	1.1E+05	9.1E+04	1.0E+05							
Total count of bacteria from water column	CFU.100ml^{-1}	1	4.4E+03	8.8E+03	1.3E+04	1.3E+04	6.2E+03	9.6E+03	0.5	0.7	0.7	**0.00**	0.69	0.85	0.50
		2	5.5E+04	6.7E+04	6.4E+04	6.5E+04	6.3E+04	6.1E+04							
		3	5.3E+04	5.3E+04	5.8E+04	5.4E+04	7.4E+04	5.9E+04							
Chlorophyll a	µg.L^{-1}	1	7.7E-03	7.5E-03	0.015	9.8E-03	1	1.1E-02	0.3	0.6	0.15	*0.07*	0.86	0.85	0.33
		2	.010	8.8E-03	1.3E-02	8.3E-03	1.6E-01	9.8E-03							
		3	9.8E-03	1.3E-02	1.5E-02	1.5E-02	3	1.1E-02							

* NF= No Feeding, LF=Low Feeding and HF=High Feeding, D=Diet and F=Feeding Level, T=Time of sampling

Chapter 3

Effect of dietary carbohydrate to lipid ratio on performance of Nile tilapia and food web enhancement in semi-intensive pond aquaculture

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Abstract

In aquaculture ponds, the application of feed may have a dual role, contributing directly to the growth of fish and indirectly through enhancing the natural food of the pond. Reducing the DP:DE ratio in tilapia feed by including higher dietary non-protein energy can help reduce feed costs and at the same time may also increase fish production. However, the impact of changing compositions of the non-protein energy on fish production in aquaculture ponds has not been tested. As a consequence, there is no well accepted range of dietary carbohydrate to lipid (CHO:LIP) ratio for tilapia pond aquaculture. This study tested two diets contrast in CHO:LIP ratio on tilapia. The aim was to see the effect of reducing dietary lipid on fish productivity, and natural food enhancement in semi-intensively managed tilapia ponds. Eight ponds, each divided into three equally-sized compartments, were assigned to test the effect of two diets, which differed in CHO:LIP ratio (4.7 *vs*. 19.5 $g.g^{-1}$) but had the same DP:DE ratio (15.5 and 15.6 $g.MJ^{-1}$). Fish were fed based on crude protein content of the feed. Three feeding levels ("no=0", "low=9 $g.kg^{-0.8}.d^{-1}$" and "high= 18 $g.kg^{-0.8}.d^{-1}$") were nested in each pond in a split plot design. Initial fish biomass was 3626 (±108) $g.compartment^{-1}$ and the experiment lasted 42 days. Increasing the CHO:LIP ratio had no impact (P>0.1) on tilapia production (*i.e.* biomass gain = 2154 *vs* 2077 $g.compartment^{-1}$); specific growth rate (1.36 *vs* 1.30 % of body $weight.d^{-1}$); FCR(1.65 *vs* 1.80); and survival (89.1 *vs* 88.5%). However, feeding level influenced both biomass gain (P<0.001), SGR (P<0.001) and survival (P<0.05). The apparent digestibility coefficient (ADC) for fat (93 *vs* 77%; P<0.001) and carbohydrate (60 *vs* 68 %; P<0.05) was influenced by dietary CHO:LIP ratio but ADC for energy (70 *vs* 67%; P>0.1) was unaffected. Body composition of tilapia was unaffected by diet except for ash (50 *vs* 46%; P<0.05). The concentration of nutrients in the fish body increased with increased feeding level. Despite of replacing the source of non-protein energy from lipid to carbohydrate, fat content in the body did not exceed 5.5% at any feeding level. Dietary CHO:LIP ratio had no impact on N, P, K, and OM in pond soil and water. It had neither an effect on the natural food except for phytoplankton diversity. However, soil and water nutrients showed a cyclic pattern of change over time while the abundance of the measured natural food increased. There was no effect of dietary CHO:LIP ratio on the organic matter composition of the faeces. The data on N gain from natural food also indicated no difference. Therefore, we postulate that changing dietary non-protein energy source from lipid to carbohydrate does not have any impact on tilapia culture in semi-intensive ponds.

3.1 Introduction

The demand for fish is increasing, because of population growth, increased wealth in developing nations and interest among consumers for a healthy and nutritious animal source food (World Bank, 2013). The fish coming from capture fisheries (60-70 million metric ton), has been static for the last couple of decades (FAO, 2018; Kobayashi *et al.*, 2015). The growth of fish production comes therefore mainly from aquaculture (Kobayashi *et al.*, 2015). This entails also a need for more fish feed. Limited availability of ingredients, competition with human food and increasing price are major challenges to secure the increased demand of fish feed (Tacon *et al.*, 2011; Tacon and Metian, 2015). Moreover, feed prices increase faster than those of fish (Rana *et al.*, 2009). This creates a pressure to make more efficient and cheaper feed (Naylor *et al.*, 2009).

The main expenditure of fish feed is protein. Dietary protein supplies amino acids as building blocks for the fish body and is also a source of energy. Dietary energy can be supplied by other sources as well. Therefore, formulation of feed by lowering digestible protein to digestible energy (DP:DE) ratio through increasing non-protein energy is a way to reduce feed cost (NRC 2011). Non-protein energy can come from lipid and carbohydrate. Fish and also vegetable oils, are major lipid sources, but are expensive. Replacing or reducing lipid (*i.e.*, oil) levels in the feed by carbohydrates can make the feed more affordable. However, minimum inclusion (specific amount depends on fish species) of lipid in the diet is required to cover the essential fatty acid (EFA) requirement and fat-soluble vitamin intake. For tilapia, limited research has been done on the carbohydrate: lipid (CHO: LIP) ratio of the diet and an optimum dietary inclusion range has not been estimated or widely accepted.

Tilapia is omnivorous and can utilize a wide range of plant-based ingredients in their diet. Studies indicate that CHO:LIP ratio ranging between 2.0-6.5 $g.g^{-1}$ provides best yield for tilapia (Ali and Al-Asgah, 2001; Coutinho *et al.*, 2018; He *et al.*, 2015; Xie *et al.*, 2017). Keeping the DP:DE ratio constant and replacing lipid completely with starch leading to CHO:LIP ratio of 20 $g.g^{-1}$ resulted in poor growth (Xie *et al.*, 2017). This indicates that a minimum inclusion of lipid is required to ensure presence of essential fatty acid in the diet of tilapia. The dietary CHO:LIP ratio also affects the fish body composition. Decreasing the CHO:LIP ratio by increasing lipid content decreased moisture and crude protein contents whereas fat and ash contents increased (Ali and Al-Asgah, 2001; Haidar *et al.*, 2018). Changing the CHO:LIP ratio can have impact on the apparent digestibility coefficient (ADC) of the nutrients, energy efficiency and waste composition (Amirkolaie *et al.*, 2006; Tran-Tu *et al.*, 2018). The first two has direct impact on fish growth, while the third factor can influence natural food in the pond. All the previous studies were either done in tanks or in cages, in absence of natural food. The effect of the CHO:LIP ratio in ponds is therefore not well studied.

Most tilapia production come from ponds located in tropical and sub-tropical regions of the world. In ponds, tilapia can obtain nutrients from the natural food and the feed works as a supplemental nutrient input. Manipulation of dietary non-protein energy by alteration of CHO:LIP ratio can also influence faecal characteristics in tilapia (Schneider *et al.*, 2004). This change in the faecal composition might steer the natural food in the pond. It has been evident that lowering the dietary DP:DE ratio in pond aquaculture enhances natural food in the pond and helps producing more fish with the same feed input (Kabir *et al.*, 2019). However, the impact of keeping the dietary DP:DE ratio constant and altering the CHO:LIP ratio on fish production in pond and influence on natural food production is not known.

Therefore, the aim of this study was to test the effect of different types of non-protein dietary energy (CHO:LIP ratio) on fish production and natural food enhancement in tilapia ponds while keeping the DP:DE ratio same between the diets. The DP:DE ratio in this study was set as recommended by Kabir *et al.*, (2019) for pond diet to maximize contribution of natural food to fish growth. The hypothesis was that fish performance will be better with a high CHO:LIP diet as dietary net energy will be higher while due to same dietary DP:DE ratio, the contribution of natural foods to fish growth will not change.

3.2 Methods

Two diets, with a contrast in the carbohydrate to lipid ratio (CHO:LIP; 4.7 *vs* 19.5 $g.g^{-1}$), were tested on Nile tilapia *(Oreochromis niloticus)* in 8 outdoor ponds for 42 days (4 repetitions per diet). Ponds were divided into three compartments to which three different feeding levels were assigned within each diet by pond in a split plot design.

3.2.1 Diets

Experimental diets were formulated to test the effect of the non-protein energy source on the performance of fish and natural food in the pond. Therefore, the diets had a contrast in CHO:LIP ratio (4.7 *vs* 19.5 $g.g^{-1}$) but had an equal DP:DE ratio (*i.e.*., C:N ratio). The contrast in CHO:LIP ratio was mainly created by replacing fish oil with multiple carbohydrate sources (*i.e.*, wheat bran, rice bran, cassava flour and wheat flour). This mixture of carbohydrate sources was used to increase both starch and non-starch polysaccharides content in the diets (*i.e.*, a mixture of digestible and non-digestible carbohydrate sources). In order to keep the DP:DE ratio equal in both diets, small alterations in the inclusion levels of protein ingredients were made (Table 1). Both diets met the nutrient requirements of tilapia (NRC, 2011). However, the DP:DE level was 15.6 $g.MJ^{-1}$, which is below the recommended level of NRC (1993). This was done to enhance the natural food in the pond (Kabir *et al.*, 2019). An inert marker, yttrium oxide (Y_2O_3), was included to test the apparent digestibility coefficients (ADC). The

experimental diets were made at the R&D facilities of De Heus (De Heus Beheer B.V.) in Vietnam. Diets were extruded to obtain floating pellets with 3 mm diameter.

Table 1. Ingredient and analysed chemical composition of the experimental Nile tilapia diets differing in carbohydrate to lipid (CHO:LIP) ratio.

		Diets	
		Low CHO:LIP ratio	High CHO:LIP ratio
Ingredients (%)			
Soybean meal		6.5	5
Wheat bran		5	7.5
Wheat		18	25.1
De-oiled rice bran (DORB)		5	7.5
Cassava		17.98	24.98
Rapeseed meal		6.5	5
Soy protein concentrate (fermented)		19	14
Meat and bone meal		6.5	5
Fish meal (CP>68%)		3	2.5
Fish oil (salmon)		10	1
Vitamin and mineral premix[†]		1	1
Mono calcium phosphate (MCP)		1.2	1.15
DL-methionine		0.3	0.25
Yttrium oxide (Y_2O_3)		0.02	0.02
Analysed chemical composition			
Dry matter (DM),	($g.kg^{-1}$)	932	892
Crude Protein	($g.kg^{-1}$ DM)	301	273
Fat	($g.kg^{-1}$ DM)	111	32
Ash	($g.kg^{-1}$ DM)	69	71
Phosphorus	($g.kg^{-1}$ DM)	10.7	11.1
Carbohydrate[‡]	($g.kg^{-1}$ DM)	519	625
Starch	($g.kg^{-1}$ DM)	273	347
NSP	($g.kg^{-1}$ DM)	240	263
Gross energy	($kj.g^{-1}$ DM)	20.8	18.8
DP:DE ratio[§]	($g.MJ^{-1}$)	15.5	15.6
CHO:LIP ratio	$g.g^{-1}$	4.7	19.5
C:N ratio[¶]	$g.g^{-1}$	9.9	10.6

[†] Commercial product.

[‡]This is calculated as follows carbohydrate= 1000 – CP – Fat - Ash

[§]Calculated based on the apparent digestibility coefficient obtained in this experiment

[¶]This is calculated C:N ratio considering 16% N content in the protein and 47, 70 and 50% C content in protein, fat and carbohydrate, respectively (Waal and Boersma, 2012).

3.2.2 Fish, rearing and housing facilities

All male, juvenile Nile tilapia *(Oreochromis niloticus)* of the 14th generation WorldFish GIFT strain were collected from the Asha Hatchery, at Bagerhat, in Bangladesh. Eight outdoor ponds, each thirty square meters, in a field experimental station were used for this experiment. Each pond was divided into three equal compartments (Fig. 1). The water column of the pond was divided by a bamboo-frame fitted with 1mm mesh sized nets allowing well mixing of nutrients and dissolved solids within the compartments but preventing that pellets and fish would pass between the compartments. At the bottom, the compartments were separated by a 8 cm thick concrete wall of 105 cm height, of which 75 cm below the soil and 30 cm extended above the pond bottom to prevent the exchange of uneaten feed and benthos between the compartments. All pond compartments were well aerated to ensure adequate dissolved oxygen in the pond water as well as good mixing of dissolved nutrients within and between the compartments.

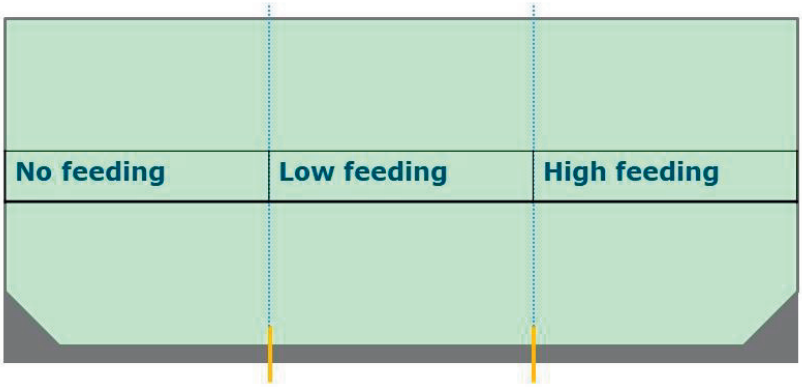


Fig. 1. Sketch of the pond compartments. The dotted line indicates the net and frame, solid bar indicates the concrete wall and the grey bottom indicates the soil layer of the pond bottom

3.2.3 Experimental procedure

Prior to the experiment, ponds were dried by pumping out the water. Two hundred fifty g $CaCO_3$ was applied at the bottom soil of each pond compartment (PC) before water filling. After water filling, 40g Dolomite ($CaMg(CO_3)_2$) was spread over the water surface of each compartment. Ten g urea (CH_4N_2O) and 20g triple super phosphate (TSP), [$Ca(H_2PO_4)_2.H_2O$], per pond compartment (Rakocy and Mcginty, 1989) were applied 1 week after liming. Fish were stocked in the ponds 5 days after fertilization (*i.e.* after 12 days of filling the pond).

Forty juvenile tilapia (4.m^{-2}) were stocked per pond compartment. Fish were fed daily at 8.00 and 16.00 hours. Fish were fed according to their metabolic body weight. Per compartment within each pond, one of three feeding levels were applied, high (18 and 20.6 g.kg$^{-0.8}$.d^{-1}), low

(9 and 10.3 $g.kg^{-0.8}.d^{-1}$) and no feeding, in a split plot design for both the diets. The high feeding level was comparable with the feeding at the semi-intensive commercial tilapia ponds. By applying these rations, ponds were fed a similar amount of protein and energy. Duration of the experiment was 42 days. Sampling for pond soil and water nutrients and natural food were done at day 1, 21 and 42.

3.2.3.1 Water quality monitoring

From day 1 onward, dissolved oxygen (DO), pH, total dissolved solid (TDS), transparency, temperature and salinity of each pond were measured daily at 6.00, 9.00, 10.00, 12.00, 14.00 and 14.30 hours; by using Lutron dissolved oxygen meter model PDO-519, Hanna instruments pocket tester HI98128-phep5, Lutron conductivity meter model PCD-431, Secchi disc, Hanna digital thermometer model HI98501 and Atago refractometer model MASTER-S28M instruments. NH_4^+, NO_2 and NO_3 were measured only at day 1, 21 and 42 by colourimetric, acc. to Neßler, with colour card and sliding comparator: 108025 | Nitrite Test, 111117 | Ammonium Test, 110022 | Nitrite Test; Merck KGaA, Darmstadt, Germany. Total suspended solids (TSS) of pond water from each compartment were also measured at day 1, 21 and 42 following the procedure of APHA methods # 2540 D (APHA, 1995)

3.2.3.2 Sampling and analysing soil and water nutrients

3.2.3.2.1 Sample collection, processing and preparation

Soil samples were collected from the top 20 cm layer of the pond bottom at three points in each pond compartment and then mixed homogeneously. Approximately 1 kg wet soil was collected from each pond compartment, labelled and packed in tight plastic bags, and transported to the laboratory. The collected samples were air dried, crumbled and grinded. The grinded samples were preserved in labelled plastic containers until analysis. Water samples were collected, with a depth sampler of 10 cm width and 25 cm length, from each pond at the same 5 soil sampling locations, within 25 cm of pond surface, transferred and sealed in airtight bottles, and preserved at -20˚C until analysed.

3.2.3.2.2 Analysis of the soil samples

Organic carbon content of the soil was determined by Walkley and Black's wet oxidation method as described by Jackson (1973). Total nitrogen of the soil was determined by Micro-Kjeldahl's method following H_2SO_4 acid digestion and alkali distillation procedures as suggested by Jackson (1962). Total phosphorus of soil was determined colourimetrically by Vanado-molybdophosphoric yellow colour method in nitric acid system (Barton, 1948). The colour intensity was determined by the spectrophotometer at 470 nm light wavelength (Jackson, 1958). The available potassium was determined after extraction the soil samples with 1N NH_4OAc, pH-7.0 solution followed by the measurement of extractable K+ by Flame

emission spectrophotometer (Model: Jenway, PEP-7) at 766 nm wave length using potassium filter, as outlined by Jackson (1973).

3.2.3.2.3 Analysis of the water samples

The organic carbon content of the water was determined by the method described by Tyrine (1995), as water commonly contains relatively smaller amounts of organic matter. Under dilute conditions Tyrine's method does not function well. So, the sample was dried first. The total inorganic nitrogen concentration was determined by the Micro-Kjeldahl method (Jones, 1991) and alkali distillation procedures as suggested by Jackson (1962). Available phosphorus was determined colourimetrically by molybdophosphoric blue colour method (Murphy and Riley, 1962). The available potassium of water was determined by a flame analyzer at 589 nm wavelength (Jackson, 1967).

3.2.3.3 Sampling and analysing plankton

Phytoplankton and zooplankton samples were collected at day 1, 21 and 42. Samples were collected between 9.00-11.00 hrs form 3 points, equally spaced on a diagonal line in each pond compartment. At each point 15 L water was passed through the 45 µm mesh plankton net, thus pulling together 45 L of water from each compartment were sampled.

The concentrated samples were preserved in small plastic bottles with 5% buffered formalin. Diversity (group.L^{-1}) and abundance (ind.L^{-1}) estimations of plankton were done using a Sedgewick– Rafter (S-R) cell containing 1000 1-mm^3 cells. A 1 ml sample was put in the S-R cell and was left 15 min undisturbed to allow plankton to settle. The plankton in 10 randomly selected cells were identified where possible up to genus level and counted under a binocular microscope (LABOMED America.inc; Lx 300). Plankton was identified using keys by Ward and Whipple (1959), Prescott (1962), Belcher and Swale (1976), and Bellinger (1992). Plankton abundance was calculated using the following formula:

N = (P×C×100)/V. Here, N is the number of plankton cells or units per litter of pond water; P= the number of planktons counted in 10 fields of the S-R cell; C= the volume of final concentrate of the sample (ml); V= the volume of the pond water sample in litter.

3.2.3.4 Sampling and analysing benthos

The benthic macroinvertebrate samples were also collected on day 1, 21 and 42 with an Ekman grab (area: 225 cm^2). In each pond compartment, bottom mud samples were collected from three different locations, which were then combined into a composite sample. Benthic macroinvertebrates were collected after filtering sediments through 4 different mesh sieves and preserved in a plastic vial containing a 10% buffered formalin. Identification keys used for

benthic macroinvertebrates were Brinkhurst (1971), and Pinder and Reiss (1983). Benthic macroinvertebrates density was calculated using the formula:
N = Y ×10,000/3A. Here, N=the number of benthic organisms per square meter; Y=total number of benthic organisms counted in 3 samples; A= Area of the Ekman dredge.

3.2.3.5 Sampling and analysing bacteria

In order to isolate and quantify bacterial communities, samples from both water and soil sediments were collected at day 1, 21 and 42. All samples were collected from three different locations of each pond compartment in sterile containers (15 ml tube, Falcon, USA), mixed homogenously before transported back to the Limnological Laboratory of the Environmental Science Discipline of Khulna University, Bangladesh. One ml water sample was transferred with a sterile pipette to a test tube containing 9 ml of phosphate buffered saline (PBS) and the tube was shaken thoroughly, while 5 g of each sediment and water samples were weighed and transferred to a sterile conical flask and made up to 50 ml with PBS and the contents were mixed thoroughly to prepare a stock solution. Serial dilution of up to 10−6 for water and 10−8 for sediment were prepared with PBS. Volumes (0.1ml) of each dilution were spread over the surface of duplicate plates of tryptone soya agar (TSA; Difco, Detroit, MI, USA) with incubation at 30 °C for 24–48 h. Plates with 30– 300 colony forming units (CFU) were counted with a Leica Quebec Dark field Colony Counter (Leica, Inc., Buffalo, NY, USA) and expressed as CFU/ ml.

3.2.3.6 Sampling and analysing Chlorophyll a

Water samples from the water column of three parts of the pond compartment were collected, well mixed, and kept into 500 ml bottles. The samples were transferred to the lab within one hour for analysis. There we filtered 250 ml of water through Whatman GF/C filter paper. We then torn the filter paper into 5-6 pieces and inserted them into a 50 ml centrifuge tube. Thereafter, we added 20 ml of methanol into each tube to cover the filter paper pieces in it, shook well and vortex until the filter paper was broken up. Kept them in the freezer overnight. Centrifuged at 3200 rpm for 10 minutes. Poured off the supernatant into a 1 cm cuvette and measured the extinction at both 665 nm and 750 nm (zero with methanol). Chlorophyll a was calculated as Chl-a ($ug.L^{-1}$) = ((Abs [665nm]-Abs [750nm]) x A x V_m)/ V_f x L. Here, A = absorbance coefficient of chlorophyll-a in methanol (12.63); V_m = volume of methanol used for extinction (ml); V_f = litres of water filtered; and L = path length of cuvette.

3.2.3.7 Sampling and analysing proximate composition of fish and feed

Initial body composition was determined in 25 fish, which were randomly selected at the start of the experiment. For final body composition, 5 fish were randomly selected per compartment at the end of the experiment. Fish, which were used for body composition analysis, were euthanized by an overdose of a phenoxy-ethanol solution (1.0ml. L^{-1}) and

stored at −20°C. Before chemical analysis, the sampled fish were cut into small pieces, homogenised by grinding in a mincing machine twice through a 4·5 mm screen grinder and subsequently oven-dried. Chemical analyses were done in triplicate. Dry matter (DM) was determined gravimetrically after drying at 103°C for 4, and 24 h until constant weight, respectively, for feed and fish samples (ISO 6496, 1983). Crude ash was determined after incineration at 550°C for 4 h (ISO 5984, 1978). Crude protein (CP) was determined by the Kjeldahl method (ISO 5983, 1979) and calculated by multiplying the measured N content by 6.25. Fat was quantified by petroleum–diethyl ether extraction (ISO 6492, 1999). Before fat analysis, feed samples were hydrolysed by boiling for 1 h with 3 M-HCl. Dietary energy content was measured by direct combustion in an adiabatic bomb calorimeter (IKA-C-7000; IKA analysentechnik, Weitersheim, Germany). Starch including free sugars was enzymatically determined in feed and faecal samples by using amyloglucosidase without the ethanol extraction step and measuring glucose content as described (Goelema, Spreeuwenberg, Hof, van der Poel, & Tamminga, 1998). NSP content was calculated as total carbohydrates – "Starch + free sugars". Yttrium, P and Ca in feed and faeces were analysed using inductively coupled plasma-mass spectrometry (ICP- OES) according to the standard NEN 15510 (2007).

3.2.3.8 Faeces collection and preservation

At the end of the pond experiment, 180 tilapia with mean body weight of 161 (±31) g were restocked in the indoor concrete tanks for faeces collection to determine apparent digestibility. Ten fish were allocated in each of 18 tanks of 1000 litter water holding capacity, filled with 700 lifter water. All tanks were aerated. Both experimental diets were fed at 6, 9 and 12 $g.kg^{-0.8}d^{-1}$ with 3 replications per treatment. Fish were fed daily at 7.00 and 15.00 hours. The first seven days fish were fed in the tank for acclimation to the tank environment. Starting from day 8, faeces were collected by siphoning 3 hours after each feeding for a total period of 10 days. Collected faeces were preserved in labelled plastic pots at -20˚C. Later all samples from the same tank were pooled together for chemical analysis.

3.2.3.9 Analysis of stomach contents

Fish for stomach content were harvested on day 43, 19 hours after the last feeding, to ensure that no pellets remained in the stomach. Fish were euthanized by an overdose of a phenoxy-ethanol solution ($1.0ml.L^{-1}$) and transported to the laboratory. In the laboratory the fish were dissected to collect the stomach and preserve it in 10% formalin. Total volume of the stomach and the number of food items were recorded. Volume of food items occupying in general and by each food group were visually estimated (Jude, 1971). Total weight of food was expressed as percentage of weight of the stomach on a wet weight basis (Gibbons & Gee, 1972). The index of relative importance (IRI) of observed natural food groups was estimated by diet to

understand the relative importance of each natural food group in the growth of the fish following the methods described by Pinkas *et al.*, (1971) and Prince (1975).

IRI = (%G_n + %G_v) x % G_f; where, G_n is percentage by group number, $G_{v\ is}$ volume of group number and G_f is frequency of occurrence by the group number.

3.2.4 Analytical procedures and calculations

3.2.4.1 Performance

Biomass gain (g) was calculated as the difference between the biomass stocked and biomass harvested per compartment. The specific growth rate (SGR) was calculated as $SGR=((\ln(IndBW_{42})-\ln(IndBW_0))/42)\times100$; where $IndBW_{42}$ and $IndBW_0$ means individual body weight at day 42 and day 0. Growth ($g.d^{-1}$) was calculated as individual gain (g) divided by duration of the experiment (d). Feed conversion ratio (FCR) was calculated per compartment using the feed given and weight gain. The survival of fish per compartment was calculated as $(N_f/N_i)\times100$, where N_f is the final number of fish and N_i the initial number at pond compartment (PC) level.

