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STIRLING

POTENTIAL OF EXOGENOUS ENZYMES IN LOW FISH MEAL DIETS TO IMPROVE NUTRIENT DIGESTIBILITY AND SUSTAINABILITY OF FARMED TILAPIA IN THAILAND

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Declaration

I declare that this thesis describes original work based on results of my own investigation and where appropriate acknowledgement has been given to other researchers based on their contribution to the project. In addition, all other background literature has been duly cited and referenced.

I also declare that this manuscript has not been submitted previously for any other degree.

Janielle L. Wallace

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In memory of

Vindell and Edmond

My first educators and parents, who taught me to believe in excellence always, grow from criticism, and to do the good I can, in all the ways I can, to all the people I can, as long as I can

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"Great is thy Faithfulness"
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Francis, Dave, Kim and Oscar

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Abstract

Intensive and semi-intensive aquaculture systems are dependent on nutrient input either in the form of supplemental or complete feeds. Most complete diets still include high fish meal (FM) levels (≥10%). However, as the industry attempts to reduce its reliance on FM, feeds must now be formulated with much lower levels especially for omnivorous species such as tilapia. By 2015, mean FM inclusion in tilapia diets was projected to fall below 3% and be further reduced to 1% by 2020. In the global context of competition for crops, finding suitable plant-based replacers for FM and meeting the increasing demand for seafood, lower-cost and under-utilised plant feedstuffs are now receiving greater attention. The study was divided into three distinct components – field survey, growth experiments, and life cycle assessment. Field surveys were used to contextualise the growth experiments and assess commercialisation opportunities for multi-enzyme inclusion in tilapia feeds. Two sets of digestibility and growth experiments were designed to evaluate the feasibility of using high inclusions of plant-based ingredients sourced from locally available feedstuffs in Thailand to substitute FM at low inclusion levels (0 – 5%). The research evaluated the hypothesis regarding the potential of exogenous enzymes (protease, xylanase and phytase) to minimize anti-nutritional effects on nutrient digestibility of proteins, polysaccharides and phosphorus in tilapia. The research also assessed the secondary effects of enzyme supplementation on economic efficiency and life cycle environmental impacts.

Tilapia is the second most cultured finfish globally and Thailand is the sixth largest producer. Based on the findings of the field survey, feeding practices of Thai tilapia farmers were confirmed to be diverse. Feed inputs included, but were not limited to, agro-industrial by-products (*e.g.* rice bran, corn bran *etc.*) and commercial diets. Commercial diets contained 15 – 30% crude protein and lower protein livestock diets (*i.e.* pig ration) were often used for supplemental feeding or "fattening". The experimental low FM diets were therefore formulated as grow-out or "fattening" diets for semi-intensive green-water systems, a prominent feature (>60%) of Thai tilapia farming.

In Phase 1, the digestibility experiment assessed the digestibility and growth in tilapia fed 0%, 3% and 5% FM diets with and without xylanase (0.385 g kg^{-1}) and phytase (0.075 g kg^{-1}). Performance decreased significantly with declining FM levels. No differences in feed intake, feed conversion ratio (FCR), specific growth rate (SGR) and weight gain were observed between the enzyme and control diets. Nevertheless, tilapia fed the enzyme supplemented 3% FM and control 5% FM performed similarly ($P < 0.05$). No enzyme-related effects were noticed for protein digestibility but phosphorus (P) digestibility improved by 9%, except at 0% FM level ($P > 0.05$). The enzymes had no apparent influence on nitrogen (N) retention contrary to previous studies, however, higher retention for P was observed. Villus length decreased with declining FM levels yet no improvements were seen in tilapia fed enzyme diets. In a simultaneous grow-out experiment, the six experimental diets were compared to an industry 10% FM standard. Conversion ratio was the lowest (1.66) in adult tilapia fed 10% FM diet however the enzyme supplemented 0% FM fed fish had a comparatively low FCR of 1.67. There were no significant enzyme-related effects on weight gain¸ SGR and protein efficiency. Proximal villi results were inconsistent. The cost of feed decreased with declining FM levels but increased with enzyme inclusion. Nevertheless, the economic returns per kg of whole fish produced were better using enzyme supplemented diets compared to the controls. Though the size of the effects on growth and nutrient utilisation were modest, the findings suggested that xylanase and phytase had some level of synergistic action on the targeted anti-nutrients. However, further research was required.

In Phase 2, two control diets (2% FM, negative control (NC) and 10% FM, positive control (PC)) were compared with three enzyme supplemented 2% FM diets (NO-PRO, 0.385 g kg^{-1} xylanase and 0.075 g kg^{-1} phytase only; LO-PRO, xylanase + phytase + 0.2 g kg^{-1} protease and HI-PRO, xylanase + phytase + 0.4 g kg^{-1} PRO). Growth performances improved with enzyme supplementation compared to the NC ($P < 0.05$). Of the enzyme supplemented diets, the LO-PRO diet showed the highest improvements in weight gain (26%) and feed intake (19%), the latter comparing statistically to the 10% FM PC diet. The HI-PRO diet had the best FCR (1.88), again comparable to the PC

(1.73). The NO-PRO diet had the highest protein, P, lipid and energy digestibility, suggesting no additive effect of protease on these coefficients. In terms of gut histomorphology, the LO-PRO and PC diets had the highest measurements and were statistically similar which may have explained similarities in feed intake. Compared to the NC, the HI-PRO diet produced the highest level of change in net profit due to gains in feeding efficiency however, the LO-PRO showed better improvements in terms of growth. Based on these findings, the ternary combination of protease with xylanase and phytase (LOPRO) has potential in limiting FM use for tilapia grow-out feeds, however, the economic efficiencies were still below that of a 10% FM diet. Future considerations for research should target the indigestible dietary components in order to optimise enzyme dosages and maximise the benefits of each enzymes.

In conclusion, a comparative life cycle assessment (LCA) was used to evaluate the environmental impacts of low FM diets and commercial feeds associated with tilapia production in Thailand. The study showed that the low FM enzyme supplemented diets had lower impact potentials and were environmental superior to the average (10% FM) commercial standard. LCA modules are recommended for least-cost formulation programmes as an option going forward. Additionally, LCA can be used as a predictive tool to guide farmers, especially small-scale producers, on the potential impacts of feed input choices and feeding practices. This will ensure higher product quality but also demonstrate environmental responsibility on the part of aquafeed and fish producers to final seafood consumers.

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List of Abbreviations

AA	Amino acid
ABP	Agricultural by-product
ADC	Apparent digestibility coefficient
ADG	Average daily gain
ALU	Agricultural land use
ANF	Anti-nutritional factor
ANOVA	Analysis of variance
ANPU	Apparent net protein utilisation
AOAC	Association of Analytical Chemists
AP	Acidification potential
CM	Cassava meal or cassava root meal
CP	Crude protein
CF	Crude fibre or commercial feed
CFC	Chlorofluorocarbon
DCB	Dichlorobenzene
DDGS	Distillers dried grains with solubles
DM	Dry matter
DO	Dissolved oxygen
DOF	Department of Fisheries (Thailand)
DMRT	Duncan multi-range test
EAA	Essential amino acids
EE	Ether extract
EFA	Essential fatty acids
EP	Eutrophication potential
ETP	Ecotoxicity potential
EU	European Union or energy use
FAO	Food and Agricultural Organisation of the United Nations
FBW	Final body weight
FCR	Feed conversion ratio
FFPS	Follow-up feeding practice survey
FI	Feed intake or Feed in
FM	Fish meal
FO	Fish oil
FTU	Fungal phytase unit
FXU	Fungal xylanase unit
GDP	Gross domestic product

GE	Gross energy
GWP	Global warming potential
HSI	Hepatosomatic index
IBP	Industrial by-product
IBW	Initial body weight
IFS	Integrated farm survey
IPT	Intensive pond tilapia
ISO	International Standardization Organization
IUB	International Union of Biochemistry
IU	International units
KJ	Kilo Joules
KW	Kruskal Wallis
LCA	Life cycle assessment
LCIA	Life cycle inventory analysis
MA	Maize or corn meal
ME	Metabolisable energy
MJ	Mega Joules
NC	Negative control
NEAA	Non-essential amino acid
NFE	Nitrogen free extract
NRC	National Research Council
NSP	Non-starch polysaccharides
OFF	On-farm feed
PC	Positive control
PER	Protein efficiency ratio
PI	Protease inhibitors
PSF	Photochemical smog formation
PUFA	Polyunsaturated fatty acid
RB	Rice bran
SBM	Soybean meal
SEAT	Sustaining Ethical Aquaculture Trade
SGR	Specific growth rate
SIPT	Semi-intensive pond tilapia
SPSS	Statistical package for the social science
TFS	Transition farm survey
TGC	Thermal growth coefficient
THB	Thai baht
WE	Water exchange
XU	Xylanase units

List of Common and Latin Names of Fish Species

Common Names	Latin Names
Nile tilapia	*Oreochromis niloticus*
Walking catfish	*Clarias batrachus*
Hybrid walking catfish	*Clarias macrocephalus x C. gariepinus*
Asian catfish	*Pangasius pangasius*
Striped catfish	*Pangasius hypophthalmus*
Channel catfish	*Ictalurus punctatus*
Common carp	*Cyprinus carpio*
Mud carp	*Cirrhinus molitorella*
Bighead carp	*Hypophthalmichthys nobilis*
Rohu carp	*Labeo rohita*
Silver barb	*Barbonymus gonionotus*
Seven striped barb	*Probarbus jullieni*
Snakeskin gourami	*Trichogaster pectoralis*
Hybrid striped bass	*Morone saxatilis x M. chrysops*
Gilthead seabream	*Sparus aurata*
European seabass	*Dicentrarchus labrax*
Atlantic salmon	*Salmo salar*
Rainbow trout	*Oncorhynchus mykiss*
Turbot	*Psetta maxima*
Cobia	*Rachycentron canadum*
African sole	*Solea senegalensis*
White shrimp	*Penaeus vannamei*
Freshwater prawn	*Macrobrachium rosenbergii*

Chapter 1
General Introduction and Literature Review

1.1 **Prologue**

By 2030, aquaculture production will contribute 62% or 93.6 million tonnes to global seafood[1] production (The World Bank 2013). Fed-aquaculture will demand 91 – 102 million tonnes of aquafeed utilising 3.2 – 3.5 million tonnes of fish meal (FM). By this time, average FM inclusion for tilapia feeds is projected to fall from 2% (in 2015) to 1 (Tacon & Metian 2008; The World Bank 2013). By 2025 however, tilapia production would have already been 100% dependent on feeds producing 12.9 million tonnes of tilapia (Tacon & Metian 2015). In light of this, are these projections of FM inclusion for tilapia feed realistic based on actual industry consumption? Are they feasible based on the required production performances needed to meet future seafood demand? And if not FM, what are the practical alternatives?

1.2 **Tilapia Production**

1.2.1 World Tilapia Production

In response to a growing demand for global seafood products and a decline in fisheries landings, aquaculture has become the fastest growing food production sector, increasing at an average annual rate of 6.7% compared with 2.7% recorded by other livestock sectors combined (FAO 2010; Tacon & Metian 2015). Tilapia is the second largest farmed finfish group by production after carps and has shown 10 – 13% growth per annum (Tacon & Metian 2015). In fact, production volumes in 2012 (4.51 million tonnes) exceeded projections (3.34 million tonnes) by approximately 35% (Lupatsch 2012; FAO 2014c). Though Nile tilapia (*Oreochromis niloticus*) is the dominant cultured species of the group (70.9%), tilapia is generally used to describe species belonging to a group of specific

[1] Seafood is defined as any fish or shellfish harvested from capture fisheries and aquaculture production in marine or freshwater (Smith et al. 2010)

genera of the Cichlidae Family *i.e. Oreochromis*, *Tilapia* and *Sarotherodon* (Trewavas 1983; Madalla 2008). Their environmental tolerances, adaptability to a broad range of culture conditions, and their ability to feed at different trophic levels makes them very desirable for commercial production (Lim & Webster 2006). From a global perspective, the culture systems used for tilapia production span the entire continuum, from earthen static green-water ponds to intensive highly sophisticated clear-water recirculation systems (Gupta & Acosta 2004). Nevertheless, a significant portion of total global production is still cultured in semi-intensive green-water systems, mostly in mainland Asia (Edwards et al. 2000).

Globally tilapia production is dominated by China, accounting for approximately 46% of total production in 2009 (Mjoun et al. 2010). Thailand is among the major producing countries, which includes Egypt, Indonesia, Brazil and the Philippines (Figure 1.2.1). The residual contribution is provided by at least 79 other countries worldwide with very different geographies demonstrating the species tolerance to different conditions (Young & Muir 2002). World tilapia production is expected to continue its upward trend surpassing the 5.0 million tonnes mark by 2015 and 12.9 million by 2030 contributing 7% to global aquaculture production (FAO 2014a; Tacon & Metian 2015).

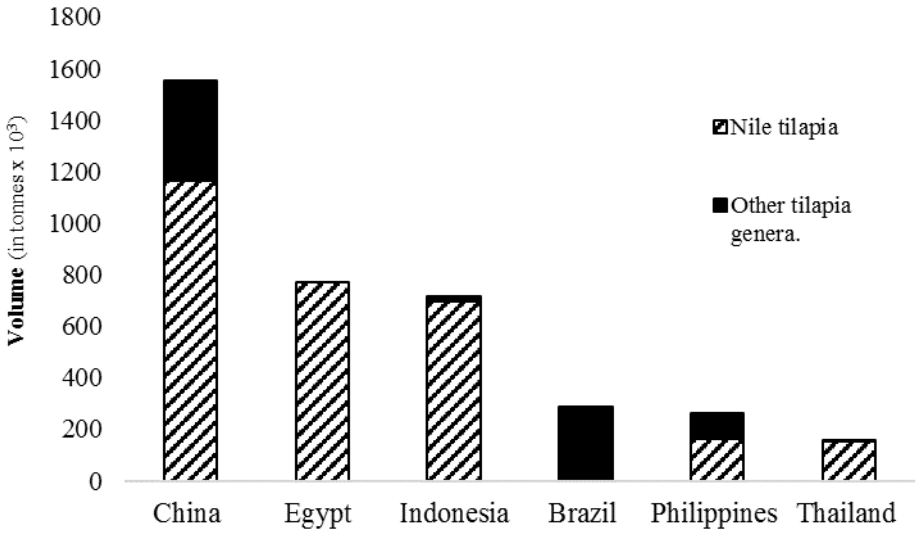


Figure 1.2.1: World-leading producers of tilapia, highlighting contribution of *O. niloticus*. Source: FAO (2015)

1.2.2 Thailand‘s Aquaculture and Tilapia Industries

1.2.2.1 Country Profile and Demographics

Thailand is located in the Indochina peninsula of Southeast Asia. From a socioeconomic perspective, the country is divided into four regions - North, Northeastern, Central and Southern (Figure 1.2.2). Thailand has three distinct climate seasons, rainy (June – October), cool (November – February) and dry (February – May) (United Nations 2008).

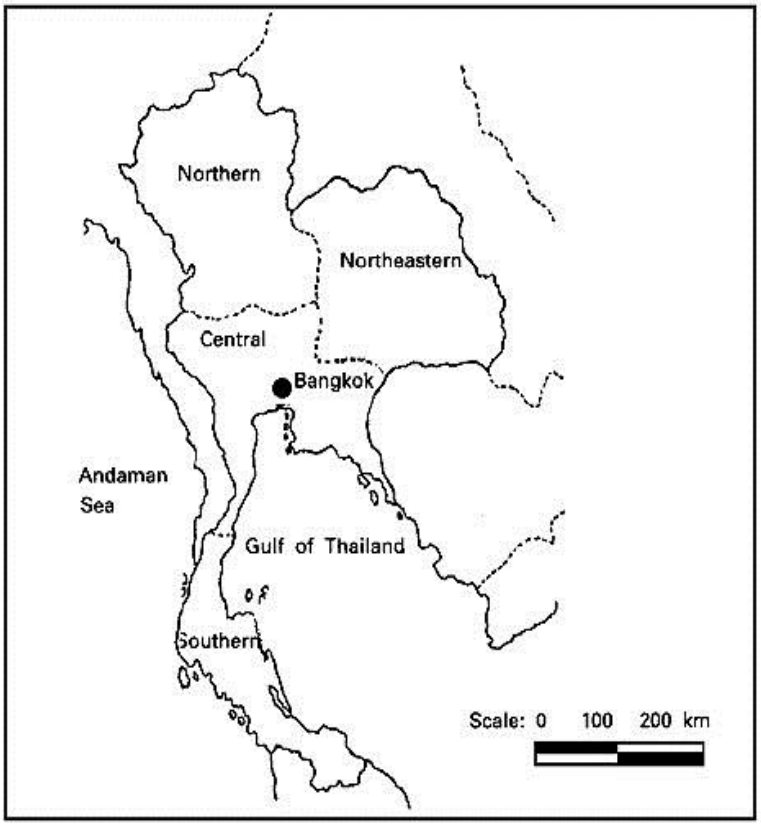


Figure 1.2.2: Map of Thailand illustrating its four regions – North, Northeastern, Central and South. Source: Agnet.org, (n.d.)

Thailand’s fisheries sector including aquaculture plays an important role in the national economy and contributed 2.9% to total GDP in 2006 (Thongrod 2007; Lymer et al. 2011). The fisheries sector employs approximately 650,000 people, 400,000 of which are involved directly or

indirectly in freshwater aquaculture (Poutiainen 2012). Those involved in aquaculture and its related industries such as feed, distribution *etc.* are from various backgrounds having different educational training and professional expertise. Both males and females participate in all activities (production, collection, processing, trading) along the value chain (FAO 2012b).

1.2.2.2 State of Thailand's Aquaculture

Thailand currently produces the fifth largest volume of aquaculture products and accounted for 2.41% or 1.28 million tonnes of global production in 2012 (FAO 2012c; FAO 2014b). Aquaculture is crucial to food security and trade providing seafood for both the export market and domestic consumption. In fact, fish has been the primary source of animal protein for the Thai population for centuries (Edwards et al. 2003; Belton & Little 2008). For example, in 1997 annual per capita fish consumption was 27 kg compared to 11.5 kg, 8.5 kg and 2.1 kg for chicken, pork and beef respectively (Piumsombun et al. 2005). By 2000, the national average consumption increased to 35 kg yr^{-1}, though it varied between regions, provinces and communities (Edwards et al. 2003).

Despite the considerable history of Thai aquaculture, expansion of small-scale finfish culture truly began in the 1970's and has, since then, developed into a dynamic and diverse sub-sector (Belton & Little 2008). Total fisheries production (including aquaculture) exceeded 2.0 million tonnes for the first time in 1977 and by 1996 increased to 3.5 million tonnes. Coastal and freshwater aquaculture contributed 10% and 5.9% respectively of total fisheries production (Piumsombun et al. 2005). By 2003, 27.2% or 1.06 million tonnes of the total fisheries production in Thailand was contributed by freshwater aquaculture; representing a significant increase from 89,800 tonnes reported in 1986 (Piumsombun et al. 2005; Tabthipwon n.d.). Freshwater fish now accounts for 70 – 90% of total fish consumed in all regions (DOF, 2012). Freshwater aquaculture records annual growth rates of 11.9% and 17.1% in volume and value respectively (Piumsombun et al. 2005).

Freshwater aquaculture is still largely traditional involving some level of integration with livestock culture or cropping activity. Ponds are fertilized using animal manure, processed agro-industrial by-products or commercial fertilisers to stimulate algal bloom. There are four types of

farming practices, pond culture (86%), cage culture (9%), paddy field culture (4%) and ditch culture (1%)(Figure 1.2.3); and two patterns of culture, monoculture and polyculture (Edwards et al. 2003; Piumsombun et al. 2005; DOF 2012). There have been over 50 relevant freshwater species cultured by small-scale aquaculture, however, the main production species are Nile tilapia (*O. niloticus*), common carp (*Cyprinus carpio*), striped catfish (*Pangasius hypophthalmus*), walking catfish (*Clarias batrachus*), silver barb (*Barbodes gonionotus*), snakehead gourami (*Trichogaster pectoralis*) and freshwater prawn (*Macrobrachium rosenbergii*) (Bhujel, 2013). The main aquaculture provinces are concentrated in the central and coastal regions of the country, and include Chachoengsao, Nakhon Pathom, Suphanburi, Chanthaburi, Samutprakarn *etc*. (Lawonyawut 2007). In the central region, fish farming accounted for 50% of the national output in 2012 (DOF, 2012).

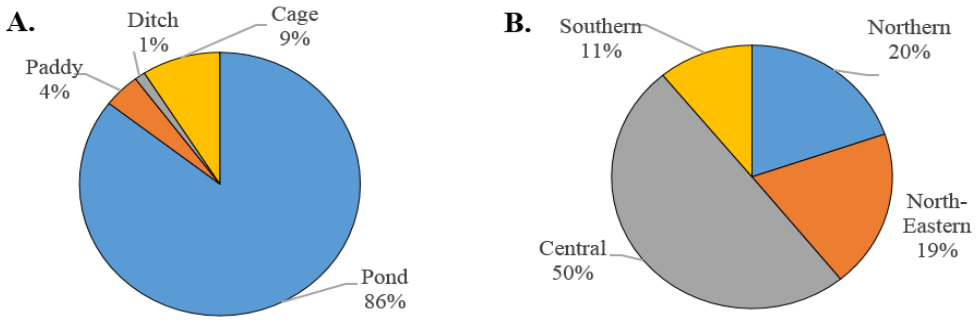


Figure 1.2.3: Freshwater aquaculture production in Thailand by systems (A) and by regions (B). Source: DOF (2012)

1.2.2.3 Thailand's Tilapia Production

After shrimp, tilapia is likely to become the second most important aquaculture species in Thailand in the near future (Bhujel & Woollard 2011). *O. niloticus* were first introduced in 1965, but it was not until 1967 that culture was fully established following distribution of fingerlings by the Department of Fisheries (DOF) (Bhujel, 2008). By the 1980s, tilapia became the focus of research by

the Asian Institute of Technology which promoted awareness of the species across Thailand (Bhujel, 2008). Tilapia production rose exponentially, volumes increased from 22,800 tonnes in 1990 to 203,700 tonnes by 2005 (Belton et al. 2009). This development was attributed to a number of factors, which included greater utilisation of agricultural by-products for fish feed and popularization of monosex cultures. In 2011, Nile tilapia contributed approximately 29% to Thailand's inland freshwater aquaculture production though the industry suffered extensive flooding (FAO 2012b). By 2013, production normalised supplying 212,772 tonnes of tilapia to Thailand's total national aquaculture output (Figure 1.2.4) (FAO 2015)

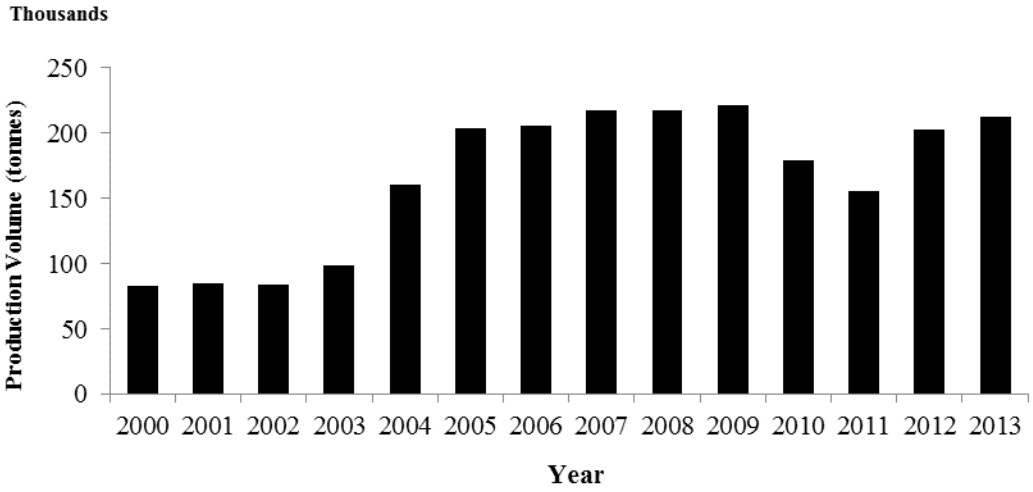


Figure 1.2.4: Tilapia production trend in Thailand. Source: http://www.fao.org/figis (2015)

Although tilapia is cultured in various systems, pond polyculture is most common (Bhujel & Woollard 2011). Nevertheless, there are a number of small and medium scale farmers who stock only monosex tilapia using two broad production strategies. The first production strategy involves growing tilapia to 400 – 500 g over an eight months period and the second is a 12 – 13 months cycle with a defined grow-out fattening phase with products averaging over 600 g (Belton et al. 2009). In terms of pond yield, on average farmers produce approximately 6.3 tonnes per hectare (6.25 rai) (Gupta & Acosta 2004). Unlike the eight month strategy where fertilisation is used throughout the production

cycle supplemented with farm-made feeds[2], the 12 – 13 month strategy relies more on complete commercial diets to assist with fattening. River or reservoir cage-based tilapia culture also utilises commercial feeds because of inability to fertilise these systems. Though cage culture represents only 9% of aquaculture systems, it accounts for >30% of the total Thai tilapia output. A small number of farmers also practice a combination of culture techniques where they culture tilapia to 200 – 300 g in green-water ponds then transfer them to cages for fattening using high quality pelleted diets (Belton et al. 2009). However, in light of global feed ingredient volatility and pressure on locally grown feedstuffs from various industries in Thailand, tilapia farmers now struggle with declining profit margins as the value of tilapia often cannot justify the use of commercial feeds for semi-intensive and intensive production (Bhujel 2013). Consequently, the tilapia farmers require more innovative feeding strategies to maintain production, but more importantly, to remain competitive.

1.3 **Nutrient Requirements of Tilapia**

1.3.1 Feeding Behaviour and Digestive Physiology

Tilapia (*Oreochromis spp*.) are largely opportunistic and omnivorous in their feeding behaviour. They have an ability to convert low quality feed into high quality protein for human consumption (Jauncey 1998; Lupatsch 2012). For these reasons, among others, tilapia are generally fed inexpensive, low nutrient dense diets (Jauncey 1998). Their digestive physiology is simple consisting of a stomach (low pH gastric environment) and intestine (high pH bile salt environment) (NRC 1983; Madalla 2008; Holphe et al. 2014). Tilapiine gross intestinal morphology consists of five distinct regions that includes a proximal major coil (Figure 1.3.1). The proximal portion is the most developed part of the intestine and was found to be the site having the highest enzymatic activities and nutrient absorption rate (Sklan et al. 2004).

[2] A farm-made or on-farm feed is a combination of two or more feedstuff, agricultural or industrial by-products used as supplemental feed for fish production excluding organic and inorganic fertilisers (Thongrod 2007)

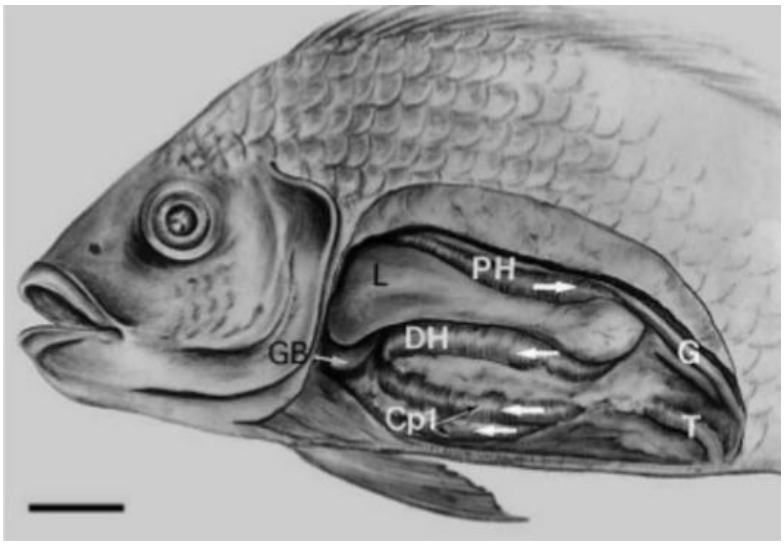


Figure 1.3.1: Visceral structure of *Oreochromis niloticus* (*in situ*). L = liver; PH = proximal limb or hepatic loop; DH = distal limb of hepatic loop; Cpl = proximal centripetal limb of proximal major coil; GB = gall bladder; G = Gonad. Bar = 2 cm. Source: Smith et al. (2000)

1.3.2 Nutrient Requirements

Since there is no strain-specific data for the hybrid red tilapia (*Oreochromis niloticus x O. mossambicus*), the nutrient requirement values for *O. niloticus* are given below. Protein and energy requirements are age and size dependent (Table 1.3.1) (Jauncey, 1998). Carbohydrates are non-essential but can be used for protein sparing and as energy source. Fish (*i.e.* tilapia) however do not require proteins and lipids but rather essential amino acids and fatty acids (Table 1.3.2) (NRC, 2011). Apparent protein and energy digestibility, and their ratio are also essential components for formulation optimization.

Table 1.3.1: Approximate dietary protein and lipid requirement for tilapia based on body weight

Fish weight (g)	Optimal protein level (%)	Optimal lipid level (%)
Fry – 0.5	40 – 45	6 – 12
0.5 – 10	30 – 35	6 – 12
10 – 30	25 – 30	6 – 12
30 – market size	25 – 30	6 – 8

Table 1.3.2: Protein and energy requirement for tilapia

Nutrients	*Oreochromis spp*
Protein and Energy Concentrations[a]	
Digestible protein (%)	29
Digestible energy (MJ kg^{-1})	14.21
Recommended DP/DE ratio[b]	20
Amino Acids (% of the diet)	
Arginine	1.2
Histidine	1.0
Isoleucine	1.0
Leucine	1.9
Lysine	1.6
Methionine	0.7
Methionine + cysteine	1.0
Phenylalanine	1.1
Phenylalanine + tyrosine	1.6
Threonine	1.1
Tryptophan	0.3
Valine	1.5
Taurine	NT
Fatty Acids (%)	
n-3 LC-PUFA	R
18:2n-6	0.5 – 1.0

[a] Typical concentrations in commercial diets; [b] Optimal P/E ratio: *O. mossambicus* (19.7 mg CP kJ g^{-1}), *O niloticus* (15 – 16 mg CP kJ g^{-1}) and *O. niloticus x O. aureus* (27.3 CP kJ mg g^{-1}) (Ng & Romano 2013)
NT – Not tested; R – Required but quantity not determined

Micro-additives (vitamins and minerals) are provided in trace quantities but are essential for metabolic and health related functions. Mineral and vitamin requirements for tilapia are given in Table 1.3.3 (NRC 2011). Available P requirement for tilapia ranges from 5 – 9 g kg^{-1} of the diet (Cao et al. 2008; Kumar et al. 2012). Optimal dietary choline requirement for tilapia ranges from 900 – 1,000 mg kg^{-1} (Shiau & Lo 2000). Adequate niacin levels are reported at 26 mg kg^{-1} (Shiau & Suen 1992).