3.2.4.2 ADC Calculation

The apparent digestibility coefficients of nutrients were measured for each tank using Y_2O_3 as an inert marker. The yttrium content of feed and faeces was analysed using inductively coupled plasma-mass spectrometry (ICP- OES) according to the standard NEN 15510 (2007). Apparent digestibility coefficients (ADCs) of the dietary components in the diets were calculated by using the following formula:

$$\%\ ADC_{diet}= 100\%*(1-[Y_{diet}/Y_{faeces}]*[N_{faeces}/N_{diet}])$$

Here, Y_{diet} and Y_{faeces} are the content of the inert marker (yttrium) in the diet and faeces, respectively ($g.kg^{-1}$ DM); and N_{faeces} and N_{diet} are the contents of the dietary components in the faeces and diets, respectively ($g.kg^{-1}$ DM).

3.2.4.3 Fish N gain

N gain in fish was calculated by the difference between the N_h and N_s. Here, N_h=amount of N in the harvested fish biomass and N_s=amount of N in the biomass at start. N feed was calculated by total feed input per compartment, multiplied by the N content in feed. Contribution of feed N to fish N gain was calculated based on the ADC of CP from this study and considering average N retention efficiency (RE) of 40% (Azevedo *et al.*, 2004) for both the diets at all feeding levels. N retained from natural food was calculated by deducting N retention from feed from the total N gain in fish.

3.2.5 Statistical analysis

The data were analysed using the IBM SPSS software package version 23. All data, except water quality and ADC, were analysed in a split plot design using the procedure general linear model (GLM). Effect of diet has been tested between ponds while effect of feeding level and diet and feeding level interaction were tested between compartments within the pond. Univariate analysis was done to see the effect of diet on water quality at pond level only. Effect of diet, feeding level and their interaction on ADC of nutrients were tested using univariate analysis by the procedure general linear model (GLM). When a significant interaction effect was found, multiple comparisons of means using Tukey's multiple range test were performed.

3.3 Results

The average individual BW at stocking was 91 (±5) g and was unaffected by diet and feeding level. There were no effects of the dietary CHO:LIP ratio and the interaction of diet and feeding level on the measured indicators for fish performance (Table 2). Biomass harvested, biomass gain, individual gain, survival, specific growth rate and daily growth rate increased with increasing feeding levels (P<0.001; except for survival P<0.05). The average overall fed compartments FCR was 1.7 and was even unaffected by feeding levels (P<0.10).

Table 2. Effect of dietary carbohydrate to lipid ratio and feeding level on performance of tilapia

		Low CHO:LIP diet			High CHO:LIP diet			Pooled	P-values		
Variables	Units	FL0	FL1	FL2	FL0	FL1	FL2	SEM	D	FL	D*FL
Initial individual BW	g	90	88	89	91	94	92	3	ns	ns	ns
Biomass stocked	$g.comp^{-1}$	3586	3534	3544	3642	3756	3692	108	ns	ns	ns
Biomass harvested	$g.comp^{-1}$	4329	5455	7341	4062	5493	7767	278	ns	***	ns
Individual BW Gain	g	44	67	100	40	63	110	9	ns	***	ns
Biomass gain	$g.comp^{-1}$	743	1921	3798	420	1737	4075	232	ns	***	ns
Survival	%	82	88	98	81	88	96	6	ns	*	ns
FCR	$g.g^{-1}$		1.54	1.77		1.81	1.79	0.19	ns	ns	ns
SGR	$\%.d^{-1}$	0.9	1.3	1.8	0.8	1.2	1.9	0.1	ns	***	ns
Growth Rate	$g.d^{-1}$	1.0	1.6	2.4	0.9	1.5	2.6	0.2	ns	***	ns

CHO:LIP ratio = dietary carbohydrate to lipid ratio , FL0= no feeding, FL1= low feeding , FL2=high feeding, D=diet and FL= feeding level, D*FL= diet and feeding level interactions, BW= body weight, FCR= feed conversion ratio, SGR= specific growth rate, Comp=compartment, d=day, P values: ns (not significant, P>0.1), * (P<0.05), *** (P<0.001).

The apparent digestibility coefficient (ADC) for fat and carbohydrate were affected by the dietary CHO:LIP ratio. ADC for fat was higher (93 *vs* 77%) with low CHO:LIP diet (4.7 *vs* 19.5 $g.g^{-1}$; P<0.001) while ADC for carbohydrate was higher (68 *vs* 60%) with high CHO:LIP diet (19.5 *vs* 4.7 $g.g^{-1}$; P<0.05). There was no effect of feeding level and the interaction of diet and feeding level on the ADC for any of the nutrients (Table 3). The ADC of ash, dry matter (DM) and minerals (*i.e.* P, Ca and Mg) were affected by the rearing facility (*i.e.* concrete tanks) and faeces collection methods (*i.e*. by siphoning). Therefore, we didn't present the outcome here.

Table 3. Effect of dietary carbohydrate to lipid ratio and feeding level on apparent digestibility coefficient (ADC) of tilapia

	Units	Low CHO:LIP diet	High CHO:LIP diet	Pooled SEM	P-values		
					D	FL	D*FL
Crude protein	%	75	73	2.4	ns	ns	ns
Crude fat	%	93	77	3.1	***	ns	ns
Energy	%	70	67	33	ns	ns	ns
Carbohydrate	%	60	68	3.6	*	ns	ns
Organic matter(OM)	%	68	69	2.0	ns	ns	ns

D=diet and FL= feeding level, D*FL= diet and feeding level interactions, P values: ns (not significant, P>0.1), * (P<0.05), *** (P<0.001).

Dietary CHO:LIP ratio only affected ash content (50 *vs* 46$g.kg^{-1}$; P<0.05) in the final fish body composition and increased with lowering the CHO:LIP ratio (19.5 *vs* 4.7 $g.g^{-1}$). DM, protein and fat content increased with increasing feeding level (P<0.001). Interaction of diet and feeding level did not affect the final fish body composition (Table 4).

Table 4. Effect of dietary carbohydrate to lipid (CHO:LIP) ratio and feeding level on body composition of tilapia

	Units	Low CHO:LIP diet			High CHO:LIP diet			Pooled SEM	P-values		
		FL0	FL1	FL2	FL0	FL1	FL2		D	FL	D*FL
DM	$g.kg^{-1}$	261	274	287	260	262	280	4	ns	***	ns
Protein	$g.kg^{-1}$	150	155	160	149	151	155	2	ns	***	ns
Fat	$g.kg^{-1}$	45	53	53	48	49	52	2	ns	***	ns
Ash	$g.kg^{-1}$	44	52	55	45	46	47	2	*	*	ns

CHO:LIP = dietary carbohydrate to lipid ratio , FL0= no feeding, FL1= low feeding, FL2=high feeding, D=diet and FL= feeding level, D*FL= diet and feeding level interactions, P values: ns (not significant, P>0.1), * (P<0.05), *** (P<0.001).

Table 5. Effect of dietary carbohydrate to lipid ratio and feeding level on accumulation of soil and water nutrients

	units	Low CHO:LIP diet			High CHO:LIP diet			Pooled SEM	P-values		
		FL0	FL1	FL2	FL0	FL1	FL2		D	FL	D*FL
Phosphorus in soil	$mg.L^{-1}$	78	141	92	10	29	63	268	ns	ns	ns
Phosphorus in water	$mg.L^{-1}$	3	4	3	6	3	2	2	ns	ns	ns
Organic matter in soil	$mg.L^{-1}$	-983	83	11	433	-417	-83	504	ns	ns	ns
Organic matter in water	$mg.L^{-1}$	-34	-5	11	-110	-61	-83	59	ns	*	ns
Nitrogen in soil	$mg.L^{-1}$	-58	12	23	23	0	35	34	ns	ns	ns
Nitrogen in water	$mg.L^{-1}$	-2	0	1	-6	-3	-4	3	ns	*	ns
Potassium in soil	$mg.L^{-1}$	-48	-96	-32	-159	-144	-128	49	ns	ns	ns
Potassium in water	$mg.L^{-1}$	-8	-6	-8	-10	-10	-8	4	ns	ns	ns

CHO:LIP = dietary carbohydrate to lipid ratio , FL0= no feeding, FL1= low feeding (, FL2=high feeding, D=diet and FL= feeding level, D*FL= diet and feeding level interactions, P values: ns (not significant, P>0.1), * (P<0.05).

Table 6. Effect of dietary carbohydrate to lipid ratio and feeding level on the mean (average of three sampling times) natural food of the pond (by compartment)

	Units	Low CHO:LIP diet			High CHO:LIP diet			Pooled	P-values		
		FL0	FL1	FL2	FL0	FL1	FL2	SEM	D	FL	D*FL
Chlorophyll a	$\mu g.L^{-1}$	0.011	0.008	0.01	0.007	0.01	0.01	0.003	ns	ns	ns
Phytoplankton abundance	$ind.L^{-1}$	22125	21806	21319	19875	16861	25333	2443	ns	ns	ns
Phytoplankton diversity	$genus.L^{-1}$	8.1	8.4	8.9	7.2	6.8	8.1	0.66	*	ns	ns
Zooplankton abundance	$ind.L^{-1}$	8958	8875	7083	7875	9000	10375	799	ns	ns	*
Zooplankton diversity	$genus.L^{-1}$	5.167	5.278	4.833	4.778	5.056	5.278	0.40	ns	ns	ns
Benthos abundance	$ind.m^{-2}$	7742	8533	6617	10075	10383	7867	2907	ns	ns	ns
Benthos diversity	$group.m^{-2}$	3	3	2	3	3	3	0.26	ns	ns	ns
Water bacteria	$CFU.\ ml^{-1}$	2804	2588	2656	2652	2799	3106	237	ns	ns	ns
Soil bacteria	$CFU.ml^{-1}$	2283	2463	2368	2394	2492	2481	115	ns	ns	ns

CHO:LIP = dietary carbohydrate to lipid ratio , FL0= no feeding, FL1= low feeding, FL2=high feeding, D=diet and FL= feeding level, D*FL= diet and feeding level interactions, P values: ns (not significant, P>0.1), * (P<0.05).

Irrespective to diets, potassium in both soil and water column of ponds showed a negative accumulation. A similar outcome was observed for organic matter in the pond soil and total inorganic nitrogen in the pond water column for most of the treatments (Table 5). The negative accumulation of organic matter (or carbon) and nitrogen from the pond water and

soil reduced with increasing feeding level (P<0.05). Over the duration of the experiment, all nutrients in the pond environment showed cyclic patterns of ups and downs (Supplementary Fig. 1) in relation to sampling time points (P<0.001).

Table 7. Effect of dietary carbohydrate to lipid ratio and feeding level on the natural food observed in the stomach content of tilapia

	Units	Low CHO:LIP diet			High CHO:LIP diet			Pooled SEM	P-values		
		FL0	FL1	FL2	FL0	FL1	FL2		D	FL	D*FL
Volumetric occurrence of natural food	%	34	34	24	34	32	18	4.3	ns	*	ns
Gravimetric occurrence of natural food	%	59	64	42	61	48	35	6.7	ns	*	ns

CHO:LIP = dietary carbohydrate to lipid ratio , FL0= no feeding, FL1= low feeding, FL2=high feeding, D=diet and FL= feeding level, D*FL= diet and feeding level interactions, P values: ns (not significant, P>0.1), * (P<0.05).

The dietary CHO:LIP ratio influenced phytoplankton diversity in the ponds; it was higher (P<0.05) with the low CHO:LIP diet. The interaction of diet and feeding level influenced the abundance of zooplankton. With low CHO:LIP diet it decreased (P<0.05) with increasing feeding level. For the high CHO:LIP diet, the opposite happened (Table 6). Except for these two components, there was no effect of diet, feeding level and their interactions on the measured parameters of natural food in the pond (Table 6). Over the duration of the experiment, except for benthos diversity, all measured components of natural food increased over time (Supplementary Table 1).

Table 8. Effect of dietary carbohydrate to lipid ratio and feeding level on pond water quality

	Units	Low CHO:LIP diet	High CHO:LIP diet	Pooled SEM	P-values for Diet
Dissolved oxygen (DO)	$mg.L^{-1}$	3.4	3.5	0.1	ns
Temp	˚C	33	34	0.1	ns
pH	-	8	8	8.0	ns
Transparency	inch	9	9	0.1	ns
Water depth	inch	35	34	2.0	ns
Salinity	ppt	3	3	0.3	ns
TSS	$mg.L^{-1}$	286	276	14.6	ns
NO_2	$mg.L^{-1}$	0.011	0.012	0.004	ns
NH_4	$mg.L^{-1}$	0.19	0.17	0.053	ns

CHO:LIP = dietary carbohydrate to lipid ratio , P value: ns (not significant, P>0.1).

There was no effect of diet on the presence of natural food in the stomach of tilapia. Both the volume of natural food compared to the volume of the fish stomach, and the weight of natural food compared to the weight of the stomach content decreased with increased feeding level

(P<0.05). Also, there was no interaction effect of diet and feeding level on the measured parameters (Table 7). Index of relevance importance (IRI) is the measure of dominant natural food group consumed by the fish. IRI from the stomach content observations for both the diets were also the same. The IRI of food groups in the fish stomach were zooplankton, phytoplankton, crustaceans and molluscs, respectively. All the measured physical parameters of pond water were unaffected by the dietary CHO:LIP ratio and were within the optimum levels for pond aquaculture (Table 8).

3.4 Discussion

We investigated the effect of dietary non-protein energy (*i.e.* CHO:LIP ratio) on performance of tilapia in pond aquaculture, as well as its role on enhancing natural food in the pond as an indirect means of contributing to fish growth. Changing the dietary CHO:LIP ratio from 4.7 to 19.5 $g.g^{-1}$ by increasing the carbohydrate level did not affect fish growth. Also, at a fixed DP:DE (or C:N) ratio, the change in dietary energy source seems to have no impact on fish growth realized on natural food.

The experimental diets were formulated with the aim to achieve better performance from the low CHO:LIP diet. In this experiment that did not happen. Very few studies have been conducted with specific focus on dietary CHO:LIP ratio on tilapia. The few ones who did so observed better yield between the range of 2.0-6.5 $g.g^{-1}$ (Ali and Al-Asgah, 2001; Coutinho *et al.*, 2018; He *et al.*, 2015; Xie *et al.*, 2017). However, in general studies on the performance of tilapia in response to different levels of dietary carbohydrate and lipid give contradictory results. Increasing lipid as energy source at a fixed DP:DE ratio increased fish performance in some studies (Haidar *et al.*, 2018; Saravanan *et al.*, 2012), while the opposite has been observed also, *e.g.*, by Amirkolaie *et al.* (2006) and Tran-Duy *et al.* (2008). In the above studies, all tested diets were within the CHO:LIP ratio between 1.5- 7.0 $g.g^{-1}$. Xie *et al.* (2017) tested an extreme diet with a CHO:LIP ratio of 20, entirely excluding lipid, and noticed very poor performance. So, in addition to diet composition what else is impacting fish growth?

In the studies of Haidar *et al.* (2018) and Saravanan *et al.* (2012), fish were fed at satiation, while Amirkolaie *et al.* (2006) and Tran-Duy *et al.* (2008) fed fish restrictively. This means that fish performance showed a similar response when the feeding level was similar with a comparable dietary nutrient composition. The extreme CHO:LIP ratio (*i.e.* 19.5 $g.g^{-1}$) in the current study did not affect fish performance, which is contradictory to the findings of Xie *et al.* (2017). This can be due to two factors,

1. Xie *et al.* (2017) completely eliminated oil from the diet. The overall lipid content was only 27g per kg of feed . It can be questioned whether a shortage of dietary EFA might

have played a role. In contrast, the high CHO:LIP diet in the current study still contained 10g of fish oil and overall 32 g of lipid per kg feed.

2. Husbandry conditions might have impacted the outcome as well. The study of Xie *et al.* (2017) was in flow through tanks, with a daily water exchange of 50% of the tank volume. So, the fish had no access to natural food. The current study was conducted in outdoor ponds. In outdoor ponds fish have access to natural food in addition to the formulated feed which can stimulate the fish performance (Porchas-Cornejo *et al.*, 2012; Pucher and Focken, 2017; Rahman *et al.*, 2008; Roy *et al.*, 2012). Algae, that are abundant in fish ponds, can be a potential source of EFA for tilapia (Mizambwa, 2017; Patil *et al.*, 2007; Teuling *et al.*, 2017). Based on the stomach content analysis, phytoplankton was the second most important natural food in the diet of tilapia, and thus most likely gave tilapia access to additional EFA.

Therefore, in addition to the dietary CHO:LIP ratio, feeding level and husbandry conditions, (*i.e.* pond or tank due to presence of natural food), may determine the fish performance.

The different feed ingredients which provide the dietary macro-nutrients in fish feed, influence the ADC of the nutrients and thus ultimately also fish performance (Teuling *et al.*, 2017; Tran-Ngoc *et al.*, 2017). In the current study increasing the CHO:LIP ratio decreased the ADC of lipid and increased the ADC of carbohydrate. Fish oil was the main lipid ingredient source in the low CHO:LIP diet (Table 1), which is more digestible compared to plant based lipid. The observed difference in carbohydrate ADC between both diets is most likely related to the changes in carbohydrate composition. In the high CHO:LIP diet, the starch content was higher by 74 $g.kg^{-1}$ (347 *vs* 273 $g.kg^{-1}$) and the NSP content only by 23 $g.kg^{-1}$ (263 *vs* 240 $g.kg^{-1}$). Since compared to starch, NSP is poorly digestible in Nile tilapia (Amirkolaie *et al.*, 2006; Haidar, Petie, Heinsbroek, Verreth, & Schrama, 2016), this most likely explains the higher carbohydrate ADC at the high CHO:LIP diet. The opposite trend in fat ADC and carbohydrate ADC between both experimental diets did compensate each other, which most likely resulted in an equal energy ADC for both diets. This may have contributed to the fact that no difference in fish growth was observed in the current study. However, between both experimental diets the composition of the digestible energy coming from fat and carbohydrate strongly differed between both experimental diets. Recently it was shown in tilapia that digested fat is energetically more efficiently utilized than digested starch (Schrama *et al.*, 2018). However, in the current study this was not reflected in growth differences, which also might be due to differences in husbandry condition between experiments. Calculated N gain in de current study indicated that more than 50% growth was attained from the natural food of the pond (Fig. 2), which may have masked the effects of differences in digestible energy composition.

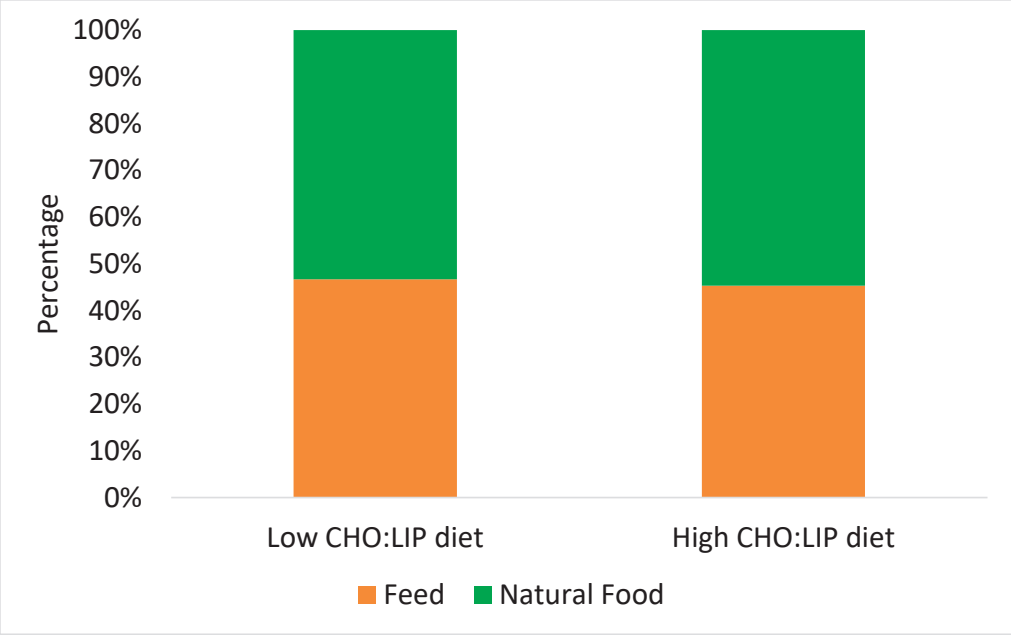


Fig. 2. Effect of dietary CHO:LIP ratio on the relative contribution of feed and natural food to N gain in fish

In pond aquaculture, natural food contributes between 40-65 % of total fish growth (Anderson *et al.*, 1987; Burford, Preston, Glibert, & Dennison, 2002; Burford *et al.*, 2004; Cam and Mariotti, 1991; Porchas-Cornejo *et al.*, 2012) depending on the amount of feed supplied. The level of contribution of natural food depends on the enhancement effect of the dietary nutrient inputs. Increasing the dietary C:N ratio to ~15, either by addition of carbohydrate besides a conventional feed (Asaduzzaman *et al.*, 2010; Avnimelech, 1999) or by lowering the dietary protein to energy ratio (Kabir *et al.*, 2019) can greatly increase this natural food contribution. In this study dietary C:N ratio was ~10 and hence the contribution of natural food (*i.e.* ~ 52%) is comparable with the lower margin of the previous studies (Kabir *et al.*, 2019). As there was no difference in the dietary C:N ratio (9.9 *vs* 11.1), the contribution of natural food to fish growth remained the same between the two diets and thus missing the potential natural food enhancement through increasing the dietary C:N ratio. Yet, in our study the composition of C was different which could impact the composition of the OM in the faeces and thus the availability of nutrients in the pond for the enhancement of natural food. In our study, the ADC of OM was unaffected by the dietary CHO:LIP ratio (Table 3). Moreover, the macro nutrient composition in the OM of the faeces (as measured for the ADC determination) did not change between the diets (Fig. 3). However, did the composition of OM in the faeces remain unaffected with changes in the CHO:LIP ratio in previous studies as well?

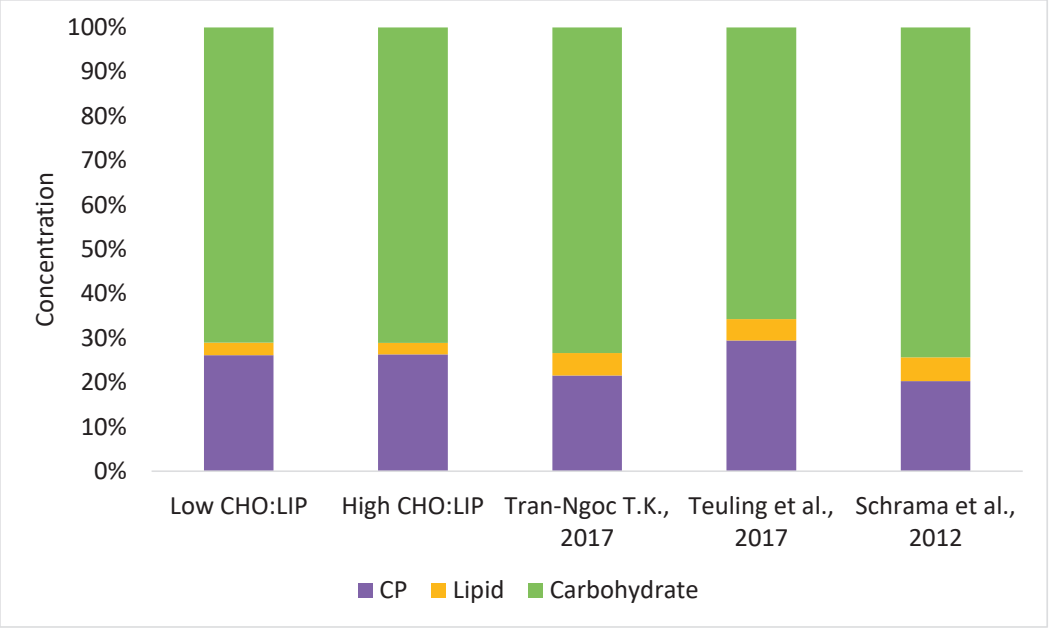


Fig. 3. Concentration of macro nutrients in the composition of organic matter (OM) of the faeces. "Low" and "high" CHO:LIP are from the current study; the other three are calculated values from the referred studies. For the referred studies, OM composition of faeces was based on the calculated mean value of all the experimental diets in these studies, where different sources of dietary non-protein energy were tested. CP=Crude Protein

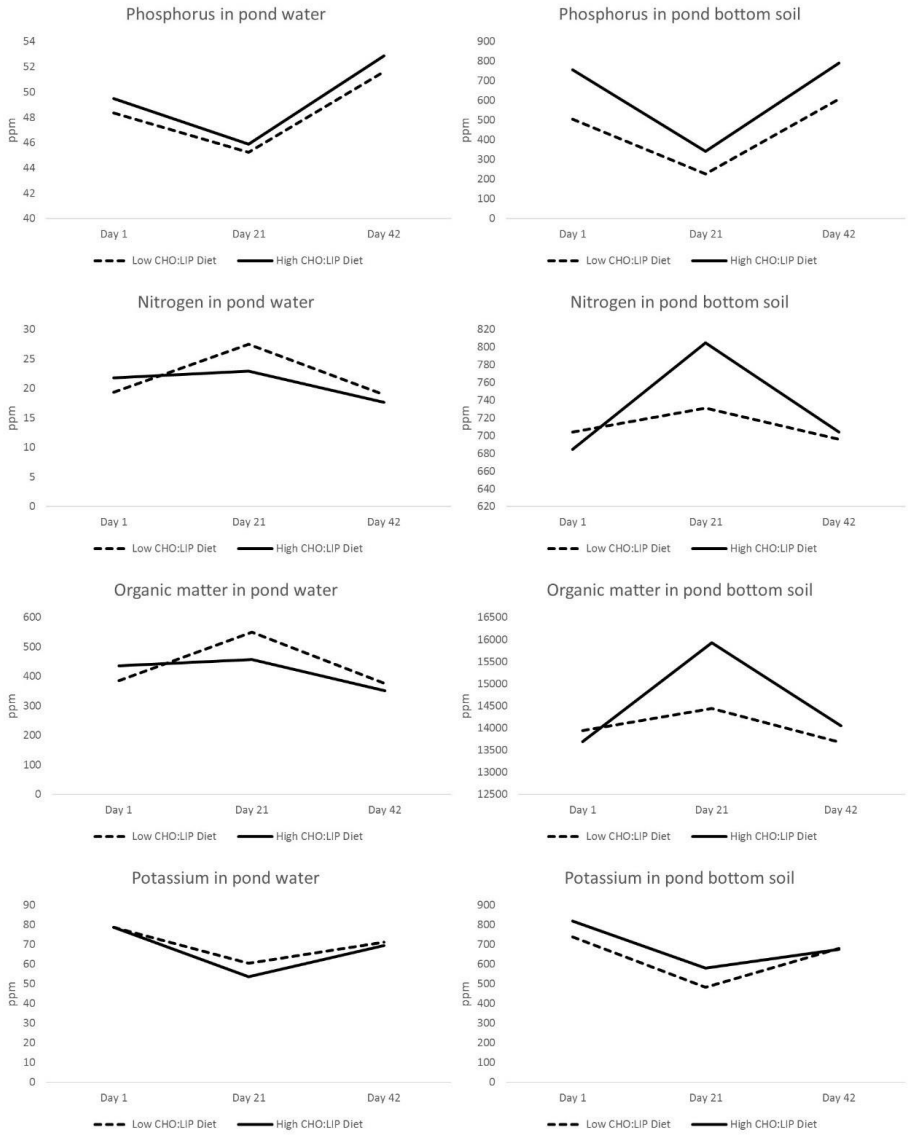
We calculated the macro nutrient concentration in the OM of the faeces in relation to different dietary CHO:LIP ratios in tilapia (Schrama *et al.*, 2012; Teuling *et al.*, 2017; Tran-Ngoc, 2017). The composition of macro nutrients in the OM of the faeces was comparable with the current study (Fig. 3). In addition to the CHO:LIP ratio, the composition of carbohydrate (starch *vs* NSP) can alter the ADC as well as the faeces composition (Haidar *et al.*, 2016). In this study NSP contents between the diets were comparable (240.2 *vs* 262.5 $g.kg^{-1}$). So, this did not impact the faeces composition.

As the nutrient input through the diets were the same, the ADC of CP and energy was the same, and the nutrients in the OM of the faeces entering in the pond were also the same, we did not notice differences in the growth of the fish (Table 2) and in the enhancement of natural food contribution to tilapia production (Fig. 2).

3.5 Conclusion

Changing CHO:LIP ratio within the tested range (4.7 *vs* 19.5 $g.g^{-1}$) does not affect the production of tilapia in pond culture in diets with a similar low DP:DE ratio. This study suggests

that lipid can be replaced by carbohydrate as a source of non-protein energy in tilapia pond culture without compromising growth performance. This finding might be useful to reduce tilapia feed cost in pond aquaculture.



Supplementary Fig. 1. Change in the concentration of pond water and soil nutrients during three sampling time (Day 1, 21 and 42)

Supplementary Table 1: Effect of dietary CHO:LIP ratio on the natural food in the pond at the different sampling time

Natural food types	Units	Low CHO:LIP diet			High CHO:LIP diet			Pooled SEM	P-values						
		ST 1	ST 2	ST 3	ST 1	ST 2	ST 3		ST	Diet	FL	D*FL	T*D	T*FL	T*D*FL
Chlorophyll a	$\mu g.L^{-1}$	0.002	0.003	0.024	0.002	0.003	0.021	0.003	***	ns	ns	ns	ns	ns	ns
Phytoplankton abundance	$ind.L^{-1}$	20069	16389	28792	14167	14236	33667	2627	***	ns	ns	ns	ns	ns	ns
Phytoplankton diversity	$genus.L^{-1}$	8.0	7.4	9.9	6.5	6.2	9.4	0.54	***	#	ns	ns	ns	ns	ns
Zooplankton abundance	$ind.L^{-1}$	6958	9042	8917	7833	9917	9500	787	*	ns	ns	*	ns	ns	ns
Zooplankton diversity	$genus.L^{-1}$	4.8	5.4	5.1	4.7	5.6	4.8	0.35	*	ns	ns	ns	ns	ns	ns
Benthos abundance	$ind.m^{-2}$	7975	5567	9350	9992	6575	11758	2088	*	ns	ns	ns	ns	ns	ns
Benthos diversity	$group.m^{-2}$	2.4	2.4	2.8	3.1	2.8	2.9	0.22	ns	ns	ns	ns	ns	ns	ns
Water bacteria	$CFU.\ ml^{-1}$	844	3137	4067	1166	3803	3589	290	***	ns	ns	ns	ns	ns	ns
Soil bacteria	$CFU.ml^{-1}$	644	1471	5000	656	1368	5343	139	***	ns	ns	ns	ns	ns	ns

CHO:LIP = dietary carbohydrate to lipid ratio , FL0= no feeding, FL1= low feeding , FL2=high feeding, D=diet and FL= feeding level, D*FL= diet and feeding level interactions, ST1=sampling time 1 (day 1), ST2=sampling time 2 (day 21), ST3=sampling time 3 (day 42), P values: ns (not significant, P>0.1), # (P<0.1-0.05), * (P<0.05), *** (P<0.001).

Chapter 4

Dietary non-starch polysaccharides influenced natural food web and fish production in semi-intensive pond culture of Nile tilapia

This chapter has been submitted for publication to “Aquaculture” as:

Kabir, K.A., Verdegem, M.C.J., Verreth, J.A.J., Phillips, M.J., Schrama, J.W., 2019. **Dietary non-starch polysaccharides influenced natural food web and fish production in semi-intensive pond culture of Nile tilapia**

Abstract

Dietary non-starch polysaccharide (NSP) changes the nutrient digestibility and faecal characteristics in fish. This study assessed the effect of the type of dietary NSPs on fish production and the contribution of natural food to the total fish production in semi-intensively managed tilapia ponds. Twelve ponds, each divided into three equally-sized compartments, were assigned to test the effect of type of dietary NSPs (*i.e.* "PecHem-Diet", a diet with easily fermentable NSP, *vs* "LigCel-Diet", a diet with slowly fermentable NSP). Fish were restrictively fed, based on the crude protein content of the feed. Three feeding levels ("no=0", "low=9 $g.kg^{-0.8}.d^{-1}$" and "high= 18 $g.kg^{-0.8}.d^{-1}$") nested in pond were analysed in a split plot design. Initial fish biomass was 3084 (±30) $g.compartment^{-1}$ and the experiment lasted 56 days. With the "LigCel-Diet" biomass gain was higher (2192 *vs* 2599 $g.compartment^{-1}$) and feed conversion ratio (FCR) was lower (1.9 *vs* 1.4; P<0.001) than with the "PecHem-Diet". Diet had no effect on fish survival and specific growth rate (SGR). For both the diets, increasing feeding level increased (P<0.001) biomass gain, fish survival, FCR and SGR. There was a significant interaction effect (P<0.05) between diet and feeding level on FCR. Fish body composition was the same in both the diets. With the "LigCel-Diet", the apparent digestibility coefficient (ADC) was higher (P<0.001) for crude protein, fat, phosphorus and calcium and lower (P<0.05) for ash compared to the other diet. Neither feeding level nor the interaction between diet and feeding level influenced the apparent digestibility coefficient (ADC) of any nutrient. Diet composition did not alter the organic matter (OM) composition of the faeces. $\Delta^{13}C$ and $\Delta^{15}N$ from the stable isotope analysis revealed that N gain in fish originated from both feed and natural food of the pond. Natural food abundance in the pond increased over time for both the diets. Chlorophyll-a was higher in the pond fed with "LigCel-Diet". Fish gut content and calculated N gain indicated an enhanced contribution of natural food to fish growth in ponds fed with "LigCel-Diet". In conclusion, the type of dietary NSP determines tilapia productivity in semi-intensive managed ponds by altering food web productivity.

4.1 Introduction

Global aquaculture production doubled during the last decade. This was mainly achieved in inland ponds. Because of the limited land area for aquaculture, growth was achieved by converting extensive into semi-intensive systems. This transformation required more feed to support the growth of aquaculture. As a result, total industrial feed production reached ~60 million metric ton (Tacon and Metian, 2015) and will continue to grow in the coming years. The supply of fishmeal and fish oil did not increase since 2000 (World Bank, 2013). So, fish feed composition shifted from fish-based ingredients to more plant-based ingredients to meet the demand. As a result of this change, aqua feeds today contain more carbohydrates, including non-starch polysaccharides (NSP) than before.

The current knowledge on nutrient requirements of fish, summarized in NRC (1993; 2011) is predominantly based on studies in which fish were kept in aquaria or cages. In these studies, the contribution of natural food to the fish production is minimal or absent. In ponds, which today are still the most common aquaculture production system, both diet composition and feeding level affect fish performance directly via digestion and absorption of the feed and indirectly via consumption of natural food, the latter stimulated by the feed waste acting as fertilizer. Kabir *et al.*, (2019) showed that a diet with a protein to energy ratio below the recommended level (NRC, 2011) increased total pond production via a stronger contribution of natural food. In a more recent study, our research team demonstrated that the type of non-protein energy (carbohydrates *vs*. lipid) in the diet did not affect the contribution of natural food to total pond production (manuscript submitted). It provides an opportunity to move to the direction of cheaper feed using carbohydrate as major source of dietary energy as lipid ingredients are expensive (Tacon and Metian, 2008). However, there are different types carbohydrate used in fish diet and can interfere the performance (Haidar *et al.*, 2016)

Starch and free sugars are dietary carbohydrates, which can be hydrolysed by fish enzymes and consequently absorbed. The remaining part of the carbohydrate fraction, the non-starch polysaccharides (NSPs), comprises among others lignin, cellulose, hemicellulose and pectin's (REF). NSPs are considered to have low nutritional value for fish because of their low digestibility and also due to their anti-nutritional properties (Francis *et al.*, 2001). Knowledge on the direct effects of dietary NSPs on fish performance is relatively scarce. However, comparison within and between studies showed that the type of NSP can have different effects on fish performance. For example, in Nile tilapia, guar gum strongly reduced growth compared to cellulose due to hampering the digestibility of protein and fat (Amirkolaie *et al.*, 2005). Additionally, the digestibility of NSPs can differ largely between different types of ingredients (Leenhouwers *et al.*, 2008; Teuling *et al.*, 2017). Such differences in digestibility between ingredients is more likely related to differences in fermentability of the type of NSPs.

This suggests that the type of NSPs can alter tilapia performance directly. The type of NSPs also alters faeces composition. In recirculation aquaculture systems (RAS), solid waste needs to be removed while in ponds it can act as an in-situ fertilizer stimulating the food web. In ponds, natural food contributes substantially to fish growth (Kabir *et al.*, 2019). Therefore, the effect on production of different types of dietary NSP can be very different in ponds compared to their effects in RAS or cages. However, information on the impact of the type of NSPs on fish performance in ponds including natural food, is absent.

In this study, the effect of type of dietary NSP on the productivity of tilapia cultured in ponds was assessed. It was hypothesised that the type of NSP regarding composition ("hemicellulose (Hem) and pectin (Pec)" versus "cellulose (Cel) and lignin (Lig)") would influence the productivity of the natural food in the pond due to difference in their fermentability. In ruminants, it is well known that the type of NSP (dietary fibre) influence the function of the rumen (microflora) through differences in fermentability (Jha and Berrocoso, 2015). The fermentability (degradation rate) between type of NSPs declines from pectin's to hemicellulose to cellulose and is lowest in lignin (Williams *et al.*, 2001). In this study, we wanted to explore if differences in types of dietary NSP regarding fermentability (slow *vs* quick) would affect pond productivity.

4.2 Methods

Two diets, with a contrast in the type of NSPs, were tested on Nile tilapia *(Oreochromis niloticus)* in 12 outdoor ponds (six per diet) for 56 days. Each pond consisted of three equally sized compartments, to which one of three different feeding levels were assigned according to a split plot design.

4.2.1 Diets

Two experimental diets were formulated that were equal regarding NSP content and the digestible protein to digestible energy ratio (DP:DE ratio; and in C:N ratio). Both diets met the nutrient requirements of tilapia, except for the DP:DE ratio being ±15 $g.MJ^{-1}$, which is below the recommended level for tilapia (NRC, 1993). This low DP:DE ratio was used to stimulate the productivity of the natural food web in the pond (Kabir *et al.*, 2019). Two diets were formulated to create a contrast in the type of NSP: A "PecHem-Diet" with quick/easy bio-degradable (fermentable) NSPs versus a "LigCel-Diet" with slow bio-degradable NSPs. For creating this contrast in the type of NSPs (fermentability) the qualification of dietary fibres by the Van Soest method (Van Soest *et al.*, 1991) was applied, which determines the acid detergent lignin (ADL), acid detergent fibre (ADF) and acid neutral detergent fibre (NDF). The ADL and ADF represent the lignin and cellulose part of the dietary fibre. Using the nutritional value ingredient tables of feedstuff database webapp (CVB, 2019), the "LigCel-Diet" was

Table 1. Ingredient and analysed chemical composition of the experimental Nile tilapia diets differing in the types of non-starch polysaccharides (NSP).

Ingredients		**"PecHem-Diet" (%)**	**"LigCel-Diet" (%)**
Soybean meal		12.00	
Wheat bran		23.57	
Wheat flour		20.90	18.97
De-oiled Rice bran (DORB)		6.30	12
Maize		18.00	17
Canola meal		12.00	
Sunflower meal			13.3
Palm kernel			18.5
Poultry meal			10.8
Fish meal (CP>68%)		2.00	3
Fish oil		2.00	4
Mono calcium phosphate (MCP)		1.50	1.50
Lime		1.00	
Vitamin/mineral premix[a]		0.45	0.45
DL Methionine (99%)		0.20	0.20
L-Lysine (HCL 79%)			0.20
Yttrium oxide (Y_2O_3)		0.08	0.08
Analysed composition			
Dry matter (DM),	($g.kg^{-1}$)	917	921
Crude protein	($g.kg^{-1}$ DM)	238	274
Fat	($g.kg^{-1}$ DM)	58	84
Ash	($g.kg^{-1}$ DM)	71	74
Phosphorus	($g.kg^{-1}$ DM)	11	13
Calcium	($g.kg^{-1}$ DM)	10	11
Carbohydrate[b]	($g.kg^{-1}$ DM)	633	568
Starch	($g.kg^{-1}$ DM)	323	277
NSP[c]	($g.kg^{-1}$ DM)	265	276
Acid detergent fiber	($g.kg^{-1}$ DM)	64	123
Acid detergent lignin	($g.kg^{-1}$ DM)	12	21
Neutral detergent fiber	($g.kg^{-1}$ DM)	189	238
Gross energy	($kj.g^{-1}$ DM)	19	20
DP:DE ratio[d]	($g.MJ^{-1}$)	14.2	15.8
C:N ratio[e]		12.3	10.8

[a] Commercial product.
[b]This is calculated as follows carbohydrate= 1000 – CP – Fat - Ash
[c]NSP, non-starch polysaccharides calculated
[d]Calculated based on the apparent digestibility coefficient obtained in this experiment
[e]This is calculated C:N ratio considering 16% N content in the protein and 47, 70 and 50% C content in protein, fat and carbohydrate, respectively (Waal and Boersma, 2012).

formulated to have a high ADL and ADF content in contrast to the “PecHem-Diet”, while keeping the NSP content of the diets equal. Thus, the contrast in the type of NSPs was mainly in the higher presence of pectin and hemicellulose in one diet and lignin and cellulose in the other diet. Therefore, the diets were defined as “PecHem-Diet” and “LigCel-Diet”. This contrast was created by including wheat bran and soya bean meal in the “PecHem-Diet” and palm kernel meal and sunflower meal in the “LigCel-Diet”. The ingredient and analysed nutrient composition of the experimental diets is given in Table 1. An inert marker, yttrium oxide (Y_2O_3), was included to test the apparent digestibility coefficient (ADC). The experimental diets were made at the R&D facilities of De Heus in Vietnam. Diets were extruded into a floating pellet with a 3 mm diameter.

4.2.2 Fish, rearing and housing facilities

All male, juvenile Nile tilapia *(Oreochromis niloticus)*, 14th generation WorldFish GIFT strain were collected from Asha Hatchery, at Bagerhat, in Bangladesh. Twelve outdoor ponds, each thirty square meters, in a field experimental station were used for this experiment. Each pond was divided into three equal compartments (Fig. 1). Water column of the pond was divided by a bamboo-frame fitted with 1mm mesh sized nets that allow well mixing of nutrients and dissolved solids within the compartments but prevents passing of the pellets and fish between the compartments. The bottom compartments were separated by concrete blocks of 105 cm height, of which 75 cm was in the soil and 30 cm extended above the pond bottom to minimize the exchange of uneaten feed and benthos between the compartments. All pond compartments were well aerated to ensure adequate dissolved oxygen in the pond water as well as good mixing of dissolved nutrients within and between the compartments.

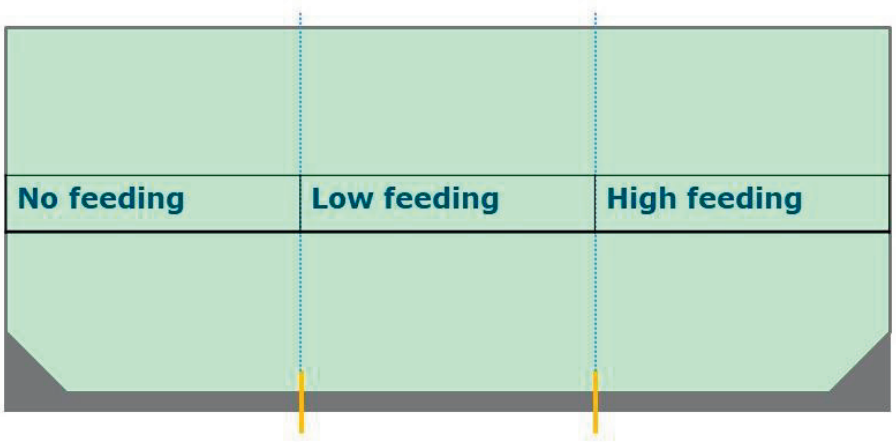


Fig. 1. Sketch of the pond compartments. The dotted line indicates the net and frame, solid bar indicates the concrete block and the grey bottom indicates the mud layer of the pond bottom

4.2.3 Experimental procedure

Prior to the experiment, ponds were dried by pumping out the water. Two hundred fifty g $CaCO_3$ was applied at the bottom soil of each pond compartment (PC) before water filling. After water filling, 40g Dolomite ($CaMg(CO_3)_2$) was spread over the water surface of each compartment. Ten g urea (CH_4N_2O) and 20g triple super phosphate (TSP), [$Ca(H_2PO_4)_2.H_2O$], per pond compartment (Rakocy and Mcginty, 1989) were applied 1 week after liming. Fish were stocked in the ponds 5 days after fertilization.

Forty juvenile tilapia ($4.m^{-2}$) were stocked per pond compartment. Fish were fed daily at 8.00 and 16.00 hours. The amount of protein (nitrogen) given to each pond was kept equal for both experimental diets. Fish were fed according to the mean metabolic body weight measured at stocking over all ponds and an expected growth rate. Per compartment within each pond, one of three feeding levels were applied: high (18 and 20.6 $g.kg^{-0.8}.d^{-1}$), low (9 and 10.3 $g.kg^{-0.8}.d^{-1}$) and no feeding. Due to differences in dry matter and protein content, the amounts of feed given differed between the diets, in order to supply the same amount of nitrogen per pond and within each feeding level.

Duration of the experiment was 56 days. Sampling for pond soil and water nutrients and natural food were done at day 1, 28 and 56.

4.2.3.1 Water quality monitoring

From day 1 onward, dissolved oxygen (DO), pH, total dissolved solid (TDS), transparency, temperature and salinity of each pond were measured daily at 6.00, 9.00, 10.00, 12.00, 14.00 and 14.30 hours by using a Lutron dissolved oxygen meter model PDO-519, Hanna instruments pocket tester HI98128-phep5, a Lutron conductivity meter model PCD-431, a Secchi disc, a Hanna digital thermometer model HI98501 and an Atago refractometer model MASTER-S28M instruments. NH_4^+, NO_2 and NO_3 were measured only at day 1, 28 and 56 by colourimetric, acc. to Neßler, with colour card and sliding comparator: 108025 | Nitrite Test, 111117 | Ammonium Test, 110022 | Nitrite Test; Merck KGaA, Darmstadt, Germany. Total suspended solids (TSS) of pond water from each compartment were also measured at day 1, 28 and 56 following the procedure of APHA methods # 2540 D (APHA, 1995)

4.2.3.2 Sampling and analysing soil and water nutrients

4.2.3.2.1 *Sample collection, processing and preparation*

Soil samples were collected from the top 20 cm layer of pond bottom at three points in each pond compartment and then mixed homogeneously. Approximately 1 kg wet soil was collected from each pond, labelled and packed in tight plastic bags, and transported to the laboratory. The collected samples were air dried, crumbled and grinded. The grinded samples

were preserved in labelled plastic containers until analysis. Water samples were collected, with a depth sampler of 10 cm width and 25 cm length, from each pond at the same 5 soil sampling locations, within 25 cm of pond surface, transferred and sealed in airtight bottles, and preserved at -20˚C until analysed.

4.2.3.2.2 *Analysis of the soil samples*

Organic carbon content of the soil was determined by Walkley and Black's wet oxidation method as described by Jackson (1973). Total nitrogen of the soil was determined by Micro-Kjeldahl's method following H_2SO_4 acid digestion and alkali distillation procedures as suggested by Jackson (1962). Total phosphorus of soil was determined colourimetrically by Vanado-molybdophosphoric yellow colour method in nitric acid system (Barton, 1948). The colour intensity was determined by the spectrophotometer at 470 nm light wavelength (Jackson, 1958). The available potassium was determined after extraction the soil samples with 1N NH_4OAc, pH-7.0 solution followed by the measurement of extractable K+ by Flame emission spectrophotometer (Model: Jenway, PEP-7) at 766 nm wave length using potassium filter, as outlined by Jackson (1973).

4.2.3.2.3 *Analysis* of the water samples

The organic carbon content of the water was determined by Tyrine's method as water commonly contains relatively smaller amounts of organic matter. As under dilute conditions Tyrine's method does not function well, the sample was dried first (Tyrine, 1965). The total inorganic nitrogen concentration was determined by the Micro-Kjeldahl method (Jones, 1991) and alkali distillation procedures as suggested by Jackson (1962). Available phosphorus was determined colourimetrically by molybdophosphoric blue colour method (Murphy and Riley, 1962). The available potassium of water was determined by a flame analyzer at 589 nm wavelength (Jackson, 1967).

4.2.3.3 Sampling and analysing plankton

Phytoplankton and zooplankton samples were collected at day 1, 28 and 56. Samples were collected between 9.00-11.00 hrs form 3 points, equally spaced on a diagonal line in each pond compartment. At each point 15 L water was passed through the 45 µm mesh plankton net, thus together 45 L of water from each compartment were sampled.

The concentrated samples were preserved in small plastic bottles with 5% buffered formalin. Diversity (group.L^{-1}) and abundance (ind.L^{-1}) estimations of plankton were done using a Sedgewick– Rafter (S-R) cell containing 1000 1-mm^3 cells. A 1 ml sample was put in the S-R cell and was left 15 min undisturbed to allow plankton to settle. The plankton in 10 randomly selected cells were identified where possible up to genus level and counted under a binocular

microscope (LABOMED America.inc; Lx 300). Planktons were identified using keys by Ward and Whipple (1959), Prescott (1962), Belcher and Swale (1976), and Bellinger (1992). Plankton abundance was calculated using the following formula:

$N = (P \times C \times 100)/V$.

Here, N is the number of plankton cells or units per litre (L) of pond water; P= the number of planktons counted in 10 fields of the S-R cell; C= the volume of final concentrate of the sample (ml); V= the volume of the pond water sample (L).

4.2.3.4 Sampling and analysing benthos

The benthic macroinvertebrate samples were also collected on day 1, 28 and 56 with an Ekman grab (area: 225 cm^2). In each pond compartment, bottom mud samples were collected from three different locations, which were then combined into a composite sample. Benthic macroinvertebrates were collected after filtering sediments through 4 different mesh sieve and preserved in a plastic vial containing a 10% buffered formalin. Identification keys used for benthic macroinvertebrates were Brinkhurst (1971), and Pinder and Reiss (1983). Benthic macroinvertebrates density was calculated using the formula:

$N = Y \times 10{,}000/3A$. Here, N=the number of benthic organisms per square meter; Y=total number of benthic organisms counted in 3 samples; A= Area of the Ekman dredge (m^2).

4.2.3.5 Sampling and analysing bacteria

In order to isolate and quantify bacterial communities, samples from both water and soil sediments were collected at day 1, 28 and 56. All samples were collected from three different locations of each pond compartment in sterile containers (15 ml tube, Falcon, USA), mixed homogenously before transported back to the Limnological Laboratory of the Environmental Science Discipline of Khulna University, Bangladesh. One ml water sample was transferred with a sterile pipette to a test tube containing 9 ml of phosphate buffered saline (PBS) and the tube was shaken thoroughly, while 5 g of each sediment and water samples were weighed and transferred to a sterile conical flask and made up to 50 ml with PBS and the contents were mixed thoroughly to prepare a stock solution. Serial dilution of up to 10−6 for water and 10−8 for sediment were prepared with PBS. Volumes (0.1ml) of each dilution were spread over the surface of duplicate plates of tryptone soya agar (TSA; Difco, Detroit, MI, USA) with incubation at 30 °C for 24–48 h. Plates with 30– 300 colony forming units (CFU) were counted with a Leica Quebec Dark field Colony Counter (Leica, Inc., Buffalo, NY, USA) and expressed as CFU/ ml.