Table 1.3.3: Mineral and vitamin requirements for tilapia (*Oreochromis spp*.)

Macro-minerals	(%)	Micro-minerals	(mg kg^{-1})
Calcium	0.70	Iron	85
Phosphorus	0.40	Zinc	20
Chlorine	0.15	Copper	5
Magnesium	0.06	Iodine	NT
Potassium	0.20 – 0.30	Manganese	7
Sodium	0.15	Selenium	0.1[a]
Fat-soluble Vitamins	mg kg^{-1}	Water-soluble Vitamins	mg kg^{-1}
A	1.8	Thiamine	2.5[a]
D[b]	9.0	Riboflavin	6.0
E	60	B_6	15
K	NT	Pantothenic acid	10
		Niacin	26
		Biotin	0.06
		B_{12}	NR
		Folic acid (folacin)	1.0
		Choline	1,000
		Myo-inositol	400
		C	20

[a] Jauncey (1998); [b] value in µm kg^{-1}; NT – not tested; NR – not required under practical conditions.

1.3.3 Feeding Rates

Feeding rates and frequencies are generally affected by various factors, which includes feeding behaviour, physiology, feed quality and environmental conditions. Table 1.3.4 gives the suggested feeding rates and frequencies for tilapia fed complete diets at their optimum growth temperature (Jauncey 1998).

Table 1.3.4: Feed rates based on tilapia body size

Average fish size (g)	Jauncey (1998)		NRC, (2011)		Bhujel (2013)
	Feed Rate (%)	Frequency (day^{-1})	Feed Rate (%)	Frequency (day^{-1})	Semi-intensive Feeding rate (%)
<50	7 to 6.5	3 – 6	4.5	3	10 to 5
50	3	3	3.7	3	3
Up to 100	2	2	3.2	3	3
Up to 200	2	2	2.8	2	2
Up to 300	1.8 to 1.5	2	2.3	2	1.5
>300	-	-	2.0 to 1.4	2	1.4 to 1.3

Feeding rates are given as % of total tilapia biomass per day

1.4 **Aquafeed Production**

1.4.1 World Aquafeed Production and Fish Meal Demand

In response to global seafood demand, aquaculture's growth has unintentionally enhanced the strain on marine capture fisheries, which provides essential ingredients for the industry. To sustain annual growth levels (~6.7%), the aquafeed industry must respond by matching or exceeding this pace. Global aquafeed production is currently growing at a mean annual rate of 10.9%. In 2008, 708 million tonnes of industrial animal feed was produced of which 29.3 million tonnes (4.1%) was aquafeed (FAO 2012a). Fed-species accounted for 70% (35.7 million tonnes) of global aquaculture production in 2012 with 68% (24.3 million tonnes) dependent on commercial aquafeeds (FAO 2014a; Tacon & Metian 2015). Tilapia production utilised 13.5% of total aquafeed in 2008 (Tacon 2010) and increased to 16.8% by 2012 (6.7 million tonnes)(Tacon & Metian 2015). Figure 1.4.1 shows the estimated aquafeed production for 2012 and consumption per species.

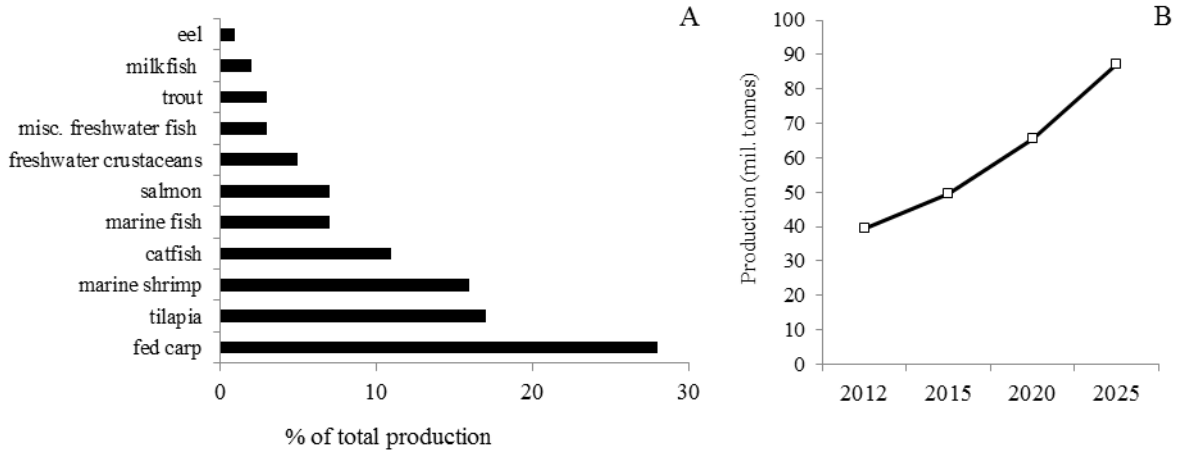


Figure 1.4.1: Global aquafeed production (39.6 million tonnes) in 2012 (A) and Projected growth in production (B). Source: (Tacon & Metian 2015)

The pattern of FM use has shifted nearly exclusively to aquafeed production from livestock (Hardy 2010). Aquaculture consumed 3.72 million tonnes or 60.8% of total FM produced (Tacon et al., 2011) and 0.78 million tonnes (73.8%) of global fish oil (FO) in 2008 (FAO 2012c), at the expense of the livestock sectors which have continued to reduce their usage of these marine commodities. By 2012, aquaculture's FM consumption rose to 68% while FO usage remained the same (74%) (Tacon & Metian 2015). Despite efforts to improve FM availability and quality, global FM production has remained static (5 – 7 million tonnes) year over year due to fully/over-exploited fisheries while the production of cereal grains and oil seeds are trending upwards at 2.9 billion and 574.1 million tonnes respectively (USDA 2015). Coupled with increasing FM prices and restricted access from major FM-producing countries, significant amount of research has been undertaken to find and secure more sustainable alternative feedstuffs (Deguara et al. 1999).

Though much success has already been realised, the aggregated effects of increased production may still impact fisheries-derived resource significantly (Naylor et al. 2009; Hardy 2010). In fact, aquaculture producing countries such as Thailand and China are consuming greater volumes of FM (imported and local) while exporting less feed and ingredients (Naylor et al. 2009). Additionally, by

2030 regionally FM usage in Southeast Asia is projected to increase by 10% and globally by 7.6% (The World Bank 2013). In light of this, mean FM inclusion levels in aquafeeds are expected to decline even further. For example, average FM inclusion level for tilapia feed was projected to decrease from 5% in 2006 to 2% in 2015 (Tacon et al. 2011). Continued industry growth and intensification will therefore depend upon highly renewable and sustainably-sourced FM replacers which can provide the nutrients required for effective conversion to high quality fish flesh at least cost with minimal impacts to the environment (Gatlin et al. 2007).

1.4.2 Thailand's Aquafeed Industry

Thailand's feed milling industry is one of the country's largest and fastest growing industries with ~50 registered mills (Roembke 2014). The industry is supported by Thailand's diverse supply of raw feedstuffs. The major feedstuffs include rice bran (RB), cassava meal (CM), corn/maize (MA), soybean meal (SBM), FM, sorghum (*Sorghum bicolor*), kapok meal (*Ceiba pentandra*) and mungbean (*Vigna radiata*) (Havanont 1993). Although aquaculture has been practiced extensively in Thailand for centuries, it has not maintained pace with livestock farming in terms of feed development and feeding practices. Feeding practices are still largely traditional using fertilizers and on-farm feeds. It was not until 1986 when Thai shrimp culture began to evolve that the demand for commercial feeds and feed mill facilities expanded (Havanont 1993). There are now reportedly over 20 feed mills producing aquatic feeds (Nietes-Satapornvanit 2014).

1.4.2.1 Raw Ingredients and Commodities

Thailand is considered almost self-sufficient in terms of the supply of feed ingredients for the agro-industry as 80 – 90% of raw ingredients are produced locally. Thailand is also one of the world's leading FM producers and contributed 8% to the global market in 2007 (OXFAM 2014). Thai FM is produced from three sources; local by-catch (*pla pet*), surimi factories and tuna processing by-products (Henriksson et al. 2014). Like China, however, Thailand consumes far more FM than produced. In fact, local FM production has been declining over the last decade (Figure 1.4.2), and catch per unit effort of fisheries raw material has decreased from 297.8 kg hr^{-1} in 1961 to 17.8 kg hr^{-1}

(OXFAM 2014). To compensate, high quality Peruvian FM is imported and is mainly used for high-valued export feed/seafood production such as shrimp due to high import tariffs (15%) (Thongrod 2007).

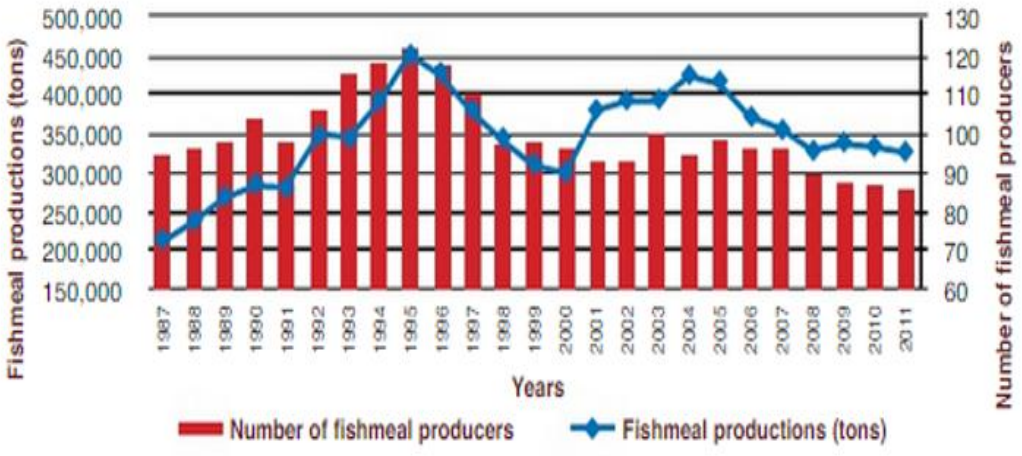


Figure 1.4.2: Thailand's FM production and producers 1987 – 2011. Source: OXFAM (2014).

Like most countries worldwide, Thai feed manufacturers are currently faced with sharp increases in ingredient prices which have forced re-formulation using lower cost ingredients. Oilseeds, legumes, cereals, and root tubers have potential value for Thailand's aquaculture, however, their use like other plant-based ingredients are limited by their nutritional content, palatability, digestibility, anti-nutritional factors (ANFs) and cost-effectiveness. Soya bean is one of the most important oilseed used, however, significant amount is imported (Table 1.4.1). Cassava or 'tapioca' has been used for monogastric feeds, however, its use is highly dependent on various other demand factors including price and availability of other alternative ingredients such as maize. The root meal is used in aquafeeds for its ability to improve water stability (Solomon et al. 2011). RB, the principal by-product of rice milling, is sold cheaply and used often as a direct feed input at all stages of aquaculture production in Thailand (Unprasert n.d.).

Table 1.4.1: Production and consumption of common commodities used by Thailand's animal feed industry (in million tonnes) (The Thai Feed Mill Association 2012; Henriksson et al. 2014)

Commodity*	Production	Import	Export	Consumption
Soya bean	0.18	1.53	0.001	1.72
Rice	34.3	0.005	9.81	24.5
Maize	4.8	0.42	0.48	4.8
Cassava	27.6	NI	18.3	8.7

* Unprocessed grains/tuber; NI – No information

1.4.2.2 Feed Availability and Use

Local feed mills now incorporate plant-based ingredients of high quality yet competitively priced to maintain benefits that can be passed on to farmers (The Thai Feed Mill Association 2012). High quality commercial feeds are therefore available, however, they are often uneconomical for some small-scale famers due high prices and low returns on investment (Bhujel & Woollard 2011). Consequently, feeding practices for the most part have remained largely traditional based on broken rice, RB and trash fish pond applications (Unprasert n.d.). Fish farmers often source and use cheaper feeds of low nutrient density (*e.g*. pig rations) or use on-farm diets based on agricultural by-products in order to minimise operational cost and boost profitability. On the other hand, given higher demands for fish and a drive to "intensify, expand and potentially export", tilapia farmers will have to rely on more consistent nutrient inputs to maximise their yields and maintain product quality. The latter is even more essential for export value chains which now have strict requirements for feeds/nutrient inputs. This is the advantage of tilapia cage farmers who generally utilise balanced commercial diets achieving optimal feed conversion ratios (FCR) of 1.4 – 1.8 (Lebel et al. 2013). These cage-based systems are often located in rivers and large reservoirs making fertilization difficult and unstable on-farm diets inadequate. Though on-farm feeds may not be appropriate for cage cultures, farmers have considered exploring this alternative to commercial diets due to high feed costs. In fact, on-farm feeds have traditionally played an important role in the production of low-value freshwater fish such as

tilapia (Thongrod 2007). Although largely undocumented, global on-farm feed production is estimated to be 18.7 – 30.7 million tonnes per year (Tacon & Metian 2015).

1.5 **Alternative Plant-based Ingredients**

Based on the nutritional properties (imbalanced AA and nutrient composition) of most plant-derived ingredients however, combing several ingredients supplemented with exogenous feed additives such as amino acids (AA) is more effective in replacing FM (Gatlin et al. 2007). Unlike FM, grain and oilseed production have increased significantly over the last decade due to more acreage, genetic improvements and higher crop yields. In 2007, production for soya bean, corn and wheat were 216, 785 and 607 million tonnes respectively (Hardy 2010) and were projected to increase to 314, 988 and 723 million tonnes respectively by 2014/2015 (United States Department of Agriculture 2015). Common plant-based ingredients used for aquafeeds include, but are not limited to, SBM and concentrates, wheat and wheat-by products, MA, barley, cottonseed meal, lupine meal, canola meal, rice, and rice by-product, cassava, jatropha kernel meal and flaxseed meal (Tibbetts et al. 2006; Madalla 2008; Naylor et al. 2009; Krome et al. 2014). Most of these ingredients have been evaluated with relative success as FM replacers, given measures to improve digestibility and counter anti-nutritive effects (Hardy 2010). Further assessments of plant-based ingredients are critical to fish nutrition research and the future development of sustainable diets for aquatic species in Thailand, Asia and the world over (Glencross et al. 2007).

1.5.1 Soybean Meal

Soya bean (*Glycine max* L.) is the leading oilseed crop produced globally and is used predominantly to produce the meal extract (Gatlin et al. 2007). SBM is the most widely used and researched oilseed meal in animal feeds (Dale 1996). It is considered the most useful partial replacer for FM in aquafeeds because of its high CP level, lysine content, abundant yet stable supply and comparatively lower price (Drew et al. 2007; Twibell & Brown n.d.). On the other hand, SBM is limited in sulphur containing AAs, methionine and cysteine (El-Sayed 1999). In addition to

challenges associated with under-processing (residual ANFs) and over-processing (reduced AA availability), SBM also has lower metabolisable energy (ME) than FM (Lovell 1989) due to its high carbohydrate fraction. SBM contains a variety of ANFs, such as protease inhibitors (PI), phytate, non-starch polysaccharides (NSP) *etc.*, which have negative impacts on fish performance (Ogunkoya et al. 2006; NRC 2011). The presence of ANFs in SBM and other plant ingredients has considerable implication for monogastric animals (poultry, swine and fish) which often lack the appropriate digestive enzymes to hydrolyse these fractions (Dale 1996). ANFs will be discussed further in Section 1.6.2.

SBM can replace up to 70% of FM depending on soy cultivar, final processing method, fish species, development stage, dietary protein requirement and culture system (El-Sayed 1999). In support, Gallagher, (1994) reported up to 75% of FM can be replaced with SBM in diets (35% CP; 47% FM) of hybrid striped bass (*Morone saxatilis x M. chrysops*) without significant effects on growth, feed conversion, protein utilisation or body composition. Dehulled, solvent extracted and full-fat SBM was incorporated at 14% inclusion in Atlantic salmon (*Salmo salar*) feed without any effect on weight gain (Olli et al. 1994 cited by Carter & Hauler 2000). Fontainhas-Fernandes et al. (1999) also cited no negative effects on specific growth rate (SGR) and feed gain ratio when FM was replaced by up to 33% plant-based ingredients including SBM in tilapia basal diet (DP 35% dry matter; 50% FM). On the contrary, Koumi et al., (2009) reported that final body weight, weight gain and SGR decreased with increasing dietary soybean inclusion (24.8 – 50%) in diets fed to *O. niloticus.*

1.5.2 Rice Bran

Large volumes of grain by-products such as RB and wheat bran are commercially available for use in animal feeds (Choct 2006). Approximately 63 – 76 million tonnes of RB is produced annually and 90% is sold as animal feed (Kahlon 2009). RB is a fine powdery, fluffy material consisting of seeds, kernels, particles of pericarp, seed coat, aleurone, germ and endosperm. It constitutes ~10% brown rice (*Oryza salva* L.) (Farrell 1994) and may contain 15 – 22% oil, 11 – 17% CP, 6 – 14%

fibre, and 8 – 17% ash (Zare-Sheibani et al. 2015). It is nutrient dense, its fatty acid (FA) profile includes approximately 74% unsaturated FA (mono- and poly-unsaturated) and is considered superior to cereal grains (Samli et al. 2006). RB proteins can be efficiently digested and has high nutritional value (Kahlon 2009; Zhang et al. 2012). RB is also rich in the Tocopherols and B-Vitamins (Samli et al. 2006).

RB contains PIs (trypsin and chemotrypsin), phytate and hemoglutinin, limiting the full potential of the product for animal feed (Samli et al. 2006). RB and wheat by-products are generally characterised by insoluble NSP content which includes hemicelluloses (*e.g.* arabinoxylans) and celluloses (Choct 2006). RB is used extensively as a feed additive in Asia (Kahlon 2009). In Thailand, RB is often used as a low cost feed input for various fish species (Thongrod 2007), however, there are no references in regards to its ideal inclusion level. Notwithstanding, RB and its concentrates have had commercial applications in swine and poultry diets. Moreira et al. (2003) used 17.2% defatted RB along with SBM and MA in the diets of swine. Samli et al. (2006) included RB up to 15% in layer feed but reported inclusions less than 10% had no adverse effect on performance, egg quality and intestinal integrity.

1.5.3 Cassava Meal

CM is a processed product of cassava (*Manihot esculenta* Crantz) root tuber. Cassava production increased from 164 million tonnes in 1999 to 228 million tonnes in 2008 (Garcia & Dale 1999; Chauynarong et al. 2009). 70% of production originates in Nigeria, Brazil, Thailand, Indonesia and the Congo Democratic Republic, the major producing countries (Garcia & Dale 1999; Chauynarong et al. 2009). Thailand is the world's leading exporter of the product (cassava chips) particularly to Europe. Production in Thailand increased significantly from 3.4 million tonnes in 1970 to 24.3 million tonnes in 1989 (Garcia & Dale 1999). Today, production exceeds 30 million tonnes per annum (Thai Tapicoa Starch Association 2015).

CM's protein content is very low accounting for 0.7 – 2.5% on a dry matter (DM) basis (Garcia & Dale 1999). The lipid content is similarly very low ranging from 0.3 – 1.2%, however, it has a high

starch content (60 – 70%). This makes CM very useful during pelleting, its excellent binding property eliminate the need for artificial feed binders (Madalla 2008). The meal is also used as a source of energy (Garcia & Dale 1999). Inclusion rates for poultry feeds range from 10% to 60% but is highly dependent on anti-nutrient levels (specifically cyanogenic glucosides), nutritional content and processing method. The ingredient is widely used in Thai commercial aquafeeds, however, there is also very little focus given to the ingredient in fish nutrition research. Madalla (2008) investigated the suitability of replacing wheat meal with CM at 25 – 75% in the diets of *O. niloticus* and found that it did not impair growth or nutrient utilisation. Recommended maximum inclusion level for Nile tilapia diets was 60% (Wee and Nag 1986 cited by Madalla 2008).

1.5.4 Maize Meal

Ground maize (corn meal) is an important ingredient for animal feed formulation. The term maize is often used interchangeably with corn depending on geographic location but generally refers to the crop, *Zea mays*. The availability of quality maize for animal feed production has decreased over the last decade due to ever increasing production of ethanol as an alternative fuel source (Wright 2011). This competition may also worsen in the face of climate change and land availability in the near future (ActionAid International 2012). Nevertheless, this situation has increased the production of alternative feed ingredient distillers dried grains with solubles (DDGS), a by-product of the fermentation process. Lumpkins et al. (2004) estimated that by 2005 there would have been 5.5 – 7 million tonnes available for use by animal feed producers. The volume of this derived ingredient alone is comparative to that of FM production annually, which adds to the appeal of using plant-based replacers.

Maize is generally characterised for its low CP (8.0%), fibre (2.2%) and ash levels (1.2%) yet high starch content (64%) (INRA/AFZ 2004). It is therefore used mainly as source of energy in animal feeds. It is fairly high in thiamine and niacin, in the bound form (Auburn University 2014) but is more lysine deficient compared to other grains and oilseeds (Gatlin et al. 2007; Hardy 2010). It is a poor source of other AAs (methionine and tryptophan) and trace minerals (Hertrampf & Piedad-

Pascual 2000). Though a common feed component, to improve its utilisation by fish, it is often incorporated in a processed form. In addition to ground maize, corn gluten meal (CGM) is another common aquafeed ingredient, particularly for carnivorous species due to its high CP content. CGM can be included up to levels of 20 – 25% but is used more sparingly within the 10 – 15% range (Gatlin et al. 2007).

1.6 **Challenges with Plant-based Ingredients**

From a market-availability and economic perspective, plant-based ingredients are practical FM alternatives for fish feed formulations (Deguara et al. 1999). Nevertheless, even the most promising alternatives, e.g. SBM, have comparatively lower nutrient digestibility and contain one or more ANFs limiting their nutritive value and usefulness (Francis et al. 2001). ANFs in plant-based ingredients are known to have adverse effects on feed consumption, fish physiology, growth and can potentially become toxic (Twibell & Brown n.d.). ANF levels differ from plant source to plant source, strain, culture conditions, harvesting time and processing methods. For example, oilseeds are purported to contain more ANFs of concern to aquatic animals compared to that of grains (Hardy 2010). Some ANFs (*e.g.* phytate) may also affect apparent nutrient availability and digestibility.

1.6.1 Lower Digestibility

Nutrient digestibility of plant-based ingredients is a critical component in determining the potential of raw feedstuffs for inclusion in fish feed. The term digestibility describes the amount of the nutrients or energy in the ingested feed that is not excreted by the animal (NRC 2011). It is essential for optimising inclusion levels and minimising resource waste. Ingredient digestibility has been reported for only a few important commercial species (NRC 2011), Atlantic salmon (*Salmo salar*), *Dicentrarchus labrax* (European seabass) (Altan & Korkut 2011), *Labeo rohita* (Rohu carp) (Asad et al. 2013), *Oncorhynchus mykiss* (rainbow trout) (Gaylord et al. 2008), *Psetta maxima* (turbot) and *Rachycentron canadum* (cobia). However, gaps still remain in the literature in regards to "uncommon" ingredient digestibility in tilapia *e.g.* cassava, RB *etc*. (Zhou et al. 2004).

Compared to FM, plant-based ingredients have relatively lower digestibility. This is due to structural components (cellulose, hemicellulose *etc.*) and metabolites (ANFs) which interfere with the animal's digestive metabolism, lowering dietary nutrients absorption. Consequently, the nutritive value of a feedstuff also includes its nutrient and energy bioavailability (Altan & Korkut 2011). Digestibility can improve, however, with additional processing (extraction/cooking) and treatment with digestibility-improving additives (*e.g.* enzymes) pre-pelleting or application to diet post-pelleting.

1.6.2 Anti-nutritional Factors

Plants commonly synthesize metabolites of low and high molecular weight called ANFs as a defence mechanism against herbivores (Khokar & Apenten, 2003). ANFs are classified as endogenous compounds found in all plant-based ingredients which may negatively influence feed intake, nutrient digestibility and utilisation, growth, affect the function of internal organs and alter disease resistance (Krogdahl et al. 2010). They include, but are not limited to, phytates, PIs, NSPs (cellulose and hemicellulose), saponins, tannins, haemagglutinins or lectins, gossypols and cyanogenic glycosides (Soetan & Oyewole 2009).

The structure and chemical composition, specifically heat-sensitivity, of ANFs can determine which physical or chemical processes may be effective in reducing their biological effects in animals (Khokar & Apenten, 2003). ANFs can be removed or inactivated by selective breeding, genetic modification, heat treatment or extraction (extrusion, pelleting, alcohol extraction), or through supplementation (enzyme, mineral *etc*.) (Krogdahl et al. 2010) (Table 1.6.1). Despite extensive ANF research, particularly phytate, in livestock (Maga 1982), researchers have acknowledged that an understanding of their antagonistic interactions in fish is still in its developmental phase and further knowledge is needed in order to counter their potentially negative impacts.

Table 1.6.1: Processing steps for removal/inactivation of ANFs (Nwanna 2007; Hardy n.d.)

Anti-Nutrient	Heat Sensitivity	Extraction	Other Treatment
Phytic Acid	No	No	Phytase
Arabinoxylans (NSP)	?	?	Xylanase
Protease inhibitors	Yes	No	Protease
Hemagglutinin	Yes	No	No
Saponin	No	Yes	No
Phytoestrogen	No	Yes	No

The following commercially important ANFs, phytate-P, arabinoxylan (NSP) and PIs on growth and nutrient utilisation in tilapia was of particular interest. Broadly speaking, phytate binds naturally occurring plant P making it unavailable to monogastrics and impairs mineral absorption; NSP (soluble and insoluble) interferes with digestive processes limiting nutrient uptake while PIs depress the digestion of protein, hindering AA absorption (Krogdahl et al. 2010). Table 1.6.2 lists their chemical name and plant origin. They will be defined and discussed further in the upcoming sections.

Table 1.6.2**:** List of anti-nutrients relevant to this study and their plant source

Antinutrients	**Chemical name**	**Plant source**	**Source**
Phytic acid *or* Phytate-P	Myoinositol 1,2,3,4,5,6-hexakisdihydrogen phosphate	Cereal and legumes	(Khokar & Apenten, 2003)
Non-Starch Polysaccharides	*e.g.* Arabinoxylans (arabinose and xylose)	Cereals (wheat, rye, barley, rice, sorghum)	(Sinha et al. 2011)
Protease Inhibitors	*e.g.* Trypsin inhibitor	Most plants particularly legumes and cereals	(Francis et al. 2001; Krogdahl et al. 2010)

1.6.2.1 Phytate-Phosphorus

Phytate is the primary storage form of P in many plants accounting for 0.4 – 6.4% by weight and 60 – 90% of total P (Khokar & Apenten, 2003) (Table 1.6.3). Phytate consists of an inositol

group, hexahydrocyclohexane in a chair configuration with six phosphate ester bonds (Figure 1.6.1) (Haros et al. 2005; Kumar et al. 2012).

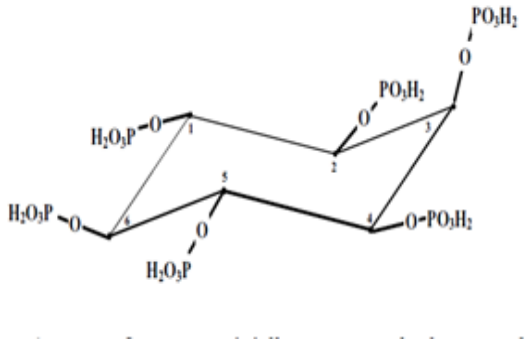


Figure 1.6.1: Chemical structure of phytate-phosphorus showing its chair-like conformation. Source: Adeola and Sands, (2003)

Table 1.6.3: Total P and phytate-P in common plant-based ingredients. Source: Kumar et al., (2012) and Ravindran et al., (1994)

Ingredients	**Total P** ($g\ kg^{-1}$)	**Phytate-P** ($g\ kg^{-1}$)	**Proportion of Phytate-P in Total P (%)**
Maize	2.40	2.05	85.4
Corn	2.50	1.70	73.0
Rice	1.20	0.80	65.0
RB	17.51	15.83	90.2
Soya bean	5.55	3.08	55.5
SBM	6.66	4.53	68.3
Cassava	1.60	0.40	25.0

The phosphate groups within the molecule carry a strong negative charge (total of twelve charges when dissociated) having high chelating ability. This causes them to bind to multivalent cations such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+} and Cu^{3+} rending them unavailable for absorption by monogastrics (Francis et al. 2001; Haros et al. 2005; Makhode 2008). This is of particular concern for trace minerals such as Zn and Fe which are normally supplied in small quantities within the diet yet

critical for metalloenzyme and haemoglobin production. Phytate also inhibits the activities of digestive enzymes such as pepsin, α-amylase and trypsin (Nwanna 2007). Phytate is known to reduce the availability of protein, fats and starches by forming complexes (Francis et al. 2001; Sugiura et al. 2001). The latter is especially true in dicotyledonous plants such as legumes, nuts and oilseeds where phytates are found closely associated with proteins and are often isolated and concentrated within the protein fraction of these feedstuffs (Khokar & Apenten, 2003). For example, in soya bean phytate is found associated with protein bodies (Bedford 2000). In monocotyledonous plants, however, phytate is present in the germ and aluerone or bran layer which are easily separated during milling. In small grained cereals, 90% of phytate is found in the aleurone layer and 10% inside the endosperm with the reverse scenario in maize (Makhode 2008). Under digestive or gut conditions, phytate interacts with protein over a range of pH forming phytate-protein complexes as a result of electrostatic interactions at low pH and forms ternary complexes with minerals as the pH approaches neutrality (Walk 2009; Makhode 2008).

Since phytate cannot be digested by non-ruminants due to the lack of intestinal phytase to hydrolyse the bound phosphate to bioavailable orthophosphate, phytate-P is usually unavailable for use by fish (Francis et al. 2001; Tudkaew et al. 2008). Nevertheless, it has been suggested that tilapia possesses the ability to degrade phytate as phytase activity has been discovered localized in small intestine brush borders. According to Kumar et al. (2012), Nile tilapia can digest ~50% of dietary phytate-P due to phytase activity originating from gut microflora, this however requires further elucidation. To compensate for poor P availability from plant-based ingredients, inorganic phosphate (*e.g* mono- or dicalcium phosphate) is generally added to animal feeds to fulfil essential P requirement. This practice has become expensive and environmentally unsustainable as unutilised plant P passes into the environment contributing to potential nutrient enrichment. These issues can be mitigated by addition of exogenous phytase to diets, a process that is supported by extensive research in poultry and swine nutrition (Moreira et al. 2003). Nevertheless, studies are still believed to be in their infancy in terms of the use of phytases in fish feed (Kumar et al. 2012).