4.2.3.6 Sampling and analysing Chlorophyll a

Water samples from the water column of three parts of the pond compartment were collected, well mixed, and kept into 500 ml bottles. The samples were transferred to the lab within an hour for analysis. There 250 ml of water was filtered through Whatman GF/C filter

paper. We then torn the filter paper into 5-6 pieces and inserted them into a 50 ml centrifuge tube. Thereafter, we added 20 ml of methanol into each tube to cover the filter paper pieces in it, shaken and vortexed until the filter paper was broken up. The samples were kept in the freezer overnight. The next day, they were centrifuged at 3200 rpm for 10 minutes. The supernatant was decanted into a 1 cm cuvette and the extinction was measured at both 665 nm and 750 nm (zero with methanol). Chlorophyll a was calculated as Chl-a ($ug.L^{-1}$) = ((Abs [665nm]-Abs [750nm]) x A x Vm)/ Vf x L. Here, A = absorbance coefficient of chlorophyll-a in methanol (12.63); Vm = volume of methanol used for extinction (ml); Vf = litres of water filtered; and L = path length of cuvette.

4.2.3.7 Sampling and analysing proximate composition of fish and feed

Initial body composition was determined in 25 fish, which were randomly selected at the start of the experiment. For final body composition, five fish were randomly selected per compartment at the end of the experiment. Fish, which were used for body composition analysis, were euthanized by an overdose of a phenoxy-ethanol solution ($1.0ml.L^{-1}$) and stored at −20°C. Before chemical analysis, the sampled fish were cut into small pieces, homogenised by grinding in a mincing machine twice through a 4·5 mm screen grinder and subsequently oven-dried. Chemical analyses were done in triplicate. Dry matter (DM) was determined gravimetrically after drying at 103°C for 4 and 24 h (h) until constant weight, respectively, for feed and fish samples (ISO 6496, 1983). Crude ash was determined after incineration at 550°C for 4 h (ISO 5984, 1978). Crude protein (CP) was determined by the Kjeldahl method (ISO 5983, 1979) and calculated by multiplying the measured N content by 6.25. Fat was quantified by petroleum–diethyl ether extraction (ISO 6492, 1999). Before fat analysis, feed samples were hydrolysed by boiling for 1 h with 3 M-HCl. Dietary energy content was measured by direct combustion in an adiabatic bomb calorimeter (IKA-C-7000; IKA analysentechnik, Weitersheim, Germany). Starch including free sugars was enzymatically determined in feed and faecal samples by using amyloglucosidase without the ethanol extraction step and measuring glucose content as described in Goelema *et al.*, (1998). NSP content was calculated as total carbohydrates – "Starch + free sugars". Acid detergent lignin (ADL), acid detergent fibre (ADF) and acid neutral detergent fibre (NDF) were estimated following the method of Van Soest *et al.* (1991). Yttrium, P and Ca in feed and faeces were analysed using inductively coupled plasma-mass spectrometry (ICP- OES) according to the standard NEN 15510 (2007).

4.2.3.8 Faeces collection and preservation

After ending the pond experiment, 180 tilapia with mean body weight of 161 (±31) g were restocked in the indoor concrete tanks for faeces collection to determine apparent digestibility. There were 18 tanks of 1000 L water holding capacity, filled with 700 L water. Ten fish were allocated in each tank. All tanks were aerated. Both experimental diets were fed at

6, 9 and 12 g.kg$^{-0.8}$d^{-1} with 3 replications per treatment. Fish were fed daily at 7.00 and 15.00 hours. The 1st seven days, fish were fed in the tank for acclimation to tank condition and diets. Starting from day 8, faeces were collected by siphoning 3 hours after each feeding for 10 days. Collected faeces were preserved in labelled plastic pots at -20˚C. Later all samples from the same tank were pooled together for chemical analysis.

4.2.3.9 Analysis of stomach contents

Fish for stomach content were harvested on day 57, 19 hours after the last feeding, to ensure that no pellet remained in the stomach. Fish were euthanized by an overdose of a phenoxy-ethanol solution (1.0ml.L^{-1}) and transported to the laboratory. In the laboratory the fish were dissected to collect the stomach and preserve it in 10% formalin. Total volume of the stomach and the number of food items were recorded. Volume of food items occupying in general and by each food group were visually estimated (Jude, 1971). Total weight of food was expressed as percentage of weight of the stomach on a wet weight basis (Gibbons & Gee, 1972). Index of relative importance (IRI) of observed natural food groups was estimated by diet to understand the relative importance of natural food group in the growth of fish following the methods described by Pinkas *et al.*, (1971) and Prince (1975).

IRI = (%G_n + %G_v) x %G_f. Where, G_n is percentage by group number, G_v is volume of group number and G_f is frequency of occurrence by the group number.

4.2.3.10 Sample collection and chemical analysis of ^{13}C and ^{15}N stable isotope:

All samples were collected on day 57 (after completion of the feeding trial). Plankton were collected by pumping pond water for 5 minutes through plankton net of 45µ mesh size. Three fish from each pond compartment were isolated, euthanized by an overdose of a phenoxy-ethanol solution (1.0ml.L^{-1}) and transported to the laboratory. In the laboratory the fish were degutted in order to take out egested feed. Afterwards, the degutted fish were oven dried and grinded by using a bullet mill (100-200 µm) to ensure isotopic homogeneity. Dry matters from three fish was pooled together to make one composite sample per pond compartment. Then, samples were analysed for dry matter (DM) according to AOAC (1990). For total nitrogen (TN), and total carbon (TC) content, and isotopic enrichment by an EA Elemental Analyzer (Euro Vector, HEKAtech, Wegberg, Germany) coupled to an isotope ratio mass spectrometer (Delta Plus Advantage, THERMO, Bremen, Germany). Isotopic ratios were expressed relative to international standards (Vienna Pee Dee Belemnite, VPDB, for carbon and atmospheric N_2 for nitrogen).

4.2.4 Analytical procedures and calculations

4.2.4.1 Performance

Biomass gain (g) was calculated as the difference between the biomass stocked and biomass harvested per compartment. The specific growth rate (SGR) was calculated as $SGR=((\ln(IndBW_{56})-\ln(IndBW_0))/56)\times100$; where $IndBW_{56}$ and $IndBW_0$ means individual body weight at day 56 and day 0. Feed conversion ratio (FCR) was calculated per compartment using the feed given and weight gain. The survival of fish per compartment was calculated as $(N_f/N_i)\times100$, where N_f is the final number of fish and N_i the initial number at pond compartment (PC) level.

4.2.4.2 ADC Calculation:

The apparent digestibility coefficients of nutrients were measured for each tank using Y_2O_3 as an inert marker. The yttrium content of feed and faeces was analysed using inductively coupled plasma-mass spectrometry (ICP- OES) according to the standard NEN 15510 (2017). Apparent digestibility coefficients (ADCs) of the dietary components in the diets were calculated by using the following formula:

$$\%\ ADC_{diet} = 100\%*(1-[Y_{diet}/Y_{faeces}]*[N_{faeces}/N_{diet}])$$

Here, Y_{diet} and Y_{faeces} are the content of the inert marker (yttrium) in the diet and faeces, respectively ($g.kg^{-1}$ DM); and N_{faeces} and N_{diet} are the contents of the dietary components in the faeces and diets, respectively ($g.kg^{-1}$ DM).

4.2.4.3 Fish N gain calculation:

N gain in fish was calculated by the difference between the Nh and Ns. Here, Nh is the amount of N in the harvested fish biomass and Ns is the amount of N in the biomass at start. N feed was calculated by total feed input per compartment, multiplied by the N content in feed. Contribution of feed N to fish N gain was calculated based on the ADC of CP from this study and considering average N retention efficiency (RE) of 40% (Azevedo *et al.*, 2004) for both the diets at all feeding levels. N retained from natural food was calculated by deducting N retention from feed from the total N gain in fish.

4.2.4.4 Calculation of isotope ratios:

Isotope ratios were compared by using a δ^hX value, obtained by using Formula 1 (Fry, 2006; Peterson and Fry, 1987).

$$\delta^H X = \left(\frac{Rsample}{Rstandard} - 1\right) * 1000\ (‰) \text{ (Formula 1)}$$

Here, H the atomic mass, X the atom, R_{sample} the isotope concentration of the sample and $R_{standard}$ a standard value which is for $^{15}N/^{14}N$ based on the concentration in the air (0.0036765) and for $^{13}C/^{12}C$ based on PeeDee Belemnite (0.011180) (Fry, 2006)

4.2.5 Statistical analysis

The data were analysed using the IBM SPSS software package version 23. All data, except water quality and ADC, were analysed in split plot design using the procedure general linear model (GLM). Effect of diet has been tested against the variation between ponds while the effects of feeding level and diet by feeding level interaction were tested against the variation between compartments within the pond. Univariate analysis was done to see the effect of diet on water quality at pond level only. Effect of diet, feeding level and their interaction on ADC of nutrients were tested by two-way ANOVA following the procedure general linear model (GLM). When a significant interaction effect was present, post hoc multiple comparisons of means using Tukey's multiple range test was performed.

4.3 Results

Average individual body weight (BW) at stocking was 77g, unaffected by diet and feeding level. At pond level, biomass gain was 18.5% higher ($P<0.05$) with the "LigCel-Diet", while FCR was 25% lower ($P<0.001$), compared to the other diet. Biomass harvested, biomass gain per compartment, individual gain, fish survival, FCR and growth rate increased with feeding level ($P<0.001$; Table 2). The interaction effect between diet and feeding levels influenced FCR ($P<0.05$), and also tended ($P<0.1$) to influence biomass harvested and biomass gain (Table 2). With increasing feeding level, the difference between both diets became larger.

The apparent digestibility (ADC) of ash, crude protein, fat, phosphorus and calcium were affected by the type of dietary NSP. The ADCs of these nutrients were higher at the "LigCel-Diet" then at the "PecHem-Diet". There was a tendency ($P<0.1$) for higher ADC of carbohydrate at the "PecHem-Diet" and for energy an opposite tendency was observed. Feeding level and the interaction between feeding level and diet did not influence any of the nutrient ADCs (Table 3).

The type of dietary NSP did not affect body composition, but feeding level influenced protein and ash content ($P<0.05$; Table 4). Protein and ash content increased with feeding level. The interaction effect between diet and feeding level also did not affect body composition (Table 4).

The comparison of the carbon (^{13}C) and nitrogen (^{15}N) stable isotope (δC:δN) signature of the experimental diets, fish and natural food web items are presented in figure 2. Diets were equal in δC and had a small difference in δN. The δC and δN content of the food web items (plankton as well as periphyton) overlap strongly, did not differ between ponds fed the different diets and overlapped with the signature of both diets. The δC and δN content of fish did not differ between both experimental diets.

Table 2. Effect of type of dietary non-starch polysaccharides (NSP) and feeding level on performance of tilapia

		"PecHem-Diet"			"LigCel-Diet"			Pooled	P-values		
	Units	FL0	FL1	FL2	FL0	FL1	FL2	SEM	D	FL	D*FL
Initial individual BW	g	77	77	77	77	77	77	0.7	ns	ns	ns
Biomass stocked	$g.comp^{-1}$	3097	3068	3089	3073	3083	3092	30	ns	ns	ns
Biomass harvested	$g.comp^{-1}$	3669	5466	6695	3824	5738	7483	139	*	***	#
Individual BW Gain	g	46	84	104	51	87	114	7.3	ns	***	ns
Biomass gain	$g.comp^{-1}$	572	2398	3607	752	2654	4391	150	*	***	#
Survival	%	76	86	93	77	88	98	3.0	ns	***	ns
FCR	$g.g^{-1}$		1.40	2.45		1.13	1.73	0.076	***	***	*
Growth Rate	$g.d^{-1}$	0.8	1.5	1.8	0.9	1.6	2.0	0.10	ns	***	ns

"PecHem-Diet", a diet with quick/easy bio-degradable (fermentable) NSP, "LigCel-Diet", a diet with slow bio-degradable (fermentable) NSP, FL0= no feeding, FL1= low feeding level, FL2=high feeding level, D=diet and FL= feeding level, D*FL= diet and feeding level interactions, BW= body weight, FCR= feed conversion ratio, Comp=compartment, P values: ns (not significant, P>0.1), # (P<0.1), * (P<0.05), *** (P<0.001).

Table 3. Effect of type of dietary non-starch polysaccharides (NSP) and feeding level on apparent digestibility coefficient (ADC) in tilapia

	Units	"PecHem-Diet"			"LigCel-Diet"			Pooled	P-values		
		FL0	FL1	FL2	FL0	FL1	FL2	SEM	D	FL	D*FL
Crude ash	%	-16	-06	-30	-01	-05	-02	7.0	*	ns	ns
DM	%	61	65	60	63	63	65	2.0	ns	ns	ns
Crude protein	%	77	79	77	81	81	82	1.0	***	ns	ns
Fat	%	87	87	86	90	91	91	1.0	***	ns	ns
Energy	%	67	70	67	69	70	72	2.0	#	ns	ns
Carbohydrate	%	61	66	62	58	59	62	2.0	#	ns	ns
P	%	39	42	40	51	53	54	2.0	***	ns	ns
Ca	%	-4	3	-5	10	14	14	4.0	***	ns	ns

"PecHem-Diet", a diet with quick/easy bio-degradable (fermentable) NSP, "LigCel-Diet", a diet with slow bio-degradable (fermentable) NSP, FL0= no feeding, FL1= low feeding level, FL2=high feeding level, D=diet and FL= feeding level, D*FL= diet and feeding level interactions, P values: ns (not significant, P>0.1), # (P<0.1), * (P<0.05), *** (P<0.001).

Table 4. Effect of type of dietary non-starch polysaccharides (NSP) and feeding level on body composition of tilapia

	Units	"PecHem-Diet"			"LigCel-Diet"			Pooled	P-values		
		FL0	FL1	FL2	FL0	FL1	FL2	SEM	D	FL	D*FL
DM	$g.kg^{-1}$	280	288	308	280	294	281	7.5	ns	#	ns
Protein	$g.kg^{-1}$	151	153	163	153	157	155	2.4	ns	*	ns
Fat	$g.kg^{-1}$	49	51	57	50	53	52	2.0	ns	ns	ns
Ash	$g.kg^{-1}$	52	58	63	55	62	60	2.4	ns	*	ns

"PecHem-Diet", a diet with quick/easy bio-degradable (fermentable) NSP, "LigCel-Diet", a diet with slow bio-degradable (fermentable) NSP, FL0= no feeding, FL1= low feeding level, FL2=high feeding level, D=diet and FL= feeding level, D*FL= diet and feeding level interactions, P values: ns (not significant, P>0.1), # (P<0.1), * (P<0.05), *** (P<0.001).

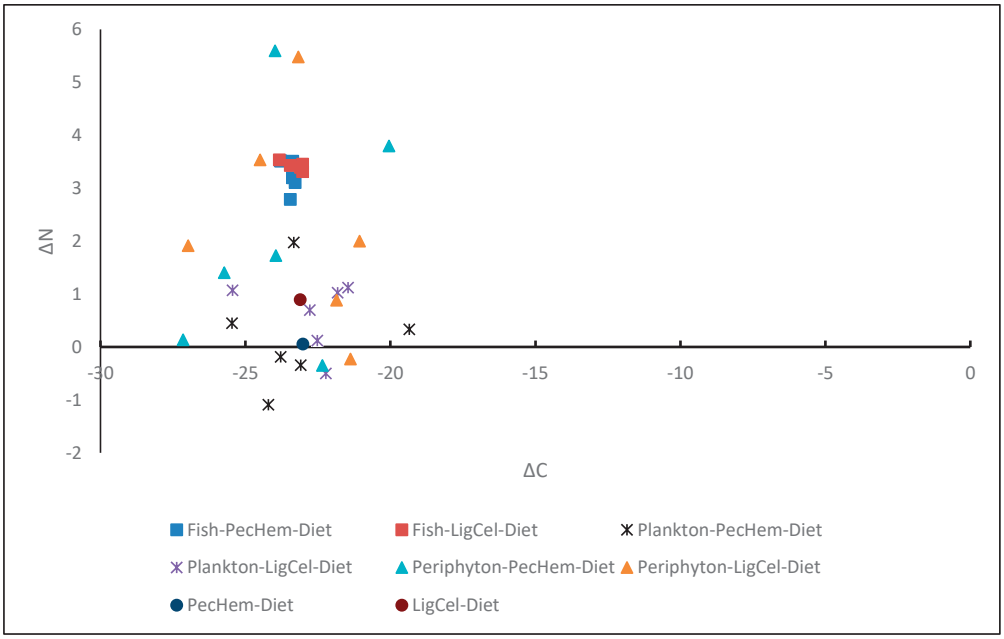


Fig. 2. Effect of type of dietary non-starch polysaccharides (NSP) on the distribution of δC:δN in feed, plankton, periphyton and fish body.

Here, Fish-"PecHem-Diet" = fish fed with "PecHem-Diet", Fish-"LigCel-Diet" = fish fed with "LigCel-Diet", Plankton-"PecHem-Diet" = plankton samples collected from the pond fed with "PecHem-Diet", Plankton-"LigCel-Diet" = plankton samples collected from the pond fed with "LigCel-Diet", Periphyton-"PecHem-Diet" = periphyton samples collected from the pond fed with "PecHem-Diet", Periphyton-"LigCel-Diet" = periphyton samples collected from the pond fed with "LigCel-Diet", PecHem-Diet"= a diet with quick/easy bio-degradable (fermentable) NSP, "LigCel-Diet"= a diet with slow bio-degradable (fermentable) NSP,

Irrespective to diets, nutrients (N, P, K and organic matter) of pond soil and water changed ($P<0.001$) over the time (Table 5). N content of pond water was higher ($P<0.05$) with the "PecHem-Diet" compared to the "LigCel-Diet", and tended to be higher for water organic matter content ($P<0.1$). The 3-way interaction effect between sampling time, diet and feeding level influenced the soil N content, while the interaction effect between sampling time and diet affected the water N content (Table 5). Furthermore, the interaction effect between sampling time and diet influenced the water organic matter content (Table 5). For all these interaction effects with time, the effect between treatments (diets) was largest at the last sampling moment (end of the experiment).

Sampling time influenced chlorophyll a, phytoplankton (abundance & diversity), zooplankton and benthos abundance and total bacterial count in both pond water and soil (Table 6). Chlorophyll a content of water was higher at the "LigCel-Diet". The difference in Chlorophyll a increased with time, indicated by the significant interaction effect between sampling time and diet. The interaction between sampling time and diet also tended ($P<0.1$) to influence benthos abundance and total count of soil bacteria. The interaction effect between sampling time and feeding level influenced ($P<0.05$) the total bacterial count of water and tended to influence ($P<0.1$) the soil bacteria count as well. Total soil bacteria count also showed a tendency ($P<0.1$) to be influenced by the interaction effect between sampling time and feeding level.

The effect of diet and feeding level on stomach fullness with natural food, both volumetric and gravimetric, is given in Table 7. Volumetrically, the presence of natural food in the stomach was higher ($P<0.05$) and gravimetrically it tended to be higher ($P<0.1$) at "LigCel-Diet" compared to the "PecHem-Diet". Gravimetrically, the interaction effect between diet and feeding level tended to influence the presence of natural food in the fish stomach, showing an increased stomach fullness at the higher feeding levels at the "LigCel-Diet". IRI, the indicator of relative importance of natural food group in the diet of fish, from the stomach content observation for both the diets showed that phytoplankton, zooplankton and crustaceans, respectively, are the important natural food groups for tilapia for both diets (data no shown).

Table 5. Effect of type of dietary non-starch polysaccharides (NSP) and feeding level on soil and water nutrients

	Nutrients	Units	"PecHem-Diet"			"LigCel-Diet"			Pooled SEM	P-values						
			ST1	ST2	ST3	ST1	ST2	ST3		ST	D	FL	D*FL	ST*D	ST*FL	ST*D*FL
Water	Nitrogen	$mg.L^{-1}$	13	22	24	13	19	22	0.7	***	*	ns	ns	*	ns	ns
	Phosphorus	$mg.L^{-1}$	37	34	38	39	35	39	1.6	***	ns	ns	ns	ns	ns	ns
	Potassium	$mg.L^{-1}$	68	42	59	70	45	63	1.5	***	*	#	ns	ns	ns	ns
	Organic matter	$mg.L^{-1}$	256	445	489	266	376	445	14	***	#	ns	ns	***	ns	ns
Soil	Nitrogen	$mg.L^{-1}$	0.08	0.09	0.08	0.08	0.10	0.09	0.002	***	ns	ns	ns	ns	ns	*
	Phosphorus	$mg.L^{-1}$	582	505	694	762	748	924	111	***	ns	ns	ns	ns	ns	ns
	Potassium	$mg.L^{-1}$	733	526	627	742	581	676	19	***	*	ns	ns	ns	ns	ns
	Organic matter	$g.L^{-1}$	15.6	17.9	17.3	16.2	18.5	17.8	0.31	***	ns	ns	ns	ns	ns	ns

"PecHem-Diet", a diet with quick/easy bio-degradable (fermentable) NSP, "LigCel-Diet", a diet with slow bio-degradable (fermentable) NSP, FL0= no feeding, FL1= low feeding level , FL2=high feeding level, ST= sampling time (day), D=diet and FL= feeding level, D*FL= diet and feeding level interactions, P values: ns (not significant, P>0.1), # (P<0.1), *(P<0.05), *** (P<0.001).

Table 6. Effect of type of dietary non-starch polysaccharides (NSP) and sampling time (ST) averaged over feeding levels natural food items in ponds (expressed per compartment)

	Units	"PecHem-Diet"			"LigCel-Diet"			Pooled SEM	P-values						
		ST1	ST2	ST3	ST1	ST2	ST3		ST	D	FL	D*FL	ST*D	ST*FL	ST*D*FL
Chlorophyll a	$\mu g.L^{-1}$	4	3	5	6	4	16	2	***	*	ns	ns	*	ns	ns
Phytoplankton abundance	$Ind.ml^{-1}$	235	419	697	252	422	913	119	***	ns	ns	ns	ns	ns	ns
Phytoplankton Diversity	$group.L^{-1}$	12	11	13	11	12	14	0.7	*	ns	ns	ns	ns	ns	ns
Zooplankton abundance	$Ind.ml^{-1}$	137	90	195	94	82	134	31	*	ns	ns	ns	ns	ns	ns
Zooplankton Diversity	$group.L^{-1}$	7	6	7	7	7	6	0.50	ns	ns	ns	ns	ns	ns	ns
Benthos abundance	$Ind.L^{-1}$	52	83	82	70	62	81	16	*	ns	ns	ns	#	ns	ns
Benthos diversity	$group.L^{-1}$	2.7	3.0	2.7	2.9	2.7	2.8	0.22	ns	ns	ns	ns	ns	ns	*
Water bacteria	$CFU.ml^{-1}$	478	704	2458	464	723	2447	165	***	ns	#	ns	ns	*	ns
Soil bacteria	$CFU.ml^{-1}$	606	1455	3849	472	1578	4481	166	***	ns	ns	ns	#	#	ns

"PecHem-Diet", a diet with quick/easy bio-degradable (fermentable) NSP, "LigCel-Diet", a diet with slow bio-degradable (fermentable) NSP, FL0= no feeding, FL1= low feeding level, FL2=high feeding level, ST= sampling time (day), D=diet and FL= feeding level, D*FL= diet and feeding level interactions, Ind. = Individual, P values: ns (not significant, P>0.1), # (P<0.1), * (P<0.05), *** (P<0.001).

Table 7. Effect of type of dietary non-starch polysaccharides (NSP) and feeding level on the amount of natural food content in the stomach of tilapia

	Units	"PecHem-Diet"			"LigCel-Diet"			Pooled SEM	P-values		
		FL0	FL1	FL2	FL0	FL1	FL2		D	FL	D*FL
Volumetric occurrence of natural food	%	26	23	21	31	28	29	2.5	*	ns	ns
Gravimetric occurrence of natural food	%	30	21	24	22	32	31	3.8	#	ns	#

"PecHem-Diet", a diet with quick/easy bio-degradable (fermentable) NSP, "LigCel-Diet", a diet with slow bio-degradable (fermentable) NSP, FL0= no feeding, FL1= low feeding level, FL2=high feeding level, D=diet and FL= feeding level, D*FL= diet and feeding level interactions, P values: ns (not significant, P>0.1), # (P<0.1), * (P<0.05).

All the measured physical parameters of pond water quality were unaffected by diet (*i.e.*., type of the dietary NSPs) and were within the accepted level for tilapia cultured in ponds (Table 8).

Table 8. Effect of type of dietary non-starch polysaccharides (NSP) and feeding level on pond water quality

	Units	"PecHem-Diet"	"LigCel-Diet"	Pooled SEM	P-values for Diet
Dissolved oxygen (at morning)	$mg.L^{-1}$	5.4	5.4	0.0	ns
Temperature	˚C	30	30	0.1	ns
pH	-	7.6	7.6	0.0	ns
Transparency	cm	33	33	0.5	ns
Water depth	cm	107	109	3.6	ns
Salinity	ppt	1.9	2.0	0.42	ns
Total suspended solid	$mg.L^{-1}$	325	323	23	ns
Total dissolved solid	$mg.L^{-1}$	4121	4062	196	ns
NO_2	$mg.L^{-1}$	0.011	0.012	0.004	ns
NH_4	$mg.L^{-1}$	0.19	0.17	0.053	ns

"PecHem-Diet", a diet with quick/easy bio-degradable (fermentable) NSP, "LigCel-Diet", a diet with slow bio-degradable (fermentable) NSP, FL0= no feeding, FL1= low feeding level, FL2=high feeding level, D=diet and FL= feeding level, D*FL= diet and feeding level interactions, P values: ns (not significant, P>0.1).

4.4 Discussion

In this study, the effect of type of dietary non-starch polysaccharides (NSP) on the productivity of tilapia cultured in ponds was assessed. It was hypothesised that the type of NSP regarding fermentability (slow *vs* quick; *e.g.*, "hemicellulose and pectin's" versus "cellulose and lignin") would influence the productivity of the pond food web. The experimental results demonstrate that the type of dietary NSPs can influence pond productivity in tilapia mono-culture. This impact on productivity seems to be related to enhancement of the natural food in ponds fed with the "LigCel-Diet", as differences were observed in concentration of water chlorophyll-a, benthos abundance and total count of soil bacteria, and natural food content in fish stomach (Table 6 and 7).

The differences in productivity at pond level between diets (*e.g.*, biomass gain; Table 2) was not due to a different input of nutrients via feeding. Ponds were all fed the same amount of protein (nitrogen) based on the analysed dietary crude protein content (Fig. 3). Although the C:N ratio of both diets were almost equal, the energy (C input) given to ponds at the "PecHem-Diet" was slightly higher compared to ponds at the "LigCel-Diet" due to a small numerical difference in C:N ratio. Studies on dietary protein to energy ratio by Kabir *et al.* (2019) demonstrated that lowering this ratio (*i.e.*, increasing the C:N ratio) increased pond productivity by enhancing the food web. Consequently, the small difference in dietary C:N ratio between the experimental diets might have reduced the observed impact of type of dietary NSP in the current study. Next to N, P input via the feed into the ponds was identical between diets.

To determine if the effects of the type of dietary NSP on pond productivity were due to differences in nutrient digestibility (ADC), the ADC of macro-nutrients were determined in fish kept in tanks without the presence of the natural food web (Table 3). The measured ADC of macronutrients showed that diets were not only different regarding the type of NSP, but also regarding their digestibility. The observed differences in ADC between both diets in protein and fat are most likely due to the fact that diets were largely different regarding the composition of ingredient that provided the dietary fat and crude protein (Table 1). It is well

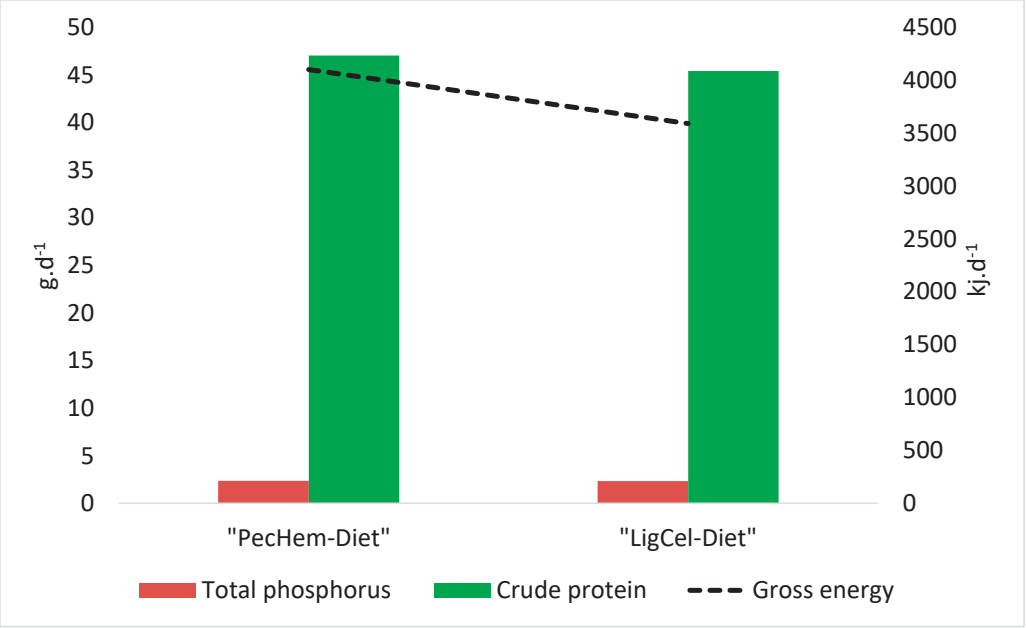


Fig.3. Daily input of dietary crude protein, gross energy and total phosphorus in each pond. "PecHem-Diet", a diet with quick/easy bio-degradable (fermentable) NSP, "LigCel-Diet", a diet with slow bio-degradable (fermentable) NSP.

known, that ingredient composition is a major determinant in feed quality, *i.e.*., digestibility (Glencross *et al.*, 2007). However, the higher crude protein and fat ADC at the "LigCel-Diet" might also be due to a direct effect of the type of dietary NSPs. Water soluble NSP, mostly originating from pectin's and hemicellulose, affect dietary viscosity (Leenhouwers *et al.*, 2007). Various studies in fish have demonstrated that increasing dietary viscosity can negatively affect digestibility of other macronutrients (Amirkolaie *et al.*, 2005; Tran-Tu *et al.*, 2019, 2018). Opposite to crude protein and fat ADC, carbohydrates tended to have a higher ADC with the "PecHem-Diet" compared to the "LigCel-diet", which is fully in line with the higher fermentability of pectin's and hemicellulose compared to cellulose and lignin. This is also in line with findings in tilapia that diets/ingredients rich in pectin's and hemicellulose have higher carbohydrate and NSP ADC compared to cellulose rich diets (Amirkolaie *et al.*, 2005; Haidar *et al.*, 2016; Maas *et al.*, 2019). Faecal starch content was not measured in this study, but when assuming a constant starch ADC for both diets of 98%, the calculated NSP ADC in the current study was 17% at the "PecHem-Diet" and 22% at the "LigCel-Diet". This shows, like in other studies in Nile tilapia (Haidar *et al.*, 2016; Leenhouwers *et al.*, 2008; Maas *et al.*, 2019), that

NSP are not inert. Besides, the available phosphorus as a result of low ADC with “PecHem-Diet” (Table 3) might have influenced also fish performance. The difference in macronutrient ADC were small between diets, but still could have played a role in the observed difference in pond productivity due to an altered faeces composition having a fertilization effect on the natural food and/or direct uptake of nutrient (especially protein) for growth.

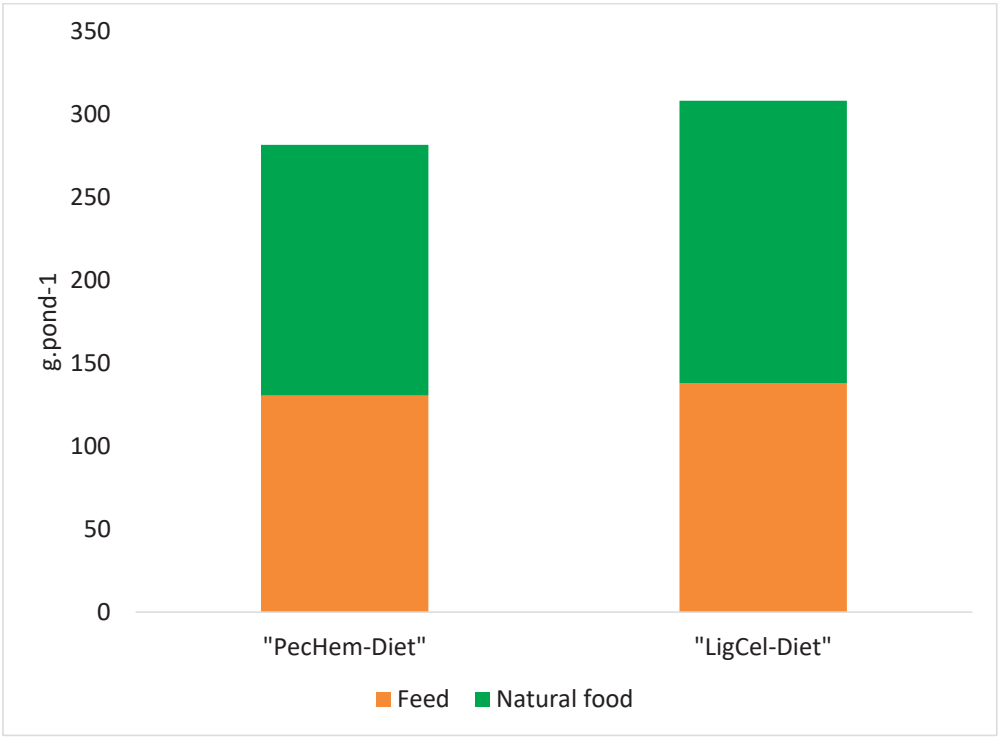


Fig.4. Effect of types of dietary NSPs on the total N gain in fish over the experimental period originating from feed and from natural food. “PecHem-Diet”, a diet with quick/easy biodegradable (fermentable) NSP, “LigCel-Diet”, a diet with slow bio-degradable (fermentable) NSP.

In Fig. 4 the N gain from feed and food web was calculated identical to Kabir *et al.* (2019). Over the whole experimental period, the total N gain per pond was 284 and 308g with respectively the “PecHem-Diet” and “LigCel-Diet” of which 46.3 and 44.8%, respectively, originated from feed-N. The difference in N-gain at pond level between both diets was for 71% related to a higher contribution coming from the food web with the “LigCel-Diet”. This indicates that the type of dietary NSP can influence the productivity of the pond food web. The higher

productivity of the food web at the “LigCel-Diet” is in line with the observed higher water chlorophyll a content (Table 6), the abundance of benthos and soil bacteria (Table 6) and stomach fullness with natural food (Table 7).

Analysis of ^{13}C and ^{15}N stable isotope (Fig. 2) also indicate that fish consumed nutrients not only from the feed but also from other sources in the pond (*i.e.*. natural food). The IRI indicates that phytoplankton (or algae) was the most important group of natural food found in the stomach of the fish for both diets. Higher chlorophyll-a levels in ponds fed with “LigCel-Diet” thus indicate that the dominant food group was more abundant in the ponds fed with this diet. Because algae is the primary producer in a pond, more likely they had also a positive impact on the other parts of the food web in the pond. The better growth performance of tilapia in the non-fed compartments of pond fed with “LigCel-Diet” (Table 2), also indicates the importance of pelagic natural food to growth of tilapia in aquaculture ponds. So, natural food, more specifically the pelagic food web led to the difference in the fish performance.

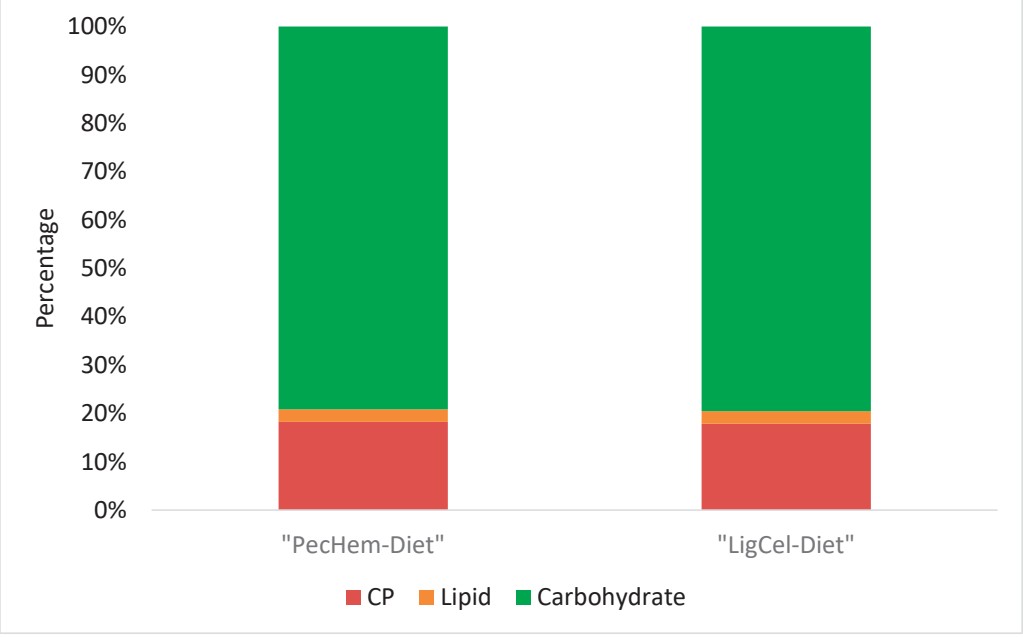


Fig. 5. Effect of types of dietary NSPs on organic matter (OM) composition in faeces. “PecHem-Diet”, a diet with quick/easy bio-degradable (fermentable) NSP, “LigCel-Diet”, a diet with slow bio-degradable (fermentable) NSP,

Still it remains the question how different types of dietary NSP steer the natural food in the pond. Enhancement of the natural food in a pond by fertilization through feed supplementation depends among others on the amount and composition of both the uneaten feed and the produced faeces by the fish. The C:N ratio of the nutrient input (Asaduzzaman *et al.*, 2010; Avnimelech, 1999) is considered to be a key factor for natural food enhancement in fish ponds. In Fig. 5 the calculated organic matter composition of faeces produced at both diets (derived from nutrient ADC values in table 3) is shown. The C:N ratio in the feed (12.3 *vs* 10.8) were slightly different but in the faeces this ratio was comparable (17.1 *vs* 17.5) (Fig. 5). Overall, organic matter composition of the faeces was also similar (Fig. 5).

The total amount of the faeces produced, calculated based on feed ration and ADC of DM, was higher with "PecHem-Diet" compared to the other (DM 1531 *vs* 1269 g). One would expect that the higher amount of faeces at the "PecHem-Diet" would be positive for stimulating the food web because this enlarged especially the C input in the ponds. However, one should realize that the type of NSP might affect the stability of the faeces. Amirkolaie *et al.* (2005) showed that soluble *vs*. insoluble NSP (guar gum *vs*. cellulose) altered the stability/characteristics of the faeces. The soluble NSP diets had more diarrhoea like faeces. Therefore, it can be hypothesised that the type of dietary NSP might also shift the place where faecal nutrients (C and N) end up in the pond: dissolved in the water column versus settled at the bottom as solids. The "PecHem-Diet" containing most likely more soluble NSP, may have created less stable faeces, which was probably emitting from the system more rapidly instead of being available to the biota of the pond as their nutrient for a prolonged time. Organic matter levels were not different between pond fed with different diet which supports this statement. On the other hand, faeces with low soluble NSPs (most likely at the "LigCel-Diet) are usually solid (Amirkolaie *et al.*, 2005) and therefore can reach to the pond sediment. We do not have data on the consequence of the faeces reaching the pond bottom. The possible explanation might be microbes released the trapped nutrients to make it available to them as well as to other components of the food web present in the pond. This may have resulted in a high natural food production in the pond fed with "LigCel-Diet". However, further research should elucidate how type of NSP is altering the productively of the natural food web.

4.5 Conclusion

The “LigCel-Diet” enhanced natural food and increased its contribution to fish growth in pond culture of tilapia while both the diets had comparable C:N ratios. Therefore, not only the amount of C that contributes to the C:N ratio, but also the composition of carbon is important for food web enhancement. The current study showed that the type of dietary NSP determines the pond food web productivity.

Chapter 5

Effect of dietary protein to energy ratio, stocking density and feeding level on performance of Nile tilapia in pond aquaculture

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Abstract

There is growing interest to understand the dietary P:E requirements for the supplemental feed used in tilapia pond culture where natural food contributes to production. In an on-farm trial, we tested the effect of lowering dietary P:E ratio on fish performance, pond nutrient utilization and economic benefit under two stocking densities and feeding levels. Forty ponds, (average size 234±112 m^2), were assigned to test the effect of two diets, which differed in P:E ratio (18 *vs* 14 $g.MJ^{-1}$), two feeding levels (14 *vs* 18 $g.kg^{-0.8}.d^{-1}$) and two stocking densities (2 *vs* 3 $fish.m^{-2}$). Initial fish biomass was 45(±21) *vs* 67(±38) $g.m^{-2}$ at 2 *vs* 3 $fish.m^{-2}$, respectively. The experiment lasted 82 days. Decreasing P:E ratio enhanced tilapia production ($P<0.05$; 459 *vs* 399 $g.m^{-2}$). Increasing stocking density of tilapia from 2 to 3 m^{-2} increased biomass gain 43% ($P<0.001$; 354 *vs* 505 $g.m^{-2}$). Averaged over both diets and stocking densities, growth and feed conversion ratio increased with increasing feeding level ($P<0.001$). Fish survival was unaffected by diet, stocking density and feeding level. Dissolved oxygen increased with increased stocking density with low P:E diet. The opposite happened for high P:E diet ($P<0.05$). Increasing the feeding level also increased the DO concentration ($P<0.001$). N retention efficiency was higher with the low P:E ratio diet ($P<0.001$; 71 *vs* 52%) and decreased with increasing feeding level ($P<0.001$). The data on N gain and N balance at the pond level suggest that the food web productivity was stimulated by reducing the dietary P:E ratio. The low P:E diet increased the gross margin by 95% ($P<0.001$; 2076 *vs* 1067 $USD.ha^{-1}$) and benefit cost ratio by 22% ($P<0.05$; 1.57 *vs* 1.29). The P:E ratio of the low P:E diet is lower than the presently advised level. Lowering the P:E ratio from 18 to 14 $g.MJ^{-1}$ in pond feeds for tilapia will increase the economic viability of pond aquaculture.

5.1 Introduction

In terms of production volume, tilapia is the second largest farmed fish group after carp, showing a fast growth in this sector, particularly in the last decade(FAO, 2018). It is grown across all the tropics, in more than 100 countries, at different culture intensities and in diverse production systems (Wang and Lu, 2016). In Southeast Asia, where the majority of Nile tilapia is produced, it is mainly farmed in extensive to semi intensive ponds. The farm gate price of tilapia in Southeast Asia is low. Therefore, the economic viability of farming tilapia is challenged in many countries. The largest expenditure for tilapia farming is feed, constituting ~70% of total operating cost (Yuan, Yuan, & Dai, 2017; Ahmed, 2007). Therefore, making feed affordable and increasing efficiency of feed utilization can help ensure good economic benefits for the producers.

Feed cost depends largely on the crude protein content in the diet. Most of the commercial diets comply with the NRC (1993 and 2011) recommendation to have a dietary digestible protein to digestible energy (DP:DE) ratio of 18-23 $g.MJ^{-1}$. This NRC recommendation is based on studies done in tanks in absence of natural food. However, in ponds where additional feeding is applied, natural foods can still contribute up to 40 – 68% to the production (Anderson, Parker, & Lawrence, 1987; Burford, Preston, Glibert, & Dennison, 2002; Burford *et al.*, 2004; Cam & Mariotti, 1991; Porchas-Cornejo *et al.*, 2012). This contribution can be enhanced by increasing the C:N ratio of nutrient inputs (Asaduzzaman *et al.*, 2010). Natural food availability depends on a well-functioning food web. Bacteria can mineralize waste and prevent ammonia accumulation form the base of the food web. To mineralize all the waste, bacteria need energy. The energy in pond aquaculture is often provided by administration of carbohydrates to raise the C:N ratio to 15-20 (Asaduzzaman *et al.*, 2008; Avnimelech and Kochba, 2009; Crab *et al.*, 2007). By increasing the carbon or energy availability in the pond, production can be increased. When the C:N ratio of the nutrient input raises above 10, heterotrophic bacteria become dominant (Boyd, 1996; Lancelot and Billen, 1985), contributing substantial amounts of bacterial biomass to the food web. Organic, but also inorganic nitrogen are taken up by heterotrophic bacteria, thus keeping ammonia and nitrite levels in the pond low (Avnimelech, 1999; Hari *et al.*, 2006, 2004)). Heterotrophic bacteria, are a protein source, stimulating the food web and the production of fish based on natural foods (Asaduzzaman *et al.*, 2008).

Kabir *et al.*, (2019) demonstrated that the tilapia yield in semi-intensive culture system was better with a diet of P:E ratio 14 $g.MJ^{-1}$ compared to a diet of P:E ratio 18 $g.MJ^{-1}$, while realizing a FCR of 0.88 and 1.02, respectively. Application of this concept could substantially reduce the cost of feed and thus total production cost, allowing to increase economic profitability and long-term sustainability of tilapia culture in ponds.

However, the study of Kabir *et al.*, (2019) was done in uniform experimental ponds. It remains to be verified if the same effects will also be obtained under less uniform rearing conditions typical for farmer ponds. The underlying mechanism of the better fish performance with low P:E diet was due to increased intake of natural food. This higher natural food intake is related to their higher prevalence in ponds, steered by the low P:E (or high C:N) ratio diet. However, enhancement of natural food in pond also depends on the quality of pond soil and water, and the availability of sunlight to stimulate the autotrophic food web. In addition, increased culture intensity (stocking density) and input of supplemental feed is believed to reduce the relative contribution of natural food to fish production. All these factors may vary among different farmer ponds.

Therefore, the current study was planned to test the effect of lowering dietary P:E ratio in an on-farm trial with two feeding levels and two stocking densities. The diets were the same as in Kabir *et al.* (2019). The high P:E diet (C:N ratio 8) was comparable to a diet similar in P:E ratio of a standard commercial tilapia diet (NRC 1993, 2011) and the low P:E diet (C:N ratio 11) was in the direction to the recommended P:E ratio for pond aquaculture by Asaduzzaman *et al.* (2010, 2008), and Hari *et al.* (2006, 2004). The feeding levels were comparable with a commercial feeding schedule for semi-intensive tilapia culture in ponds. The high stocking density was selected considering the reported carrying capacity for tilapia in non-aerated ponds, 5800 kg/ha (Xu *et al.*, 2011). Effect of diet, stocking density and feeding level on fish production; nitrogen retention; accumulation of nutrients in pond water and soil, and economic return were assessed.

We hypothesised that:
1. With low P:E diet, more energy (carbon) will be available to enhance natural food in the pond;
2. Enhanced natural food will compensate for lowering the dietary P:E ratio for fish performance; 3. Increasing stocking density, will contribute to increased fish production; and
4. If stocking density is not too high feed utilization efficiency will remain same.

5.2 Methods

5.2.1 Experimental Design

Two diets contrasting in P:E ratio (18 *vs* 14 $g.MJ^{-1}$), 2 stocking densities (2 *vs* 3 m^{-2}) and 2 feeding levels (14 *vs* 18 $g.kg^{-0.8}.d^{-1}$) were tested in a 3-way full factorial design, with 5 replicates per treatment. The feeding levels aimed to represent levels commonly applied in ponds. Because within semi-intensive ponds, stocking densities and feeding levels vary, the high feeding level was chosen to represent a standard semi-intensive system and the low feeding

level to represent a low intensity semi-intensive pond culture system. All-male juvenile Nile tilapia were stocked and grown for 82 days. Feeding was gradually reduced assuming 80% fish survival in each pond at the end of the culture period.

5.2.2 Preparation of Diets

Experimental diets were contrast in P:E ratio, extruded pellets of 3 mm size. The high P:E diet was formulated to have a P:E ratio of 18 $g.MJ^{-1}$, which is at the lower range of the recommendation

Table 1. Ingredient and analysed chemical composition of the experimental Nile tilapia diets differing in protein to energy (P:E) ratio.

		Diets	
		High P:E ratio	Low P:E ratio
Ingredients (%)			
Maize		20	20
Soybean meal		12	6
Wheat bran			15
Wheat flour		20	20
Rice bran			12
Sunflower meal		12	6
Rapeseed meal		12	6
Meat & bone meal		15	8
Fish meal		5	3
Fish oil		2	2
Vitamin & Mineral premix[a]		1	1
Mono calcium phosphate (MCP)		0.7	0.8
DL Methionine		0.3	0.2
Chemical composition			
Dry matter (DM),	($g.kg^{-1}$)	893	889
Crude Protein (CP)	($g.kg^{-1}$ DM)	322	255
Fat	($g.kg^{-1}$ DM)	37	34
Ash	($g.kg^{-1}$ DM)	124	96
Phosphorus	($g.kg^{-1}$ DM)	15	13
Carbohydrate[b]	($g.kg^{-1}$ DM)	518	615
Gross energy	($kj.g^{-1}$ DM)	18	18
P:E ratio	($g.MJ^{-1}$)	17.5	14.1
C:N ratio[c]	($g.g^{-1}$ DM)	8	11

[a] commercial product made by ACI Godrej Agrovet Private Limited.

[b]This is calculated value where Carbohydrate= 1000-CP-Fat-Ash

[c]This is calculated C:N ratio considering 16% N content in the protein and 47, 70 and 50% C content in protein, fat and carbohydrate respectively (Waal and Boersma, 2012).

for tilapia (NRC, 1993). This High P:E diet had a C:N ratio of 8 and was comparable regarding nutrient content to currently used commercial diets in Southeast Asia (formulated in compliance with NRC, 1993). The Low P:E was formulated to have a higher C:N ratio of 11. This was achieved by replacing protein ingredients (*i.e*., soybean meal, sunflower meal, rapeseed meal, meat and bone meal, and fishmeal) with carbohydrate ingredients (*i.e.*., wheat barn and rice bran). The inclusion of rice bran and wheat bran resulted in an increase of the non-starch polysaccharide content at the Low P:E diet compared to the High P:E diet. Both diets were identical in energy content. This was further confirmed by the chemical analysis of the feed (Table 1).

5. 2.3 Study area, Fish rearing and housing facilities

Forty outdoor ponds, average surface area 234 (±112) m^2, in farmer fields in south-western Bangladesh (Figure 1) were used for this experiment. Though the ponds belong to two different sub-districts, they actually lie two sides of river Bhadra under the same agro-ecological zone of lower Ganges tidal flood plain. During the experiment, the ponds were exclusively dedicated for the experiment and the protocol was strictly followed by the project field research assistants. All male sex reversed 30 days old Nile tilapia fry, 14th generation WorldFish GIFT strain, were collected for this experiment from Asha Hatchery, a GIFT Tilapia Multiplication Center in Bangladesh.

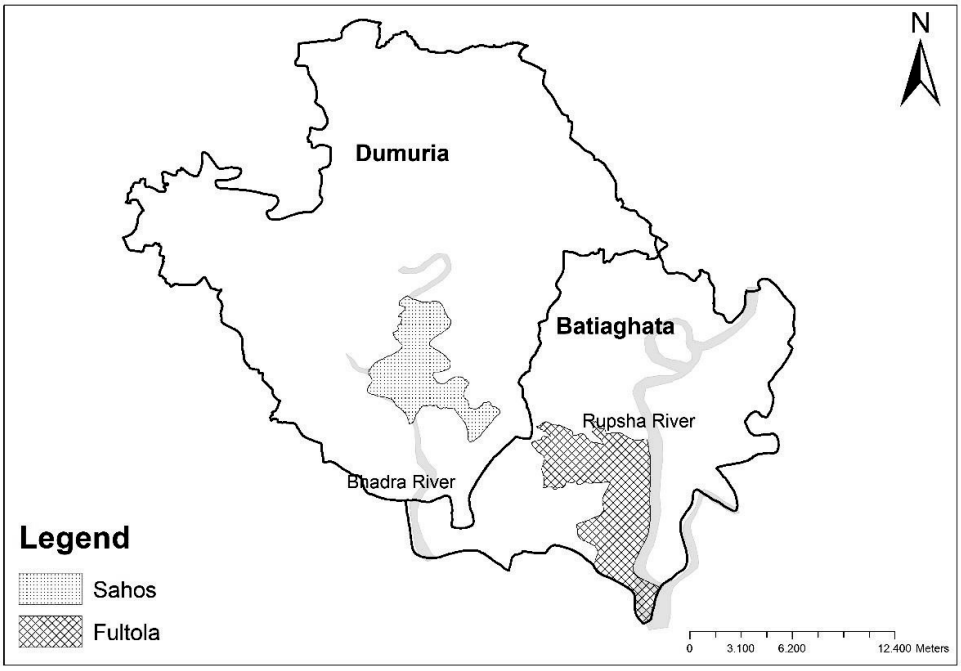

Fig. 1. Map of the study areas; 20 ponds in each location – in Batiaghata and Dumuria sub-district lying on opposite side of Bhadra river of Khulna District, Bangladesh

5.2.4 Experimental procedure

5.2.4.1. Pond preparation

Ponds were dried by pumping out the water. Twenty-five $g.m^{-2}$ $CaCO_3$ was applied at the bottom soil of each pond before water filling. After water filling, 4 $g.m^{-2}$ dolomite ($CaMg(CO_3)_2$) was spread over the water surface of each pond. One $g.m^{-2}$ urea (CH_4N_2O) and 2 $g.m^{-2}$ triple super phosphate (TSP), [$Ca(H_2PO_4)_2.H_2O$], per pond (Rakocy and Mcginty, 1989) were applied 1 week after liming. Fish fries were stocked in a small pen, 2.25 m^{-2} frame covered with 1 mm mesh sized nylon net, in each pond, 5 days after fertilization. Fish fries (0.03 g) in the pen were fed a commercial nursery diet until the mean body weight was 22.6 (±11.6) g, sufficient to eat 3 mm pellet.