1.6.2.2 Non-Starch Polysaccharides

Dietary fibre is the portion of plant nutrient containing lignin and polysaccharides (cellulose and hemicellulose) (McDonald et al. 2002; NRC 2011). NSPs are, hemicellulose, a complex group of polysaccharides (with the exception of starch) containing several hundred linked monomers of hexoses and pentoses (Sinha et al. 2011). The main constituents are rhamnose, arabinose, xylose, glucose, galactose, mannose, glucuronic acid and galacturonic acid. Arabinoxylans (the arabinose and xylose fractions) make up 60 – 70% of the endosperm wall and aleurone layer in most cereals with the exception of rice and barley where the percentages are 40% and 20% respectively. They are classified as non-cellulosic polysaccharides composed primarily of the two pentosans in a linear configuration (Figure 1.6.2). They are classified according to their water-binding capacity, viscous or water soluble and non-viscous water insoluble compounds (NRC 2011) Water soluble arabinoxlyans are thought to play the most important role in the anti-nutritive effects in monogastrics (Tapingkae et al. 2008).

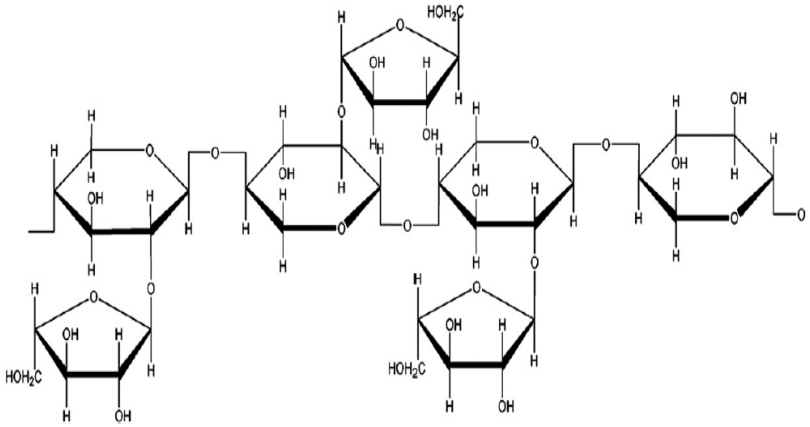


Figure 1.6.2: Chemical structure of arabinoxylan. Source: Sinha et al., (2011)

SBM, the most highly utilised plant-based ingredient, contains significant amounts of NSPs (Table 1.6.4) (Ogunkoya et al. 2006). Raw soya beans contain approximately 200 g kg^{-1} NSP (Refstie & Svihus 1999) and cereals 100 – 200 g kg^{-1} of NSPs in soluble and insoluble forms (Castanon et al.

1997). RB contains approximately 20 – 25% NSP which consist of equal portions of cellulose and arabinoxylans (Choct 1997). Arabinoxylans are also the major NSP in maize (NRC 2011).

Table 1.6.4: NSP comparison of major plant-based ingredients (in g kg^{-1}), Adapted from Choct (1997), McDonald et al., (2002) and NRC (2011)

Ingredients	Total NSP	Arabinoxylans[a]	Other fractions[b]
RB	218	85	133
Corn	81	52	29
Maize	97	52	45
Soya beans	192	47	145
SBM	196	42	154

[a] Arabinoxylan = arabinose + xylose; [b] Other fractions – Rhamnose, fucose, mannose, , glucose and uronic acids
RB – rice bran; GF – gluten feed; SBM – soybean meal

Unlike the structure of starch, NSPs are composed of different monomers linked by β-glycosidic bonds. This difference in bonding structures has profound effects on nutrient digestibility as different classes of enzymes are required to hydrolyse β-glycosidic bonds versus α-glycosidic bonds. The digestion of starch is facilitated by α-amylase, α-glucosidase and oligo-1,6-glucosidase, specialized enzymes for hydrolysing α-glycosidic bonds (Sinha et al. 2011). In herbivores and some omnivores, the activities of these enzymes range from high to medium, negating the need for exogenous additives. Monogastrics, however, do not produce enzymes such as β-xylanase or β-glucanase that can hydrolyse the bonds found in NSPs (Sinha et al. 2011).

Effects of NSP associated with gut viscosity have been extensively documented for poultry and swine. Simon (2000) reported pronounced viscosity reduction when cereal rations containing high levels of soluble pentosans were treated with NSP hydrolysing enzymes in broilers. Similar results were obtained in pigs fed diets containing wheat and triticale. In broilers, DM digestibility coincided with indigestible soya bean NSP which resulted in increased viscosity of the intestinal content and overall negative effects on digestion (Refstie & Svihus 1999). Cereal grains were also found to decrease digesta DM and increase digesta viscosity in the stomach of *O. niloticus* (Leenhouwers et al.

2007). High intestinal viscosity is also known to affect feed intake due to slower passage of feed along the gastrointestinal tract (GI) (Hetland et al. 2004). The findings of Amirkolaie et al. (2005) also support the observation that increased digesta viscosity caused by soluble NSPs resulted in lower growth and apparent digestible coefficient (ADC) of nutrients in tilapia. Intestinal enteritis in salmon has been widely reported in cases of prolonged exposure to SBM at dietary inclusions >30% (Hardy 2010). This is linked to secondary effects of NSP that results in the shortening of the villi and mal-absorption of intestinal content (Sinha et al. 2011). Sinha et al (2011) also noted specific gaps in research as it relates to the effects of NSP in fish in general. There appears to be even less information on the direct NSP effects on gut morphology, especially in tilapia, though this has been documented in other monogastrics.

1.6.2.3 Protease Inhibitors

One of the main limitations of using high inclusions of plant-based feedstuff is their comparatively low quality protein content (López et al., 1999). The presence of PIs compounds this problem because they reduce the activities of proteolytic digestive enzymes (*i.e.* protease). Proteases are enzymes that catalyse the hydrolytic cleavage of specific peptide bonds in their target proteins (Habib & Fazili 2007). PIs are therefore proteins that form complexes with specific proteases (*e.g.* trypsin, chymotrypsin *etc.*) and suppress their activities along the GI tract (Krogdahl et al. 2010). In essence, PIs are natural anti-metabolic proteins which interfere with the digestive process in animals as a plant defence mechanism (Habib & Fazili 2007). Their presence therefore negatively affects digestive processes and protein utilization, similar to the effects seen with phytate (Alarcón et al. 1999).

PIs are found in nearly all plants accounting for 1 – 10% of total protein and are abundant in storage organs such as seeds and tubers (Wati et al. 2009). In fact, PIs represent 6% of the protein present in soya bean and despite the efficiency of processing, residual levels may remain (Mikic et al. 2009). Although some PIs are heat-labile and can be eliminated using thermal treatments (*i.e.* pelleting), some researchers argue that technological treatments do not always guarantee complete

elimination of trypsin inhibitor in feeds (López et al., 1999). However, other studies have confirmed that heat treatment typically used in the extrusion process (>120 ^{o}C) for fish feed may be sufficient to inactivate most of the trypsin inhibitor activity in untreated SBM (Romarheim et al. 2005)

The inhibitory effects of SBM, corn gluten meal and wheat bran on the protease activity in Nile tilapia (*O. niloticus*), gilthead seabream (*Sparus aurata*) and African sole (*Solea senegalensis*) were evaluated (López et al., 1999). The authors found that tilapia showed the greatest sensitivity to PIs, which was unexpected based on its omnivorous feeding habit. In salmonids, PIs were found to reduce the apparent digestibility of protein and lipids while in rainbow trout (*Oncorhynchus mykiss*), they indirectly caused a negative apparent digestibility in the intestinal pyloric region due to increased secretion of pancreatic enzymes (Krogdahl et al. 2010). These authors also noted that salmonids and Nile tilapia were sensitive but may be able to compensate when inhibitor activity in the final feed is below 5g kg^{-1}

1.7 **Exogenous Enzymes for Feed Production**

1.7.1 Commercial Enzymes

Enzymes are biological catalysts that accelerate biochemical reactions using alternative reaction pathways (Cech and Bass, 1986). They can be produced commercially for use in various industries including the animal feed sector. This is due to their high specificity and efficiency in accelerating biochemical processes which otherwise would not occur under normal conditions (Nielsen & Wenzel 2006). The use of commercial enzymes as feed additives is now fully established in poultry and swine (Kirk et al. 2002) since the first reported study by Chickner and Follwell (1925) using protozyme in pullet feed (Brufau 2006; Choct 2006). Continued development since then has been linked to parallel developments in biotechnology. For the livestock industries, commercial enzyme application began in the early 1990's and has come of age (Chesson 1993). Consequently, enzyme technology has progressed significantly in terms of efficacy and substrate specificity (Choct 2006). The availability of commercial feed-grade enzymes has now encouraged changes in formulation and higher inclusion

of plant-based ingredients for animal diets. They have also promoted the use of lower cost, less-processed, raw ingredients, increasing the choice and flexibility of feed manufacturers (Deguara et al. 1999). The major feed enzyme categories include phosphatases (phytase), carbohydrases (xylanase, β-glucanases *etc*), proteases, lipases and oxidoreductases (Cosson et al. 1999; Pariza & Cook 2010).

1.7.2 Phytases

Phosphatases are a diverse group of enzymes that catalyse the hydrolysis of phosphomonoester bonds of various phosphate esters. Phytases are a sub-group of phosphatases with specificity for hydrolysing phytate into phosphoric acid and myo-inositol phosphate (Haros et al. 2005), with complete hydrolysis yielding one molecule of inositol and six molecules of inorganic phosphate (Makhode 2008) (Figure 1.7.1). This action reduces the chelation capacity of phytate (Kumar et al. 2012).

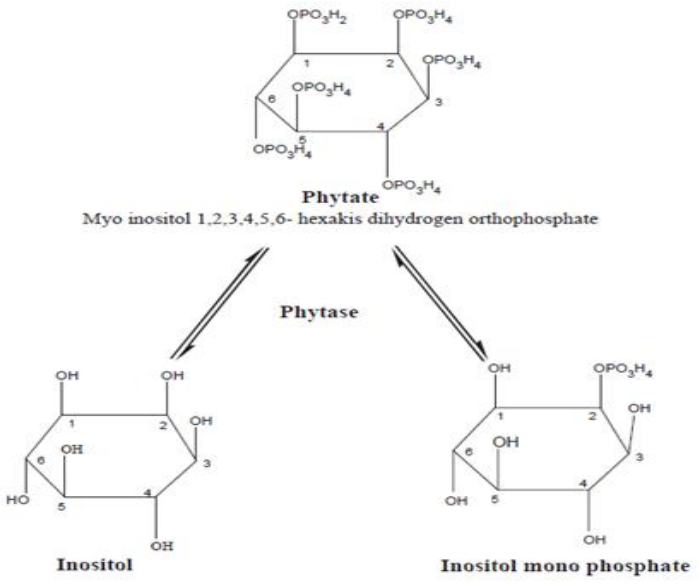


Figure 1.7.1: Hydrolysis of phytate by phytase. Source: Kumar et al., (2012)

The efficiency of phytase is highly dependent on dietary content, plant-based ingredient and the associated storage site for phytate (Kumar et al. 2012). In addition, the greater the dietary calcium

content, the poorer the efficacy of the phytase. In fact, calcium not only interacts with phytate but binds soluble substrates reducing interaction of nutrients to enzymatic attack (Bedford 2000). Nevertheless, the use of phytases in animal feeds has been linked to improvements in P availability. Addition of microbial phytase to diets of growing pigs increased apparent P bioavailability by 24% and reduced excretion by 35%. In support, a reduction of 50% faecal P was observed in broilers fed diets treated in a similar manner (Chesson 1993). Choct, (2006) also reported that phytase increased phytate-P digestibility from ~25% to 50 – 70% in poultry.

Early studies using phytase focused primarily on ingredients pre-treatment due to previous instability of enzymes during feed manufacturing process. Pre-treatment of plant-based ingredients has been useful especially for ingredients (RB, soya bean *etc*.) that have relatively high phytate-P content. Liang et al. (2009) remove 92% of phytate-P in RB by pre-treating with phytase at pH 5.5 and 50^{o}C for 30 mins. In support, Cao et al. (2008) showed phytase pre-treatment of soya bean significantly increased P availability in a dose response manner; 1000 U[3] kg^{-1} was most efficient. Incubation of soy protein concentrate with phytase also improved protein digestibility, FCR, protein retention, and reduced N excretion in Atlantic salmon (Storebakken et al. 1998).

Based on several related studies, optimal phytase inclusion for channel catfish (*Ictalurus punctatus*), Nile tilapia (*O. niloticus*), Common carp (*Cyprinus carpio*) and Asian catfish (*Pangasius pangasius*) diets ranged from 500 – 1000 FTU kg^{-1} (Kumar et al. 2012). Yan et al. (2002) reported higher bone ash, Ca, P, and Mg in channel catfish fed diets supplemented with 500 U kg^{-1} phytase. The digestibility of P, Mg, Na, K, Cu and Zn was enhanced in rohu carp (*Labeo rohita)* fingerlings fed corn-gluten diets with 750 FTU kg^{-1} phytase compared to reference diets (Hussain et al., 2011). Similarly, digestibility coefficient for CP, lipid and apparent gross energy (GE) of a sunflower meal based diet improved by 13 – 23% with 750 FTU kg^{-1} in *L. rohita* (Hussain et al., 2011)

[3] Activity of phytase is expressed as FYT, FTU, FU or U (means the same). One unit of phytase is defined as the quantity of enzyme that liberates 1 mmol of inorganic-P per minute from 0.0015 mol/l sodium phytate at pH 5.5 and 37^{o}C (Kumar et al. 2012)

1.7.3 Xylanases

Monogastrics lack the appropriate digestive enzymes to degrade NSPs, however, this can be mitigated through addition of exogenous enzymes to their diets (Sinha et al. 2011). Among the various NSPases[4], xylanase has been used successfully in poultry and swine diets because it has an ability to randomly cut the arabinoxylan backbone creating fragments of smaller molecular weight (Tapingkae et al. 2008). Their mode of action (Figure 1.7.2) is still debatable because interactive processes are often disregarded in individual studies (Bedford & Schulze 1998). Nevertheless, the addition of xylanase to NPS-rich diets decreases viscosity and increases cell wall permeability (Sinha et al. 2011). This directly improves digestive processes such as motility and endogenous enzyme access to substrates (Walk 2009). Xylanases have also been used in rye-wheat, cereal-based and maize-SBM diets commercially to improve their nutritive value (Kirk et al. 2002; Choct 2006; Angel et al. 2010).

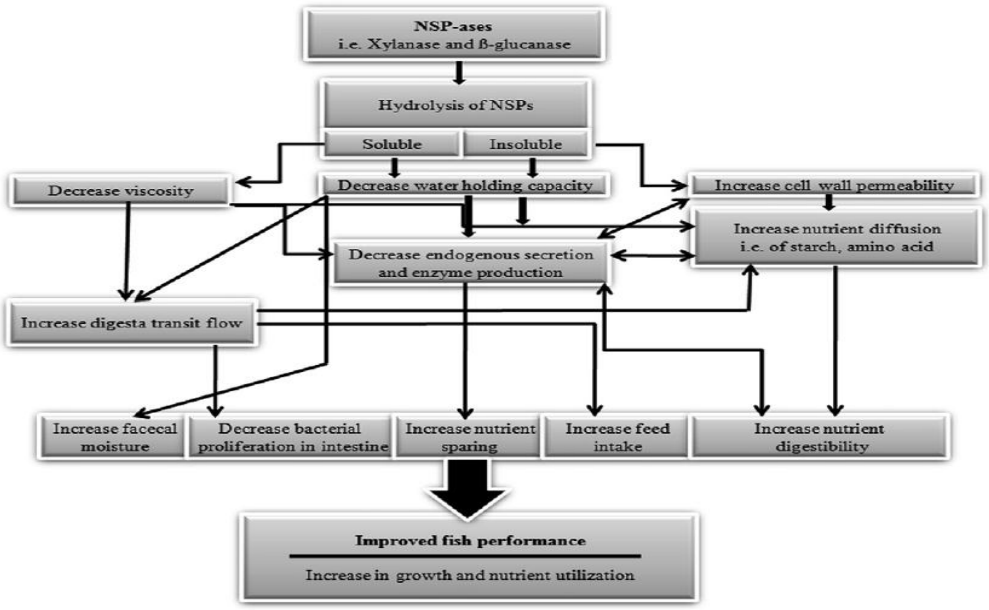


Figure 1.7.2: Mode of action of NSP degrading enzyme such as xylanase. Source: Sinha et al., (2011)

[4] NSPases are carbohydrases that degrade non-starch polysaccharides

Xylanase also increased β-glucanase activity in the posterior gut of broilers through beneficial modifications of microflora which had direct impacts on overall NSP degradation (Schurz 2000). On the contrary, general responses to xylanase use in pigs have been inconsistent due to differences in diet composition and enzyme choice. Nevertheless Tapingkae et al. (2008) reported positive improvements in *in vitro* protein digestibility and performance of piglets post-weaning when fed practical diets containing SBM, RB, maize and CM supplemented with crude xylanase. In fish, xylanase supplementation (200 – 2470 U kg^{-1}) of Jian carp (*Cyprinus carp var. Jian*) diets improved growth performance and enhanced intestinal enzyme activity (Jiang et al. 2014). No information is available regarding the effects of xylanase on changes to tilapia gut morphology (Sinha et al. 2011).

1.7.4 Proteases

Proteases perform a variety of biological functions in homeostasis, apoptosis, signal transduction, reproduction and immunity. Though from a nutritional perspective, the hydrolysis of protein to individual AA and peptides during digestion is thought to be their key function (Angel et al. 2010). They account for 60% of global enzyme production for industrial purposes, however, for animal feed production it merely accounts for 5% of the global feed enzyme market (Smith, 2014). Most published research involving protease in animal feed generally include the enzyme as an additive within a cocktail making it difficult to assess the direct impacts of the enzyme on animal performance (Angel et al. 2010).

Deguara et al. (1999) treated SBM-based sea bream diets with low and high pH active protease and α-galactosidase and found some growth parameters improved at low pH (3.0) as opposed to high pH (8.5). Drew et al. (2005) reported 0.25 g kg^{-1} protease significantly increased the coefficient of total tract digestibility of rainbow trout fed a canola-pea diet but no effect was seen with a coextruded flax-pea diet. The authors concluded that protease's benefits are dependent on the selected ingredients in support of Lopez et al (1999). Supplementation of pea-based diets with protease and α-galactosidase resulted in poorer growth and FCR in growing pigs, however, supplementation with protease alone showed nutritional benefits of improved FCR (O'Doherty & Forde 2013). A cocktail

of protease and carbohydrases (E1) or single protease (E2) was used to promote growth improvements in broilers fed maize-soybean diets (Yu et al. 2007). The cocktail produced better weight gain but the protease inclusion (E2) resulted in improved feeding efficiency compared to E1 and the controls.

Continued development of product formulations and stability may further improve the application of enzymes, especially proteases, for animal feed production (Chesson 1993). This may also widen the scope of alternative plant-based ingredients in the future. Furthermore, as more knowledge become available in terms of the chemical structures of anti-nutrients (specifically NSPs) and their interactive effects, highly sophisticated enzyme cocktails can be developed to target these ANFs in a more precise manner (Choct 2006).

1.7.5 Benefits of Combining Enzymes

The cooperativity of enzymes to degrade feedstuff and their interactions require much research. The benefits of combining phytases and xylanases have been demonstrated to some extent in broilers (Bedford 2000). Several enzyme companies (Novozyme/Royal DSM, Alltech, Ameco-Bio & Co., Canada Bio-Systems Inc. *etc*.) are now producing enzyme cocktails to improve, even further, the efficiencies of feed utilisation, particularly those with high inclusions of plant-based ingredients, and the synergistic benefits for animal performances. Combining enzymes may provide additional benefits, in that, different enzymes act in different location along the GI tract and target different substrates (Walk 2009).

Ogunkoya et al. (2006) reported significant effects of multi-enzyme supplementation on ADC of DM, CP, nitrogen free extract (NFE), P and GE in SBM-based diets fed to rainbow trout. Using a similar commercial enzyme complex, higher FI was recorded with tilapia fed diets containing 0.15 g kg^{-1} but no difference were observed in protein, lipid and GE ADCs between treatments (0, 0.15 and 1.0 g kg^{-1}) (Lin et al. 2007). Khalafalla et al. (2010) also showed the addition of Amecozyme in diets at 0.5% and 1.0% enhanced the growth performance of *O. niloticus* fingerlings. Similarly, a cocktail containing protease, xylanase, glucanse, lipase, amylase and cellulase was used to supplement five

grain diets fed to tilapia which improved fish performance, nutrient digestibility, carcass characteristics and faecal recovery (Soltan 2009). Table 1.7.1 compares enzyme inclusions between studies.

Table 1.7.1: Comparison of enzyme inclusion levels in different monogastric diets (in g kg^{-1})

Products	Protease	Xylanase	Phytase	Size/Species	Sources
Amecozyme[a]	0.6	0.16	0.05	10g Tilapia	Khalafalla et al., (2010)
Cocktail[b]	1.0-1.5		-	17g Tilapia	Lin et al., (2007)
Cocktail[c]	-	0.1	0.1	148-160g Tilapia	Li et al., (2009)
WX, NP	-	0.1-0.25	0.12-0.15	Tilapia	Dias & Verlhac (2007/8)*
Superzyme CS[d]	1.0-2.5		-	5g R. trout	Ogunkoya et al., (2006)
Cocktail[e]	0.228	0.208	-	73-110g R. trout	Dalsgaard et al., (2012)
Poultry GRO-250	0.25	-	-	>190g R. trout	Drew et al., (2005)
Cocktail[f]	0.1	-	-	50g S. bream	Deguara et al. (1998)
PRO, Cock., PHY	0.2	0.2	0.2	89g S. bream	Ayhan et al., (2008)
WX, NP, VP[g]	-	0.8	0.2	6g J. seabass	Ai et al., (2007)
Cocktail[h]	1.0	-	-	Pigs	O'Doherty and Forde, (2013)
Cocktail[i]	0.02-0.5	-	0.1	Broilers	Simbaya et al., (1996)
PRO[j]	0.125-0.5	-	-	Broilers	Yu et al., (2007)
XYL (pure)		0.2-0.4		Broilers	Schurz, (2001)

[a] cocktail with amylase, lipase, glucanase & galactosidase; [b] cocktail with glucanase; [c] cocktail with citric acid; [d] contains xylanase, protease, amylase, cellulose, glucanase; [e] cocktail with β-glucanase; [f] cocktail with galactosidase; [g] VP – glucanase, pentonase, cellulase (0.4g kg^{-1}),; [h] cocktail with galactosidase; [i] cocktail with proteases and carbohydrases; [j] PRO – protease; WX – xylanase and NP - phytase
*Unpublished

1.7.5.1 Economic Benefits of Supplementation

The use of enzymes must sufficiently demonstrate substantial improvements in feed conversion or product quality to cover any adjustments in formula cost resulting in higher profit margin (Chesson 1993). In other words, they must somehow improve upon least-cost formulation by lowering input cost while maximizing outputs in terms of animal performance, health and cost to produce one unit of animal protein. The economic benefits of using exogenous phytases are by far more straightforward than those of xylanases and proteases. Phytase delivers direct cost benefit by replacing the need for inorganic phosphate (Bedford 2000). The benefits of reducing P loading and feed formulation cost are

clear, and as a result phytase is now considered a standard feed additive. Though most enzyme studies acknowledge supplementation-related formula cost savings, rarely are these figures published for reference.

In rare cases, a few studies have included the economic advantage of exogenous enzymes particularly as it relates to saving in cost per kg feed used. Iyayi & Davies (2005) reported the cost of feed per kg weight gain in broilers (starters) was lower when they were fed Avizyme supplemented diets containing palm kernel meal and brewer's dried grain. No benefit was realised in finishers, however, enzyme supplementation had reduced maize utilisation required for both diet types. Using the same enzyme complex (Avizyme) in pullet diets, Novak et al. (2008) reported significant effects on the cost of raising pullets to 126 days when a low CP, low ME enzyme supplemented diet was fed compared to commercial controls. In addition to those economic gains, environmental benefits of enzyme supplementation are equally important. Though a tangible economic value may not be easily quantified, the reduction of potential impacts to the environment due to reduced nutrient load are just as meaningful.

1.8 **Life Cycle Assessment of Aquaculture**

1.8.1 Environmental Impacts of Aquaculture

With the rapid increase in aquaculture production, stakeholders have grown increasingly concerned about the industry's environmental impacts. As the global industry expands, it is likely to place greater demands on finite natural resources (water, land, ingredients *etc*.) which provide ecosystem goods and services. It follows, therefore, that with greater demands on these resources, potentially greater negative environmental impacts will arise. Nevertheless, through hotspot[5] analysis and mitigatory efforts, aquaculture's sustainability can potentially improve (Cho & Bureau 2001). Sustainable aquaculture is multidimensional, however, loosely defined it is the culture of aquatic

[5] For the purpose of this study, a "hotspot" can be defined as an activity of interest which causes disportional sustainability impacts along a value chain (Hospido & Tyedmers 2005)

species to meet current demands for seafood without compromising future social, economic and environmental development.

Prior to the 1990's, aquaculture impacts were narrowly viewed from a *two*-dimensional perspective, direct inputs (fish, feed, fertilizers and chemicals) and outputs (uneaten feed, faeces, and residues). Consequently, most of the associated impacts were dissolved and particulate nutrients, in the form of N, P and suspended solids (Jauncey 1998). In fact, aquaculture wastes are still largely dietary in origin with estimates of >52 % of feed N alone ending up in the environment (Preetha et al. 2012)(Boyd 2015). Fortunately, the industry has evolved and does not operate exclusively within a box. The use of chemicals in aquaculture has also raised concerns owing to their potential impacts on downstream aquatic systems (Rico et al. 2012), nevertheless this was outside the scope of this study. However, since feed is considered one of the major sources of aquaculture waste, sustainability strategies should prioritise feed ingredient choices, diet composition and on-farm feeding practices as key components (Amirkolaie et al. 2005). This will require broad frameworks and systems to manage cumulative impacts along the entire value chain.

Ecological footprint was initially developed to measure aquaculture resource use but was found inadequate to address the various aspects of industry sustainability (Kautsky et al. 1997). Using consultative approaches, Caffey et al. (2001) engaged aquaculture stakeholders (producers, researchers, regulatory agencies and non-governmental organizations) to develop a comprehensive list of sustainability indicators that was appropriate to the sector at the time. The environmental indicators had two main foci, resource use and environmental pollution impacts, *e.g.* land and water conservation, effluent biochemical oxygen demand, suspended solids and use of non-native species (Caffey et al. 2001). Since then some of these indicators have been incorporated into newer methodologies of assessing the potential industry impacts with one such method being Life Cycle Assessment (LCA).

1.8.2 LCA as a Predictive Tool

The first LCA study was conducted for the packaging industry in 1969 and through a series of methodological developments over 40^{+} years; it is now widely applied to food and agricultural production processes including aquaculture (Pelletier 2006; Caffrey & Veal 2013). LCA has become a widely accepted method of evaluating and identifying aquaculture impacts and assessing its overall environmental performance (Henriksson et al., 2011). LCA addresses all the processes in a production chain and offers a convenient means of quantifying impacts associated with energetic and material inputs and outputs (Pelletier et al. 2007; Nielsen & Wenzel 2006). The limited, but increasing, amount of LCA research in aquaculture indicates a growing interest in LCA, particularly to understand and improve the sustainability of its supporting industries *e.g.* feed (Aubin et al. 2006; Pelletier et al. 2007; Iribarren et al. 2012). Its integrative approach considers impacts on both local and global scales (Aubin et al. 2006). Despite its increased popularity as a standardized tool of assessing ecological impacts (Cao et al. 2011), there remains a need for methodological harmonization as it relates to choice of aquaculture-appropriate indicators and resource allocation.

LCA is based on four critical steps, Goal and Scope Definition, Inventory Analysis, Impact Assessment and Interpretation; formalized and standardized by the International Standard Organization (ISO) 14040/44:2006 Environmental Management – Regulations and Guidelines. Goal and scoping defines the functional unit and establishes a system boundary based on research objectives. A functional unit is an established reference used to assess system performance and is the reference point to which indicators are compared. Common aquaculture functional units are one tonne of live weight or fillet while system boundaries often include feed and fish production to the farm gate, market or consumers (Henriksson et al., 2011). Life cycle inventory phase includes data collection of all material and energetic inputs, and process outputs and emissions.

The impact assessment phase models different production scenarios by translating the inventory data into selected impact categories (Pelletier 2006). This is done using one of two approaches, attributional or consequential allocation. Attributional LCA emphases the inputs and outputs of

relevant process flows in a production or value chain while consequential LCA focuses on the (environmental) effects caused when one or more aspect(s) of that process flow/system changes. There are two types of impact categories, end-point and mid-point, however, the latter is considered more reliable (Bare et al. 2000). Common fisheries and aquaculture-related mid-point impact categories are listed below (Table 1.8.1). Mid-point impact categories are critical points in the cause and effect chain where characterization factors can be calculated to reflect the impact on the end-point category, *e.g.* ecosystem damage (Bare et al. 2000). Characterisation factors are equivalent scales used to convert the impacts of different pollutants and emissions (*e.g.* ammonia, methane, nitric oxide *etc.*) to potential impact categories. For example, 1 kg of ammonia is equal to 0.35 kg PO_4 *equivalent* (EP) or 1.88 kg SO_2 *equivalent* (AP).

Table 1.8.1: Impact categories commonly used for fisheries and aquaculture LCA studies. (Pelletier et al., 2007; Ayer and Tyedmers, 2009; Cao et al., 2011; Henriksson et al., 2011)

Mid-point Impact Categories	**Units**
Global Warming Potential (GWP)	CO_2 equivalent
Acidification Potential (AP)	SO_2 equivalent
Eutrophication Potential (EP)	PO_4 equivalent
Energy Use (EU)	MJ[a]
Agricultural Land Use (ALU)	m^2 $year^{-1}$
Abiotic Resource Use	Sb[b]
Biotic Resource Use	NPP [c]
Photochemical Oxidant Formation	C_2H_4
Aquatic and Terrestrial Ecotoxicity	1,4 DCB[d] equivalent
Human Toxicity	1,4 DCB equivalent
Ozone Depletion	CFC[e]

[a] – Mega joules; [b] – Antimony, [c] – Net Primary Productivity; [d] – 1, 4 Dichlorobenzene; [e] – Chlorofluorocarbon

Due to its general applicability to production industries, LCAs are now being used to merge process chains of two or more associated industries, *i.e.* one offering a product or service to the other(s). Though limited, a few LCA have been published linking feed to fish production

(Papatryphon et al. 2004; Iribarren et al. 2012; Samuel-Fitwi et al. 2013; Avadí et al. 2015) and commercial enzymes to animal production (Nielsen & Wenzel 2006; Nielsen et al. 2007b; Oxenboll et al. 2011). In fact, the latter studies support the use of multi-enzymes for animal production due to their associated environmental benefits (Skals et al. 2008).