Fish were fed daily at 8.00 and 16.00 hours. Fish were fed according to their metabolic body weight. Two feeding levels were applied, high (18 $g.kg^{-0.8}.d^{-1}$) and low (14 $g.kg^{-0.8}.d^{-1}$). The feed rations were adjusted fortnightly based on body weight sampling in all ponds. Cast nets were used for sampling. Fish were harvested from four corners and center of the pond by single through of the cast net. 5-15% fish were sampled during each sampling. The amount of feed given was based on the measured DM content of the diet. Therefore, the crude protein input in the ponds at each diet was different. This experiment mimicked low intensity semi-intensive production in non-aerated ponds.

5.2.4.2 In-situ water quality monitoring

Dissolved oxygen (DO), pH, total dissolved solid (TDS), transparency, temperature and salinity of each pond were measured daily between 9.00 -12.00 hours; by using Lutron dissolved oxygen meter model PDO-519, Hanna instruments pocket tester HI98128-phep5, Lutron conductivity meter model PCD-431, Secchi disc, Hanna digital thermometer model HI98501 and Atago refractometer model MASTER-S28M instruments.

5.2.4.3 Sampling and analysing soil and water nutrients

5.2.4.3.1 Sample collection, processing and preparation

Soil samples were collected from the top 20 cm layer of pond bottom at five points of each pond and then mixed homogeneously. Approximately 1 kg wet soil was collected from each pond, labelled and packed in tight plastic bags, and transported to the laboratory. The collected samples were air dried, crumbled and sieved through a 2 mm to separate the coarse (>2 mm) and fine (<2 mm) fractions. The sieved fractions were then preserved in labelled plastic containers until analysis. Water samples were collected, with a depth sampler of 10 cm width and 25 cm length, from each pond at the same 5 soil sampling locations, within 25 cm of pond surface, transferred and sealed in airtight bottles, and preserved at -20˚C until analysed. These samples were collected at day 1, 41 and 82 of the experiment. Accumulation

of nutrients over time in the culture pond were calculated by deducting the observation of day 1 from day 82.

5.2.4.3.2 Analysis of the soil samples

Organic carbon content of the soil was determined by Walkley and Black's wet oxidation method as described by Jackson (1973). Total nitrogen of the soil was determined by Micro-Kjeldahl's method following H_2SO_4 acid digestion and alkali distillation procedures as suggested by Jackson (1962). Total phosphorus of soil was determined colourimetrically by Vanado-molybdophosphoric yellow colour method in nitric acid system (Barton, 1948). The colour intensity was determined by the spectrophotometer at 470 nm light wavelength (Jackson, 1958). The available potassium was determined after extraction the soil samples with 1N NH_4OAc, pH-7.0 solution followed by the measurement of extractable K+ by Flame emission spectrophotometer (Model: Jenway, PEP-7) at 766 nm wave length using Potassium filter, as outlined by Jackson (1973).

5.2.4.3.3 Analysis of the water samples

The organic carbon content of the water was determined by Tyrine's method as water commonly contains relatively smaller amounts of organic matter. As under dilute conditions Tyrine's method does not function well, the sample was dried first (Tyrine, 1965). The total inorganic nitrogen concentration was determined by the Micro-Kjeldahl method (Jones, 1991) and alkali distillation procedures as suggested by Jackson (1962). Available phosphorus was determined colourimetrically by molybdophosphoric blue colour method (Murphy and Riley, 1962). The available potassium of water was determined by a flame analyzer at 589nm wavelength (Jackson, 1967).

5.2.4.4 Sampling and analysing proximate composition of fish and feed

The initial body composition was determined on 200 fingerlings with 22.6 (±11.6) g mean body weight. Five fish fingerlings were collected from each pond at the start day of the feeding trial. They were euthanized by an overdose of a phenoxy-ethanol solution (1.0ml. L^{-1}) and stored at −20 °C. For final body composition, 5 fish were randomly selected from each pond at the end of the experiment. Fish, which were used for body composition analysis, were euthanized by an overdose of a phenoxy-ethanol solution (1.0 ml.L^{-1}) and stored at −20 °C. Before chemical analysis, the sampled fish were cut into small pieces, homogenised by passing them twice through a 4·5 mm screen grinder and subsequently oven-dried. Chemical analyses were done in triplicate. Dry matter was determined gravimetrically after drying at 103 °C for 4 and 24 h for feed and fish samples respectively (ISO 6496, 1983). Crude ash was determined after incineration at 550°C for 4 h (ISO 5984, 1978). Crude protein (CP) was determined by the Kjeldahl method (ISO 5983, 1979) and calculated by multiplying the measured N content by

6·25. Fat was quantified by petroleum–diethyl ether extraction (ISO 6492, 1999). Before fat analysis, feed samples were hydrolysed by boiling for 1 h with 3 M-HCl. Dietary energy content was measured by direct combustion in an adiabatic bomb calorimeter (IKA-C-7000; IKA analysentechnik, Weitersheim, Germany).

5.2.5 Data Analysis

5.2.5.1 Performance

Biomass gain ($g.m^{-2}$) was calculated as the difference between the biomass stocked and biomass harvested (in $g.m^{-2}$). The specific growth rate (SGR) was calculated as $SGR=((\ln(IndBW_{82})-\ln(IndBW_0))/82)\times100$; where, $IndBW_{82}$ and $IndBW_0$ means individual body weight at day 82 and day 0 of the experimental feeding. Feed conversion ratio (FCR) was calculated as FCR = weight of the total feed applied/fish produced (wet weight basis). The survival of fish per pond was calculated as $(F_h/F_s)\times100$, where F_h is the number of fish harvested and F_s is the number of fish stocked at the pond.

5.2.5.2 Nitrogen (N) retention

N gain in fish was calculated by the difference between the N_h and N_s. Here, N_h=amount of N in the harvested fish biomass and N_s=amount of N in the biomass at start. N feed was calculated by total feed input per square meter multiplying the N content in feed; N balance was calculated by deducting N gain in fish from the feed N input based on proximate composition. Contribution of natural food to fish growth can be measured using stable isotope of N15 and C13 (Michener and Lajtha, 2007; Smyntek *et al.*, 2010). However, we could not use inert stable isotope in the feed for this analysis due to limited funding and the scale of the experiment. The current experiment focused on testing of the P:E concept under field/practical conditions. Therefore, N retained from natural food was calculated by deducting N retention from feed from the total N gain in fish. In this study it was not possible to measure the apparent digestibility as the experiment was in ponds. So, the calculation of N retention resulting from direct feed consumption was based on a 90% apparent digestibility coefficient (ADC) for N (Azevedo *et al.*, 2004; Kaushik *et al.*, 1995) and N retention efficiency (RE) of 40% (Azevedo *et al.*, 2004). The difference between total N retention in fish biomass gain and the N retention based on feed is considered as N retention from natural food.

The ADC and RE of N may vary based on the concentration of CP and feeding levels. However, the simulation with different ADC and RE from Kabir *et al*. (2019) indicated that those changes do not result in major changes in the contribution of natural food and supplementary feed on the N gain in fish. As this study is a validation of the proof of concept of the previous study of Kabir *et al.* (2019), we kept the analysis simple and used the most common level of ADC and

RE in this study. As such, the analysis is intended to give the reader an impression of importance of natural food to fish production in ponds.

5.2.5.3 Calculation of economic benefit

The total cost was estimated as the sum of the depreciated pond construction cost considering a pond life of 20 years, rent of the land area of the pond for one production cycle (0.5 year), fish seed, feed, labour and other inputs and contingency cost. Rent of the land and pond construction cost was based on the local context of the research area for the year of the study. Return is the value of the sold fish after harvest. The price of the fish was set based on the wholesale price of tilapia in the local auction center on the day of harvest. Gross margin was calculated by deducting the total cost from the return. Benefit cost ratio (BCR) was calculated by dividing the return with the total cost.

5.2.5.4 Statistical analysis

All parameters were analysed for the effects of diet and stocking density. Though we designed the experiment with two feeding levels (14 *vs* 18 $g.kg^{-0.8}.d^{-1}$), the actual feed ration varied due to difference in biomass at stocking and adjustment of the feed ration based fortnightly body weight sampling. Therefore, instead of using feeding level as a fixed factor, feed ration ($g.fish^{-1}.m^{-2}.d^{-1}$) was used as a continuous variable in a covariate analysis in applying univariate ANOVA using the procedure general linear model (GLM). Before using feed ration as a covariate, the effect of diet, stocking density and their interaction on feed ration ($g.fish^{-1}.m^{-2}.d^{-1}$) was tested by two-way ANOVA. None of these factors were significant, which indicates that the feed ration ($g.fish^{-1}.m^{-2}.d^{-1}$) can be considered as an independent (continuous) variable, making a similar effect as feeding level. When significant interaction found multiple comparisons of means using Tukey's multiple range test were performed. Deviation from the mean has been expressed as standard error throughout the analysis.

As we had varying pond environment, we wanted to see how pond environment explains the variation in fish growth in different ponds. For this analysis dissolved oxygen, pH, temperature, water transparency, pond water depth, and organic matter, total nitrogen, total phosphorus and available potassium of pond water and bottom soil were included as environmental explanatory variable. Individual weight gain, biomass gain per square meter, fish survival, feed conversion ratio and specific growth rate were grouped as response variables. These data were fitted into a multivariate distance based linear model (DistLM) using BEST procedure in Primer 6 and Permanova^{+}.

In the arrangement of pond distribution, the ponds belong to two different administrative zone. However, these are ponds in the same floodplain on both sides of a river and all situated

within a 10 km radius. We tested the effect of location on performance indicators (*i.e.*. biomass gain ($g.m^{-2}$), FCR ($g.g^{-1}$) and growth ($g.d^{-1}$) growth). As there was no effect of pond location on these indicators, we have excluded location from the model.

5.3 Results

In the design of the study, it was intended to have 2 distinct feeding levels (14 *vs* 18 $g.kg^{-0.8}.d^{-1}$). The feeding rations applied were calculated based on the measured initial BW and were adjusted based on the fortnightly body weight sampling. The initial individual BW of tilapia was 22.6 (±11.6) g. Thus, differences in mean BW per pond, concurred with differences in feed ration between ponds, ranging from 1 to 3.8 $g.fish^{-1}.m^{-2}.d^{-1}$. Considering differences in feed ration, it was included into the statistical model as a covariate and expressed as $g.fish^{-1}.m^{-2}.d^{-1}$. Feed ration was unaffected by diet and stocking density, confirming that the fixed effects and the covariate in the statistical model used were independent of each other. Moreover, preliminary analysis showed that no interaction effects were present between feeding ration and both fixed effects (diet and stocking density) for any of the parameters related to fish performance. This implies that the effect of feeding ration (if present) was similar for both diets and also for both stocking densities.

5.3.1 Fish performance

At stocking, the average BW was 22.6 (±11.6) g and was unaffected by diet and stocking density. Final BW and individual fish growth were not influenced by stocking density and tended to be higher with the low P:E diet ($P<0.10$). Feeding ration strongly affected final BW and individual BW gain ($P<0.001$). Increasing the feeding ration with 1 $g.fish^{-1}.m^{-2}.d^{-1}$ increased the final BW with 72 g (Table 2). Survival rate averaged over all treatments was 74% and equal between treatments ($p>0.1$) (Table 2).

As expected, the stocked biomass per pond (in $g.m^{-2}$) was only affected by the stocking density. Increasing stocking density from 2 to 3 $fish.m^{-2}$ increased the harvested biomass 42% and biomass gain with 43% ($P<0.001$). Lowering the dietary P:E ratio from 18 to 14 $g.MJ^{-1}$ increased weight gain of tilapia from 399 to 459 g/m^{-2} ($P<0.05$; Table 2). This diet effect on biomass gain tended to depend on stocking density, being reflected by the interaction effect between diet and stocking density ($P<0.10$). Whether this tendency remains as a significant effect should be tested by further research. The impact of dietary P:E ratio on biomass gain tended to be higher at the high stocking density (Table 2). Similar to individual BW gain, performance at pond level strongly increased with increased feeding ration. Increasing the feeding ration with 1 $g.fish^{-1}.m^{-2}.d^{-1}$ increased biomass gain with 120 $g.m^{-2}$.

Table 2. Effect of dietary protein to energy ratio (P:E), stocking densities and feed input on performance of tilapia

Variables		Units	Low P:E Diet		High P:E Diet		Pooled SEM	P-values			Beta for Feed ration ($g.fish^{-1}.m^{-2}.d^{-1}$)	P value of Beta
			SD2	SD3	SD2	SD3		Diet (D)	Stocking density (SD)	D*SD		
Individual performance	Initial body weight	G	22	22	24	23	3.8	ns	ns	ns	--	--
	Final body weight	G	265	273	254	235	14	#	ns	ns	72 (±10)	***
	Weight gain	G	243	252	230	213	14	#	ns	ns	60 (±10)	***
	Growth	$g.d^{-1}$	2.958	3.09	2.799	2.602	0.175	#	ns	ns	0.7 (±0.1)	***
Pond level performance	Biomass stocked	$g.m^{-2}$	43	64	47	69	9.9	ns	*	ns	--	--
	Biomass harvested	$g.\ m^{-2}$	393	607	384	499	29	#	***	#	150 (±20)	***
	Biomass gain	$g.m^{-2}$	360	559	347	451	28	*	***	#	126(±19)	***
	Survival	%	74	76	76	71	2.5	ns	ns	ns	2.9(±1.8)	ns
	FCR	$g.g^{-1}$	0.99	0.95	0.99	1.12	0.064	ns	ns	ns	0.16(±0.04)	***

D=Diet and SD=Stocking density, D*SD=Diet and stocking density interactions, P values: ns (not significant, P>0.1), # (P<0.1), * (P<0.05), *** (P<0.001); SEM=standard error of the mean; Beta for feed ration represents the estimated regression coefficient of the feeding ration (expressed in $g.fish^{-1}.m^{-2}.d^{-1}$) on the respective dependent parameter (*e.g.*, biomass gain, FCR *etc.*).

Average over all treatments, FCR was 1.01. Diet and stocking density did not affect FCR. Increasing the feeding ration increased the FCR; per 1 g.fish^{-1}.m^{-2}.d^{-1} the FCR at the pond level increased by 0.16 g.g^{-1} (Table 2).

5.3.2 Fish body composition

At stocking, dry matter (DM), crude protein (CP), crude fat (CFat), and ash content of the tilapia were 293, 132, 27, and 68 g.kg^{-1}, respectively. Lowering the dietary P:E ratio from 18 to 14 g.MJ^{-1} decreased DM from 302 to 297 g.kg^{-1}and ash content from 61 to 57 g.kg^{-1} (P≤0.05). Feeding ration, stocking density, and the interaction of diet and stocking density had no influence on the final body composition of the fish (Table 3).

Table 3. Effect of dietary protein to energy ratio (P:E), stocking densities and feed input on the final body composition of tilapia

Variables	Units	Low P:E Diet		High P:E Diet		Pooled SEM	P-values			Beta for Feed ration (g.fish^{-1}.m^{-2}.d^{-1})	P value of Beta
		SD2	SD3	SD2	SD3		Diet (D)	Stocking density (SD)	D*SD		
Dry matter (DM)	g.kg^{-1}	288	294	302	302	5.4	*	ns	ns	0.3(±3.8)	ns
Crude Protein (CP)	g.kg^{-1}	154	155	158	154	1.9	ns	ns	ns	0.96(±1.3)	ns
Crude Fat (CFat)	g.kg^{-1}	51	53	54	52	1.4	ns	ns	ns	0.4(±1)	ns
Ash	g.kg^{-1}	55	59	62	61	1.5	*	ns	ns	1.4(±1.1)	ns

D=Diet and SD=Stocking density, D*SD=Diet and stocking density interactions, P values: ns (not significant, P>0.1), * (P<0.05); SEM=standard error of the mean; Beta for feed ration represents the estimated regression coefficient of the feeding ration (expressed in g.fish^{-1}.m^{-2}.d^{-1}) on the respective dependent parameter (*e.g.*, biomass gain, FCR *etc.*).

5.3.3. Feed nitrogen (N) input and output

The nitrogen balance at pond level is given in Table 4. Averaged over all treatments, 18 g of N.m^{-2} was added to the pond via the feed during the 82 days of the experiment. This N input via feed was higher at the high P:E diet (P<0.001); was higher at the high stocking density (P<0.001); and increased with feeding level (P<0.001). The interaction effect between stocking density and dietary P:E ratio influenced the amount of N retained in fish harvested per m^2. At the low stocking density (2 fish.m^{-2}), N gain in fish did not differ between both diets, but at high stocking density (3 fish.m^{-2}), the N gain in fish was higher with the low P:E ratio diet (14g.MJ^{-1}) (P≤0.05). Feed N retention efficiency with the low P:E diet was 71% compared to 51% with the high P:E diet (P<001); but did not differ between stocking densities (P>0.1). With the low P:E diet, the N input into the pond was lower than at the high P:E diet, but the amount

of N gained in fish was equal or higher. This was due to an increased gain of N originating from the food web. Similar to total N gain in fish, the amount of N gain from the food web was affected by the interaction effect between diet and stocking density (P<0.05). The increased N gain from the food web with the low P:E diet was stronger at high stocking density than at low stocking density.

Increasing the feeding ration, increased the N input via feed into the ponds (P<0.001), which directly related to an increased N gain in fish (P<0.001). The increased N gain in fish concurred with the increased N gain from feed (P<0.001), since increasing the feeding ration did not increase the N gain in fish from food web (P>0.1; Table 4).

Table 4. Effect of dietary protein to energy ratio (P:E), stocking densities and feed input on feed N input and gain in fish

Variables	Units	Low P:E Diet		High P:E Diet		Pooled SEM	P-values			Beta for feed ration ($g.fish^{-1}.m^{-2}.d^{-1}$)	P value of Beta
		SD2	SD3	SD2	SD3		Diet (D)	Stocking density (SD)	D*SD		
N input via feed	$g.m^{-2}$	12.5	19.3	16.3	23.9	0.47	***	***	NS	9(±0.3)	***
N gain in fish	$g.m^{-2}$	8.7	13.5	8.7	10.8	0.67	*	***	*	3(±0.5)	***
Feed N retention efficiency	%	70.3	72.3	55.9	47.5	4.50	***	NS	NS	(-11(±3))	***
N loss (not retained in fish)	$g.m^{-2}$	3.9	5.8	7.6	13.1	0.78	***	***	*	6(±0.5)	***
N gain in fish from feed[1]	$g.m^{-2}$	4.5	6.9	5.9	8.6	0.17	***	***	NS	3.2(±0.12)	***
N gain in fish from food web[1]	$g.m^{-2}$	4.2	6.6	2.9	2.2	0.68	***	NS	*	(-0.26(±0.48)	NS

D=Diet and SD=Stocking density, D*SD=Diet and stocking density interactions, P values: ns (not significant), # (P<0.1), * (P<0.05), *** (P<0.001); SEM=standard error of the mean; Beta for feed ration represents the estimated regression coefficient of the feeding ration (expressed in $g.fish^{-1}.m^{-2}.d^{-1}$) on the respective dependent parameter (*e.g.*, biomass gain, FCR *etc.*). [1]Calculated values based on ADC of CP 90% and RE 40% for all diets, stocking densities and feeding levels.

5.3.4 Accumulation of soil and water nutrients

Organic matter content of water increased with increasing feed input (P≤0.05). Except for this, there was no effect of diet, stocking density, and feeding ration on accumulation of nutrients in pond soil and water during the 82 days of culture period (Table 5).

5.3.5 Economics of Fish Production

Lowering the dietary P:E ratio from 18 to 14 $g.MJ^{-1}$ increased both gross margin (P<0.05) and benefit cost ratio (P<0.05). Gross margin was higher (P<0.05) at high stocking density and there was a tend towards interaction between dietary P:E ratio and stocking density (P<0.07).

Stocking density did not affect the benefit cost ratio (BCR). Also, feeding ration did not influence gross margin and BCR (P>0.1) (Table 6).

5.3.6 Pond water quality

Dissolved oxygen content increased with increased feeding level (P<0.001) and there was an interaction (P<0.05) between diet and stocking density. At high stocking density with low P:E diet the dissolved oxygen content was highest. Dissolved oxygen tended to increase while the transparency decreased with high stocking density. Stocking density also influenced (P<0.05) NH_4 and NO_3 concentration in the water and there was a tendency (P<0.1) to increase NO_2 with increased stocking density. There was no effect of diet on pond water quality (Table 7).

Table 5. Effect of dietary protein to energy ratio (P:E), stocking densities and feed input on accumulation of nutrients at pond water and soil.

Variables	Units	Low P:E Diet		High P:E Diet		Pooled SEM	P-values			Beta for feed ration ($g.fish^{-1}.m^{-2}.d^{-1}$)	P value of Beta
Accumulation of -		SD2	SD3	SD2	SD3		Diet (D)	Stocking density (SD)	D*SD		
Soil organic matter	$g.m^{-3}$	2453	2050	281	907	1547	ns	ns	ns	(-806(±1102))	ns
Soil Nitrogen	$g.m^{-3}$	3	-84	34	-2	42	ns	ns	ns	(-11(±30))	ns
Soil phosphorus	$g.m^{-3}$	184	662	-582	1198	814	ns	ns	ns	(-803(±580))	ns
Available Soil Potassium	$g.m^{-3}$	-11	37	8	29	29	ns	ns	ns	8(±20)	ns
Water organic matter	$g.m^{-3}$	-40.7	74.1	-16.1	-0.7	67	ns	ns	ns	93(±48)	*
Water Nitrogen	$g.m^{-3}$	0.4	-1.5	0.6	1.7	1.4	ns	ns	ns	(-0.5(±1)	ns
Available water phosphorus	$g.m^{-3}$	-0.1	0.1	0.0	0.0	0.1	ns	ns	ns	(0.02(±0.07))	ns
Available water Potassium	$g.m^{-3}$	-77	-35	-15	-116	48	ns	ns	ns	(-43(±34))	ns

D=Diet and SD=Stocking density, D*SD=Diet and stocking density interactions, P values: ns (not significant, P>0.1), * (P<0.05); SEM=standard error of the mean; Beta for feed ration represents the estimated regression coefficient of the feeding ration (expressed in $g.fish^{-1}.m^{-2}.d^{-1}$) on the respective dependent parameter (*e.g.*, biomass gain, FCR *etc.*).

5.3.7 Pond environment and fish performance

The effect of lowering the dietary P:E ratio when tested in experimental ponds (Kabir *et al.*, 2019), was confirmed in farmers ponds, operated under more variable environmental conditions. Of the environmental parameters monitored, dissolved oxygen influenced fish production performance (P≤0.001) and water temperature had a tendency to influence the fish production(P≤0.1).

Table 6. Effect of dietary protein to energy ratio (P:E), stocking densities and feed input on the production economics of Tilapia in pond aquaculture

Variables	Units	Low P:E Diet		High P:E Diet		Pooled SEM	P-values			Beta for feed ration ($g.fish^{-1}.m^{-2}.d^{-1}$)	P value of Beta
		SD2	SD3	SD2	SD3		Diet (D)	Stocking density (SD)	D*SD		
Feed Cost	$USD.ha^{-1}$	1902	2834	2112	3173	63	***	***	ns	1240(±44)	***
Total Cost	$USD.ha^{-1}$	3018	4035	3228	4374	63	***	***	ns	1240(±44)	***
Return	$USD.ha^{-1}$	4391	6814	4236	5500	337	*	***	#	1542(±236)	***
Gross Margin	$USD.ha^{-1}$	1373	2779	1008	1126	340	*	*	#	301 (±237)	ns
BCR	$USD.ha^{-1}$	1.4	1.7	1.3	1.3	0.1	*	ns	ns	(-0.02(±0.07))	ns

D=Diet and SD=Stocking density, D*SD=Diet and stocking density interactions, BCR=Benefit cost ratio (Return/Total Cost), Gross Margin=Return-Total Cost, Return=Total sale value. P values: ns (not significant, P>0.1), # (P<0.1), * (P<0.05), *** (P<0.001); SEM=standard error of the mean; Beta for feed ration represents the estimated regression coefficient of the feeding ration (expressed in $g.fish^{-1}.m^{-2}.d^{-1}$) on the respective dependent parameter (*e.g.*, biomass gain, FCR *etc.*).

Table 7. Effect of dietary protein to energy ratio (P:E), stocking densities and feed input on the pond water quality

Variables	Units	Low P:E Diet		High P:E Diet		Pooled SEM	P-values			Beta for feed ration ($g.fish^{-1}.m^{-2}.d^{-1}$)	P value of Beta
		SD2	SD3	SD2	SD3		Diet (D)	Stocking density (SD)	D*SD		
Dissolved oxygen (DO)	$mg.L^{-1}$	2.7	3.5	3.0	2.9	0.21	ns	#	*	0.7(±0.1)	***
pH		8.1	8.3	8.2	8.1	0.10	ns	ns	ns	0.002(±0.07)	ns
Transparency	cm	32.6	25.7	34.1	27.1	3.76	ns	#	ns	(-0.5(±2.6))	ns
NH_4	mg.L-1	0.86	0.95	0.85	0.92	0.03	ns	*	ns	0.025(±0.02)	ns
NO_2	mg.L-1	1.92	2.02	1.84	1.99	0.07	ns	#	ns	0.046(±0.05)	ns
NO_3	mg.L-1	3.84	3.67	4.51	3.66	0.24	ns	*	ns	0.106(±0.17)	ns

D=Diet and SD=Stocking density, D*SD=Diet and stocking density interactions, P values: ns (not significant, P>0.1), # (P<0.1), * (P<0.05), *** (P<0.001); SEM=standard error of the mean; Beta for feed ration represents the estimated regression coefficient of the feeding ration (expressed in $g.fish^{-1}.m^{-2}.d^{-1}$) on the respective dependent parameter (*e.g.*, biomass gain, FCR *etc.*).

5.4 Discussion

In this on farm trial we confirmed that the effects of lowering the dietary P:E ratio in pond diets on production performance observed in the experimental ponds remains same. Biomass gain, food web contribution to fish growth, nitrogen retention efficiency and economic benefit of tilapia aquaculture in the farmer ponds increased with low P:E diet (14 $g.MJ^{-1}$) compared to the high P:E diet (18 $g.MJ^{-1}$).

Higher biomass gain found with the lower P:E diet (14 g.MJ^{-1}) confirms the findings of Kabir *et al.* (2019). A P:E ratio of 14 g.MJ^{-1} is lower than the recommended dietary P:E ratio range (18-23 g.MJ^{-1}) for tilapia (El-Sayed and Teshima, 1992; Kaushik *et al.*, 1995; NRC, 1993). Several recent studies (Abdel-tawwab, 2012; Abdel-Tawwab *et al.*, 2010; Fernandes *et al.*, 2016; Liu *et al.*, 2018) observed better performance with high P:E ratio diets compared to the low P:E diets. However, all these studies were done in clear water tanks. We therefore hypothesize that in ponds, the presence of natural food makes the difference. Nitrogen gain based on direct feed consumption was higher with the high P:E diet (7.2 g.m^{-2} *vs* 5.7 g.m^{-2}). However, the total N gain in fish was 9.7 g.m^{-2} with the high P:E ratio diet, which is less than the 11.0 g.m^{-2} with the low P:E ratio diet. The influence of the feed N was superseded by the stronger contribution of N coming from the natural food (Table 4, Figure 2). Overall depletion of inorganic N from the pond environment with the low P:E diet (Table 5) also indicates that this N was used to compensate for the lower dietary N inclusion. Similar observations were also reported by several pond studies where dietary C:N ratio was increased by adding carbohydrate besides the regular diet (Anderson *et al.*, 1987; Burford *et al.*, 2002, 2004; Cam and Mariotti, 1991; Porchas-cornejo *et al.*, 2012) to enhance effect of natural food. In the high P:E dietary treatment, the proportion of feed N that was not deposited in the fish was high. On the other hand, reducing the dietary P:E ratio (*i.e.* increasing dietary C:N ratio in this study), demonstrated a higher N retention efficiency in fish biomass. This corroborates the results of Kabir *et al.* (2019). However, it remains to be seen if a low P:E ratio diet will maintain a similar effect with an increasing stocking density and feeding level, as it is believed that the culture intensity reduces contribution of natural food.

In this experiment, increasing the stocking density from 2 to 3 tilapia.m^{-2} also increased fish biomass gain. Such an effect of stocking density was also observed by Wu *et al.*, (2018) and Abdel-Tawwab *et al.*, (2014). However, this gain was mainly derived by the increased feed input in response to the higher stocking density (Table 4) as increasing stocking density did not influence the contribution of natural food to fish N gain or on feed N retention efficiency. The rate of increase of biomass gain in relation to stocking density with the low P:E diet was 25% higher than the high P:E ratio diet. This large increase in biomass gain due to the interaction effect between diet and stocking density was probably influenced by the 23% difference in the C:N ratio of the pond soil observed between the low and high P:E ratio diets at high stocking density (59.9 *vs* 48.7). The 607 g.m^{-2} harvested tilapia biomass is more than the reported 588 g.m^{-2} carrying capacity for tilapia in non-aerated ponds by Xu et al., (2011). This indicates that application of this concept might increase the carrying capacity of non-aerated ponds for tilapia aquaculture, and thus capacity to digest more waste resulting from increased feeding.

In this experiment, increasing the feed ration increased fish growth ($g.d^{-1}$), biomass gain ($g.m^{-2}$) and FCR ($g.g^{-1}$). Literature shows contradictory results regarding the relation between FCR and feed ration. Liu, Wen and Luo (2018) and El-Sayed (2002) reported the same outcome as we observed, while Haidar et al., (2018) and Deyab and Hussein, (2015) observed that FCR first decreased with increasing feed ration before starting to increase when further increasing of feed ration. In the current experiment, growth increased with increasing feed ration, but also FCR increased. The N balance parameters at pond level (Table 4) demonstrated that increasing the feeding level (*i.e.*. increasing N feed input) resulted in a higher N gain. This increase in N gain was not related to an increased N intake from the natural food but fully due to a higher N intake by the fish. The estimated beta for feed ration for the N gain originating from the food web was almost zero (slightly negative; Table 4). This suggests that in the current range of production intensity, feeding level has no stimulating impact on the productivity of the natural food web. This may explain why increasing the feeding level by 1 $g.fish^{-1}.m^{-2}.d^{-1}$ only slightly increased the gross margin by 301 $\$US.ha^{-1}$ and that is was not statically significant (Table 6). However, economically on a yearly basis (2 production cycles) and for the Bangladesh setting it is still a relevant increase in income. Moreover, from a local perspective of food security, increasing the feed level by 1 $g.fish^{-1}.m^{-2}.d^{-1}$ still can increase 1260 $kg.ha^{-1}$ biomass gain (*i.e.*., yield; Table 2). Although, advising for increasing the feeding level should be handled cautiously, because the above conclusion is only valid within the range of feed ration applied in the current study. Increasing the feeding level beyond the maximal level in the current study, might even lead to collapse of production of the natural food web and deterioration of water quality. As a consequence, fish yield might decrease as well. Additionally, a much larger field experiment, involving large numbers of farmers, is needed to confirm the economic benefit of applying increased feed ration in non-aerated ponds.

The economic benefit of applying low P:E ratio diet was mainly due to low the feed cost (as well as reduced total production cost) while increasing the yield (and return) at the same time. In aquaculture, feed cost is the main factor determining economic return (Hebicha, El Naggar and Nasr-Allah, 2013; Yuan and Dai, 2017). On the other hand, Yuan and Dai (2017) also mentioned that fish price also plays an important role in the economic profitability as the profit margin is low and fish price varies due to season and location. In the present study calculation of economic return was based on the wholesale price of tilapia at the day of harvest. Price fluctuation was not considered. Our calculations were only intended to give an idea of economic benefit that can be achieved by using the low P:E diet for tilapia aquaculture in ponds.

5.5. Conclusion

Lowering the P:E ratio from 18 to 14 g.MJ^{-1} in formulated feeds applied in farmers pond improved tilapia production. Repetition of this result from experimental ponds to farmers pond indicates that the requirement of P:E ratio in the supplemental diet for tilapia aquaculture in ponds are lower from the known standard. Apparently, fish consumed more natural foods to compensate for the lower nutrient input through the formulated feed. Better yield with high stocking density without compromising the feed efficiency indicates possibility of further intensification even in non-aerated ponds. Increasing feeding level increased growth and yield but also created more pollution (in terms of feed N not retained in fish). The economic assessment indicates that using low P:E diet will increase farmers economic benefit and will increase economic viability of tilapia farming in areas of low profit margin. Additional research to test the performance at higher intensity will help to understand effect of this concept on more commercial implication as well as on improving carrying capacity of the pond system.

Chapter 6

General discussion

6.1 Overview

The aim of this research was to develop better understanding on how dietary macronutrients determine fish production in ponds directly, as a nutrient source for fish, and indirectly, via fertilization of the pond's food web (Fig. 1). The second aim was to determine how the impacts of macronutrients on fish performance interact with culture intensity (*e.g.* feeding level and stocking density). In the current study, Nile tilapia was used as model species.

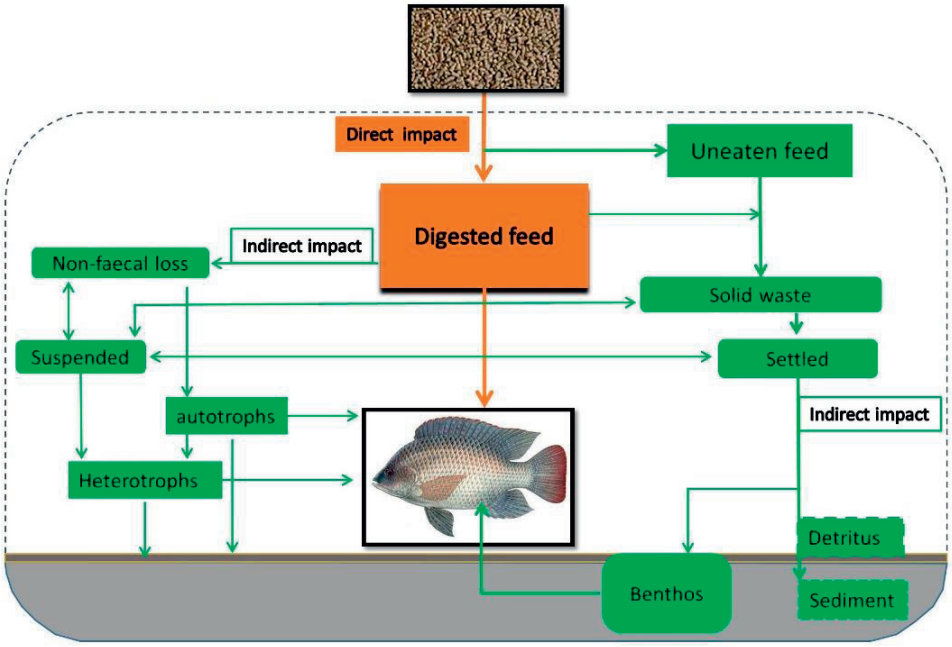

Fig. 1. The concept of direct and indirect impacts of feed on fish performance in pond

The dietary protein to energy (P:E) ratio influenced pond productivity. Fish performance was better with a low P:E diet. Changing the dietary non-protein energy source from lipid to carbohydrate had no effect. However, fish performance (*i.e.*, pond productivity) was better when the diet contained low degradable non-starch polysaccharides (NSP) compared to high fermentable NSP. As we observed better performance with a low P:E diet which had a lower P:E ratio than recommended by NRC (1993), we validated the outcome in an on-farm trial. In the on-farm trial the positive effects of lowering the dietary P:E ratio were again present. There was no interaction either between dietary P:E ratio and feeding level or between dietary P:E ratio and stocking density which implies that the positive impacts of the low P:E ratio was unaffected by culture intensity.

6.2 Fish, feed, food and culture intensity

6.2.1 Effect of dietary protein to energy (P:E) ratio

In chapter 2 and 5 we have noticed that the optimum level of the dietary P:E ratio for pond feeds is different from the NRC (1993) recommendation. Lowering the dietary P:E ratio below the optimal levels recommended by NRC (1993) increased fish production at pond level (chapter 2, table 2; chapter 5, table 2) and greatly stimulated the natural food production in the pond which not only compensated for reduced dietary protein inclusion but also contributed to higher growth (chapter 2, fig. 2). Haidar *et al.* (2018) found that the optimal digestible protein to digestible energy ratio for Nile tilapia is lower than the NRC (1993) recommendation even in absence of natural food. However, in the Haidar *et al.* (2018) study, the dietary protein level was much higher (> 38%) compared to the low P:E diet of this study (24%). Therefore, we assume that the requirement of dietary protein to energy ratio and/or optimal dietary protein level is related to the intensity of the culture system. In the on-farm trial (chapter 5), we again observed better pond productivity at the "low" P:E diet even at the higher stocking density and also at higher feeding levels. Therefore, we hypothesise that up to a certain (critical) level of intensity, the optimal dietary P:E ratio does not change with culture intensity (Fig.2). Above this critical point of culture intensity, the optimal P:E ratio might increase towards the optimal level recommended by NRC (1993) measured in experiments without the presence of a natural food web. To test this hypothesis and to understand the combined effect of culture intensity and presence of natural food, (more) nutritional studies should be carried out in ponds with a functional food web.

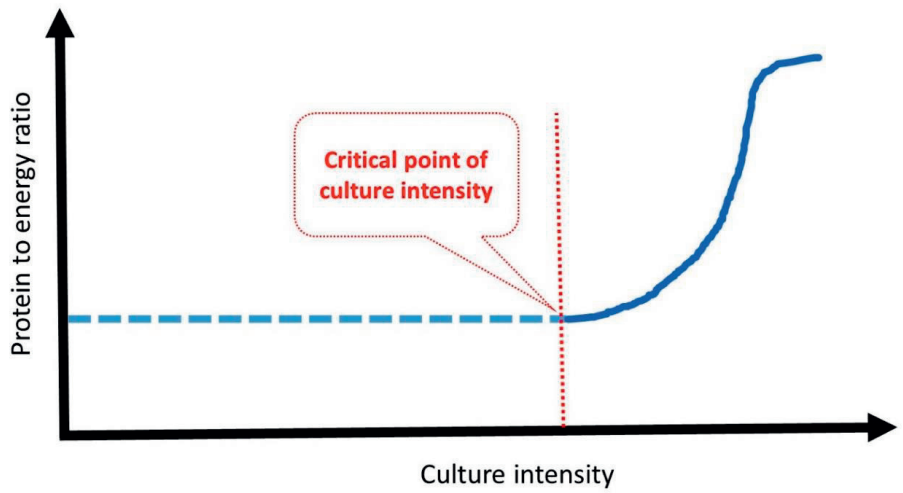

Fig. 2. Change in the optimal dietary protein to energy (P:E) ratio in relation to the intensity of culture system

6.2.2 Effect of dietary carbon

In experimental ponds but also in the on-farm trial, we observed consistently that carbon plays a vital role in enhancing the contribution of natural food to fish production in the pond. Considering this importance of dietary carbon (or energy), we further investigated whether the type of non-protein energy (*i.e.*, carbohydrate or fat) has a different effect on the contribution of feed to the fish growth in ponds. In chapter 3, we found that the source of dietary non-protein energy makes no difference in fish production (chapter 3, table 2), directly nor indirectly (chapter 3, fig. 2). In that study, the impact of non-protein energy was studied in terms of dietary carbohydrate to lipid ratio. As most lipid sources have a comparable apparent digestibility coefficient (ADC), we did not explore different lipid sources further. However, carbohydrates show a broad range of ADCs. Starch and sugar are considered easily digestible sources of energy for the fish, while non-starch polysaccharides are not desirable. With increasing tendency of using plant-based ingredients in fish feeds, inclusion of NSPs will become inevitable. NSPs can be very different in terms of ADC by fish but also in terms of resistance to microbial decomposition in ponds. In chapter 4, we noticed that low fermentable (slow degradable) NSPs influenced fish production in ponds better than a diet with high fermentable (fast degradable) NSPs (chapter 4, table 2). This better performance was achieved indirectly through the contribution of natural food to the total fish production in the pond (chapter 4, fig. 4).

6.2.3 Influence of feeding level and stocking density

Across all the experiments, feeding level increased fish production but reduced feed utilization efficiency (*i.e.*, the feed conversion ratio- FCR – increased). Fish production increased with changing stocking density from 2 to 3 tilapia.m^{-2}. The effect of stocking density on production was numerically more pronounced with a low P:E diet (chapter 5, table 2). The contribution of natural food as a consequence of the type of feed given decreased with feeding level (chapter 2, fig. 3). At high feeding level the contribution of natural food to fish growth was much higher with a low P:E diet (~50 *vs* 30%). This tells us that if we can make a proper feed for pond culture, the natural food will always have a strong effect on fish production even when feeding intensity is high. In the on-farm trial (non-aerated ponds), the maximum fish biomass with the low P:E diet was 6000 kg.ha^{-1} (chapter 5, table 2) which was higher than the reported carrying capacity for tilapia in non-aerated ponds (Wang and Lu, 2016). Therefore, we hypothesised that if the synergetic effect of supplemental feed (formulated for feeding the pond) and natural food can be optimized properly, the carrying capacity of the pond might increase (Fig. 3).

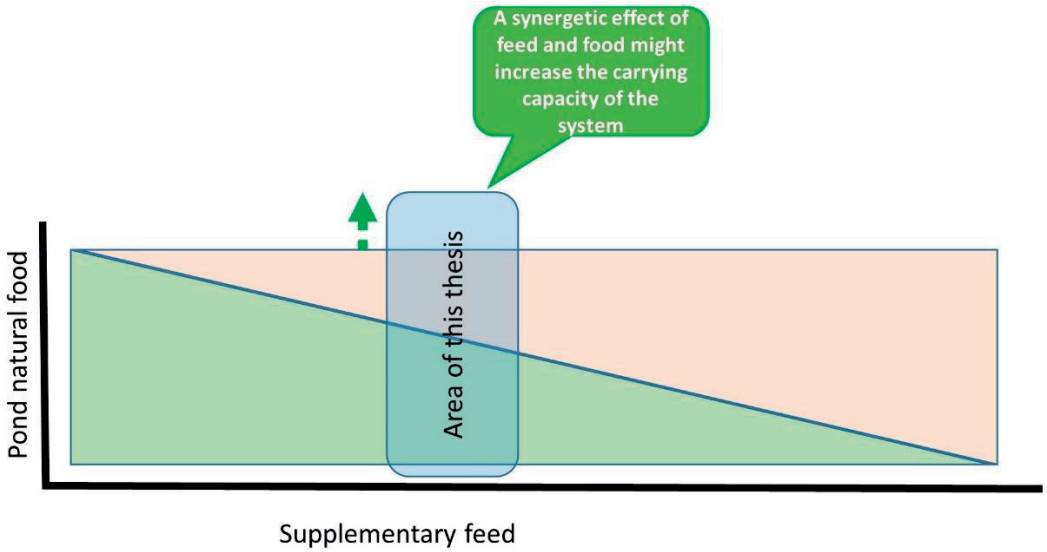

Fig. 3. Assumption of the synergetic effect of supplemental feed and natural food on the carrying capacity of the pond

6.3 Feeding fish or pond

The above findings indicate that the composition of dietary macronutrients for pond aquaculture is different from the known NRC recommendations and current industrial practice. Now the question is what we should focus on: feeding the fish or feeding the pond as a system including fish?

6.3.1 Dietary nutrient requirements for feeding fish

To feed fish, ignoring the effect of natural food, in any type of production system, we have to make a diet, which entirely meets the nutrient requirements of the fish. For example, the optimum dietary protein to energy ratio for Nile tilapia ranges between 18-23 $g.MJ^{-1}$ on digestible nutrient basis (NRC, 1993) and the recommended concentration of crude protein is always above $300g.kg^{-1}$ (NRC, 2011). Lipid based high energy diets are often recommended for better fish performance. In these high energy diets, lipids can be complemented by easily digestible starch sources. As starch is coming from plant ingredients, it comes along with more or less NSPs. NSPs are not well digestible by fish and hence are usually not desired. Feed also requires adequate vitamins and minerals (*i.e.*, phosphorus) in addition to a dense concentration of macro-nutrients. This high-quality diet is efficient but also more expensive. Under this concept, feed is the only available nutrient provider for fish and the amount of feed per kg fish production is more likely higher compared to a system where half of the fish growth is achieved from natural food. Moreover, a diet with a high concentration of protein and with a higher ADC produce faeces with insufficient energy (or carbon) for microbiota to fully

degrade the organic waste resulting from feeding the fish. This leads to pollution and incomplete mineralization.

6.3.2 Carbon (energy) plays a key role in feeding the pond

An advantage of pond aquaculture is the presence of natural food that also contributes to the nutritional requirements of the fish (Porchas-Cornejo *et al.*, 2012; Pucher *et al.*, 2014; Pucher and Focken, 2017; Roy *et al.*, 2012). For an aerobic breakdown of organic matter, on a molar basis, the amount of carbon in the matter (for example, faeces) needs to be minimally twice the amount of its nitrogen (Avnimelech, 1999). Faeces resulting from a high protein diet, in which all macro-nutrients are highly digestible, lacks this carbon (Haidar *et al.*, 2018). Increasing the C:N ratio of the feed has been found effective in enhancing both autotrophic and heterotrophic food webs (Kabir *et al.*, 2019). For certain species this concept was effective in both mono-culture and polyculture systems (Asaduzzaman *et al.*, 2010b; Uddin, 2007; Wahab *et al.*, 2011). In chapter 2 and 5 we have seen that the contribution of natural food increased with increasing dietary C:N (or decreasing P:E) ratio. When the aim is to provide carbon to the food web through the fish diet, a part of the carbohydrate in the diet must better not be digested so that the fish faeces gets a low P:E ratio (or high C:N ratio). Among the carbon types, less degradable NSPs enhanced the natural food most. Therefore, the macro-nutrient composition for feeding the pond is different from the one for feeding only the fish. In addition, algae can be a source of essential unsaturated fatty acids (Teuling *et al.*, 2017) and microbes may liberate bound (*i.e.*, non-available) phosphorus into free/available phosphorus for fish (Da Silva *et al.*, 2013). The natural food most likely contains also vitamins and other minerals (Abd El-Hady *et al.*, 2016). Therefore, we believe that the extent of micro-nutrient inclusion in the pond feeds may also change. This may have positive side consequences since feed cost may decrease and the aqua feed industry gets the opportunity to diversify their range of ingredients.

6.3.3 How to feed the pond

If we consider carbon as a critical input for feeding the pond, we need to find better ways of doing this. Several studies (Asaduzzaman *et al.*, 2010a; Hari *et al.*, 2006, 2004; Magondu *et al.*, 2015; Yogev *et al.*, 2017) focused on enhancing the contribution of natural food, increasing the C:N ratio of the nutrient input by feeding a commercial feed and parallel to it, adding a carbohydrate source (such as tapioca flour) to the pond. In this study, we took a different approach of feeding the pond by putting all extra carbon for the pond into the diet. The diet was formulated with the intension to increase the C:N ratio by reducing the crude protein and increasing the carbohydrate content. A regular diet combined with extra carbon does not involve industry in the process of innovation and expansion. It is also difficult for a small-scale farmer to calculate the necessary amount of carbon to achieve the proper C:N ratio in parallel

to the diet. Commercial diet compositions are not identical and hence making a general guideline cannot be used. This approach requires also that the farmer sources and purchases dual inputs. In this respect, developing novel feeds which lead to the right C:N ratio in the pond constitutes a more elegant solution. It includes the feed industry, stimulating it to focus on innovations, it helps solving the sustainability challenges of sectoral growth and can bring a comprehensive solution to the end-user.

The proposed approach provides other advantages (Fig.4). When carbohydrate is included within the diet, it first goes through the fish digestion and absorption process. So, the energy and nutrient input is primarily contributing to fish growth. The urinary, branchial and faecal wastes are utilized by the organisms in the pond's food web and contribute to fish growth as an indirect pathway of nutrient deposition. When carbohydrate is applied in the pond separate from the diet, the nutrient (or energy) goes straight to the food web and the energy becomes available to fish only through transfer of one or more trophic level. Roughly 90% of the energy is lost when is transferred from one trophic level to the next in an ecosystem (Feng *et al.*, 2018). Therefore, the added extra carbohydrate is less efficient. When adding energy by providing carbohydrates separately form the feed to raise the C:N ratio to 12 – 20, much more energy is given to the food web than through the faeces, thus demanding much more oxygen also. In addition, the extra carbon is easily and quickly removed from the system via the effluents. On the other hand, faeces produced with a high protein fish diet lack sufficient energy to stimulate natural food production in the pond. Therefore, adding carbon in parallel to a regular diet is less effective than adjusting the energy distribution in the feed considering the food web in addition to the fish requirements. Indeed, as the supplementary energy (carbon) becomes only available to fish after passing through one or more trophic levels, less of its original energy will be ultimately available for fish. So, feeding the pond solely via the diet is a better option in terms of ecological functioning, management and application.

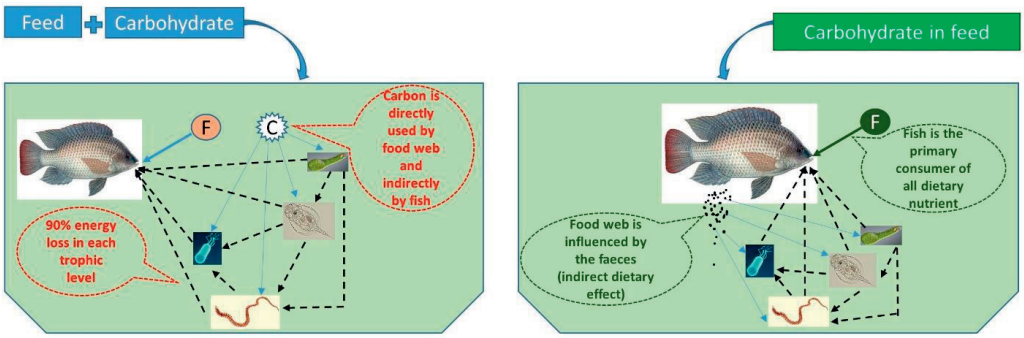

Fig. 4. Comparison of feeding the pond with a combination of commercial diet and additional carbohydrate versus with a diet rich in carbohydrate

6.3.4 Challenges of feeding the pond

The concept of feeding the pond, as illustrated above, is based on a few experimental observations only. Our study is a first step to generate more detailed knowledge in this area.

6.3.4.1 Monoculture *vs* polyculture

Most of the current fed-pond aquaculture in South Asia are polyculture systems. Our study however has been tested in monoculture conditions. Although few studies looked at natural food production and its role in polyculture systems (Asaduzzaman *et al.*, 2010b; Rahman *et al.*, 2008; Wahab *et al.*, 2001), their focus was not on feeding the pond solely with diet, rather on enhancement of natural food via fertilization. It means that the application in polyculture systems of the concept presented in our study is still poorly understood. In principle, if more than one species is used, and each species partially overlaps in how they feed on natural foods, the benefit from feeding the pond in polyculture systems might in some species combinations be higher than in monoculture ponds, in other species combination lower than in monoculture ponds. More research will be needed to develop polyculture systems that make efficient use of dietary nutrients.