1.8.3 LCA Applied to Enzymes and Animal Feed Production

Traditionally, enzyme biotechnology was confined to the pharmaceutical, food and beverage industries (Skals et al. 2008). They have been adapted extensively for swine and poultry diets for over 20 years yet they are still considered relatively novel feed additives for aquafeed. They are produced on a large scale through fermentation, filtration and granulation processes (Nielsen & Wenzel 2006). The commercial production of enzymes often requires high inputs of energy and raw materials. In an assessment of enzyme products, Nielsen et al. (2006) focus on four mid-point impact categories to comparatively assess the environmental profiles of enzyme assisted processes using consequential allocation or system expansion, contrary to attributional or mass allocation used in most LCA studies.

The cradle-to-gate assessment revealed the main hotspots in enzyme production were the ingredient production and fermentation. Nielsen and Wenzel (2006) concluded that the application of phytase as an alternative to inorganic phosphate in swine feed for intensive systems was justified by improvements in GWP, AP, EP, photochemical oxidant formation, energy use and phosphate rock savings. A small quantity of phytase displaced a large amount of inorganic phosphate, and the results showed that the impacts of the latter exceeded that of the former. In a follow-up to their study with phytase, Nielsen et al. (2007a) assessed the environmental burdens of xylanase supplementation on Danish swine production. Feed demand was reduced by 2.5% and 12.4 kg less protein was required when xylanase was included in the diet to produce 280 kg of animal meat. There were also reductions in ammonia (NH_3) and nitric oxide (N_2O) as a function of N excretion from manure linked to dietary xylanase inclusion.

Proteases are used primarily to augment endogenous supply in order to improve protein hydrolysis and nitrogen utilisation which positively affects CP digestibility and excretory N

(Oxenboll et al. 2011). The authors investigated the environmental benefits of lowered protein content and the resulting N content of manure associated with protease use in poultry feed. Significant improvements were obtained in all three impacts categories used *i.e.* GWP, AP and EP. The results also showed net savings in N emissions (NH_3, N_2O) with protease supplementation. In addition, there was net reduction in feed consumption which in turn lowered FCR and manure emissions.

1.9 **Research Hypothesis and Thesis Structure**

1.9.1 Hypotheses

Based on the anti-nutritive effects of phytate-P, NSPs and possibly PIs on animal growth and performance, the potential benefits of commercial enzymes in monogastric diets, and the application of LCA in aquaculture research, the following were hypothesised:

a) Multi-enzyme supplementation of plant-based low FM diets will improve nutrient utilisation and growth performance in tilapia,

b) There will be higher economic benefits in using multi-enzymes diet formulations, and

c) There will be overall lower environmental impacts associated with using multi-enzymes for tilapia feeds and production.

1.9.2 Research Objectives

The objectives of the project were to:

- Evaluate the feeding practices of tilapia farmers in central Thailand and potential commercialization opportunities of enzyme supplemented (ES) low FM diets
    a. Evaluate the trends in feed ingredient prices, use of commercial feed and feed additives (specifically enzymes) in Thailand
- Evaluate growth performance of, and nutrient utilisation, in tilapia fed ES low FM diets
    a. Formulate sustainable low FM tilapia grow-out diets using locally available lower-cost plant feedstuff (Phase 1 and 2).

b. Investigate the effects of declining FM (10%, 5%, 3% and 0%) at fixed enzyme (phytase and xylanase) dosage on feed utilization and growth (Phase 1)

c. Investigate the interactive effects of protease with phytase and xylanase on nutrient digestibility, protein utilisation and growth in 2% FM diet (Phase 2)

d. Evaluate enzyme effects on different growth and economic matrices (Phase 1 and 2)

- Assess the environmental impacts associated with enzyme supplementation

a. Conduct life cycle audit of value chain processes (enzyme → feed → fish) to comparative assess the environmental impacts of ES low FM diets and commercial feeds associated with tilapia production in Thailand.

1.9.3 Thesis Structure

This dissertation contains eight chapters; General Introduction, General Methodology, five Experimental/Results chapters and General Discussion. Experimental chapters (3 – 7) were written up in manuscript format and so some of the text in Chapters 1 and 2 may be repeated briefly in the introduction and method sections of the former chapters.

Chapter One provided a general introduction to the thesis' multidisciplinary topics – tilapia production, aquafeed production, plant-based feedstuffs, challenges associated with plant-based ingredients, commercial enzymes, and LCA of feed and animal production. It included an in-depth literature review of key gaps in monogastric animal research, the major objectives and research hypotheses.

Chapter Two details the general methods and materials. It covers the sample frame and data collection for the field surveys, experimental designs for the on-farm growth and digestibility trials, and LCA modelling. It also presents baseline information used in the formulation of the experimental low FM tilapia grow-out diets. It supports the diet compositions used for experiments in Chapters 4, 5 and 6.

Chapter Three provides background information on feeding practices of tilapia farmers in central Thailand and seek to identify underlining trends. Finally it attempts to pinpoint possible

commercialization opportunities and justify how low FM enzyme supplemented diets may be positioned in the context of Thai aquafeed market and industry.

Chapters Four, Five and Six present the findings of two sets of on-farm digestibility and growth experiments using low FM diets supplemented with phytase, xylanase and protease. The chapters explore their effects on growth and economic performance indicators as well as interactions between enzymes.

Chapter Seven merges the four preceding chapters by comparatively assessing the environmental impacts of the best performing experimental diets with an average commercial feed associated with tilapia production in Thailand (LCA).

Chapter Eight discusses the overall findings of the research and presents the final conclusions regarding the application of exogenous enzymes in low FM diets on nutrient utilisation, growth and sustainability of farmed tilapia in Thailand

Chapter 2
General Methodology

2.1 **Field Survey**

The field survey component was based upon the structure of an EU-funded inter-disciplinary project, Sustaining Ethical Aquaculture Trade (hereafter referred to as SEAT). The project involved broad and in-depth integrated farmer surveys focused primarily on four export commodities including tilapia and were carried out in four Asian countries including Thailand. The overall aim was to evaluate how different types and scales of farming operation impacted production, environment, socio-economics and marketing (Murray et al. 2013).

2.1.1 Sample Frame

The SEAT surveys were implemented over four years (2010 – 2013) and began with a piloting exercise evaluating relevance, redundancy, comprehension, logical flow and coding. Independent variables were stratified according to the following primary factors chosen based on export-value chain characteristics – country, species, farm-scale and farming system. In Thailand, there were two species of interest (shrimp and tilapia), three farm-scales (small, medium and large), and three types of farming systems (extensive, intensive, semi-intensive). *A posteriori* secondary stratification factors related to the primary variables were also chosen *e.g.* monoculture *versus* polyculture systems. Farms were selected using a randomised probabilistic approach and multi-stage process (purposive and cluster sampling). The farms were first selected using clusters or grouping within provinces after which they were stratified based on DOF farm numbers (Murray et al. 2013). There were two SEAT surveys, a baseline integrated farm survey (IFS) consisting of 199 tilapia farmers followed by a transition farm survey (TFS) of a smaller subgroup of 81 respondents who had expressed an interest for long-term follow-up interviews. These were supplemented with a follow-up feeding practice survey (FFPS; 2014) using Nam Sai (local tilapia hatchery/seed supplier) customers as the sample

frame (due to time limitation). Nevertheless, it was a more purposive approach to the previous method although it introduced certain biases related to location and seed type. It involved 20 tilapia farmers focusing primarily on their feeding practices. Data from each survey was collected using qualitative survey methods and managed using Microsoft® ACCESS/EXCEL (Murray et al. 2013).

2.1.2 Data Collection

Data collection was a collaborative effort of the SEAT project team. Data was collected using in-depth telephone and follow-up face-to-face interviews guided by semi-structured questionnaires. Recall period was restricted to the last completed production cycle. Enumeration and interpretation errors were minimised through the use of supplemental definitions for key terms and detailed coding for the database (Murray et al. 2013). See Chapter 3, Section 3.2.2/3.2.3 for further details.

2.1.3 Statistical Analysis

Initial data evaluation was facilitated by Microsoft® ACCESS and where appropriate the data was exported to Microsoft® EXCEL and SPSS for further analysis (See Chapter 3, Section 3.2.5).

2.2 **Digestibility and Growth Experiments**

2.2.1 Experimental Model and Statistical Justification

2.2.1.1 Statistical Hypothesis

Based on the requirements for an effective experimental design (Ruohonen et al. 2001), each biological hypothesis (Chapter 1) was converted to a statistical hypothesis.

1. There are no differences between treatment means of low FM diets supplemented with and without enzymes on nutrient utilisation and growth in tilapia.
2. There are no differences in cost benefits between diets with and without enzyme supplementation.
3. There are no differences in environmental impacts of tilapia production using feeds supplemented with and without enzymes.

2.2.1.2 Experimental Model

A complete randomised design (CRD)[6] was chosen based on *a priori* knowledge of growth experiments. The treatments (including FM inclusion levels) were chosen from literature, previous studies and product specification (*i.e.* enzymes). The effects of each treatment were evaluated based on response variables for digestibility, nutrient utilisation, growth and economics. Based on resource and space constraints, each treatment was replicated (4 – 6 replicates[7]) and randomly assigned to experimental units using a lottery method (Bhujel, 2008).

2.2.1.3 Statistical Power Analysis

The power of test used for *a priori* evaluation of data analysis is a function of the following parameters – the significance level (α), number of treatments (k), number of replicates (n), standard deviation (s) and effect size (*f*) (Searcy-Bernal 1994). Statistical significance was considered at alpha level $P = 0.05$, based on conventional value used in previous aquaculture research. A statistical power of 80% is generally recommended which represents a probability of $\beta = 0.2$ of committing a type II error (*i.e.* accepting a false null hypothesis H_0). This is important when non-significant or negative results are obtained (Searcy-Bernal 1994). It is, however, highly dependent on the effect size[8] and required a compromised in both research phases. It was believed that setting a high power of test (0.80) at the expense of detecting small effects between treatment (above 0.40 - detection of large effect) (Cohen, 1988 cited by Searcy-Bernal, 1994), given the replication constraints, would allow for an overall higher probability of the test reaching the correct conclusion as opposed to committing a Type II error which may have significant implications (Fowler et al. 1998). In designing experiments for evaluating the effects of several diets on growth it is believed that values of *f* (effect size) equalling to or greater than 0.40 can be considered meaningful since lower values would not have any relevance to commercial aquaculture (Searcy-Bernal 1994). Power analysis was conducted *a*

[6] A CRD is the basic experimental design that is used to study the effects of one factor i.e. treatment or fixed factor keeping others constant (often called a single factor experiment)
[7] Replicates are experimental units which receive the same treatment independently
[8] Effect size is the smallest difference to be detected reliably between treatments (Ruhonen *et al.*, 2001)

posteriori to validate the power of test. Based on the F value (ANOVA), the effect size was calculated and validated using the following equation [$f = \sqrt{(k\text{-}1/kn)\ F}$] (Searcy-Bernal 1994).

2.2.1.4 Type of Data

Table 2.2.1 lists data collected (*i.e.* response variables) and derived (See Section 2.1.2.9 for equations).

Table 2.2.1 Data collection

Primary data	Derived data
Proximate Composition (g kg^{-1} or %)	**ANF (phytate and arabinoxylan)** (g $100g^{-1}$ or %)
• Ingredients	• Plant-based ingredients
• Diets/	• Experimental diets
• Fish (whole carcass)/Faeces	
	Growth and Nutrient Utilisation
Enzyme Recovery Levels (g kg^{-1})	• Condition Factor
• Diets	• Hepatosomatic Index
	• Arithmetic mean (g)
Growth	• ADG (g day^{-1}) or AWG (g)
• Total fish length (cm)	• SGR (% day^{-1})
• Weights (g)	• TGC
	• FI (g)
Water Quality	• FCR
• pH	• PER
• Temperature ^{o}C	• ANPU (%)
• DO mg L^{-1}	• N & P Retention (mg g^{-1} or %)
• Ammonia NH_3 mg L^{-1}	• ADC (%)
• Nitrite NO_2 mg L^{-1}	• EE (%)
• Nitrate NO_3 mg L^{-1}	• ME (KJ)
• Alkalinity mg L^{-1} $CaCO_3$	• Δ U ($ kg^{-1})

DO – dissolved oxygen; ADG – average daily gain; AWG – average weight gain; SGR – specific growth rate; TGC – thermal growth coefficient; FI – feed intake; FCR – feed conversion ratio; PER – protein efficiency ratio, ANPU – apparent net protein utilisation; ADC – apparent digestibility coefficient; EE – Energy efficiency; KJ – kilo joules; Δ U – change in unit profit

2.2.2 Criteria for Diet Design

Diet design is defined as the process of combining base ingredients according to a formula to meet a specific production objective (Bhosale et al. 2010). Design includes feed formulation and pellet production which are driven by the nutrient requirements of the target species, ingredient availability (quality and price) and the type of culture system. In essence, formulation addresses the animal's requirements and how these can be met cost effectively, while pelletization addresses the culture system and method of feed delivery. In Thailand, there are legislative controls that govern the nutritional standard for herbivorous feeds (Table 2.2.2) (Thongrod 2007).

Table 2.2.2 Standards for tilapia pelleted feeds in Thailand

Fish Size	CP (min. %)	CL (min. %)	CF (max. %)	Moisture (max. %)
Fingerling	28	3	8	12
150 – 250 g	25	3	8	12
>250g	20	3	12	12

Average shelf life is three month and size of pellets not defined

2.2.3 Chemical Analysis

2.2.3.1 Proximate Analysis

250 g of each ingredient or experimental diet was weighed and refrigerated (4^{o}C) on farm prior to being transferred to a laboratory. Fish and faecal samples were collected by replication units and frozen (minus 14 – 20^{o}C). Fish samples were then pooled by treatment, ground up and divided into triplicates prior to chemical analyses.

Chemical analyses of feed ingredients, experimental diets, fish whole body and faeces were conducted according to standard methods (Association of Official Analytical Chemists; AOAC 1990; 2005). Moisture level was determined by drying pre-weighed samples (2g each) at 135^{o}C for 2 hours. After cooling (desiccator), the final dried sample weights were measured and deducted from initial sample weights (Dry method AOAC 1990, 934.01). Crude protein (CP) was determined using the

Kjeldahl method (Semi-automated method AOAC 1990, 954.01). Samples (0.25g each) were digested using sulphuric acid at 410^{o}C for 45 minutes. Samples were then transferred to auto-analyser for washing and titration. Percentage protein levels were thereafter calculated from the ratio of the nitrogen content by multiplying by 6.25. Ether extract (EE) was determined using Indirect method (AOAC 1990, 954.02). HCl was added to samples (2g each) and set in water bath (70 – 80^{o}C) for ~40 minutes. Equal portions of ether and petroleum ether (25 mL) were added and tubes shaken vigorously for one minute in between. Solutions were then centrifuged at 600 rpm for 20 mins. Samples were filtered, dried (90 minutes at 100^{o}C) and fat residues weighed immediately. Crude fibre (CF) was determined using the Asbestos free method (AOAC 1990, 978.10). Samples (2g each) were defatted using petroleum ether and digested by boiling with sulphuric acid (1.25%) for 30 minutes. Solutions were funnelled through preheated filters and washed with near boiling water (four 40 mL portions). NaOH was then added to extraction vessel and boiled, the samples filtered and washed. Samples were then cooled (desiccator) and ashed for 2 hours at 550^{o}C. Percentage crude fibre was calculated by dividing the loss in weight multiplied by 100 divided by the sample weight. Ash level was analysed according to the Official final action method (AOAC 1990, 942.05). Crucibles containing pre-weighed samples (2g) were placed in a furnace (600^{o}C) for 2 hours. Crucibles were thereafter cooled and weighed immediately. Ash percentage were reported to the nearest decimal place.

Samples were also analysed for phosphorus (Photometric method; AOAC 2005, 986.24). calcium (Dry ash method; AOAC 2005, 984.27), zinc and iron (ICP-OES AOAC 2005, 999.10). Gross energy (GE) was determined using analytical methods for oxygen bomb calorimeter (Parr 6200). Analysis of chromic oxide levels in the diets and faeces were done using a photometric method after Bolin et al., (1952). Nitrogen free extract (NFE) was calculated by subtracting the sum of moisture, ash, CP, lipid (EE) and CF from 100. Carbohydrate was calculated by summing CF and NFE. All analyses were carried out independently by the Nutrition and Aquafeed Laboratory, Kasetsart University, Bangkok, Thailand**.**

2.2.3.2 Anti-nutrient Analysis

Phytic acid (phytate-P) and arabinoxylan (NSP) were determined for the plant-based ingredients and experimental diets. Trypsin inhibitor was ignored based on *a priori* knowledge as they are often degraded through pre-processing of ingredients and high extrusion temperatures for feed production. This is supported by Levic & Sredanovic (2010) study which suggested that 85 – 100% of trypsin inhibitor activity can be reduced through cooking under pressure at 121^{o}C (>15 mins) and 78 – 98% by extrusion at 145 ^{o}C (>16 s). Extrusion temperature in the present study ranged from 110 – 130 ^{o}C (Section 2.2.6). Phytic acid content of plant-based ingredients were determined from reference values provided by Royal DSM (formerly DSM Nutritional Products Inc.), France (Verhlac, personal communication)(Section 2.2.4.7). Dietary phytate-P levels were then calculated for base formulations.

Arabinoxylan was quantified indirectly from the D-xylose content of plant-based ingredients using spectrometry (MEGAZYME® K-XYLOSE 01/12). Samples were all pre-processed (as meals) and so did not require grinding. 100 mg of each sample was transferred to Corning screw-cap culture tubes (16 x 125 mm) to which 5 ml of HCl (1.3 M) was added then capped. Tubes were incubated at 100^{o}C for 1 hour and stirred intermittently. Tubes were then cooled at room temperature after which 5 ml of NaOH (1.3 M) was carefully added. The samples were quantitatively transferred to a 100 ml volumetric flask and diluted up to the mark with distilled water. The contents of the flask were then mixed thoroughly by inversion and an aliquot of the solution centrifuged at 1,500 rpm for 10 minutes. A sample of 0.1 ml was then used for the assay. Arabinoxylan was calculated according to the following series of equations using MEGAZYME's preprogrammed excel solver. When analysing solid samples, D-xylose content (g 100g^{-1}) is calculated from the amount weighed (Megazyme 2012).

Xylose concentration (c) = $[(V \times MW)/ (\varepsilon \times d \times v)] \times \Delta_{AD\text{-}xylose}$ (g L^{-1}), where:

V	Is the final volume (ml)
MW	Molecular weight of D-xylose (g mol^{-1})
ε	Is the extinction coefficient of NADH at 340 nm = 6,300 (L x mol^{-1} x cm^{-1})
d	Is the light path (cm)
v	Is the sample volume (ml)

It follows therefore for D-xylose

$$c = [(2.97 \times 150.1)/ (6{,}300 \times 1.0 \times 0.1)] \times \Delta_{AD\text{-}xylose} \text{ (g L}^{-1}\text{)}$$
$$= 0.7076 \times \Delta_{AD\text{-}xylose} \text{ (g L}^{-1}\text{)}$$

Content of D-xylose (g $100g^{-1}$) = [$c_{D\text{-}xylose}$ (g L^{-1} sample solution)/weight of the sample (g L^{-1} sample solution)] x 100

Arabinoxylan content (g $100g^{-1}$) = Content of D-xylose (g $100g^{-1}$) x (100/D-xylose content of the polymer)

2.2.4 Ingredients and Proximate Compositions

2.2.4.1 Protein Sources

FM (56% CP) was sourced locally in Thailand and is a by-product of tuna fisheries processing. SBM (solvent extracted, 40 – 45% CP) is the most suitable potential replacer for FM and was chosen as the major plant protein supplement. Apparent protein digestibility for FM and SBM in tilapia are 86 – 90% and 87 – 94% respectively (NRC 2011).

2.2.4.2 Carbohydrate Sources

The carbohydrate sources (RB, CM, and MA; < 35% CP) were also sourced locally and added to the formulation for energy, protein-sparing, pelletability and bulking. RB was used as a low-cost filler, CM as a natural binder, and MA for energy and protein sparing. Apparent carbohydrate digestibility for corn in tilapia is 45 – 58% (NRC 2011). No information was available for RB and CM.

2.2.4.3 Lipid Sources

Lipid was added to the diet in the form of palm oil to provide essential fatty acids and energy. 1 ml of the product delivered 0.06g of Linolenic acid (18:3n-3), 0.53g of Linoleic acid (18:2n-6) and 0.23g of Oleic acid (18:1n-9). For the formulation matrix, each ml delivered 33.4 kJ (8.0 kcal). The

use of saturated palm oil is thought to reduce oxidative stress and associated pathological conditions in tilapia. Furthermore, it is suggested that tilapia can synthesize important long chain polyunsaturated fatty acids (LC-PUFA), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), from linolenic acid (18:3n-3) and linoleic acid (18:2n-6) (Tocher et al. 2002). Therefore feeding of diets containing palm oil prior to harvest can potentially add beneficial n-3 PUFA, tocopherols and tocotrienols to tilapia flesh which are highly beneficial to consumers (Ng & Chong 2004; Kapateh 2009).

2.2.4.4 Micro-Ingredients

Vitamins and minerals were added as a complete concentrated premix (ADVANCE Vitapond, Thailand; Table 2.2.3). For semi-intensive (green-water) systems, however, premixes are generally not required to supply all the essential micro-nutrients (NRC 2011).

Table 2.2.3 Vitamin and mineral mix content per 1000 g diet

Vitamins	Content	Minerals	Content
A (Retinol)	36,000 IU	Mn (Manganese)	105 mg
D_3 (Cholecalciferol)	9,000 IU	Cu (Copper)	9 mg
E (Tocopherol)	187 mg	Fe (Iron)	90 mg
K_3 (Menadione)	19 mg	Zn (Zinc)	90 mg
B_1 (Thiamine)	52 mg	I (Iodine)	1.8 mg
B_2 (Riboflavin)	97 mg	Co (Cobalt)	450 mcg
B_6 (Pyridoxine)	46 mg	Mg (Magnesium)	1,900 mg
B_{12}	60 mcg	Se (Selenium)	150 mcg
C (Ascorbic acid; coated)	69,800 mg activity	Na (Sodium)	117 mg
Niacin	130 mg	K (Potassium)	3,600 mg
Pantothenic acid	93 mg	Ca (Calcium)	219 mg
Folic acid	10 mg		
Inositol	225 mg		
Biotin	450 mcg		
Choline	500 mg		

Diets were supplemented with di-calcium phosphate, however, there was no information on its P-availability in tilapia (NRC 2011). Availability in other species (trout, sea bass and yellowtail) ranges from 52 – 71%. The product was assumed to deliver 24% Ca and 18% P.

2.2.4.5 Enzymes

Ronozyme NP® (phytase), Ronozyme WX® (xylanase) and Ronozyme ProAct® (protease) (Table 2.2.4) are commercial enzymes produced by Novozyme A/S and marketed by Royal DSM globally. They are recommended for use as feed additives for broilers, swine and fish (tilapia, salmon, trout and shrimp). The use of such enzymes as feed additives places certain demands on the products, particularly their thermostability and optimum pH ranges (Makhode 2008). Some loss of enzyme activity is inevitable, however, it is thought that in most cases, the enzymes (especially in granular form) are sufficiently protected by organic and inorganic material in the feed for adequate amounts of activity to remain (Chesson 1993). Additionally, enzyme thermostability has improved through sophisticated coating systems which makes them better able to withstand the heat, moisture and friction during feed processing (Brufau 2006). Nevertheless, the liquid form of the enzymes were chosen for the study and used post-extrusion to ensure maximum recovery levels were achieved.

Table 2.2.4 Enzyme properties and specification (Royal DSM)

Prod. Name	IUB No.	Enzyme	Origin	Substrate Specificity	Activity (min)	Recovery
ProAct	3.4.21	Protease (serine)	*Bacillus licheniformis*	Proteins	75,000 PROT/g[a]	> 80%
WX	3.2.1.8	Endo-1, 4-β-xylanase	*Thermomyces lanuginosus*	Arabinoxylans and xylans	650 FXU/ml[b]	> 90%
NP	3.1.3.26	6-Phytase	*Peniophora lycii*	Phytate-P	20,000 FYT/g[c]	> 90%

[a] PROT refers to one protease unit and is defined as the amount of enzyme that releases 1 mmol of p-nitroaniline from 1mM substrate (Suc-Ala-Ala-Pro-Phe-pNA) per minute at pH 9 and 37 ºC (Fru-Nji et al. 2011).
[b] XU refers to one xyla)nase unit and is defined as the amount of enzyme required to liberate 1 µmol xylose per minute at pH 6 and assay temperature (Sunna et al. 2000). Alternatively it is defined as the amount of enzyme that releases 1 µmol of reducing moieties from 1.5% arabinoxylan substrate solution per minute at pH 5.0 and 40 ºC (Ruckebusch & Glitsoe 2013). XU = FXU
[c] Phytase activity is expressed as FYT for this product and is defined as the quantity of enzyme that liberates 1 mmol of inorganic-P per minute from 0.0015 mol/l sodium phytate at pH 5 and 37 ºC (Kumar et al. 2012) FTY, FTU, U are considered similar.
IUB – International Union of Biochemistry

Ronozyme NP® is a fungal derived phytase extracted from *Peniophora lyci* and transferred to *Aspergillus oryzae* by gene technology. The enzyme is produced on a large scale through fermentation, filtration and granulation processes (Nielsen & Wenzel 2006). Ronozyme NP® is fairly stable under normal pelleting and extrusion temperatures with recovery rates of above 80% (Royal DSM 2010a). In the formulation matrix, Ronozyme NP is assigned a relative value of 0.12% available-P based previous internal studies. Ronozyme WX® is an endoxylanase derived from *Thermomyces lanuginosus* spp. and is used to hydrolyse NSPs, specifically arabinoxylans and xylans, in animal feeds. Ronozyme WX® has a broad spectrum activity against soluble and insoluble arabinoxylans irrespective of ingredient source. The product is highly thermostable with recovery percentage of above 80% (Royal DSM 2010c). Ronozyme WX was expected to deliver an additional 13.6 kcal or 56.85 kJ of energy per kg feed. Ronozyme ProAct® is an alkaline serine protease derived from *Nocardiopsis prasina* and the production strain *Bacillus licheniformis*. Pepsin stability experiments find that 97% of this protease remains intact and active after being exposed to pepsin for 1.5 hours, pH 3 and at 40ºC (Angel et al. 2010). It is thought to be effective across a wide range of peptide bonds and is therefore a non-specific protease. Its optimal pH range is 5 – 6, and continues into the area of alkalinity. This range in operating pH complements existing endogenous proteases such as pepsin and others that operate optimally in an acidic pH (Angel et al. 2010). ProAct® has a recovery rate of 90% or above (Royal DSM 2010b).

2.2.4.6 Proximate Composition of Ingredients

Major protein and carbohydrate sources were analysed according to Section 2.2.3.1 and nutrient profiles detailed in Table 2.2.5.

Table 2.2.5 Nutrient composition of macro-ingredients used for experimental diets

Composition (g kg^{-1} as fed basis)	FM	SBM	RB	CM	MA
Phase 1					
CP	568.9	401.6	161.6	26.9	84.6
Lipid (EE)	78.9	38.8	21.0	8.8	41.7
Carbohydrate	62.7	410.9	633.1	827.9	759.9
CF	2.4	47.4	75.5	33.5	17.6
NFE	60.3	363.5	557.7	794.5	744.5
Moisture	58.3	85.9	80.6	97.0	95.6
Ash	231.3	63.0	103.8	39.5	18.3
P	39.2	5.5	21.0	1.2	3.0
GE (MJ kg^{-1})	19.4	19.8	17.4	16.7	18.5
Phase 2					
CP	565.9	459.6	70.8	15.0	78.6
Lipid (EE)	82.7	5.7	29.0	0.4	38.7
Carbohydrate	58.4	397.6	716.1	849.0	778.2
CF	57.0	59.0	286.9	1.3	17.5
NFE	1.5	338.6	429.3	847.7	760.7
Moisture	55.4	74.1	57.4	128.7	89.9
Ash	237.7	63.2	126.8	7.0	14.8
P	36.6	5.0	4.1	1.2	4.3
GE (MJ kg^{-1})	18.3	19.8	16.9	16.2	18.3
Cost kg^{-1} (US$)	1.07	0.69	0.39	0.31	0.41

Phase 1 – Chapters 4 and 5; Phase 2 – Chapter 6

The amino acid content of the basal ingredients were derived from a recent study (Kaewmanee 2009) using similar ingredients at Nam Sai Farms due to resource constraints. These values were benchmarked against NRC (2011) and Khempaka et al. (2009) (Table 2.2.6).

Table 2.2.6: Amino acid profile of major raw ingredients

Amino Acids (%)*	FM[a]	SBM[a]	RB[a]	CM[b]	MA[a]
EAA					
Arginine	4.74 (3.43)	4.16 (3.23)	1.22 (1.00)	0.08 (0.07)	0.51(0.40)
Histidine	2.76 (1.75)	1.66 (1.17)	0.19 (0.34)	0.03 (0.02)	0.30 (0.25)
Isoleucine	2.52 (2.45)	2.12 (1.99)	0.46 (0.44)	0.07 (0.07)	0.31 (0.29)
Leucine	4.81 (3.79)	3.66 (3.42)	0.94 (0.92)	0.10 (0.11)	1.00 (1.00)
Lysine	2.58 (4.22)	1.90 (2.83)	0.59 (0.57)	0.07 (0.11)	0.24 (0.26)
Methionine	2.14 (1.47)	0.70 (0.61)	0.24 (0.26)	0.02 (0.02)	0.18 (0.18)
Phenylalanine	4.05 (2.15)	3.69 (2.18)	0.67 (0.56)	0.08 (0.06)	0.47 (0.42)
Threonine	4.37 (2.31)	2.81 (1.73)	0.40 (0.48)	0.09 (0.08)	0.44 (0.30)
Tryptophan	0.64 (0.57)	0.70 (0.61)	0.16 (0.14)	0.02 (NI)	0.10 (0.07)
Valine	4.35 (2.77)	3.29 (2.40)	1.07 (0.68)	0.13 (0.09)	0.65 (0.42)
NEAA					
Cysteine	0.01(0.47)	0.02 (0.07)	0.57 (0.27)	0.07 (NI)	0.20 (0.18)
Tyrosine	3.51 (1.69)	2.68 (1.69)	0.00 (0.40)	0.00 (0.05)	0.41 (NI)
Glycine	3.22 (NI)	1.49 (NI)	0.39 (NI)	0.05 (0.08)	0.42 (NI)
Aspartic Acid	2.29 (NI)	3.28 (NI)	0.98 (NI)	0.11 (0.13)	0.47 (NI)
Serine	2.38 (NI)	2.25 (NI)	0.60 (NI)	0.07 (0.10)	0.40 (NI)
Glutamic acid	3.07 (NI)	5.03 (NI)	1.37 (NI)	0.18 (0.16)	1.19 (NI)
Alanine	3.06 (NI)	1.65 (NI)	0.76 (NI)	0.13 (0.14)	0.57 (NI)
Proline	4.28 (NI)	3.28 (NI)	0.77 (NI)	0.13 (0.10)	0.79 (NI)

[a] NRC (2011) reference values in parenthesis;
[b] Khempaka et al., (2009) reference values in parenthesis
*As a percentage of the ingredient. NI – No information

2.2.4.7 Anti-nutrient Composition

Phytate-P levels in plant-based ingredients were calculated from analysed phosphorus (Total-P) levels in Table 2.2.5 and the reference values of % Phytate-P in Table 2.2.7. Arabinoxylan levels were calculated from D-xylose concentrations (Section 2.2.2.2).