6.3.4.2 Stocking density

In this thesis, we tested the interaction of diet and stocking density in the lower range (3 fish.m^{-2}). Therefore, the question how strong natural food can contribute to fish production in very intensive systems needs still to be verified. Initially we need to determine the carrying capacity of a pond for tilapia (both in monoculture and polyculture conditions) fed with pond feed in a non-aerated pond. Later we can test the effect of pond feeding under more intensive conditions with manipulation of the system (*e.g.* aeration, water exchange, sludge removal *etc.*). Intensification of the pond system above its natural carrying capacity would require landscape planning to enable high water exchange and provide the infrastructure needed for wider scale application of aeration. Also, including these changes to the pond feeding concept might alter its economic and environmental performance as indicated in this thesis (chapter 5, table 6; chapter 2, table 4 and fig. 4).

6.3.4.3 Variation in agro-ecology

Aquaculture ponds are mainly concentrated across the whole tropical and sub-tropical belt of the world. Water and soil conditions vary largely in this wide geographical spread. In our thesis, we provided only evidence from Bangladesh. However, the farmers pond trial was done across a larger area, outside the experimental station, with variable pond water and soil conditions. Yet it reproduced similar outcomes (chapter 5, table 2) as in the experimental ponds presented in chapters 2 to 4. However, we acknowledge that this was not yet comparable to global scale variation. Therefore, the concept also needs to be tested at larger

geographical scale, especially in areas where aquaculture is an important activity and in areas where it is proposed to grow.

6.3.4.4 Adaptation with climate change

In the tropics, there are many areas where the monsoon has strong effects on water availability and salinity. Moreover, freshwater fish are mostly produced in ponds in the deltas. Most of the deltas in the tropics are highly vulnerable for climate change including sea level rise, salinity intrusion, global warming, interrupted upstream flow and uncertain pattern of rain (IPCC, 2014). Temperature and salinity are known to interfere with dietary nutrient utilization in fish (Keembiyehetty and Wilson, 1998; Moreira *et al.*, 2008; Tran-Ngoc *et al.*, 2017) and also influence the composition of natural food in ponds (Shurin *et al.*, 2012; Van Meter *et al.*, 2011). Therefore, the above situation might alter the effectiveness of the proposed concept of feeding the pond.

6.3.4.5 Culture species

Natural feeding habit differs among species. The concept of pond feeding presented in this thesis is based on the performance of Nile tilapia, which is an omnivorous species. The effectiveness of the same concept on other major pond culture species such as carp, catfish and shrimp are not known. In principle it should work for carp and shrimp ponds as well but of course there may be some variations among the species. The latter requires further research.

6.3.4.6 Flexibility in selecting ingredients

One major advantage in this concept is its flexibility in selecting locally sourced plant-based ingredients and co-by products from other food system. The nutrient composition of many of those ingredients are not known. They may contain anti-nutritional factors and might require further processing to be used in fish feeds. Developing information on local ingredients for pond diet formulation will require more research, investment and industrial collaboration within and between different food production systems.

6.3.4.7 Nutrient extraction from the environment

In some treatments (chapter 3, table 5; chapter 5, table 5) we observed depletion of nutrients, such as organic matter, nitrogen, phosphorus and potassium from the pond environment. For instance, in treatments where the N stock in water column or sediment reduced, while 70 – 85% of the dietary input was retained in harvested fish, consecutive production cycles might lead to reduced natural food production. This might be topped up by carefully formulating the pond diet and optimizing the feeding level or providing extra nutrients via inorganic fertilization. Run-off from neighbouring agricultural field can also balance possible nutrient

shortage. However, receiving run-off might also introduce toxins from other food and non-food sectors of the larger landscape, leading to contamination of the fish. A wide range of studies needs to be conducted to understand all these mechanisms. Therefore, careful and clear knowledge based development is needed, before this concept can be widely applied.

6.3.4.8 Develop methods to quantify the contribution of natural food to fish production

The advantage of feeding the pond system is basically due to the indirect dietary impact on fish growth via natural food (Kabir *et al.*, 2019). The process involved in utilizing uneaten food, faecal waste and branchial and urinary loss by the natural food of the pond is not well studied. In this thesis we monitored the concentration of chlorophyll a, abundance and diversity of phytoplankton, zooplankton, benthos and total count of bacteria in the pond (chapter 2,3, and 4 respectively in table 5, 6 and 6). Although we monitored differences in the contribution of natural food to fish growth in response to different dietary macro-nutrient compositions, these were barely reflected in our measurements. We have also observed the natural food content in the fish stomach (chapter 3, table 7; and chapter 4, table 7). In chapter 4 we measured stomach fullness by volumetric (%) and gravimetric (%) methods, and observed differences among treatments. Analysis of $\Delta^{13}C$ and $\Delta^{15}N$ in the feed, fish, plankton and periphyton from the pond indicated that fish consumed nutrients other than feed. But it did not allow to distinguish between the contribution of feed and natural food to fish growth. Unfortunately, none of the above methods is very precise. Increasing the sampling frequency and the duration of experiment might provide more precise observations. Mathematical modelling can also be developed considering fish nutrition physiology and food web interactions to predict the contribution of diet and natural food to pond production. Future research might explore this and develop methods which can explain the functioning of a pond's food web, and total production.

6.4 Sustainability

In our experiments, when feeding diets with a low protein to energy ratio, 70 to 85% of the nitrogen supplied with the diet was retained in the harvested fish (chapter 2 and 5). The nitrogen retention efficiency in traditionally fed ponds is normally 25 to 50%. As a consequence, the use of diets with a low protein to energy ratio, reduces the accumulation and discharge of nitrogen. Despite of this low environmental impact from pond farming, farmers using the low protein to energy ratio diet achieved an (extrapolated) annual production of 12 to 14 thousand $kg.ha^{-1}$ in household ponds (chapter 5). This is much higher than the average annual production of 3 to 5 thousand kg/ha presently obtained in fed fish ponds in Bangladesh (Belton and Azad, 2012). These data from Bangladesh are comparable with global fish productivity data for ponds (FAO, 2018). So, using diets with a low protein to energy ratio can help to double fish yields from ponds while having a minimal impact on

nutrient accumulation and discharge from ponds, thus contributing to the sustainable use of marine resources (SDG 14). Intensification will also help to reduce greenhouse gas (GHG) emission from ponds, as extensive ponds contribute relatively more to GHG emission than intensive ponds (Robb *et al.*, 2017). Additional reductions can be achieved by using presently unused by-products from local crops as carbohydrate source in diets with a low protein to energy ratio. This combination of valorising (1) faeces and excreta in-situ, resulting in very high nutrient retention efficiency and (2) incorporating local crop waste into aquaculture feeds will firmly integrate aquaculture into the circular economy, without negatively affecting the profitability of pond farming (chapter 5, table 6).

Above concept was tested on tilapia monoculture. For wider application, there is a need to test the concept also with other species, specially on shrimp and carps, and to explore polycultures for different combinations of these species.

6.5 Conclusion

Overall the following conclusions can be drawn from this thesis:

- In ponds with a functional natural food web, the optimal macronutrient composition of supplementary feeds for tilapia differs from the optimal composition as recommended by NRC (1993, 2011) for tilapia. This may be related to the fact that the NRC recommendations were developed without the presence of natural food, and that the effect of the latter may be related to a possible enhancement of its production due to the extra nutrient input in the ecosystem via the waste of the fish (fertilizing effect) .
    a. Lowering the dietary protein to energy ratio increased fish production at the pond level, mainly via the indirect impact of diet by enhancing the natural food in the pond.
    b. The type of non-protein energy (lipid versus carbohydrate) neither directly affected fish performance nor indirectly via influencing the natural food web of the pond.
    c. The contribution of natural food to fish growth was affected by the types of dietary non-starch polysaccharides (NSP).
- Feeding level increased fish production in semi-intensive pond culture of tilapia for all the tested diets but also increased FCR.
- Fish production increased with increasing stocking density (within the tested levels).
- Culture intensity (feeding level and stocking density) did not interact with the influence of the dietary macro-nutrient composition (*i.e*. P:E ratio) on pond

productivity. In other words, enhancement of the natural food web through diets is possible even with increasing culture intensity.

- Better approach of quantification of natural food to fish production might be useful for matching the effect of natural food with diet composition.
- Feeding the pond system (including the fish) will increase economic profitability and environmental sustainability of pond aquaculture.

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Summary

Aquaculture is one of the fastest growing food producing sectors. In 2016, aquaculture production reached 80 million metric tonne and contributed 53% to the global fish consumption. The majority of cultured fish comes from ponds. In pond culture systems, the natural food web provides part of the fish's nutritional requirements. Studies in which nutrient requirements of fish are estimated are mainly done in the absence of such a natural food web. Even more, (optimal) diet formulation for fish pre-dominantly ignores the potential intake of natural food from ponds and thus also miss the possible indirect (fertilizing) effects of feed on the food web. Therefore, we hypothesise that current feeds and fish production approaches are suboptimal because of ignoring the possible role of natural food in the growth of pond fish. Matching natural food and supplementary feeding can create synergetic effects for growing more fish with less nutrient inputs. However, the knowledge how to optimize diet composition, taking into account that the natural food of the pond can be enhanced by the amount and type of waste produced by the fish, is very limited. The aims of this research were to develop a better understanding on how dietary macronutrients determine the growth of fish when cultured in ponds, as a direct nutrient source for fish, and indirectly, via fertilization of the pond's food web. A second objective was to determine how the impacts of macronutrients on fish performance interact with feeding level and culture intensity.

In **chapter 2** we assessed if lowering the dietary protein to energy (P:E) ratio (and thus increasing the C:N ratio of the feed input in the pond) below the optimal P:E ratio affects fish productivity, food web dynamics and nitrogen balances in semi-intensively managed tilapia ponds. Twelve ponds, each divided into three equal compartments, were assigned to test the effect of two diets, which differed in P:E ratio (19 *vs* 14 $g.MJ^{-1}$). Three feeding levels (no feed, "low" and "high") were nested in each pond in a split-plot design. The duration of the experiment was 60 days. Decreasing the P:E ratio enhanced tilapia production and specific growth rate ($P<0.05$; 1195 *vs*. 986 $g.compartment^{-1}$ and 1.76 *vs* 1.55 $\%.d^{-1}$). Body composition of tilapia was unaffected by diet and feeding level. Averaged over both diets, survival and feed conversion ratio increased with increasing feeding level ($P<0.001$). Diet composition did not alter water quality, nor abundance and diversity related parameters of the food web. With the low P:E diet, 87% of the combined feed and fertilizer N input was retained in the fish compared to 59% from the high P:E diet. As a result, total N accumulation in the pond was lower with the low P:E ratio diet (*i.e.*, low protein diet). The data on N gain and N balance at the pond level suggest that the food web productivity was stimulated by reducing the dietary P:E ratio below the reported optimal levels in the literature. Our results suggest that the optimal dietary P:E ratio is dependent on the culture intensity (extensive, semi-extensive or intensive pond culture).

In **chapter 3** we tested if the type on non-protein energy in the diet (lipid *vs* carbohydrate) affected fish productivity, and natural food enhancement in semi-intensively managed tilapia ponds. The carbohydrate to lipid (CHO:LIP) ratio of the two test diets were 4.7 *vs* 19.5 $g.g^{-1}$. The experimental approach was the same as in chapter 2 and the duration was 42 days. Increasing CHO:LIP ratio had no impact on tilapia production (*i.e.*. biomass gain = 2154 *vs* 2077 $g.compartment^{-1}$); specific growth rate (1.36 *vs* 1.30 $\%.d^{-1}$); FCR(1.65 *vs* 1.80); and survival (89%). However, feeding level influenced both biomass gain, SGR and survival. Apparent digestibility coefficient (ADC) for fat and carbohydrate was influenced by the dietary CHO:LIP ratio but ADC for (overall) energy was unaffected. Despite of replacing non-protein energy source from lipid to carbohydrate, fat content in the body didn't exceed 5.5% at any feeding level. Dietary CHO:LIP ratio had no impact on N, P, K, and OM of pond soil and water and measured natural food except for phytoplankton diversity. There was no effect of dietary CHO:LIP ratio on the faeces composition. The data on N gain from natural food also indicated no difference. The results show that changing the type of dietary non-protein energy source from lipid to carbohydrate did not have any impact on tilapia production in semi-intensive ponds.

In **chapter 4** we determined the effect of the type of dietary non-starch polysaccharides (NSP)s on fish production and the contribution of natural food to total fish production in semi-intensively managed tilapia ponds following the same experimental approach of chapter 2. Two experimental diets were "PecHem-Diet" (pectin and hemicellulose), a diet with easily fermentable NSP, and "LigCel-Diet" (lignin cellulose), a diet with slowly fermentable NSP. The experiment lasted 56 days. With the "LigCel-Diet" fish biomass gain was higher (2192 *vs* 2599 $g.compartment^{-1}$) and feed conversion ratio (FCR) was lower (1.9 *vs* 1.4) than with the "PecHem-Diet". The type of dietary NSP had no effect on fish survival and specific growth rate (SGR). Averaged over both diets, increasing the feeding level increased biomass gain, fish survival, FCR and SGR. There was a significant interaction effect between diet and feeding level on FCR. Fish body composition at harvest was the same between diets. With the "LigCel-Diet", the apparent digestibility coefficient (ADC) was higher for crude protein, fat, phosphorus and calcium and lower for ash compared to the other diet. Neither feeding level nor the interaction between diet and feeding level influenced the apparent digestibility coefficient (ADC) of any nutrient. Diet composition did not alter the organic matter (OM) composition of the faeces. $\Delta^{13}C$ and $\Delta^{15}N$ data from the stable isotope analysis revealed that N gain in fish originated from both feed and natural food of the pond. The abundance of natural food in the pond increased over time for both diets. Chlorophyll-a was higher in the pond fed with "LigCel-Diet". Fish gut content and calculated N gain indicated an enhanced contribution of natural food to fish growth in ponds fed with "LigCel-Diet". In conclusion, the type of dietary NSP determines tilapia productivity in semi-intensive managed ponds by altering food web productivity.

In **chapter 5** in an on-farm trial, we tested the effect of lowering dietary P:E ratio on fish performance, pond nutrient utilization and economic benefit under two stocking densities and feeding levels. Forty ponds were assigned to test the effect of two diets, which differed in P:E ratio (18 *vs* 14 g.MJ^{-1}), two feeding levels (14 *vs* 18 g.kg$^{-0.8}$.d^{-1}) and two stocking densities (2 *vs* 3 fish.m^{-2}). Initial fish biomass was 45(±21) *vs* 67(±38) g.m^{-2} at 2 *vs* 3 fish.m^{-2}, respectively. The experiment lasted 82 days. Decreasing the P:E ratio enhanced tilapia production (P<0.05; 459 *vs* 399 g.m^{-2}). Increasing the stocking density from 2 to 3 tilapia.m^{-2} increased biomass gain 43% (P<0.001; 354 *vs* 505 g.m^{-2}). Averaged over both diets and stocking densities, growth and feed conversion ratio (FCR) increased with increasing feeding level (P<0.001). Neither the interaction of diet and feeding level nor the interaction of feeding level and stocking density influenced any of the indicators of fish performance. Fish survival was unaffected by diet, stocking density and feeding level. Dissolved oxygen increased with increased stocking density with the low P:E diet while the opposite happened with high P:E diet (P<0.05). N retention efficiency was higher with the low P:E ratio diet (P<0.001; 71 *vs* 52%) and decreased with increasing feeding level (P<0.001). The data on N gain and N balance at the pond level suggest that the food web productivity was stimulated by reducing the dietary P:E ratio. The low P:E diet increased the gross margin by 95% (P<0.001; 2076 *vs* 1067 USD.ha^{-1}) and benefit cost ratio by 22% (P<0.05; 1.57 *vs* 1.29). The P:E ratio of the low P:E diet is lower than the presently advised level. Lowering the P:E ratio from 18 to 14 g.MJ^{-1} in pond feeds for tilapia will increase the economic viability of tilapia pond culture.

In **chapter 6** the main outcomes of the studies of this thesis were summarized and discussed in the context of feeding the pond and its role in sustaining aquaculture growth, and working towards a circular food production system for pond aquaculture.
Overall the following conclusions can be drawn from this thesis:

- In ponds with a functional natural food web, the optimal macronutrient composition of supplementary feeds for tilapia differs from the optimal composition as recommended by NRC (1993, 2011) for tilapia. This may be related to the fact that the NRC recommendations were developed without the presence of natural food, and that the effect of the latter may be related to a possible enhancement of its production due to the extra nutrient input in the ecosystem via the waste of the fish (fertilizing effect) .
    a. Lowering the dietary protein to energy ratio increased fish production at the pond level, mainly via the indirect impact of diet by enhancing the natural food in the pond.

b. The type of non-protein energy (lipid versus carbohydrate) neither directly affected fish performance nor indirectly via influencing the natural food web of the pond.
c. The contribution of natural food to fish growth was affected by the types of dietary non-starch polysaccharides (NSP).

- Feeding level increased fish production in semi-intensive pond culture of tilapia for all the tested diets but also increased FCR.
- Fish production increased with increasing stocking density (within the tested level)
- Culture intensity (feeding level and stocking density) did not interact with the influence of the dietary macro-nutrient composition (*i.e.*. P:E ratio) on pond productivity. In other words, enhancement of the natural food web through diets is possible even with increasing culture intensity.
- Better approach of quantification of natural food to fish production might be useful for matching the effect of natural food with diet composition.
- Feeding the pond system (including the fish) will increase economic profitability and environmental sustainability of pond aquaculture.

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About the author

Kazi Ahmed Kabir was born on 31 December 1980 in a small village in the coastal region of Southwest Bangladesh. After completing the higher secondary school, he studied Zoology, at the faculty of Biological Science in the University of Dhaka and passed MSc in 2007. His specialization in BSc and MS degree was on fisheries. During his student life he was actively involved in co-curricular and social activities. He was one of the founder of Young Biologists Association (YBA) in the University of Dhaka and Riverine People in Bangladesh. He was national focal point for "Roots and Shoot", the student wing of Jane Goodall Institute and Commonwealth Youth Environment Network in the University of Dhaka. He was strongly connected with nature conservation and worked as volunteer in the projects of Ramsar Center Japan and UNDP. After graduation he started his career as a Lecturer in the School of Environmental Science and Management (SESM) at the Independent University of Bangladesh (IUB). He also have worked as consultant in UNDP and USAID. While getting more involved in the development programs, he shifted his career focus from conservation to food security, specially from Bangladesh country perspective. He then started working as a program manager in Bangladesh Shrimp and Fish Foundation and soon moved to WorldFish. He was also involved with several national NGOs and was successful in getting development grants. While working in WorldFish in different roles he was involved in country programs in Bangladesh, India, Indonesia and Sierra Leone. During his career in aquaculture he was focused on coastal aquaculture, integrated agriculture and aquaculture and small holders homestead farming. His research interest was nutrient management at system level and making aquaculture more harmonious with environment. He started his PhD in late 2014 at the Aquaculture and Fisheries Group of Wageningen University. He resumed his employment in WorldFish from March 2019. During his career he published several book chapter, communication products, technical report, policy papers and peer reviewed science papers.

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Publications

Peer reviewed science articles

Kabir, K. A., Schrama, J. W., Verreth, J. A. J., Phillips, M. J., & Verdegem, M. C. J. 2019. Effect of dietary protein to energy ratio on performance of nile tilapia and food web enhancement in semi-intensive pond aquaculture. Aquaculture, 499(July 2018), 235–242. https://doi.org/10.1016/j.aquaculture.2018.09.038

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Peer reviewed conference proceeding:

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K.A. Kabir , S.B. Saha, M. Karim, C.A. Meisner and M. Phillips. 2015. Productivity, diversification and resilience of saline aquaculture systems in coastal southern Bangladesh. In Humphreys, E., T.P. Tuong, M.C. Buisson, I. Pukinskis and M. Phillips. 2015. Revitalizing the Ganges Coastal Zone: Turning Science into Policy and Practices Conference Proceedings. Colombo, Sri Lanka: CGIAR Challenge Program on Water and Food (CPWF). 600pp

Karim, M. Ullah, M.H., Kabir, K. A., Meisner, C., Phillips, M. , 2015. Does homestead food production holds promise to improved household food security?: empirical evidence

from the southwest coastal zone of Bangladesh. In Humphreys, E., T.P. Tuong, M.C. Buisson, I. Pukinskis and M. Phillips. 2015. Revitalizing the Ganges Coastal Zone: Turning Science into Policy and Practices Conference Proceedings. Colombo, Sri Lanka: CGIAR Challenge Program on Water and Food (CPWF). 600pp

Kabir, K. A., Faruque, G, Sarwar, R., Barman, B., Choudhury, A., Hossain, M., Hossain, E., Aleem, N. A., Karim M., Kamp, K., Phillips M., 2015. Producing fish in the homestead shaded ponds: Finding solution with rural women. In Humphreys, E., T.P. Tuong, M.C. Buisson, I. Pukinskis and M. Phillips. 2015. Revitalizing the Ganges Coastal Zone: Turning Science into Policy and Practices Conference Proceedings. Colombo, Sri Lanka: CGIAR Challenge Program on Water and Food (CPWF). 600pp

Working Paper/Policy Briefs:

Kabir, K.A., Toufique, K. A., Genschick, S., Tran, N., Thilsted, S., and Phillips, M.J., 2018. Aquaculture and the poor: improving fish production, consumption and nutrition in Bangladesh Policy Brief. WorldFish

Douthwaite B, Kabir KA, Karim M, Lando LA, Longley C, Muyaule C, Perez M, Siota F and Sukulu M . 2015. More inclusive science for the poor: linking farmers to researchers using the rind approach. In Douthwaite B, Apgar JM, Schwarz A, McDougall C, Attwood S, Senaratna Sellamuttu S and Clayton T, eds. 2015. Research in development: Learning from the CGIAR Research Program on Aquatic Agricultural Systems. Penang, Malaysia: CGIAR Research Program on Aquatic Agricultural Systems. Working Paper: AAS-2015-16

Articles in International Aquaculture magazine:

K.A. Kabir , S.B. Saha, M. Karim, C.A. Meisner and M. Phillips. 2016. Improving the Productivity, Diversification and Resilience of

Saline Aquaculture Systems in Coastal Southern Bangladesh. World Aquaculture. World Aquaculture Society Magazine. March,2016.

K.A. Kabir , S.B. Saha, M. Phillips., M. Karim, and C.A. Meisner. 2016. Rice-fish integration for high saline, coastal areas of Bangladesh: Learning from the Challenge Program for Water and Food (CPWF). Advocate, Global Aquaculture Alliance Magazine. February, 2016

K.A. Kabir, N. Khan, M. Karim, C.A. Meisner and M. Phillips. 2015. Portable Pond for Communities in Need. Info Fish International. 6/2015

Book chapter and booklet

Kabir, K.A ., Karim, M, 2015. Case study of empowering women through participatory action research. Boru D, Kamp, K Edts. More accessible science for the poor: linking farmers to researchers through PAR. CGIAR-AAS

Baby, R.L., Kabir, K.A, Chakrabarti, T.R. and Siddiki, M.H., 2007. Jibobaichitro O Jolbau Poriborton (Biodiversity and Climate Change), Booklet, Climate Change Cell. (in Bangla)

Popular articles in newspapers/blogs:

Kabir, K.A. 2014. Are aquaculture and fisheries a solution to food insecurity? The Guardian Development Professional Blog. http://www.theguardian.com/global-development-professionals-network/2014/nov/06/aquaculture-sustainable-solution-food-

insecurity?CMP=twt_gu
Kabir, K.A. 2013. Vulnerable coastal aquatic biodiversity in South-western Bangladesh. The Daily Samakal. http://www.esamakal.net/pop_up.php?img_name=2013%2F05%2F22%2Fimages%2F04_102.jpg

Extension guidebook (based on primary research):

Kabir, K.A. 2017. "Story of Bakul" – a guide book of tilapia aquaculture in ponds with focus on feed management (in Bangla)
Kabir, K.A. 2014. Farmers' Guidebook for improved management of diversified shrimp polyculture farming (in Bangla)

Training and Supervision Plan (TSP)

Section 3. EDUCATION AND TRAINING

A. The Basic Package	Year	Credits
WIAS Introduction Day	2014	0.3
Course on philosophy of science and/or ethics	2014	1.5
Course on essential skills	2014	1.2
Subtotal Basic Package		***3***

B. Disciplinary Competences	Year	Credits
WIAS proposal	2015	6.0
Basic Statistics	2015	1.5
Advanced statistics course Design of Experiments	2015	0.8
Knowledge exchange visit to Japan	2016	1.5
Resilience of living systems – from fundamental concepts to interdisciplinary applications	2018	1.5
Subtotal Disciplinary Competences		***11***

C. Professional Competences	Year	Credits
Imaging Science	2015	3.0
Essentials of scientific writing and presenting	2017	1.2
Systematic approaches to reviewing literature (SLR)	2017	4.0
Writing the General Introduction and Discussion	2018	0.6
Subtotal Professional Competences		***9***

D. Societal Relevance	Year	Credits
Capacity building of 500 farmers on newly developed feed and feed management practice	2017	2.5
Day long workshop to share research result with industry and engaging them in the R&D process	2015-17	1.5
WIAS course Societal impact of your research	2018	1.5
Subtotal Societal Relevance		***6***

E. Presentation Skills	Year	Credits
World Aquaculture Society (American Chapter) Conference "Aquaculture America 2015" in New Orleans, Louisiana, USA (oral presentation)	2015	1.0
International Fishery Symposium 2016, Phu Quoc Island, Vietnam, 2016 (oral presentation)	2016	1.0
World Aquaculture Society and European Aquaculture Society joint conference "AQUA 2018", Montpellier, France (oral presentation)	2018	1.0
12th Asian Fisheries and Aquaculture Forum, a triannual conference of Asian Fisheries Society, Iloilo, Philippines (oral presentation)	2019	1.0
Subtotal presentations		***4***
F. Teaching competences *(max 6 credits)*	year	credits
Organizing and facilitation of training workshop for WorldFish staffs on research methods and scientific writing	2015-17	1.0
Co-supervising MSc students at home country and in AFI	2015-18	4.0
Subtotal Teaching competences		***5***
Education and Training Total		**38**