Table 2.2.7 Phytate and arabinoxylan content of plant-based ingredients

Ingredients	Total-P[1] (%)	Phytate-P as % of Total-P[2]	Phytate-P (%)	Phytate[3] (%)	Arabinoxylan (g kg^{-1})	D-xylose as % of Arabinoxylan[4]
CM	0.12	25	0.03	0.11	0.07	65.9
RB	2.10	72	1.51	5.66	1.17	74.7
MA	0.30	73	0.22	0.83	0.63	59.3
SBM	0.55	59	0.32	1.20	0.56	40.0

[1]Proximate analysis
[2]Based on reference values for phytate-P as a percentage of Total P (Royal DSM and Ravindran et al. 1994)
[3]Phytate = Phytate-P x 3.75 (Kumar et al. 2012)
[4]Based on reference values for D-xylose content as a percentage of arabinoxylan (McDonald et al., 2002; Ngoc et al., 2012)

2.2.5 Diet Formulation

2.2.5.1 Phase One – Chapter 4 and 5

Feed formulation was carried out using a pre-programmed excel-solver designed by Kasetsart University (Kaewmanee 2009). Four basal formulations containing 0%, 3%, 5% and 10% FM were used for Phase 1 experiments (Table 2.2.8). The diets were formulated to be isonitrogenous (25% CP), isoenergetic (18 kJ^{-1}) and contained marginal available phosphorus (0.4%). Essential AA (lysine and methionine) and di-calcium phosphate were supplemented according to tilapia's nutrient requirements (NRC 2011). Chromic oxide (Carlo Erba Reagents SpA, France) was added to the basal formulations as the indigestible marker for the digestibility experiment. The diets were coated post-extrusion with liquid enzymes (Ronozyme® phytase and xylanase; Royal DSM) to form the enzyme supplemented treatments (Table 2.2.9).

Table 2.2.8: Formulation and proximate composition of basal diets for Phase 1 experiments

	0% FM	3% FM	5% FM	10% FM
Ingredients (g kg^{-1})				
SBM (CP 40%)	497.5	455.0	425.0	355.0
MA	102.5	115.0	125.0	150.0
Tuna FM (CP 56%)	0.0	30.0	50.0	100.0
CM	150.0	150.0	150.0	150.0
Fine RB	200.0	200.0	200.0	200.0
Vegetable oil	30.0	30.0	30.0	30.0
Vitamin/Mineral Mix	10.0	10.0	10.0	10.0
Dicalcium phosphate	5.0	5.0	5.0	5.0
Lysine	5.0	5.0	5.0	5.0
Methionine	1.0	1.0	1.0	1.0
Chemical composition (g kg^{-1} *as fed basis*)				
DM	945	938	946	924
CP	258	253	276	252
Lipid (Ether Extract)	44	35	26	29
CF	39	39	40	33
Ash	73	79	81	83
Total-P	8.2	8.7	9.0	10.9
GE (MJ kg^{-1})	20.8	19.7	19.9	20.0
Anti-nutrients (g kg^{-1})				
Phytate-P	4.9	4.8	4.7	4.5
Arabinoxylan	0.59	0.57	0.56	0.53

Table 2.2.9: Enzyme supplementation of experimental diets and treatment codes (Phase 1)

Experimental Diets	C0FM	E0FM	C3FM	E3FM	C5FM	E5FM	C10FM
FM levels (%)	0	0	3	3	5	5	10
Enzyme Inclusion (g kg^{-1})							
Phytase[1]	-	0.075	-	0.075	-	0.075	-
Xylanase[2]	-	0.385	-	0.385	-	0.385	-

C – Control; E – Enzyme supplemented; [1] Ronozyme NP (L); [2] Ronozyme WX (L)

2.2.5.2 Phase Two – Chapter 6

Two basal formulations containing 2% and 10% FM were designed for Phase 2 experiments (Table 2.2.10).The diets were formulated to be isonitrogenous (28% CP) and isoenergetic (18 kJ^{-1}). They were fortified with feed-grade lysine, methionine, dicalcium phosphate and coated post-extrusion with liquid enzymes (Ronozyme® protease, phytase and xylanase; Royal DSM) to form the enzyme supplemented treatments (Table 2.2.11)

Table 2.2.10: Formulation and proximate composition of basal diets for Phase 2 experiments

	2% FM	10% FM
Ingredient composition (g kg^{-1})		
SBM (CP 45%)	499.0	370.0
MA	82.9	127.5
Tuna FM (CP 56%)	20.0	100.0
CM	150.0	150.0
Fine RB	200.0	200.0
Vegetable oil	20.0	30.0
Dicalcium phosphate	10.0	5.0
Vitamin premix	10.0	10.0
Lysine	5.4	5.6
Methionine	2.7	1.9
Proximate Composition (g kg^{-1} *as fed basis*)		
DM	952	957
CP	280	292
Lipid (EE)	20	33
CF	106	105
Ash	73	79
Total-P	8.0	13.0
GE (MJ kg^{-1})	18.7	19.2
Anti-nutrients (g kg^{-1})		
Phytate-P	2.4	2.1
Arabinoxylan	0.58	0.53

Table 2.2.11: Enzyme supplementation of experimental diets and treatment codes (Phase 2)

Experimental Diets	NC	NOPRO	LOPRO	HIPRO	PC
FM levels (%)	2	2	2	2	10
Enzyme Inclusion (g kg^{-1})					
Phytase[1]	-	0.075	0.075	0.075	-
Xylanase[2]	-	0.385	0.385	0.385	-
Protease[3]	-	-	0.200	0.400	-

NC – Negative control, NOPRO – No protease inclusion; LOPRO – Low protease inclusion; HIPRO – High protease inclusion. PC – Positive control; [1] Ronozyme NP (L); [2] Ronozyme WX (L); [3] Ronozyme ProAct (L)

2.2.6 Feed Production and Storage

Dry ingredients were weighed manually and mixed on-farm using a floor loading industrial mixer (max. capacity 500 kg; Pakthongchai Pasusat, Thailand) for ten minutes (Figure 2.2.1). Micro-ingredients (premix, Cr_2O_3) were added during mixing to improve homogeneity. They were bagged (by diet) and transported to Kasetsart University (AquaFeed Department) for processing and extrusion. Each diet (25 kg batch) was re-mixed and homogenized using water and palm oil (Hobart Mixer; max. capacity 50 kg). Homogenized mixtures were then transferred to a floating feed extruder for pelleting at 110 – 130^{o}C (Pakthongchai Pasusat; 250 kg hr^{-1}). The pellets (2mm; moisture level 30 – 35%) were sun dried for 3 – 5 hours then re-dried using a forced-air oven for an additional hour to reduce the moisture levels to < 8%. Feeds were transferred back to farm and stored in large plastic containers in feed warehouse under farm conditions. Enzyme supplemented diets were stored in large plastic bags at -4^{o}C after coating each week until fed.

Figure 2.2.1: Feed production (mixing, pelleting and drying). A. Major raw feedstuff – CM, RB, FM, SBM and MA (left to right). B. On-farm industrial mixer (Nam Sai Farm). C. Homogenized feed mix (Kasetsart University). D. Floating feed extruder E. Extruded pellets F. Sun drying of pellets

2.2.7 Post Extrusion Coating and Enzyme Recovery

The enzyme treatments were prepared by coating the pellets by hand weekly due to on-farm space constraints (See Section 8.6). The liquid enzymes were diluted in 200 ml of distilled water (carrier) and sprayed onto the pellets (5 kg batch) as they rotated in a small industrial mixer (Belle

Mini 150) to improve homogeneity. Pellets were then top-coated with a layer of oil (50 ml palm oil) to seal the enzymes inside, air dried indoor for a day and stored at 4^{o}C until fed. Analytical support for enzyme recovery was provided by Royal DSM Thailand through an independent laboratory in Germany (Biopract, GmbH). Post-coating, each feed type was tested separately for each of the enzymes added. Enzyme activity in 300 g of experimental diet was analysed per 5 ml of each enzyme.

2.2.8 Experimental Facility and Systems

Growth and digestibility experiments were conducted at Nam Sai Farms (Figure 2.2.2) located in Ban Sang, Prachinburi, approximately 90 km due east of Bangkok, Thailand. The systems for Phase 1 comprised twenty four (24) circular plastic tanks (1.22 m x 1.01 m x 0.80 m[9]; 800 L max. volume; Figure 2.2.3 A) and twenty eight (28) 5m^2 mesh[10] hapas (2.80m x 1.80m x 0.90m; Figure 2.2.3 B) erected within an un-fertilized green-water pond (Pond 10; Figure 2.2.2) using bamboo frames (hapa-in-pond system). The systems for Phase 2 comprised fifteen (15) circular plastic tanks (as above) and thirty (30) 1m^3 nylon cages (1m x 1m x 1m; Figure 2.2.3 C) erected within an un-fertilized green-water pond (Pond 9; Figure 2.2.2) using bamboo frames (cage-in-pond system). The tank system was installed in a small warehouse (Feed Store, Figure 2.2.2), filled with filtered pond water and allowed to sit for a week before stocking. The tanks were aerated to maintain optimal DO levels (>5mg L^{-1}). Once stocked, water lost through evaporation, husbandry activities and siphoning was replenished. Water exchange (WE) was done manually and varied between 15 – 50% per week (both experimental phases). Ponds were aerated using an electrical blower between 4 pm and 8 am to maintain adequate system DO. Water quality was monitored weekly (Section 2.2.11) and systems were exposed to natural photoperiod (~ 12hrs light: dark).

[9] surface diameter x base diameter x depth
[10] 4mm raschel PE

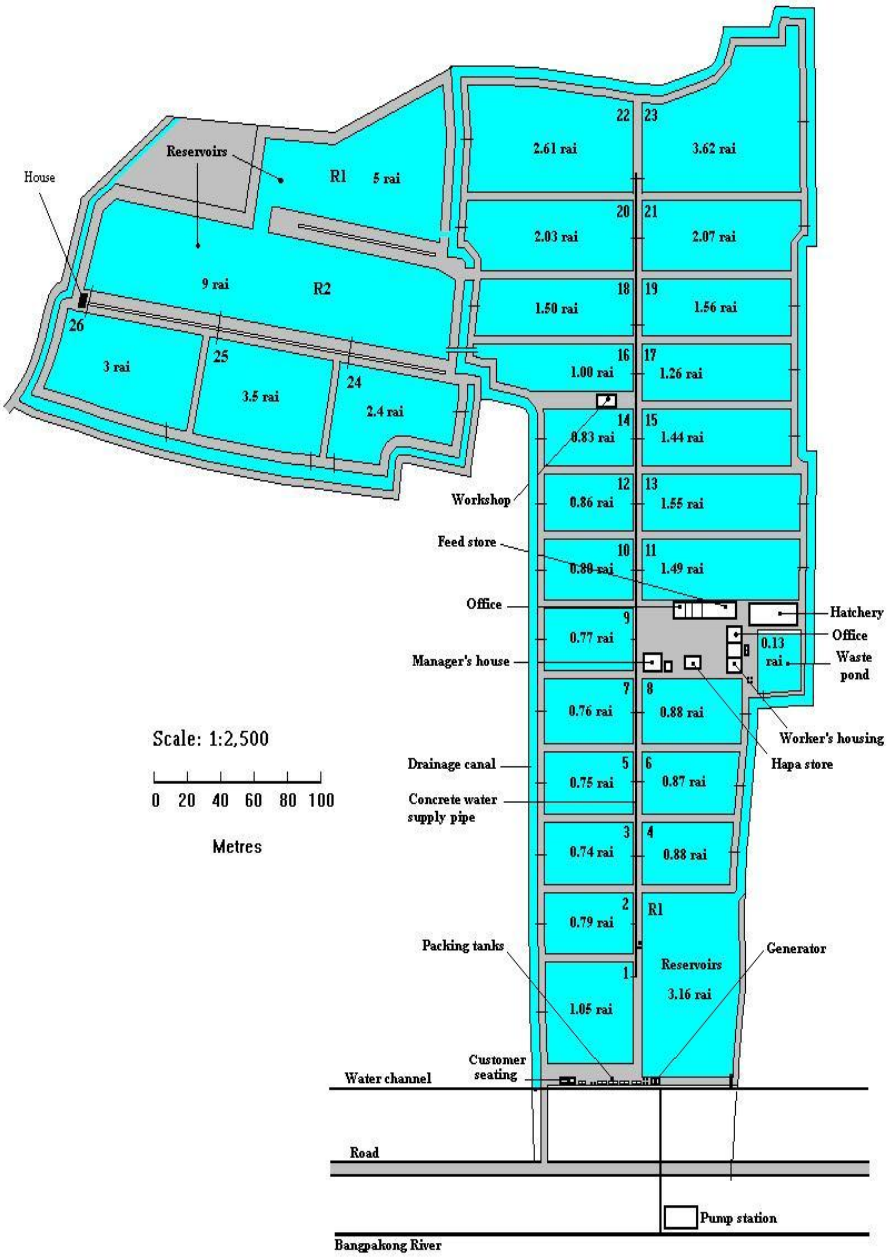


Figure 2.2.2: Layout of ponds (Nam Sai Farms, Thailand)

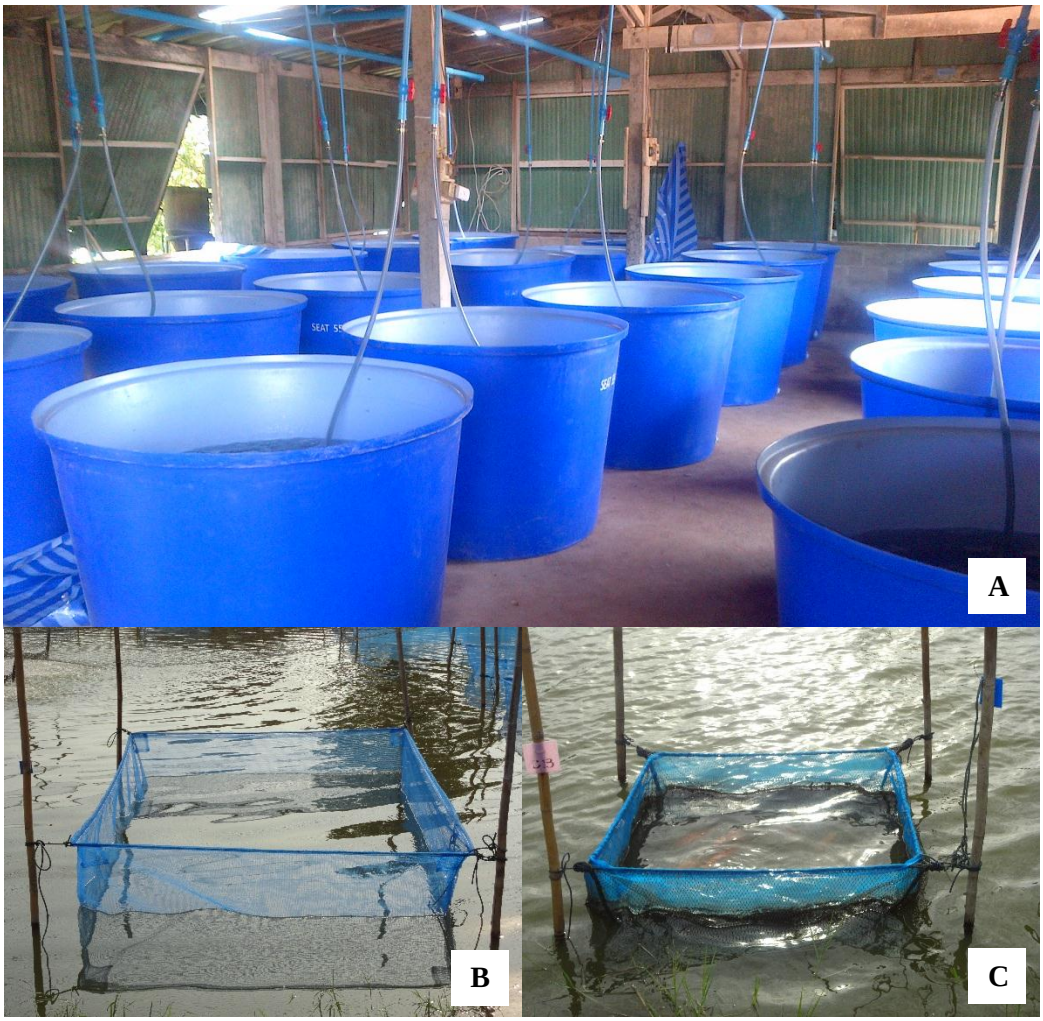


Figure 2.2.3: Experimental units: tank (A) and pond: Phase 1 hapas (B) and Phase 2 cages (C)

2.2.9 Experimental Fish and Handling

The tilapia (*Oreochromis niloticus x Oreochromis mossambicus)* stocks were provided by Nam Sai Farms. Fish were weighed (UWE Series counter-top scale) and measured (measuring board) individually prior to and during each trial. Prior to handling, they were anesthetized (1:10 solution of clove oil and 95% ethanol; dilution factor 2 ml per 5 L of water) and dried gently to remove excess water (Figure 2.2.4). Clove oil (4-allyl-2-methoxyphenol) is an effective natural plant anaesthetic and is believed to have antifungal properties (Hoskonen et al. 2013). After the initial grading process, fish were randomly distributed to experimental units and allowed to acclimate for one week prior to start

of each experiment. They were fed a commercial diet (30% CP) for that period and reweighed prior to feeding of experimental diets. 10 fish from the starter-population were randomly selected and sacrificed for carcass composition, intestine and liver samples. Tissue samples (gut and liver) were fixed in 10% formalin for histology (Section 2.2.12). Liver samples were also used for hepatosomatic evaluation. At the end of each trial, 5 – 16 fish per treatment were taken for proximate composition and tissue samples.

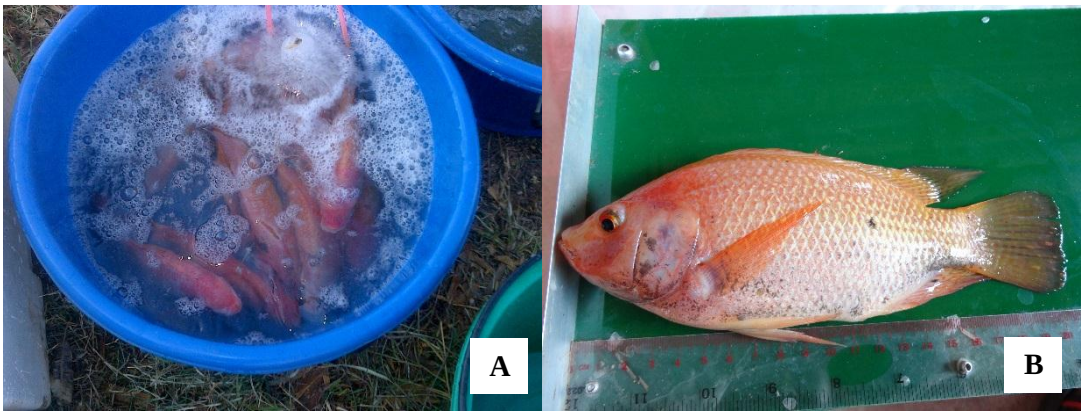


Figure 2.2.4: Sampling fish: Aerated anaesthetic bath (A) Measuring fish (B)

2.2.10 Water Quality Monitoring

Water quality parameters (Table 2.2.1) were monitored for each system weekly and recorded. Temperature, DO and pH were measured every other day using a YSI multi-parameter probe (556 MPS model, YSI Incorporated, USA). Ammonia, nitrite, nitrate and alkalinity were measured once weekly using photometry techniques. During Phase 1, this was done manually using SERA® (Hersteller, Germany) and SALINA® (Prima Tech Co., INTEQC, Thailand) water quality test kits, and during Phase 2 using a multiparameter bench photometer for aquaculture systems (Hach Instrument 83203).

2.2.11 Faecal Collection – Digestibility

In Phase 1, faecal material was collected from each tank two hour after the midday feeding daily, between weeks 2 and 12, using a fine hand held mesh (Figure 2.2.5). Faeces were pooled per tank and stored at -20^{o}C until analysed (Chapter 4). In Phase 2, faecal samples were collected daily at least one hour after the midday feeding between weeks 2 and 8. Samples were pooled by tank and stored at -14^{o}C until analysed (Chapter 6).

Figure 2.2.5: Collection of floating faeces using hand-held net

2.2.12 Histomorphology

Histology assessments of proximate intestine and liver samples (preserved in 10% formalin) were carried out to evaluate the changes in morphology due to dietary treatment effects. Small portions (~5 mm) of soft tissue samples were wrapped in biopsy tissue and soaked in tap water for 5 hours to remove residual formalin. Samples were dehydrated in series of graded methylated spirits, cleared using xylene then embedded using paraffin wax (Shandon Citadel 2000 Autoembedder). Samples were then transferred to a Leica Histoembedder for blocking. The solidified wax blocks (containing samples) were trimmed then soaked in distilled water for 30 minutes. Five micron (5µm) sections were trimmed (Microtome, Jung Biocut 2035 or Leica RM 2035), mounted and affixed to glass microscope slides using a warm water bath (Raymond A Lamb) and hot plate. Slides were then dried in an incubator (Windsor, Sandrest UK) at 60^{o}C for at least one hour prior to staining. Sample

slides were stained using haemotoxylin and eosin (H&E) and examined using a light microscope (Olympus BX51). 10 well oriented villi from five fish per treatment were measured and means calculated after Borgeson et al., (2006). Sections that were irregularly orientated or with partially disintegrated tips were omitted from the calculations. Imaging and measurements were done using AxioVision imaging software (Ref. 4.8; Carl Zeiss 2009, Germany)

2.2.13 Calculations

Condition Factor and Hepatosomatic Index

- Condition Factor = Body weight (g)/ body length (cm^3) x 100
- Hepatosomatic Index = Liver weight (g)/Body weight (g) x 100

Nutrient Digestibility (Burel et al. 2000)

- Apparent Digestibility Coefficient (ADC %) = 1 – ((% Cr_2O_{3diet}/ % $Cr_2O_{3faeces}$) x (Conc. or % $Nutrient_{faeces}$/Conc. or % $Nutrient_{diet}$))

Growth Matrices

- Arithmetic Mean (g or cm) = [∑x]/n, where x are individual weights and n is no. of fish. Applied to weight and length data.
- Average Weight Gain $_{per\ fish}$ (AWG g) = [Σ(W_f – W_i)(g)]/ no. of fish per experimental unit, where W_f is final body weight and W_i is initial body weight.
- Average Daily Gain (ADG g) = AWG (g)/experimental period in days
- Specific Growth Rate (SGR % day^{-1}) = 100 x (Log_e W_f (g) – Log_e W_i (g)/no. of days)
- Thermal Growth Coefficient (TGC) = 1000 x [($W_f^{1/3}$ – $W_i^{1/3}$)/ (Σ Temperature x exp. days)]
- Survival (%) = (Final no. of fish/Initial no. of fish) x 100

Feed Utilisation (as fed basis)

- Feed Intake $_{per\ fish}$ (FI g) = Feed consumption (g)/ no. of fish per experimental unit
- Feed Conversion Ratio (FCR) = FI (g)/AWG (g).

Protein Utilisation

- Protein Efficiency Ratio (PER) = AWG (g)/Protein intake (g).
- Apparent Net Protein Utilisation (ANPU %) = 100 × (final fish body protein (g) - initial fish body protein (g))/crude protein intake (g)

Nutrient Retention and Loading

- Nutrient retention efficiency NRE (%) = 100 x $[(W_f \times N_{final}) - (W_i \times N_{initial})]/(FI \times N)$,
  where N is nutrient *e.g.* phosphorus
- P Loading (P_e) = $(P_f \times FCR) - P_a$;
  where P_e is the environmental P load (kg tonne $fish^{-1}$), P_f is P concentration in the feed (kg tonne $feed^{-1}$) and P_a is the concentration of of P in the harvested fish (kg tonne $fish^{-1}$)

Energy Utilisation

- Energy Efficiency (%) = 100 x energy deposition (kJ)/ energy intake (kJ)

Economic Matrices (Kankainen et al. 2012; El-Sayed 1998)

- Conversion Cost ($\$ kg^{-1}$) = Feed cost per kg ($) x FCR
- Profit Index = Value of the fish stock/ cost of feed consumed
- Change in Unit Profit (Feed Efficiency) ΔU_{FE} ($\$ kg^{-1}$) = $- \Delta$ FCR x α^1P, where α^1 is the feed cost as a proportion of producer price in % and P is producers price in $\$ kg^{-1}$
- Change in Unit Profit (Growth) ΔU_G ($\$ kg^{-1}$) = $[1- (W_f/ ((W_f - W_i) \times (1 + \Delta G) + W_i))] \times P \times \alpha$, where α is the fingerling cost as a portion of the producers price in %, P is the producers price in $\$ kg^{-1}$ and ΔG is change in growth in %.

2.2.14 Statistical Analysis

Prior to analysis of variance and test of correlations, the data was assessed for normality, homogeneity of variance and independence of error to determine whether parametric or non-parametric analysis should be applied. Normality was assessed using Kolmogorov-Smirnov (KS) test and homogeneity of variances was performed using Levene's test. Data that did not meet the assumptions for parametric analysis were either normalized or kept as is. Analysis of Variance (ANOVA) was performed followed by *post hoc* analyses (Duncan Multi-Range Test; DMRT) when statistical differences were reported. For data that could not be normalized, Kruskal-Wallis (KW; H-test) non-parametric test was used followed by Mann-Whitney *post hoc*. Percentage data was transformed using arcsine square root. Statistical significance was reported at alpha level P = 0.05 or P = 0.01 where outcomes were highly significant. Statistics was performed using SPSS® version 19

and 21 (IBM 2013, 2015). Results are presented as means ± STD (standard deviation) or SEM (mean standard error) where appropriate.

2.3 **Life Cycle Analysis**

2.3.1 Goal and Scoping

An LCA model was designed to comparatively assess the environmental impacts of using low FM enzyme supplemented diets and commercial feeds associated with tilapia production in Thailand. The boundaries of the model included enzyme, feed and fish production, and the functional unit was one tonne of market-ready tilapia at farm gate. Mid-point impact categories considered were global warming potential, eutrophication potential, acidification potential and energy use. In some cases photochemical smog formation, ecotoxicity and agricultural land use were also considered.

2.3.2 Life Cycle Inventory and Data Collection

Baseline data for feed production in Thailand was collected via face-to-face interviews with manufacturers (Henriksson et al. 2014). This was supplemented with secondary data on relevant raw feedstuff and additives from research literature and online databases (*e.g.* Ecoinvent®). Enzyme production data was collected through personal communication (Novozyme A/S) and published research. Primary data for tilapia grow-out was taken from the present study's growth experiments.

2.3.3 Life Cycle Impact Assessment

Life cycle impact modelling was facilitated using CMLCA 5.2 software (University of Leiden, www.cmlca.eu) and CML database interfaces (*e.g.* EXCEL). See Chapter 7 for more details.

2.3.4 Analysis and Interpretation

Statistical analysis was done using sensitivity and uncertainty analyses (See Chapter 7, Section 7.2.4).

Chapter 3
Feed Management and Feeding Practices of Tilapia Farmers in Central Thailand: A Case Study

3.1 **Introduction**

Thailand contributes ~1.2 million tonnes to world aquaculture production (FAO 2014b). Thai aquaculture, dominated by freshwater culture, contributes ~2% to their national GDP (FAO 2012a; Thongrod 2007). In 2011, the industry consumed 810,000 and 600,000 tonnes of shrimp and fish feed respectively to produce 1,008,049 tonnes of fisheries product (FAO 2011; Aramsiriwat 2013). Nevertheless, Thai aquaculture also consumes other feed inputs such as cheaper agricultural by-products (ABP) in order to subsidize feed-related expenses (Bhujel 2013). Thailand's feed milling industry is one of the country's largest and fastest growing industries (Aramsiriwat 2013). It is the 13th largest globally, producing 16.9 million tonnes of animal feed in 2014 (Alltech 2015). The industry is supported by Thailand's diverse local feedstuffs, the major ones include RB, rice by-products, cassava, maize, soya beans, SBM, FM, sorghum, kapok meal and others (Havanont 1993; Aramsiriwat 2013). The livestock feed industry also utilises commercial feed additives such as enzymes, particularly phytase (Amornthewaphat 2009). Xylanase has seen experimental uses in monogastric and ruminant research in Thailand (Tapingkae et al. 2008; Phakachoed et al. 2012), however, there is no information regarding the use of proteases in animal feed. In fact, Tapingkae et al., (2008) pointed out that enzymes produced specifically to enhance the nutritive value of feed ingredients commonly used in Thailand requires more focus.

Aquafeed production, both shrimp and fish, represents a mere 5% of Thailand's total animal feed production annually (Roembke 2014). This may be due in part to the slow development of intensive aquaculture systems over the years. In fact, it was not until 1986 when shrimp culture began to evolve did the demand for commercial compound feeds and feed mills expand (Havanont 1993). Additionally, feeding practices have remained largely semi-intensive due to the diversity of cultured

species and markets (Bhujel 2013). Feeds and feeding practices also vary depending on farming system (Thongrod 2007). Pond (polyculture) production systems account for >80% of total industry output though cage monoculture has emerged as a recent trend (Bhujel 2013; Edwards et al. 2003; Lebel et al. 2013). Although polyculture is common, tilapia has persisted as the main freshwater finfish cultured since the early 1990s (Boonchuwong et al. 2007). Since then, tilapia production has increased exponentially and is now practiced throughout Thailand with specific clusters inland (central provinces) (Ferreira et al. 2014). This central region accounts for >50% of the volume and value of freshwater production (DOF 2012). It also has well established farming activities supported by good transportation networks to rural/urban markets and feed suppliers (Bhujel 2013).

To evaluate whether modern biotechnology, such as feed enzymes, will be effective within a fairly new market such as Thailand, a clear understanding was required of the physical, biological, socio-economic and institutional environments into which it is being proposed, and the capacity for integration into the existing system(s) (Dey et al. 2000). The objective of this case study was to assess the drivers influencing feed management and feeding practices of tilapia pond farmers in Thailand's central provinces by addressing the following research questions.

1. What are the current feeding practices and have they changed over the last 5 years? What are the underlying reasons for these changes and/or consistencies?
2. What are the linkages to management practices, feed availability and farming experience?

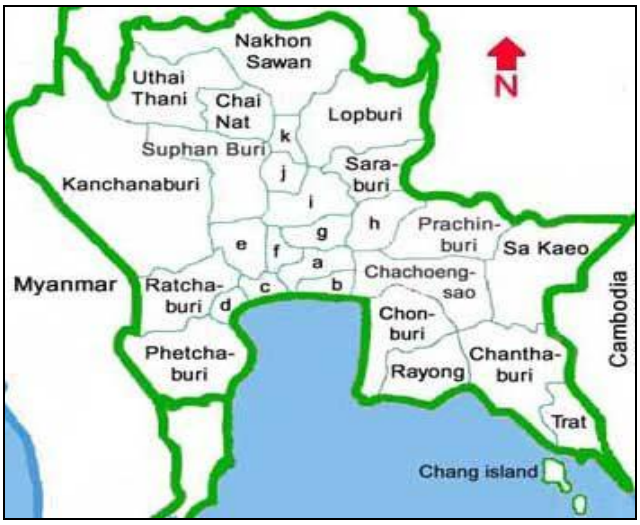
The hypotheses were:

1. Due to the saturation of the domestic tilapia market, feeding practices of tilapia farmers will involve greater usage of commercial feeds in order to meet local demand and requirements (*i.e*. standards) of potential export markets.
2. Due to access of cheaper ABP, small-scale farms will rely more on alternative feed inputs, limiting commercial feeds as supplemental inputs.
3. Feeding practices are linked to age, education levels, years of farming experience, resource access and location.

3.2 **Methods**

3.2.1 Study Area and Temporal Scope

The research involved quantitative and qualitative data derived from three surveys using mixed method approaches conducted over a period of five years (2009 – 2014) involving tilapia farmers in the central provinces of Thailand. The first involved an integrated farm survey (IFS) conducted by Sustaining Ethical Aquaculture Trade (SEAT) inter-disciplinary team between 2009 – 2012, followed by a second transition farm survey (TFS) May – June 2013 (secondary data)(Murray et al. 2013). The former evaluated general farm management practices while the latter evaluated the socio-economic and feed management changes in aquaculture farming in responses to key variables over two years (Section 3.2.2). Respondents were interviewed from four provinces, Chachoengsao, Nakhon Pathom, Suphanburi and Petchburi (Figure 3.2.1). The third survey involved a smaller group of tilapia farmers and was conducted between February – June 2014 and focused primarily on understanding the drivers of feeding practices and on-farm feed management (primary data; Section 3.2.3). Respondents were interviewed from Chachoengsao, Prachinburi, Nakhon Nayak and Chonburi.



Key:

a – Bangkok
b – Samut Prakan
c – Samut Sakhon
d – Samut Songkhram
e – Nakhon Pathom
f – Nonthaburi
g – Pathum Thani
h – Nakhon Nayok
i – Ayutthaya
j – Ang Thong
k – Singburi

Figure 3.2.1: Central Provinces of Thailand. *Source:* http://www.trekthailand.net/p3/

3.2.2 SEAT Surveys – General Farming Practices

Two surveys were conducted with tilapia farmers in central Thailand – a baseline integrated farm survey (IFS) and a transition farm survey (TFS). All SEAT surveys were based on a common sample frame derived through a multi-stage randomised cluster-based stratified process using aggregate farm number statistics to district level and DOF farm registration number thereafter (Murray et al. 2013). They were conducted using systematic telephone and qualitative face-to-face interviews with tilapia farmers identified through purposive and random sampling. Interviews were conducted using semi-structured questionnaires as guide (Appendix A). For the IFS, 199 farmers (177 pond and 22 cage farmers) were initially interviewed in 2009 – 2010 and 166 respondents indicated an interest in long-term follow-up surveys (TFS). 81 of 166 farmers which made up the TFS sample frame were interviewed, the remaining 85 did not participate for various reasons (discontinuation *etc*.). The TFS subgroup comprised 68 pond and 13 cage farms. The farms were stratified based on farm scale criteria given in Table 3.2.1. The quantitative information collected focused on differences in management, production and feeding practices across the four provinces. Furthermore, qualitative interviews of a smaller subgroup taken from Chacheongsao and Nahkon Pathom narrowed the focus areas based on specific feed management criteria (*e.g.* type of feed input *etc.*). In addition to farm scale, the data was also analysed based on feeding intensity: extensive, semi-intensive and intensive practices.

Table 3.2.1: Classification of tilapia systems in Thailand (SEAT)

No	AquaScale	1. Small	2. Medium	3. Large
1	Ownership of business (NOT land)	Household or extended family	Household or external/absentee owner	Corporate (i.e. joint stock company)
2	Full-time labour (non-family)	< 3	>3 and <15	>15
3	Management	Household or extended family	Household or salaried manager	Salaried manager
4	Total culture area (Ha)	<0.26 (1.625 rai)	>0.26	NA

3.2.3 Follow-up Feeding Practice Survey

Additional information on feeding practices was obtained through in-depth face-to-face interviews. 121 farmers were contacted and 20, expressing an interest to participate in the survey were visited and interviewed. 19 pond and 1 cage farmers were visited using Nam Sai customer list as the sample frame (due to limited time frame). Each interview lasted between 1 – 2 hours. This was a more purposive approach to the previous method though it potentially introduced certain biases *e.g.* geographic, seed type. Nevertheless, it allowed for the opportunity to communicate with random farmers. Detailed production information was collected for the last completed culture cycle using a semi-structured questionnaire (Appendix A). Additionally, a feed manufacturer was interviewed (based on availability) to purposefully provide an alternative perspective of the general state of the aquafeed industry and feed usage trends in Thailand.

3.2.4 Definition and Reclassification of Feed Categories

To reduce ambiguity of feed classes between SEAT IFS and TFS, and FFPS studies, the feed categories were reclassified to improve trend analysis and comparison between and within studies.

- An agricultural (ABP) or industrial (IBP) by-product is a single feedstuff, ingredient or industrial waste *e.g*. RB. This group also includes kitchen and slaughter house waste.
- On-farm feeds (OFF) are simple mixtures of two or more feedstuffs or ABPs produced on farm and may be further processed into a wet dough, moist feed, or extruded using a mincer with die and sun dried (Thongrod et al. 2004).
- Commercial feeds (CF) are industrial formulated, complete diets with balanced nutrient profile and generally composed from several feedstuffs. They are floating or sinking in form.
- Supplemental feeds are considered any feed inputs used in addition to fertilization.
- Natural food is natural pond productivity enhanced through fertilisation with either organic manure and/or inorganic fertilizers or a combination of both (Thongrod et al. 2004).
- Extensive farms utilise only natural food, semi-intensive farms use natural food with supplemental feed inputs while intensive farms use only commercial feed inputs.

3.2.5 Statistical Analysis

Data from interview transcripts were collated, coded and analysed initially using Microsoft® Access and Excel 2010. Results are presented using descriptive and inferential statistics (SPSS *v* 21, IBM). SEAT IFS data was used to describe farm profiles, farmer demography and farm systems while the SEAT TFS data was used to highlight significant changes in farming practices and feed management over two years. Both data sets were subjected to non-parametric test based on outcomes of K-S normality test and Levene's test for homogenous variance. Correlations between age, education and feed categories were determined using Spearman Rank Correlation. Due to the biased selection process, FFPS data was not subjected to rigorous data analysis and was used primarily to support the findings of the previous SEAT surveys.

3.3 **Results**

3.3.1 Integrated Farm Survey

3.3.1.1 Farm Profile

The demography of the SEAT IFS sample population are presented in Table 3.3.1 (n = 199 farms). Figure 3.3.1 illustrates the age class distribution of respondents within each of the province surveyed. 52.1%, 54.7%, 51% and 47.4% of respondents fell below the average age (~50 years) in Chachoengsao, Nahkon Pathom, Petchburi and Suphanburi respectively. Nahkon Pathom had the highest number of younger farmers. 66% of the total respondents were male and 83.5% owned/managed their farms. In addition to tilapia farming, respondents engaged in other business ventures which included agriculture and livestock, manufacturing, casual work, government and private sector jobs. However, only 30% of the respondents used aquaculture as their primary and sole income source.

Table 3.3.1: Profile of tilapia farms and respondents in Thailand's central provinces (SEAT)

Characteristics	SEAT IFS	Characteristics	SEAT IFS
No. of Respondents	199	**No of Provinces**	4
Average Age (Yrs.)	49.9 ± 12.3	**Avg. Aquaculture Experience** (Yrs.)	17.0 ± 9.2
Age (%)		**Experience** (%)	
≤ 30	5.0	≤ 5	5.1
31 – 39	13.6	6 – 10	23.6
40 – 49	33.2	11 – 15	25.6
50 – 59	22.1	16 – 20	16.4
≤ 60	26.1	≥ 21	29.2
Gender (%)		**Primary Income Sources (%)**	
Male	66	*Agriculture/Livestock*	38.9
Female	34	*Business/Manufacturing*	14.1
		Casual Labour	20.7
Education (%)		*Pub. Sector/Government*	5.6
Pre-Primary	0.5	*Private sector/Salaried*	6.6
Primary	60.3	*Other income*	8.1
Secondary	16.6	**Roles of respondents (%)**	
Intermediate	10.1	*Owner/Household Head*	83.5
Prof. Degree/Vocational	3.5	*Spouse or Child*	4.5
Higher Degree	7.0	*Other Relation*	3.5
Did not respond	2.0	*Did Not Respond*	8.5

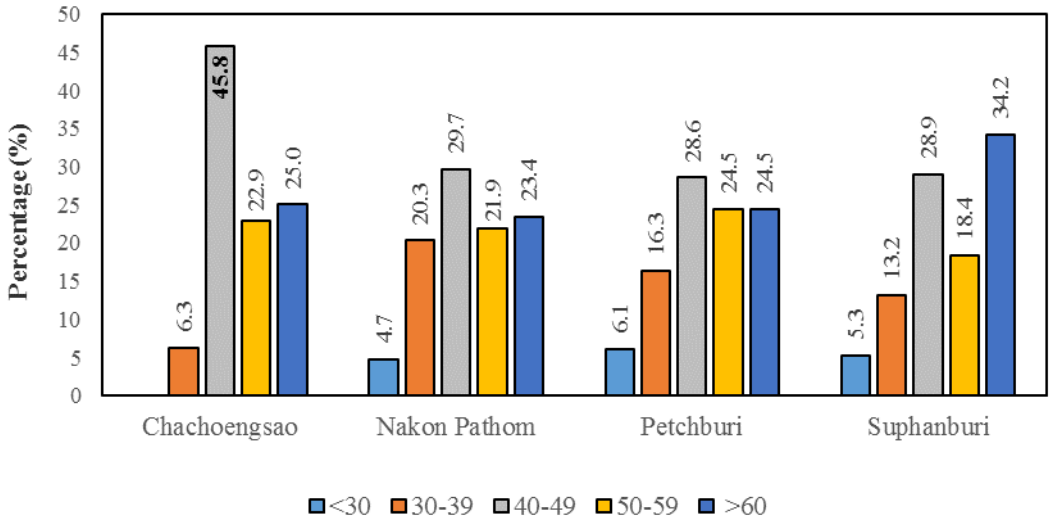


Figure 3.3.1 Age class distribution of respondents by province (SEAT IFS; n = 199)

Most respondents had modest level of education. Only 39.7% had secondary level education and above, however, 45.6% had over 16 years of aquaculture farming experience. There were more farmers in Nakhon Pathom and Suphanburi having professional/vocational and higher degrees, 14.1% and 18.4% respectively (Figure 3.3.2). Additionally, these two provinces had higher numbers of younger farmers (respondents) compared to Chachoengsao and Petchburi. Figures 3.3.3 and 3.3.4 highlight the age class and gender distribution in relation to education level within those four provinces. Higher degrees were more common among respondents 49 years and younger, as well as male farmers.

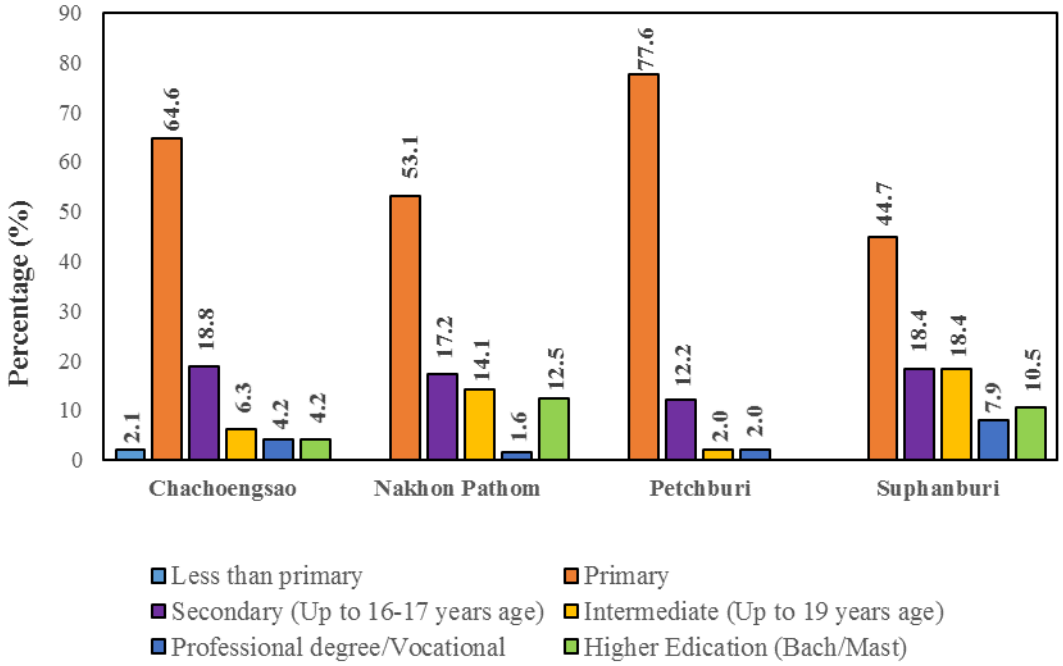


Figure 3.3.2: Distribution of respondents by province based on educational status (SEAT IFS n = 199)

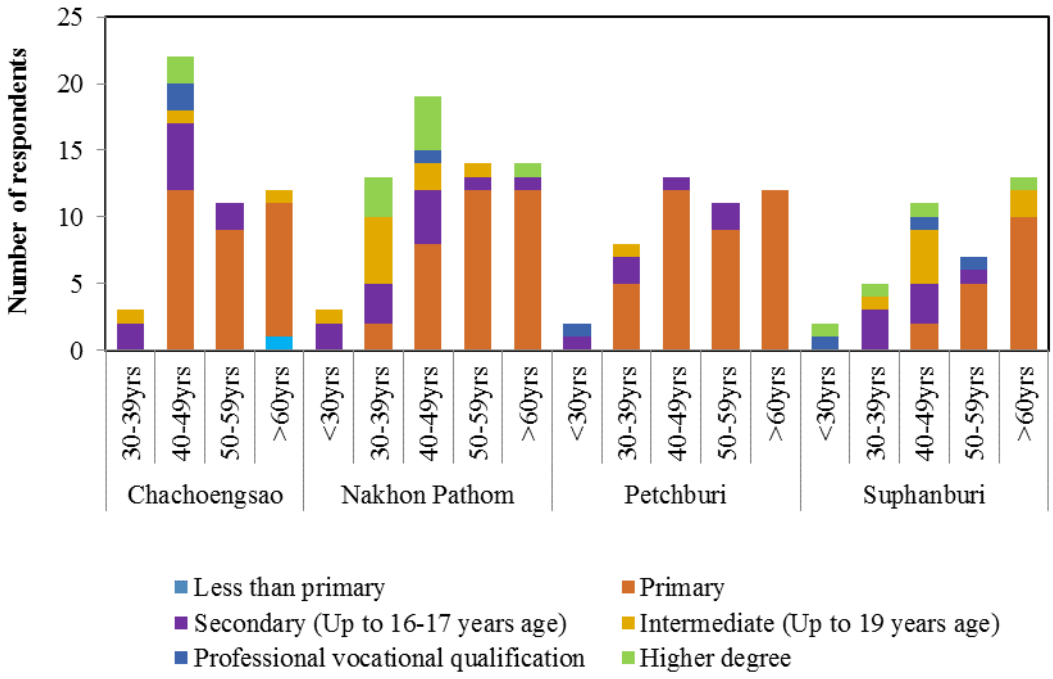


Figure 3.3.3: Education profile by age classes across four provinces (SEAT IFS n = 199)

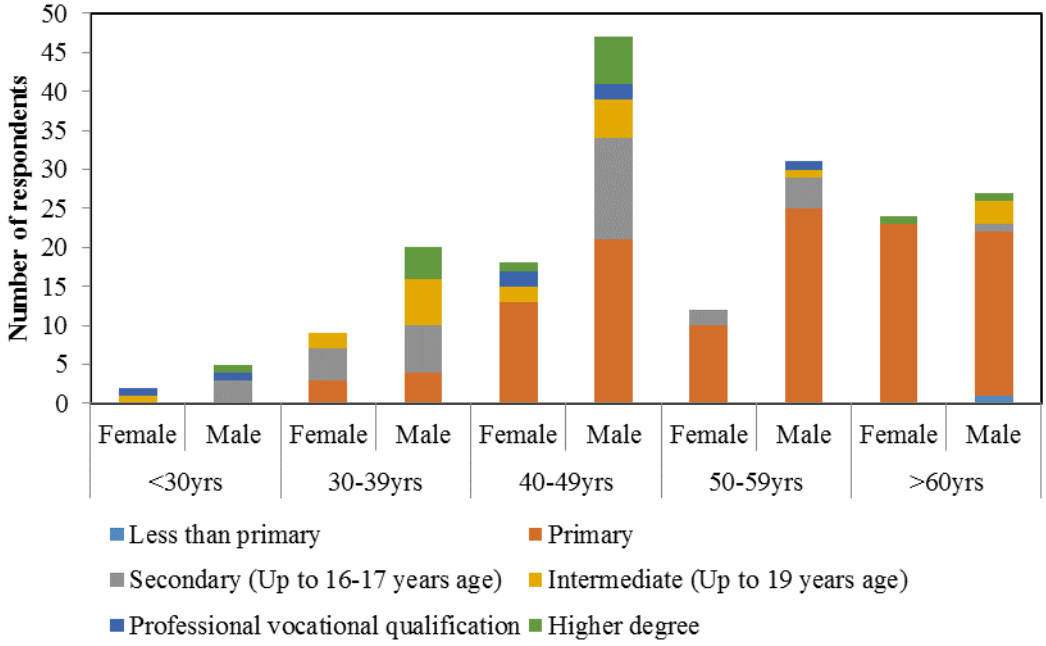


Figure 3.3.4: Gender distribution and education levels of respondents (SEAT IFS n = 199)

3.3.1.2 Production Systems

The characteristics of the SEAT IFS production systems are given in Table 3.3.2. The number of ponds per farm ranged from 1 – 18. Average pond size was 14.2 rai (2.3 ha). The number of cages per farm ranged from 1 – 76, averaging 232.5 m^2. 9.5% of the respondents had dedicated nurseries separate from grow-out ponds, a feature of multiphase system. Average number of cycles per year and culture period were 1.2 cycle and 8 months respectively. 78% of farms interviewed were small-scale producers. There was a relatively even distribution of respondents practicing monoculture (40.7%) and polyculture (59.3%). In fact, there was only one farm interviewed that practiced traditional extensive farming. Table 3.3.3 details the culture systems by production intensity. Nakhon Pathom had the highest number of semi-intensive polyculture farms while Suphanburi had the highest number of intensive monoculture farms (Figure 3.3.5).

Table 3.3.2: Characteristics of the SEAT production systems

Characteristics	**SEAT**	**Characteristics**	**SEAT**
No. of Respondents	199	**No of Provinces**	4
Avg. No. of Cycles (per year)	1.2 ± 0.5	**Avg. Culture Duration** (months)	8.0 ± 2.8
Avg. Pond No.	2.3 ± 2.1	**Farm scale** (%)	
Avg. Pond Size (rai)	14.1 ± 30.95	Small	78.9
Nursery	5.0	Medium	20.6
Grow-out	6.1	Large	0.5
Avg, Cage No.	17.0 ± 16.0	**Production Intensity** (%)	
Avg Cage Size (m^2)	232.5 ± 270.8	Extensive	0.5
Type of culture (%)		Semi-Intensive	79.9
Monoculture	40.7	Intensive	19.6
Polyculture	59.3	**Feed Input** (%)	
Seed Source (%)		ABP or IBP	15.6
Monosex	65.8	CF	65.9
Mixed sex	31.6	CF + ABP	13.3
Avg. Stocking Size (g)		OFF	1.2
Nursery	35.93 ± 30.5	No Feed Input	4.0
Grow-out	ND	**Yield per rai** (kg)	-
Avg. Stocking Density (rai)		**Mean Survival** (%)	61.5 ± 23.1
Nursery	3639	**Harvest Size** (g)	524 ± 257
Grow-out	ND	**Farm Gate Price** (THB kg^{-1})*	33.3 ± 22.5

ABP – agricultural by-product; IBP – industrial by-product; CF – commercial feed; OFF – on-farm feed; THB – Thai Baht; ND – No determined * Iced tilapia

Table 3.3.3 Culture type based on farm scale and feeding intensity (SEAT IFS n = 199)

Farm scale and intensity	Monoculture	Polyculture	Grand Total
Large	0	1	1
	(0.00%)	(0.50%)	(0.50%)
Semi-Intensive	0	1	1
	(0.00%)	(0.50%)	(0.50%)
Medium	**9**	**32**	**41**
	(4.52%)	**(16.08%)**	**(20.60%)**
Intensive	1	0	1
	(0.50%)	(0.00%)	(0.50%)
Semi-Intensive	8	32	40
	(4.02%)	(16.08%)	(20.10%)
Small	**72**	**85**	**157**
	(36.18%)	**(42.71%)**	**78.89%**
Extensive	0	1	1
	(0.00%)	(0.50%)	(0.50%)
Intensive	38	0	38
	(19.10%)	(0.00%)	(19.10%)
Semi-Intensive	34	84	118
	(17.09%)	(42.21%)	(59.30%)
Grand Total	**81**	**118**	**199**
	(40.70%)	**(59.30%)**	**100.00%**

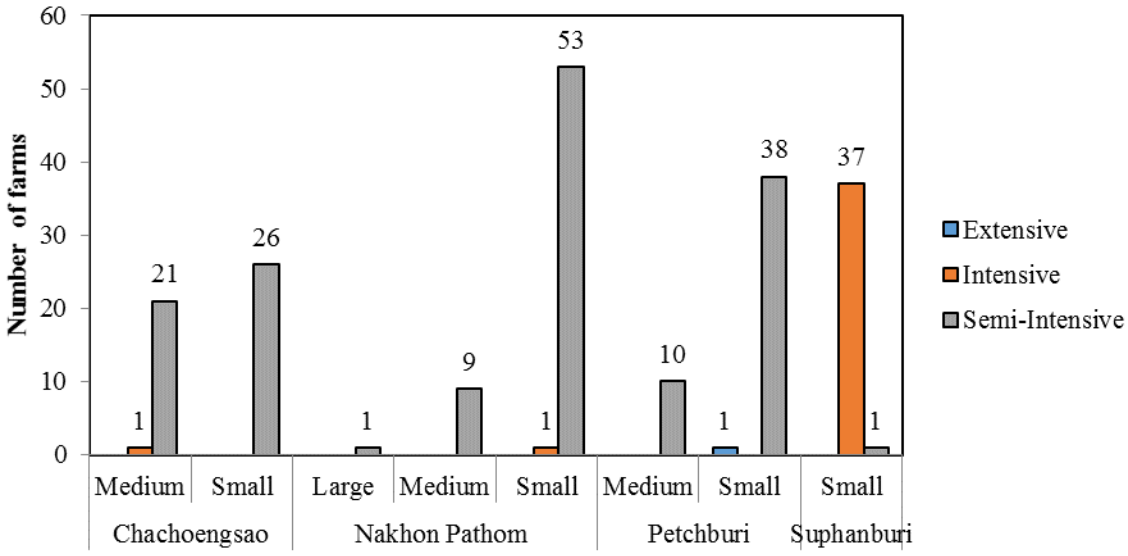


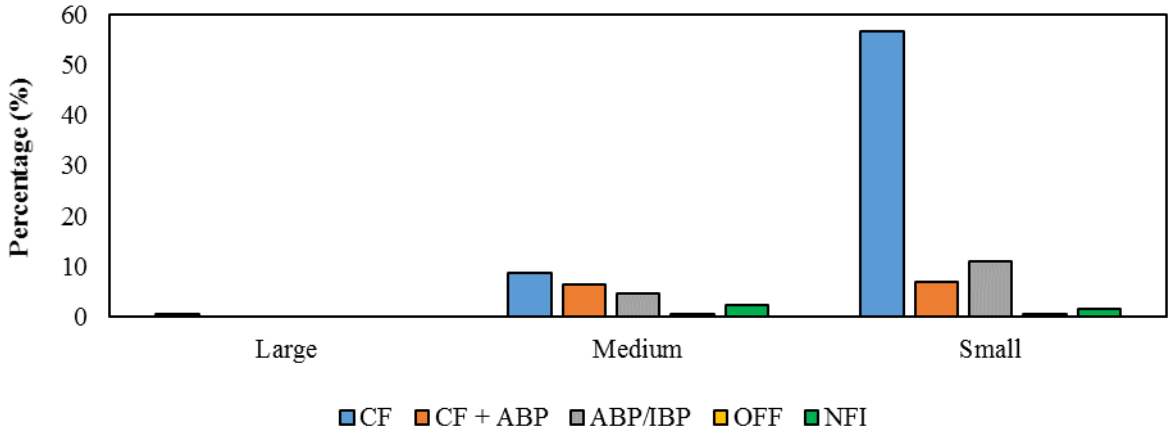
Figure 3.3.5: Farming intensity within provinces (SEAT IFS n = 199)

3.3.1.3 Feed Inputs and Management

The feed inputs included commercial feeds, APBs (RB and grass), industrial products/by-products (soybean milk, palm oil, fish sauce, fish silage and beef fat), slaughter house waste (trash fish, cow skin) and restaurant waste. Fertilisation, using organic and inorganic products, was common, however, only 27% of SEAT IFS respondents reported the type of fertilizers used. These products included organic livestock manures (poultry, swine, cattle *etc.*), effective microorganism (EM)[11], industrial/agricultural waste (*e.g.* rice straw, *ami-ami*) and inorganic products such as urea, ammonium sulphate, NPK and ammophos.

The feed inputs from the SEAT database were re-categorised based on the definitions given in Section 3.2.4. Fertilization was excluded because majority of the farms applied fertilizers. Figure 3.3.6 illustrates the feed input categories by farm scale. 56.6% and 8.6% of small and medium scale farms used commercial feeds respectively (n = 173). The single large farm interviewed also used commercial feed. Five types of commercial feeds were utilised with CP levels ranging from 12 – 35%, averaging 25.5 ± 8.5%. Feeds are produced in two forms, floating and sinking. Of the respondents that used commercial feeds, 80.7% bought floating pellet while 17.8% and 1.5% used sinking pellet or both respectively. Feeds were kept for mean storage time of 19.8 ± 20.9 days. 95% of SEAT IFS respondents hand fed from pond dykes or boats and/or used feeding stations. Only 1.2% of respondents reported the use of on-farm feed (OFF). 43% of the respondents fed fish *ad-lib* while 48% used crude estimates of biomass to determine feed quantity. 7% of respondents reported FCRs, average value for pond and cage farming were 1.36 ± 0.22 and 1.38 ± 0.14 (CV) respectively (Henriksson et al., 2014).

[11] EM is a commercial product consisting of a mixed culture of beneficial and naturally occurring microorganisms that can be applied as an inoculant to increase beneficial microbial diversity of the soil (Higa & Parr 1994)



Farm Scale	CF	CF + ABP	ABP/IBP	OFF	NFI
Large	0.58	0.00	0.00	0.00	0.00
Medium	8.67	6.36	4.62	0.58	2.31
Small	56.65	6.94	10.98	0.58	1.73
Total	**65.90**	**13.29**	**15.61**	**1.16**	**4.05**

Figure 3.3.6: Feed inputs based on farm scale (SEAT IFS n = 173)

Figures 3.3.7 and 3.3.8 illustrate the choice of feed inputs based on education level and geographic location respectively. As education level increased, so did the use of commercial feeds. Suphanburi (100%) and Nakon Pathom (93%) had the highest levels of commercial feed use.

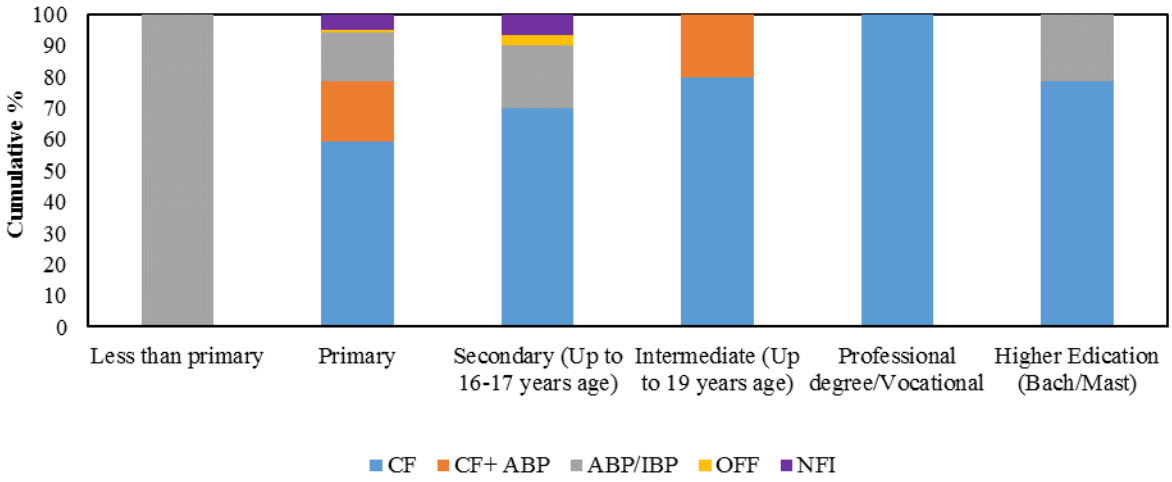


Figure 3.3.7: Feed inputs by education class (SEAT IFS n = 173)

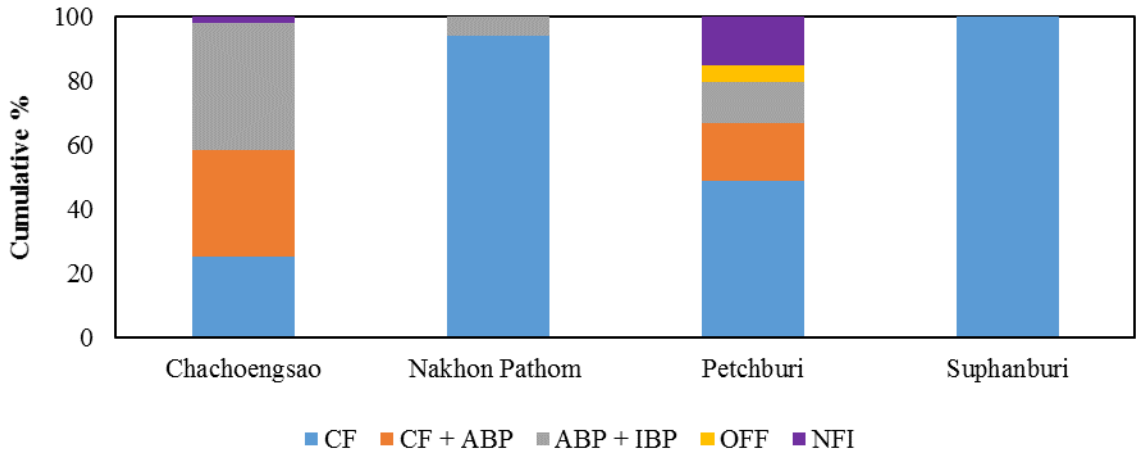


Figure 3.3.8: Feed inputs by provinces (SEAT IFS n = 173)

3.3.1.4 Market Outlets

Tilapia market size varied significantly depending on the target market. Mean harvest size was 524 ± 257g. Whole tilapia was sold in two forms, on ice or live through various market channels. 46% of respondents sold their products via wholesale spot markets while 39.7% sold directly to "middlemen" or agents. Other respondents used a combination of market outlets which included retail markets (farm gate, self retail or third party retail), and selling directly to feed providers. The average farm gate price for tilapia (iced) was 33.3 ± 22.5 THB (US$1.04 ± 0.71).

3.3.2 Transition Farm Survey

3.3.2.1 Farm Management Changes

The farm production data has been previously presented in Burana-osod (2013), and therefore only data related to changes in feed management relevant to this study will be presented here. Changes in general farm management practices were related to production, feed, land, chemical use, infrastructure, labour and water. The feed management changes included discontinuation of feed input, changes in feed source due to increased feed price, change in feed brand for better quality product, change in feed input due to system changes, required a different feed (commercial diet) due to poor animal growth and high FCR. Majority of the respondents, however, cited an increase in feed

prices as the main reason for change. Figure 3.3.9 highlights the changes in grow-out feed prices in Nakhon Pathom (SEAT TFS) as an example (Burana-osod 2013).

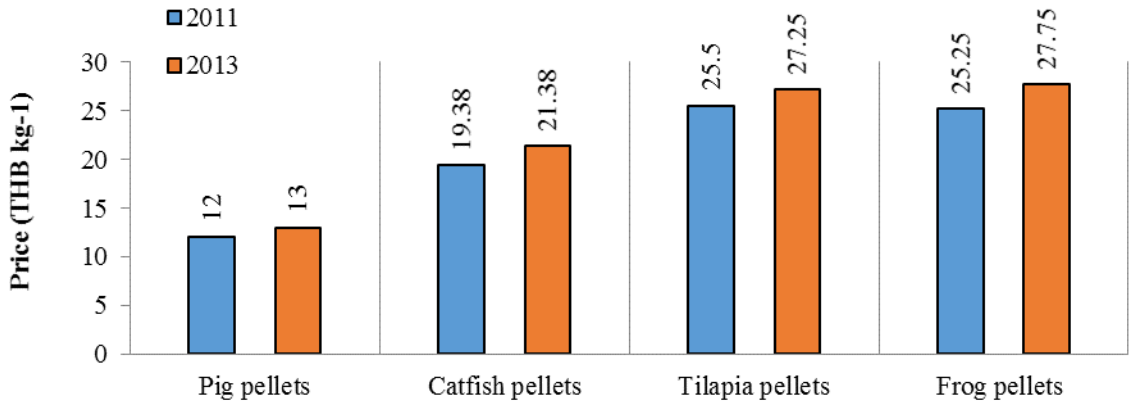


Figure 3.3.9: Change in feed price in Nakhon Pathom over 2 years. 31.91 THB = 1 US$

3.3.3 Follow-up Feeding Practice Survey

3.3.3.1 Farm Profile

The average age of respondents and years of aquaculture farming experience were 50 and 14 years respectively (Table 3.3.4). 80% of the respondents were male. Whilst, 60% of the farms were owned and managed by the respondents.

Table 3.3.4: Tilapia farm profile (FFPS)

Characteristics	FFPS	Characteristics	FFPS
No. of Respondents	20	**No of Provinces**	4
Average Age (Yrs.)	50.2 ± 8.7	**Avg. Aquaculture Experience** (Yrs.)	14.5 ± 10.6
Age (%)		**Experience** (%)	
≤ 30	0.0	≤ 5	27.8
31 – 39	13.3	6 – 10	22.2
40 – 49	33.3	11 – 15	11.1
50 – 59	33.3	16 – 20	16.7
≤ 60	20	≥ 21	22.2
Gender (%)		**Land Ownership (%)**	
Male	80	*Owned*	60
Female	20	*Leased*	30
		Both	10

3.3.3.2 Production Systems

The size of FFPS farms ranged from 15 – 700 rai (2.4 – 112 ha) in culture area. The number of ponds ranged from 1 – 40, averaging 8.2 ± 9.5 (Table 3.3.5). 80% of the farms were polyculture and semi-intensive respectively. 45% farmed over three additional species along with tilapia. The secondary species included mud carp (*Cirrhimus molitorella*), common carp (*Cyprinus carpio*), bighead carp (*Hypophthalmichthys nobilis*), silver barb (*Barbonymus gonionotus*), seven striped barb (*Probarbus jullieni*), striped catfish (*Pangasius hypophthalmus*), rohu carp (*Labeo rohita*) and whiteleg shrimp (*Litopenaeus vannamei*). 40% of FFPS respondents stocked crustaceans, 25% of which stocked tilapia with shrimp only. Average culture duration was 9.8 ± 2.4 months. Overall, 15% of FFPS farms had a history of integration (Figure 3.3.10). Majority of farmers used sex-reversed monosex tilapia seeds (80%) and had one continuous culture (55%), *i.e.* without transferring fish from nursery to grow-out. 75% of the farms that stocked monosex seeds also used commercial feed.

Table 3.3.5: Characteristics of tilapia farms (FFPS)

Characteristics	Current Study	Characteristics	Current Study
No. of Respondents	20	**No of Provinces**	4
Avg. cycles per year	1.2	**Avg. Culture Duration** (months)	9.8 ± 2.4
Farm Size (rai)*	137.3 ± 175.4	**Avg. Fattening period** (months)	3.4 ± 1.2
Avg. No. of Ponds	8.2 ± 9.5	**Type of culture (%)**	
Avg. Culture Area (rai)	20.58 ± 16.6	Monoculture	20
Nursery	9.4 ± 11.1	Polyculture	80
Grow-out	155.1 ± 197.2	**Prod. Intensity** (%)	
No. Species cultured (%)		Extensive	0
1	30	Semi-Intensive	80
2	5	Intensive	20
3	20	**Feed Input** (%)	
4	15	IBP or ABP*	35
≥5	10	CF	10
Avg. stocking size (g)		CF + ABP	55
Nursery	0.25	**Fattening System** (%)	
Grow-out	158.8 ± 134.8	Yes	50
Avg. stocking density (rai)		No	50
Nursery	3931	**Yield per rai** (kg)	736 – 1,296
Grow-out	ND	**Harvest size** (g)	300 – 1300
		Farm Gate Price (THB kg^{-1})	41.2 ± 10.8

ND – Not determined.

Figure 3.3.10: Non-operational integrated broiler/tilapia system in Prachinburi (A). Operating pullet/tilapia system in Chonburi (B)

3.3.3.3 Feed Inputs

Fertilization was also a common practice among the FFPS farmers, 80% of farms applied organic fertilizers. Manures were ordered in bulk (2 – 3 tonnes) from local livestock farmers, delivered directly to fish farm in pick-ups and applied immediately to ponds (Figure 3.3.11). Application rates varied with water quality on farm and were adjusted as needed. Average manure costs were ~1,000 THB (US$ 31.33) per tonne. In addition to fertilization, FFPS respondents also used a diverse range of readily available feedstuffs. These included other ABPs (corn bran, rice husk), agricultural waste (rice straw), kitchen waste, industrial and slaughterhouse waste (fish silage, spoiled bread, cow skin) along with commercial feeds (both aquatic and livestock). In the FFPS, six different commercial feeds were identified which varied in CP level and cost per kg (Table 3.3.6). The agri- and industrial by-products used by tilapia farmers are also presented in the table. Commercial feeds are generally stored in small warehouses on farm and prior to feeding were kept pond side in large covered containers., ABPs or IBPs were either kept pond side or applied immediately to systems depending on shelf-life and storage conditions. Irrespective of feed input, tilapia farmers broadcasted feed by hand or used feeding stations (Figure 3.3.12).

Figure 3.3.11: Application of manure to pond by supplier

Table 3.3.6: Crude protein contents and average cost kg^{-1} of feeds and other inputs (FFPS)

Feed Type	CP (%)	Average Cost kg^{-1} (THB)	Usage by farmers (%) (n = 20)	Other Feed Input	Average Cost kg^{-1} (THB)	Usage by farmers (%) (n = 20)
Frog	40	35	5	RB	8.44	75
Carnivorous	40	31	5	Corn bran	6.60	5
Catfish	16 – 30	22	35	Fish silage	1.12	5
Tilapia	22	20	10	Cow Skin	1.40	10
Herbivorous	12.5 – 15.5	15	15	Bread waste	6.00	5
Pig	12	9	10	Manure*	1.17	80

* Chicken (broiler and layer), pig and cow manure; 31.91 THB = 1 USD

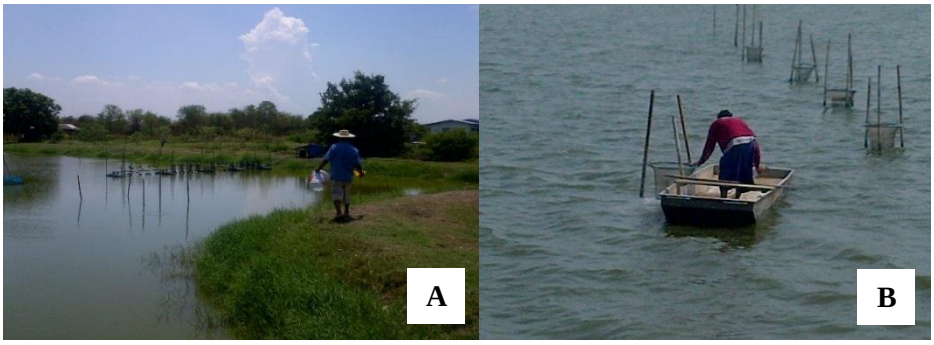


Figure 3.3.12: Broadcast feeding (A) and the use of feeding station (B)

45% of the FFPS respondents switched from shrimp culture due to disease challenges in previous years. The farmers who crossed over from shrimp culture generally continued their use of commercial feed for the production of tilapia. 66% of these farmers used commercial diets, all of which were engaged in fattening practices. In comparison, 63% of traditional tilapia farms used commercial feed and of those, only 36% practiced fattening. Overall, 65% of FFPS respondents used commercial feeds and 50% of farms had distinct periods of fattening during the grow-out stage. The 15% that did not participate fattening mixed their commercial feed (usually of low nutrient density and low CP *e.g.* pig ration) with rice or corn bran prior to feeding. The average period of fattening was 3.4 ± 1.3 months. Figure 3.3.13 illustrates a comparison of feed input categories between SEAT IFS and FFPS.

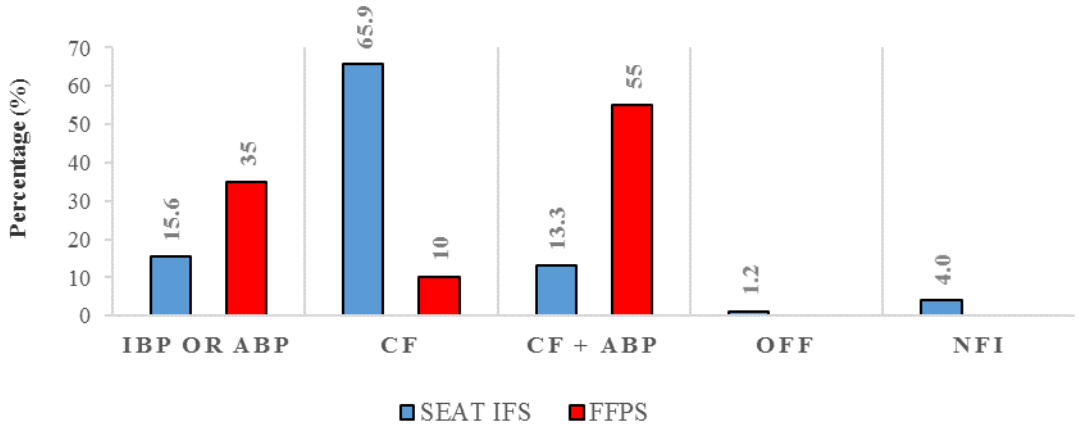


Figure 3.3.13: Comparison of feed inputs used by tilapia farms in central Thailand. SEAT IFS (n = 199) and FFPS (n = 20). IBP – industrial by-product, ABP – agricultural by-product, CF – commercial feed, OFF – on-farm feed and NFI – no feed input

3.3.3.4 Market Outlets

Final products (live and iced tilapia) were mainly marketed via "middlemen" who were often contracted to harvest. Some farmers opted to market their own products however via local markets. Iced tilapia were sold for 26.16 – 44.35 THB kg^{-1} (US$ 0.82 – 1.39) while live fish fetched 39.57 –

59.35 THB kg^{-1} (US$1.24 – 1.86)(Figure 3.3.14). The mixtures of secondary species stocked were sold for 19.78 – 23.61 THB kg^{-1} (US$ 0.64 – 0.74).

Figure 3.3.14: Fish held in cage to be sold live in Chachoengsao (A). Fish sold on ice in local market in Chachoengsao (B).

3.4 **Discussion**

3.4.1 Farm Profiles and Systems

3.4.1.1 Farmers Demography

Aquaculture farming is an age old practice in Thailand (Tabthipwon 2005). It plays an important role in the national economy and socio-economic status of rural and peri-urban communities (Thongrod 2007; Belton 2006). Tilapia farms, like most agriculture operations, are characterised by an owner-manager business model and are generally located outside of large municipalities (Bhujel 2013; Belton 2006). There are strong traditions of land and farm ownership within families. Back in 1995/6, 99% of tilapia farms were own and 100% family operated (Dey et al. 2000). In comparison, 83.% and 60% of the SEAT IFS and FFPS farms were owned and family operated respectively. 51.9% and 46.7% of the farmers were below the average age (~50 years) according the SEAT IFS and FFPS surveys respectively. The average age was consistent with Belton

(2006) and Lebel et al. (2013), and has increased by 5 years over the last 20 years (Dey et al. 2000). This potentially raises concerns about future dissemination of new technology (Bryant & Gray 2005).

More than 80% of respondents had over 5 years experience and above 35% had greater than 15 years expertise. Studies in Europe suggest that farming communities often develop unique experienced-based rules which govern their agricultural practices (Burton et al. 2008). Ifejika et al., (2007) argued, however, that experience reduces management risk but does not necessarily correlate with production practices. Thai farmers, nevertheless, value experience as an important part of success, irrespective of gender (Lebel et al. 2009). Farming is male dominated though men and women equally participate in various activities along the value chain (Piumsombun 2001). The current findings showed there were more male respondents than females in agreement with Sermwatanakul et al., (2013). Nevertheless, no gender discrimination exists in the Thai aquaculture trade and women make substantial contributions to feeding, caring for fish and post-harvesting (Lebel et al. 2009; Piumsombun 2001). Fish farming is often used in Thailand to complement other economic activities in order to diversify household income and resilience (Lebel et al. 2013). Based on SEAT IFS findings, farmers engaged in various other business ventures and livelihood activities, however, only 30% used aquaculture as their sole income source. This suggests potential economic risks associated with fish culture, which may have indirect implications for management practices, including feed input choices.

The attitudes and behaviours of farmers are generally conservative compared to other sectors (Lee 1997). A concomitant lack of willingness to develop is thought to be associated to age and education/poor training. This reluctance or inability to modernize by adopting new technology, including commercial feeds, is also believed to worsen with age (Lee 1997). Nevertheless, evidence shows that other demographic variables such as awareness and attitude also influence technology adoption behaviour (Ifejika et al. 2007). In the present study, most farmers had modest levels of education consistent with Lebel et al., (2013). In fact, only 10% of SEAT IFS tilapia farmers had professional and graduate level training. In comparison, Lebel's 2013 study of tilapia cage culture

showed only 5.5% of farmers had tertiary education. The correlation of social variables (*i.e.* age and education) with production and feeding practices will be discussed later.

3.4.1.2 System Types

Tilapia is cultured at different scales and intensity based on farm economics and resource availability. 89% of freshwater culture occurs in static pond systems with minimal water exchange except after harvest. Culture systems have gradually changed from extensive to semi-intensive and intensive over the last 70 years (Thongrod 2007). In support, there was only one farm interviewed that practiced traditional extensive culture (SEAT IFS). Semi-intensive polyculture systems dominate freshwater aquaculture (Bhujel & Woollard 2011). 53.3% of SEAT IFS farms were semi-intensive polyculture (0.05% Large, 16.08% Medium and 42.21% Small). There was, however, a relatively even distribution of SEAT respondents practicing monoculture (40.9%) and those practicing polyculture (59.1%). The distribution was, however, skewed towards polyculture (80%) in the FFPS survey, possibly due to sample frame.

Monosex sex-reversed tilapia fry accounts for 80% of seed stocked in central Thailand (Belton et al. 2009; Bhujel 2013) consistent with the findings of the SEAT IFS and FFPS. Average stocking densities for nurseries were consistent in both surveys, ~3700 fingerling/fry per rai. Stocking densities for grow-out, on the other hand, varied significantly. Farmers cultured between 1 – 5 other species at lower densities along with tilapia, taking advantage of several niches within the pond to maximise outputs. 25% of FFPS respondents stocked tilapia with shrimp only. This was often done to improve continuity/consistency of household income because shrimp had a shorter culture period and is a higher value commodity (Poutiainen 2012). Integrated aquaculture systems with poultry and/or swine were widely practiced prior to 2004 (Thongrod 2007). 1n 1995/6, 84% of tilapia systems were integrated (Dey et al. 2000). This practice has declined in recent years particularly due to risks associated with disease transmission (*e.g.* avian influenza) and stricter food safety regulations. Notwithstanding, there were remnants of this practice at several farms, however, only one farmer had a fully functioning integrated system with pullets and tilapia (FFPS).

Tilapia production is believed to be slowly moving away from green-water fertilized systems towards pellet-fed intensive systems in order to meet increasing demand. Belton (2006) concluded that post-1990, this was largely due to increased resource availability, increased farmer access to resources and improved markets. Though advanced technology and farming techniques are available in Thailand, small profit margins in tilapia production do not currently incentivise further intensification (FAO 2012b). In fact, one of the main constraints to any further expansion will likely be the development and wide-scale adoption of cost effective feeds and proper feed management protocols (Thongrod 2007). In light of this, commercial feed use is somewhat restricted by domestic market drivers.

3.4.2 Market Outlets

A farmer's choice of feed inputs is largely influenced by markets and fish farm gate prices (Piumsombun et al. 2005). Marketing channels in Thailand are not straightforward (Lebel et al. 2013) and the fish cultured rarely goes directly to the consumers unless consumed by the farm household. Fish are therefore sold through various market channels and intermediaries. These include fish collectors and brokers or "middlemen" who often go directly to farms with independent harvest teams. 93% of SEAT farmers sold fish to middlemen without any prior contractual arrangements (Burana-osod 2013). 50% of FFPS farmers also contracted middlemen to handle harvesting, particularly when there was insufficient farm labour. "Middlemen" are often engaged because they claim to have more reliable long-term resale points into markets (Lebel et al. 2013). Fish were also sold at local fish markets and sometimes directly to wholesalers (intermediate market) and retailers (terminal market) (Piumsombun 2001). FFPS farm products went to local markets in Talad Thai, Bang Kla and Chiang Mai. Others sold their products to processors and restaurants. 4% of SEAT TFS farmers sold their products themselves (Burana-osod 2013). One FFPS farmer further admitted, he gets higher returns by excluding "middlemen".

Tilapia market size varied significantly (300 – 1000g) depending on target market. Average tilapia harvest weight increased from 232g in 1995/6 (Dey et al. 2000) to 300 – 500g in 2010 (Bhujel

2013) indicating an increased consumer demand for bigger product. In support, SEAT IFS and FFPS average harvest sizes were 524 ± 257g (Henriksson et al. 2014) and 630 ± 254g respectively. Whole tilapia was sold in two forms, on ice or live, the latter commanding a higher market price. SEAT IFS results showed that tilapia sold for 33.31 ± 22.52 THB kg^{-1} (US$1.04 ± 0.71). Despite higher market price for live fish, most farmers marketed their products on ice. There was also a price divergence between cheaper Nile tilapia (*Pla Nin*) and more expensive red tilapia (*Thap Thim*) consistent with Lebel et al. (2013).

The central provinces of Thailand are the major tilapia producing region (Belton 2006). This region contributes 8.9 million THB to the total value of freshwater aquaculture in Thailand; tilapia accounting for ~5.0 million (DOF 2012). Better infrastructure and communication facilitated by economic growth has significantly improved product access and competitiveness in the domestic market (Belton 2006). The author recalled the market were dominated by wholesalers in the 1980s who controlled market prices. Now there is relatively an equal split between wholesalers and retailers (SEAT IFS). The farmer's share of retail price is merely 50 – 60% (Singh et al. 2015). The rest (~40%) is considered the marketing margin which covers marketing cost and traders profit (Piumsombun 2001). Long-term trends in farm gate price of pond cultured tilapia have, however, remained relatively consistent with cyclical fluctuation despite increase in production (Belton 2006). Production, however, peaked in 2009 and began trending downwards (DOF 2012). This signalled at the time a potential saturation of the domestic market in spite of the advantages of diverse market outlets. Thai tilapia's low farm gate value should ideally make it very competitive for export marketing, this is however not the case. In fact, tilapia exports (chilled and frozen fillets only) accounts for >10% of total production in 2013 (Nietes-Satapornvanit 2014). This suggests that there may be additional barriers to export such as poor product quality e.g. off-flavour or inadequate management strategies e.g. feeding practices.

3.4.3 Feeds and Feeding Practices

Feeding practices for Asian semi-intensive aquaculture are said to be as diverse as the feed inputs themselves (De Silva 1993). Irrespective of system type, it is undebatable that nutrient input is the single largest farm operating expenditure. In countries that practice semi-intensive aquaculture, feed choices are driven largely by the type of culture system, market, value of fish cultured, and in some instances, access to and availability of feeds/feedstuffs. The first three factors were previously discussed therefore feed access and availability will now be explored.

3.4.3.1 Choice of Feed Inputs

Natural pond productivity is generally enhanced through one-off or continuous fertilization (Yakupitiyage 1993). Increasing natural productivity through fertilization throughout the culture cycle facilitates lower levels of supplemental feeding. Fertilization, using organic or inorganic products, is common in tilapia semi-intensive culture. 80% of the FFPS systems were fertilized, all of these used organic or livestock manure. Though integrated poultry systems were not now fully accepted because of the 2003/4 bird flu pandemic (Thongrod 2007), interestingly poultry (broiler and layer) manure was the main organic fertilizer used by SEAT IFS and FFPS farmers. Nevertheless, not all farmers practiced fertilization. The farms that did not fertilize reported an inability to control planktonic blooms and changes in pond microbial community as the main reasons not to.

To supplement fertilization, pond polyculture production relies on a range of supplemental feed inputs, from simple kitchen waste to nutritionally complete commercial diets (De Silva 1993). Respondents of SEAT IFS and FFPS used a range of feed inputs consistent with Bhujel (2013) and Thongrod (2007). A total of 79.2% of SEAT IFS farmers reported commercial feed use, however, there was no differentiation in terms of which aspect of the production cycle they were used for or whether they were used solely or combined with other products prior to feeding. On the other hand, 65% of FFPS farms used commercial feeds, 55% of these farms mixed their commercial feed (usually having low protein levels) with cheaper ABPs primarily RB. In the past trash fish was also a popular input for tilapia farmers (Thongrod et al. 2004) but there was no evidence of this practice in the

present study. Trash fish is increasingly being used for FM production and may now be more common for feeding high value carnivorous species (Bundit 2007).

On-farm feeds (OFF) are produced exclusively for farm purposes and are generally not for commercial sale or profit (Bundit 2007). They have been widely promoted throughout Asia for many years (Yakupitiyage 1993; De Silva 1993), however, there was also little evidence of their use in the current study (1.2% of SEAT IFS/TFS). This may be due to their nutritional inconsistency and water instability (Thongrod 2007). Thongrod, (2007) also highlighted that farmers have limited knowledge of feed preparation and little control over ingredient quality. These reasons may have also influenced farmers' choice in using OFFs.

Most of the inputs described above were readily accessible and were sourced from neighbouring factories. In agreement with Belton, (2006), there was clearly no evidence of access restrictions to feeds in any province. Feed mills were centrally located close to "fish belt" in central Thailand with strategically positioned sales offices and distribution points (Nietes-Satapornvanit 2014). RB was the most abundant ABP and was often delivered directly to farms by rice mills/agents. 75% of FFPS respondents used RB contrary to Bhujel's (2013) finding which suggested that most tilapia farmers used maize meal as the main supplemental input. RB was also most popular among SEAT TFS farmers. SEAT IFS/TFS farmers used five types of commercial feeds with average CP levels of 25.5 ± 8.5%. This was consistent with Bhujel (2013) who established that most farmers use feeds containing 25% CP particularly during the final stages of production because this protein level was most cost effective. The author also reported the price range of pelleted feed used was 15 – 20 THB kg^{-1} (US$0.47 – 0.62). Comparatively the cost per kg of feeds and other inputs (FFPS) was 9 – 35 THB (US$0.28 – 1.09).

3.4.3.2 Commercial Feeds

Commercial feed production has increased significantly over the years with parallel increases in demand for aquafeeds (Thongrod 2007). There are several commercial aquafeeds available in Thailand designed for herbivores, carnivores and crustaceans (shrimp). Pelleted or extruded feeds are

used intensively by shrimp farmers but are less popular among tilapia farmers who rely more on fertilization and ABPs (Thongrod 2007). Use of commercial feeds for tilapia production may be limited or impractical due to their cost as a percentage of operating expenditures. Commercial feed cost per kg as a percentage of fish sale price per kg ranged from 16.7 – 66.7%, averaging 39.6%. This figure does not include fertilization. Consequently, it is not economically worthwhile to feed pond grown tilapia commercial feeds because the price of good quality feed is almost the same as the farm gate price of the fish (Bhujel 2013). In fact, cheaper feeds typically prepared for catfish and carp are commonly used for tilapia (Bhujel 2013). In support, 35% of tilapia farmers in the FFPS used catfish feed. Their reasons varied, some preferred catfish diets due to lower cost while others believed the diet quality was superior to tilapia feeds. In addition to tilapia and catfish feeds, few farmers used carnivorous/frog diets and others used swine diets. In contrast, Burana-osod (2013) found that 65% of TFS farmers used tilapia diets but concluded that farmers also used non-tilapiine formulation (catfish and frog) during nursery and pig rations during grow-out. The latter finding was consistent with FFPS. Farmers used high quality, high CP diets (starter catfish/frog) to improve survival during nursery and low CP diets (swine ration) in the final production stage to reduce grow-out cost and improve final product quality. 22% and 10% of SEAT TFS farmers used catfish and pig feeds respectively.

3.4.3.3 Feed Delivery

Nutrient delivery and feeding strategies are important aspects of animal production (NRC 2011). Feed, as previously mentioned, represents the single largest expense in semi-intensive and intensive fish culture operations, consequently optimal delivery and management becomes essential from both economic and environmental perspectives (NRC 2011).

Tilapia farmers used two methods of feed delivery; broadcast feeding and feeding stations. Broadcasting or feeding by hand to apparent satiation generally allow farmers to observe their fish stock and reduce overfeeding. Feeding stations, on the other hand, are centrally placed bags or containers suspended within ponds which facilitate slow feeding by fish. The use of feeding stations

assists with minimising feed waste and allow the fish to graze slowly, thereby improving feeding efficiency. The practice of using feeding stations is less labour intensive which may be more beneficial for small-scale farmers with less hired help. No mechanical feeders were reported in either survey, possibly due to high capital and operating cost. Feeding frequency and volume were generally adjusted over the culture cycle to suit harvest size and desired culture period. During grow-out fish are fed once or twice daily. Larger farms generally fed once per day due to limitations of time and labour while smaller farms fed twice daily.

3.4.3.4 Feed Records

One of the prevailing issues with Thai tilapia aquaculture is that most farmers do not keep proper records of feed use and input and rarely, if at all, are these analysed to improve feeding efficiencies and overall farm performance (Chong 1993). Though farmers are generally aware of the level of economic investment in feed, they fail to keep proper records that would enable more cost-effective application of this expensive input. This was true for most farms visited. Only 7% of SEAT IFS reported FCRs (Henriksson et al. 2014), however, these values were calculated from supplemental feed inputs only. For this reason, all estimated FCRs reported were used with caution because they cannot often be validated by estimates of feed-in and yield due to poor record keeping. Additionally, and in practical terms, true FCR values for polyculture systems based on the different feed inputs used (including manure) are difficult to calculate with accuracy.

3.4.4 Feed Inputs and Links to Management Practices

In the grow-out phase, farmers typically fed a low CP ABP (*e.g.* RB 8% CP) along with fertilization followed by a higher (>20%) CP commercial feed. In support, Diana et al. (1996) and Yi et al. (2002) suggested that the most efficient culture system to grow tilapia involves using fertilization up to 100 – 150 g followed by supplemental feeding. Tilapia grow-out feeds contained 22 – 30% CP. In comparison, CP levels for catfish pellets ranged from 16 – 30% while those for pig, herbivorous fish and frogs were 12, 15 and 40% respectively. Higher CP diets are used for nursery

and lower CP for grow-out. The overall choice of commercial feed and in turn CP levels, however, were highly dependent by feed cost and in some cases another primary culture species (*e.g.* shrimp).

Respondents with higher education were more inclined to supply commercial feed to their fish. Correspondingly, the number and variety of feed inputs, increased as education level decreased. Younger and less experienced farmers tend to use higher levels of commercial feed. One theory is that they lacked the confidence to experiment with various feed inputs. Conversely, older and more experienced farmers adhered to tradition and stuck more rigidly to their feeding practices of utilising by-products and alternative inputs. There was a correlation between education level and feed category ($P = 0.05$) though none was found between age and feed category ($P>0.05$). Nevertheless, there was a strong correlation found between age and education ($P<0.05$). In terms of geographic location, Nahkon Pathom and Suphanburi, which had highest percentages of younger and more educated farmers, were found to have higher commercial feed usage than Chachoengsao and Petchburi. This has important and hopefully positive implications for future adoption of commercial feeds in Thailand. Similarly, Rahman, (2007) found negative and positive correlations between the desire to adopt new technologies among pig farmers with age and education respectively. Reluctance increased with age and decreased with education level. The author further found that 63% of respondents were low adopters in respect to feeding practices. There were also no correlation in the present study between farming experience and feed inputs contrary to the hypothesis.

In terms of FM inclusion, an industry source revealed that FM levels for standard commercial tilapia diets ranged from 5 – 10% but some premium brands may contain as much as 20%. Thailand produces an estimated 248 thousand tonnes of by-product FM annually (OXFAM 2014), from fish trimmings (*i.e.* processing waste *etc.*) which finds its way back into the feed production chain. This is likely to have significant effects on life cycle impacts and will be discussed further in Chapter 7. When farmers were asked about their perspective on the heavy FM reliance by the aquaculture industry, the responses varied. Some often judged feed quality based on its "fishy" smell, *i.e.* characteristics of high FM inclusion. A common perception among farmers that requires change.

Some were keen on using more sustainable feeds made from plant-based ingredients but acknowledged that the diets would have to perform equally to the feeds they currently used.

3.4.4.1 Changes in feed Management

45% of SEAT TFS farmers made changes to feed management between 2010 – 2013. In fact, majority of the respondents cited an increase in feed prices as the main reason for change. The average tilapia feed price per kg increased by 6.8% in keeping with inflation of raw ingredients/feedstuffs. Feed prices in Nakhon Pathom (SEAT TFS) increased by 6 – 10% over that period (Burana-osod 2013). According to Ng & Chong (2004), fish feed accounts for 45 – 85% of tilapia farm gate prices which drives an interest in lowering feed cost through the use of local alternative ingredients. Despite this, Belton, (2006) argued that changes in tilapia market price have a more significant impact on farmers than changing feed prices. The author suggested that feed cost increases were smaller in magnitude compared to reduced revenues as a result of fluctuating tilapia price. Interestingly, 4% of SEAT TFS respondents gave low farm gate price as the reason for discontinuation of tilapia farming and as important as feed inputs are, no one cited changes in feed price. This is probably due to a higher flexibility in choice of feed inputs available to farmers while tilapia prices are more or less fixed by external market forces.

3.4.5 Aquaculture Legislation and Farm Certification

The fisheries industry is governed by the Fisheries Act 1947 (*amended* 1953 and 1985) which addresses the propagation of aquatic animals. A secondary legislation (The National Fisheries Development Plan) encourages the strengthening of aquaculture techniques and management, promotes the cost-effective and environmentally friendly aquaculture, upgrading of product quality and expansion of export markets (FAO 2012b). Also, the code of conduct (COC) and Good Aquaculture Practice (GAP), initially developed for the shrimp sector, are now adopted voluntarily by the tilapia sector. In addition to local certification standards, there are three independent third party certification schemes used by producers which supports aquaculture trade *i.e.* Global GAP's

GLOBALG.A.P. (Good Aquaculture Practices), Aquaculture Stewardship Council's ASC Tilapia Standard and Global Aquaculture Alliance's BAP (Best Aquaculture Practices) certification schemes.

While most farmers acknowledged the importance of good aquaculture/feeding practices, in Thailand they are not mandatory. There were 38 (20%) SEAT IFS farms with DOF's Thai GAP certifications (Nietes-Satapornvanit 2014). On the contrary, there were no third party certification among tilapia farmers which meant no stringent implication for on-farm feed management, feeding practices, record keeping, traceability *etc*.. Firstly, limited or lack of strict certification processes suggested that there is currently no institutional impetus for improvement in feed management practices as seen with the Thai Shrimp Industry; products of which are destined for export markets. Secondly, and most importantly, is the current challenge faced by small-scale farmers to access third party certification due to financial constraints and lack of technical competence to achieve international traceability/sustainability standards for tilapia production. Finally, third party certification schemes currently do not make provisions for non-commercial feed inputs (*e.g.* ABPs, OFFs) and polyculture systems (Nietes-Satapornvanit 2014); both of which are intrinsic to Thai tilapia production. With a drive to intensity within the context of a potentially saturated domestic market, product quality and production practices (*e.g.* record keeping) will have to improve. This will require special legislative reform promoting changes from voluntary to mandatory GAPs if tilapia exports to major overseas markets (specifically US and Europe) are to increase.

3.4.6 Feed Milling and Aquafeed Industry

There are currently ~50 active members of the Thai Feed Mill Association and an estimated 20 feed mills producing aquatic feeds (Roembke 2014; Nietes-Satapornvanit 2014). High quality commercial feeds are therefore readily available, however costly. There are national quality control standards for commercial feed production including aquafeeds (Thongrod 2007). Quality standards and minimum CP levels are set and regulated by the government's fisheries department (DOF). CP and FM inclusion levels vary and depend largely on the life stage of the animal and fish species. Tilapia starter feeds have high CP levels (42%) with 20% of the protein provided by FM. Tilapia

grow-out diets are formulated to contain a minimum of 20% CP and usually contain 5 – 10% FM (Thongrod 2014, personal communication). The feed mill interviewed used its own FM produced from by-products of the Company's tuna fisheries operation. 50% of its FM requirement is met from this source. The Company, however, does not use imported FM in tilapia feeds due to high import taxes (15%) which makes it uneconomical for both themselves and their customers. They also do not foresee any immediate challenges with lowering FM levels in their diets to 1 – 2 % in keeping with the direction of the global aquaculture industry because they have the ability to utilise other animal by-products (poultry by-product, offal meal *etc*.) and other plant-based ingredients (SBM, RB and CM) for their tilapia feeds. Furthermore, they are not subjected to strict export market regulations as with their shrimp diets. The Company does not use enzymes at the moment though they are equipped to do so. They cited no significant differences in performance of enzyme supplemented diets compared to their standard diet when trialled.

The feed company interviewed was ISO (9001:2008) and GAA-BAP certified as well as HACCP and GMP certified by the DOF. They were previously Global-GAP certified but discontinued because other stakeholders along the supply chain were not (hatcheries and farms *etc.*) and so this had no immediate value chain benefit. Notwithstanding, they are currently pushing to have the FM production/supply Global-GAP certified. 5.5% of the Company's production was tilapia feeds but, like most other companies, it was difficult to estimate the true demand for tilapia feeds because majority of their customers also used catfish brands for tilapia grow-out. The Company admitted that one of the main complaints from farmers was feed cost which was consistent with SEAT IFS and FFPS surveys. Their standard tilapia grow-out diet cost an average of 27.5 THB kg^{-1} (US$0.86) which represented a 29 – 47% increase over 2005 prices (14.5 – 19.5 THB kg^{-1}). Culturing tilapia using high quality commercial feeds is not considered profitable in Thailand, therefore the selection of appropriate feeds is one of the most important decisions that famers make to ensure continued profitability of their farming operations (Bhujel, 2013).

3.4.7 Conclusions

Tilapia culture systems (>60%) in Thailand are semi-intensive polyculture using primarily monosex seeds. Farmers use a diverse set of feeds (including agricultural and industrial by-products) and feeding practices were just as diverse. These practices have, however, remained consistent over the last five years. Although access and availability to alternative inputs were not an issue, these are less nutritive and cannot efficiently support intensive culture. Surveys confirmed that commercial feed use is more popular among small-scale farmers contrary to the hypothesis, however, their use is still largely restricted by cost. Use of commercial feeds was more prevalent among younger and educated farmers and to some extent defined by geographical location. Whilst some farmers understood the need for quality feeds, financial limitations, farm gate price and the returns on investments were strong deterrents to intensification and adoption of commercial feeds. Though fattening was not an established practice in Thailand, a large number of tilapia farmers did engage in this practice to improve the value of the final commodity prior to harvest. In light of the findings, diets should be tailored to suit the market based on feeding practice trends and also good feed management standards must be institutionalized as opposed to being voluntary. Finally, cost-effective low FM, low CP (25%) diets formulated for the final stages of tilapia grow-out for semi-intensive systems have significant potential in domestic Thai markets but more so, for good consumer acceptance if products are to be sold on the export markets.

Chapter 4

Effects of Xylanase and Phytase on Digestibility and Growth in Tilapia Fed Declining FM Diets

4.1 **Introduction**

Global FM use has been increasingly re-directed to aquafeed production (Hardy 2010). Continued industry growth, therefore, hinges in-part on lowering FM reliance particularly for herbivorous and omnivorous aquafeeds. As FM replacers, plant-based ingredients such as SBM, RB, CM and MA have been studied as potential alternatives individually (Borgeson et al., 2006; Deguara et al., 1999; Gallagher, 1994; Kaushik et al., 2004; Wang et al., 2006) but are often used in combination supplemented with micro-additives to achieve economic growth performance (Gatlin et al. 2007). Furthermore, plant-based ingredient mixtures are thought to be beneficial as they reduce the fish's exposure to individual ANFs such as phytate, hemicellulose, PIs *etc.* (Borgeson et al. 2006). ANFs negatively influence feed intake, nutrient digestibility, growth, and affect the function of organs (Krogdahl et al., 2010; Soetan and Oyewole, 2009). ANFs limit the value of plant-based ingredients for formulation unless their impacts can be moderated (Francis et al. 2001).

This experiment focused on two commercially important classes of ANFs – phytates and NSPs. Phytate binds 60 – 90% of natural plant P rendering it unavailable for use by monogastrics. The negatively charged phosphate groups strongly chelate multivalent cations, such as Ca^{2+} *etc.*, impairing mineral uptake in fish (Francis et al. 2001; Haros et al. 2005; Makhode 2008).. Deficiencies of essential minerals may cause poor growth, lower PER and poor carbohydrate utilisation particularly in juvenile fish (Ng & Romano 2013; Lopez et al. 2002). NSPs are compounds containing several hundred linked hexoses and pentoses. They make up a portion of dietary fibre, which is generally indigestible. Arabinoxylans interfere with digestion by changing digesta viscosity, altering food

passage rate, gut physiology, morphology and microflora (Sinha et al. 2011). However, one readily detectable effect is the shortening of intestinal villi.

Some ANFs can be deactivated or their effects reduced through enzyme supplementation (Krogdahl et al. 2010). The development of commercial enzymes with high specificity for ANFs improves the potential for higher plant-based ingredients levels in monogastric diets. Phytases are a sub-group of phosphatases with specificity for hydrolysing phytate-P (Haros et al. 2005; Makhode 2008). Phytase efficiency, however, is dependent on dietary composition and storage sites for phytate within different plant-based ingredients. Furuya et al. (2001) found that 500 – 1500 FTU kg^{-1} phytase supplementation of SBM-based diet was effective in increasing Ca and P availability, protein digestibility and the performance of Nile tilapia. Xylanases have been used with success in other monogastric diets owing to their ability to cut arabinoxylan backbone randomly creating fragments of smaller molecular weights (Tapingkae et al. 2008). Tapingkae et al. (2008) reported positive improvements in *in-vitro* digestibility of DM, CF, EE and protein utilisation in piglets fed diets containing SBM, RB, maize and CM supplemented with 600 – 900 U kg^{-1} xylanase. Nevertheless, little information exists on NSP's effects on tilapia gut morphology and the effects of xylanase in their diets (Ai et al. 2007). Moreover, there is less evidence of the synergy of xylanase and phytase on tilapia performance. The experiment was therefore designed to assess the combined effects of xylanase (250 FXU kg^{-1} or 0.385g kg^{-1}) and phytase (1,500 FYT kg^{-1} or 0.075g kg^{-1}) in diets containing 0%, 3% and 5% FM on digestibility and growth in juvenile tilapia.

4.2 **Material and Methods**

4.2.1 Experimental Diets

The basal diets were formulated to be isonitrogenous (250 g kg^{-1} CP), isoenergetic (18 kJ g^{-1} GE) and contained marginal available-P (0.4%; minimum tilapia requirement determined by NRC (2011)). The diets contained four plant-based ingredients of which the SBM and MA were adjusted to substitute FM at three levels (Table 4.2.1). The diets were divided into two equal portions and half

coated with enzyme mixture containing 0.385 g kg^{-1} xylanase and 0.075 g kg^{-1} phytase to form a total of six treatments. See Section 2.2.5/6 for diet formulation and feed production.

4.2.2 Digestibility Experiment

Twenty (20) sex-reversed juvenile tilapia (48.85 g ± 13.96, mean ± STD) were stocked in each of twenty four (24) 800L indoor circular static tanks. Each tank contained 650 L of sand-filtered pond water and was continuously aerated to maintain DO above 5 mg L^{-1}. Tanks were subjected to ambient temperature and photoperiod (~12hr light: 12hr dark). The six experimental diets were each randomly assigned to four replicate tanks. Fish were acclimated for 2 weeks to a commercial diet (30% CP) before being fed the experimental diets for 12 weeks (80 days). Fish were fed three times daily (8:30, 13:00 and 16:30) to apparent satiation, 7 days per week except on sample days. Weekly water exchange rate was 15% per tank (at once) for the 1st four weeks then 30% until the end of the experiment.

Individual fish weights and total lengths were taken at the start of the experiment and at 2 week intervals. Prior to sampling, fish were anesthetized with a 1:10 solution of clove oil and ethanol (Section 2.2.9). Mortalities were recorded daily and water quality monitored weekly (Section 2.2.11). For proximate analysis, 10 fish from the starter-population were sacrificed prior to and 16 fish pooled per treatment at the end of the experiment. Intestine samples were collected and fixed for histomorphology (Section 2.2.12) while liver samples were weighed and discarded. For digestibility analyses, faecal matter was collected 2 hours after midday feeding from each tank daily (between weeks 2 – 12) and stored at -20 ^{o}C until analysed (Section 2.2.10)

Table 4.2.1 Formulation and proximate composition of experimental diets

	C0FM	E0FM	C3FM	E3FM	C5FM	E5FM
Ingredient (g kg^{-1})						
SBM	497.5	497.5	455.0	455.0	425.0	425.0
MA	102.5	102.5	115.0	115.0	125.0	125.0
FM	0.0	0.0	30.0	30.0	50.0	50.0
CM	150.0	150.0	150.0	150.0	150.0	150.0
RB	200.0	200.0	200.0	200.0	200.0	200.0
Palm oil	30.0	30.0	30.0	30.0	30.0	30.0
Vitamin/Mineral mix	10.0	10.0	10.0	10.0	10.0	10.0
Cr_2O_3	5.0	5.0	5.0	5.0	5.0	5.0
Dicalcium phosphate	5.0	5.0	5.0	5.0	5.0	5.0
Lysine	4.9	4.9	4.9	4.9	4.9	4.9
Methionine	1.0	1.0	1.0	1.0	1.0	1.0
Phytase	-	0.075	-	0.075	-	0.075
Xylanase	-	0.385	-	0.385	-	0.385
Proximate analysis (g kg^{-1}as fed)						
DM	945.0	907.3	938.0	923.7	946.2	915.3
CP	257.6	242.9	252.9	246.9	276.5	263.5
Lipid (EE)	44.1	45.9	34.8	41.8	26.2	39.8
Carbohydrate	570.0	548.4	571.7	557.5	562.7	533.8
CF	38.8	36.3	38.8	36.6	39.9	35.7
NFE	531.3	512.1	532.9	520.9	522.8	498.2
Ash	73.4	70.2	78.7	77.7	80.9	78.3
Total-P	8.2	8.0	8.7	8.6	9.0	9.2
Ca	6.6	5.2	7.3	8.3	8.9	8.0
Zn	0.510	0.542	0.679	0.582	0.517	0.521
Fe	0.025	0.036	0.048	0.048	0.030	0.039
Cr_2O_3	4.0	3.9	4.3	4.8	4.0	3.4
GE (MJ kg^{-1})	20.8	22.1	19.6	19.2	19.9	19.2
Enzyme Recovery (g kg^{-1})						
Phytase	NT	0.104	NT	0.076	NT	0.087
Xylanase	NT	0.447	NT	0.550	NT	0.557

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4.2.3 Trial Parameters

Growth performance was assessed using mean body indices, condition factor, hepatosomatic index, FI, FCR, SGR, ADG, PER, EE, nutrient retention efficiency and mean villi lengths,

4.2.4 Histomorphology

Five (5) µm sections of formalin-fixed proximal intestine samples were processed and stained using H&E (Section 2.2.12). Imaging and measurements were made using light microscopy and AxioVision imaging software. The lengths of 10 well-oriented villi per intestinal section (5 sections per treatment) were measured and means calculated after Borgeson et al. 2006.

4.2.5 Chemical Analysis

Raw ingredients, experimental diets, fish carcass (pooled per treatment) and faecal material were assessed for moisture, CP, EE, CF, ash, P. Ca, Zn and Fe using standard methods (AOAC 2005; AOAC 1990). Dietary chromic oxide was analysed according to Bolin et al., (1952). Carbohydrate and NFE levels were calculated. Arabinoxylan was quantified indirectly from the D-xylose using spectrometry (Megazyme D-XYLOSE 11/12) (Section 2.2.3)

4.2.6 Calculations and Statistical Analysis

Apparent digestibility was calculated according to the following equation.

$$ADC\ (\%) = 100 * (1 - [(\%Cr_2O_{3\ Feed}/\%Cr_2O_{3\ Faeces}) * (\%Nutrient_{\ Faeces}/\%\ Nutrient_{\ Feed})])$$

Statistical analysis was performed using SPSS *v* 19 and 21 (IBM, USA). Data was tested for normality (K-S test) and homogeneity of variance (Levene's Test). Based on the outcomes, results were subjected to either non-parametric KW or parametric ANOVA. KW was used to assess villi lengths and survival. ANOVA was used to examine means for FI, FCR, SGR, ADG, PER, EE and ADC. *Post-hoc* analyses were conducted to determine the loci of any significant differences; Mann-Whitney for significant KW χ^2 values and DMRT for ANOVA *F* values. Statistical significance was reported at $P = 0.05$. The replication rate, though 4 per treatment, contributed to a large effect-size (> 0.40). Results are presented as means ± STD or SEM.

4.3 **Results**

4.3.1 Water Quality

System temperature, DO and pH ranges were 28.98 ± 0.73°C, 5.96 ± 0.51 mg L^{-1} and 6.87 ± 0.57 respectively. NH_3 (< 0.03 mg L^{-1}), NO_2 (0.5 – 5.0 mg L^{-1}), NO_3 (25 – 100 mg L^{-1}) and alkalinity (34 – 68 mg L^{-1}) were within acceptable ranges for tilapia culture (DeLong et al. 2009) (Appendix B).

4.3.2 Growth Performance

Growth response indicators and feed utilization efficiencies are given in Tables 4.3.1 & 4.3.2. Condition factor and HSI were similar among treatments. Mean weight gain diverged progressively between treatments by the end of the trial ($P < 0.01$). FI was unaffected by enzyme inclusion ($P > 0.05$). There were highly significant differences in FCR, SGR and ADG between diets at graded FM levels ($P < 0.01$), however, differences due to enzyme supplementation were insignificant. FCRs decreased with higher FM levels ($P < 0.05$). FCR, ADG and SGR were not significantly different between C5FM and E3FM diets. Survival rates were within acceptable ranges and had no treatment-related effect.

Table 4.3.1 Initial and final body weight, condition factor and hepatosomatic indices of red tilapia fed declining FM diets with and without enzyme supplementation

Diets	IBW (g)	FBW (g)	Condition Factor	HSI (%)
C0FM	49.4 ± 12.8	121.3 ± 35.4	1.99 ± 0.20	1.61 ± 0.52
E0FM	48.7 ± 14.6	120.4 ± 32.9	1.83 ± 0.13	1.48 ± 0.42
C3FM	47.3 ± 13.5	132.9 ± 32.8	1.96 ± 0.22	1.34 ± 0.54
E3FM	49.1 ± 16.2	146.5 ± 46.9	1.96 ± 0.17	1.12 ± 0.74
C5FM	48.4 ± 14.4	150.5 ± 38.4	1.98 ± 0.19	1.51 ± 0.71
E5FM	50.3 ± 11.9	160.2 ± 40.7	1.86 ± 0.14	1.44 ± 0.46
ANOVA				
F-value	5.64	67.10†	1.27	0.69
P-value	0.342^{NS}	0.000**	0.294^{NS}	0.634^{NS}

IBW – Initial Body weight; FBW – Final Body Weight; HSI – Hepatosomatic Index
Mean values ± STD of individual weights and lengths. Means in the same column with similar letters are not significantly different ($P > 0.05$); † Kruskal Wallis χ^2*value*

Table 4.3.2 Feed intake, feed conversion, growth and survival of tilapia after 80 days

Diets	FI (g fish^{-1})	FCR	SGR (% day^{-1})	ADG (g day^{-1})	Survival (%)
C0FM	143.4 ± 8.4	1.99 ± 0.09^{a}	1.12 ± 0.06^{a}	0.90 ± 0.09^{a}	92.5 ± 1.02
E0FM	139.8 ± 4.8	1.95 ± 0.04^{a}	1.13 ± 0.05^{ab}	0.89 ± 0.05^{a}	97.5 ± 1.03
C3FM	148.5 ± 6.3	1.74 ± 0.06^{b}	1.29 ± 0.04^{bc}	1.07 ± 0.06^{ab}	100.0
E3FM	164.9 ± 13.3	1.72 ± 0.04^{b}	1.34 ± 0.06^{c}	1.20 ± 0.12^{bc}	100.0
C5FM	167.4 ± 7.9	1.64 ± 0.02^{bc}	1.42 ± 0.04^{c}	1.28 ± 0.06^{bc}	100.0
E5FM	169.3 ± 10.0	1.54 ± 0.05^{c}	1.44 ± 0.07^{c}	1.37 ± 0.12^{c}	98.7 ± 1.01
ANOVA					
F-value	2.18	10.67	6.62	5.14	10.28†
P-value	0.102 ^{NS}	0.000**	0.001**	0.004**	0.068^{NS}
FMLEVEL		*P < 0.05*	*P < 0.05*	*P < 0.05*	
ENZYME		NS	NS	NS	
*FML*ENZ*		NS	NS	NS	

FI – feed intake; FCR – Feed conversion ratio; SGR – Specific growth rate; ADG – Average daily gain. Mean values ± SEM of four replicate. Means in the same column with similar letters are not significantly different (P>0.05). ^{NS} Not significant (P>0.05); * Significant (P<0.05); ** Highly significant (P<0.01); † Kruskal Wallis Chi-square value

4.3.3 Carcass Composition

Analyses of whole-carcass proximate (Table 4.3.3) indicated differences in DM, CP, lipid, ash, P, Ca and GE., however, differences in DM, CP and lipid content had no direct linkages to either enzyme supplementation or dietary FM levels. Lipid levels were, however, higher in fish fed control diets, with exception of the 3% FM diet, corresponding with higher GE values. Carcass P content showed no trend related to enzyme supplementation though the values were slightly higher for the enzyme diets. There were higher deposition of Ca in fish fed enzyme supplemented diets except at the 5% FM level.

Table 4.3.3 Whole body composition of tilapia fed declining FM diets with and without enzymes (g kg^{-1} wet weight basis)

Parameters	Experimental diets						
	Initial	C0FM	E0FM	C3FM	E3FM	C5FM	E5FM
DM	288.5 ± 3.3	278.6 ± 0.0	287.3 ± 0.0	278.0 ± 0.0	257.1 ± 0.1	295.1 ± 2.4	26.92 ± 0.10
CP	160.2 ± 1.3	149.6 ± 0.1	159.6 ± 0.5	155.0 ± 0.8	131.9 ± 0.7	157.5 ± 0.9	14.51 ± 0.13
Lipid (EE)	84.6 ± 0.6	89.3 ± 0.5	84.3 ± 0.4	77.4 ± 0.0	83.8 ± 0.3	90.1 ± 1.2	7.63 ± 0.07
Ash	39.2 ± 0.7	36.0 ± 0.3	39.0 ± 0.3	35.4 ± 0.2	34.9 ± 0.2	41.4 ± 0.7	3.86 ± 0.05
P	5.9 ± 0.1	5.3 ± 0.1	5.9 ± 0.0	5.4 ± 0.0	5.2 ± 0.1	6.0 ± 0.4	0.61 ± 0.01
Ca	10.8 ± 0.0	11.0 ± 0.0	11.2 ± 0.2	10.0 ± 0.1	10.5 ± 0.2	12.1 ± 0.0	1.17 ± 0.07
GE (MJ kg^{-1})	6.69 ± 0.01	6.81 ± 0.03	6.81 ± 0.01	6.58 ± 0.0	6.31 ± 0.1	7.37 ± 0.01	6.35 ± 0.0

Mean of triplicate samples (composite of treatment) ± SEM

4.3.4 Digestibility

Faecal matter composition from experimental fish are given in Table 4.3.4. Correspondingly, ADC for DM, organic matter (OM), CP, lipid, GE, ash, and phosphorus are given in Table 4.3.5. DM ADC values improved with graded FM levels ($P < 0.05$), however, no differences were observed between the 3% and 5% FM diets, irrespective of enzyme supplementation. Xylanase and phytase had no effect on OM and CP digestibility. Lipid ADC results were inconsistent with little variability among treatments. Digestible energy trends mirrored DM and CP digestibility. P digestibility increased by 9% at the 3% and 5% levels with enzyme supplementation, however, there were no significant differences between these diets. There was also no difference seen in fish fed the all plant diets (0% FM) ($P > 0.05$).

Table 4.3.4 Faecal composition of tilapia fed declining FM diets with and without enzymes

Parameters	Experimental diets					
(g kg^{-1} DM)	C0FM	E0FM	C3FM	E3FM	C5FM	E5FM
CP	146.4 ± 1.8	161.3 ± 2.3	151.6 ± 1.2	154.8 ± 0.6	166.9 ± 0.5	177.8 ± 0.3
Lipid (EE)	24.8 ± 0.4	27.1 ± 0.5	17.8 ± 0.5	28.0 ± 0.4	18.4 ± 0.8	27.9 ± 1.5
CF	200.0 ± 0.3	208.6 ± 2.1	167.9 ± 0.9	214.9 ± 2.3	177.2 ± 0.3	167.3 ± 0.6
Ash	185.4 ± 1.0	147.7 ± 0.4	188.7 ± 0.5	173.6 ± 2.0	187.7 ± 0.3	185.5 ± 0.5
P	18.4 ± 2.0	19.7 ± 0.1	19.7 ± 0.1	18.0 ± 0.1	18.8 ± 0.8	18.0 ± 0.0
Ca	32.3 ± 0.3	25.1 ± 0.0	27.5 ± 0.2	27.9 ± 0.1	27.8 ± 0.1	29.2 ± 0.1
Cr_2O_3	10.8 ± 0.1	11.1 ± 0.4	14.9 ± 0.1	17.9 ± 0.0	14.1 ± 0.3	13.3 ± 0.2
GE (MJ kg^{-1})	16.83 ± 0.04	17.89 ± 0.09	16.18 ± 0.0	17.62 ± 0.03	16.42 ± 0.01	17.53 ± 0.09

Mean of four replicate tanks

4.3.5 Nutrient Retention

PER decreased with lower FM levels but improved with xylanase and phytase (Table 4.3.6). There were no differences in energy efficiency due to supplementation. N retention values were higher for control diets except at the 0% FM level. P retentions were 5 – 7% higher in diets supplemented with enzymes.

Table 4.3.5 Apparent digestibility of nutrients and energy of practical diets in tilapia fed declining FM diets

Experimental Diets	ADC $_{DM}$	ADC $_{OM}$	ADC $_{CP}$	ADC $_{CL}$	ADC $_{GE}$	ADC $_{CARBO}$	ADC $_{ASH}$	ADC $_{P}$
C0FM	60.81 ± 0.61^{a}	63.09 ± 0.60^{a}	78.95 ± 0.12^{a}	79.17 ± 0.32^{a}	70.07 ± 0.60^{a}	58.18 ± 0.74^{a}	6.45 ± 1.42^{a}	16.89 ± 9.60^{a}
E0FM	61.20 ± 4.21^{a}	59.82 ± 4.31^{a}	76.67 ± 2.13^{a}	79.26 ± 1.86^{a}	71.51 ± 3.33^{a}	57.46 ± 4.86^{a}	26.08 ± 8.06^{ab}	13.45 ± 9.70^{a}
C3FM	69.23 ± 1.08^{b}	70. 72 ± 1.02^{b}	82.70 ± 0.52^{b}	85.24 ± 1.15^{b}	76.24 ± 0.73^{b}	67.59 ± 1.02^{b}	30.80 ± 2.43^{bc}	34.65 ± 1.32^{b}
E3FM	70.97 ± 0.84^{b}	71.57 ± 0.91^{b}	83.19 ± 0.54^{b}	82.04 ± 0.32^{ab}	75.42 ± 0.68^{b}	69.04 ± 1.00^{b}	40.09 ± 0.78^{c}	43.87 ± 3.09^{b}
C5FM	70.02 ± 0.61^{b}	71.68 ± 0.58^{b}	82.88 ± 0.41^{b}	80.08 ± 1.13^{a}	76.66 ± 0.40^{b}	68.39 ± 0.59^{b}	34.18 ± 1.39^{c}	40.74 ± 0.68^{b}
E5FM	72.07 ± 2.34^{b}	72.44 ± 2.32^{b}	82.75 ± 1.34^{b}	82.08 ± 0.47^{ab}	76.64 ± 2.00^{b}	70.83 ± 2.62^{b}	39.44 ± 4.94^{c}	49.98 ± 3.63^{b}
ANOVA								
F-value	*6.17*	*6.27*	*6.57*	*5.21*	*3.15*	*6.57*	*11.48*	*4.940*
P-value	*0.005***	*0.004***	*0.004***	*0.009***	*0.048**	*0.004***	*0.000***	*0.011**
FMLEVEL	*P < 0.05*	*P < 0.05*	*P < 0.05*	*P < 0.05*	*P < 0.05*	*P < 0.05*	*P < 0.05*	*P < 0.05*
ENZYME	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*P < 0.05*	*NS*
*FML*ENZ*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*

ADC OM (Organic matter) = ADC DM – ADC Ash. Mean of four replicates ± SEM. NS – not significant

Table 4.3.6 Effects of dietary treatments on protein efficiency ratio, apparent net protein utilisation, energy efficiency and nutrient retention.

Diets	PER	EE	Nutrient Retention	
		(%)	*Nitrogen*	*Phosphorus*
C0FM	1.94 ± 0.10^{a}	17.52 ± 0.87^{a}	27.64 ± 1.50^{a}	32.35 ± 1.63^{a}
E0FM	2.10 ± 0.05^{ab}	17.70 ± 0.39^{a}	33.49 ± 0.77^{b}	37.69 ± 0.86^{bc}
C3FM	2.27 ± 0.08^{bcd}	20.34 ± 0.76^{b}	34.58 ± 1.31^{b}	35.68 ± 1.32^{ab}
E3FM	2.34 ± 0.05^{cd}	19.99 ± 0.50^{b}	27.42 ± 0.86^{a}	34.99 ± 0.81^{ab}
C5FM	2.21 ± 0.03^{bc}	24.86 ± 0.27^{d}	34.49 ± 0.41^{b}	40.70 ± 0.47^{cd}
E5FM	2.45 ± 0.08^{d}	22.76 ± 0.76^{c}	33.84 ± 1.19^{b}	42.80 ± 1.34^{d}
ANOVA				
F-value	*6.89*	*20.31*	*10.42*	*11.44*
P-value	*0.001***	*0.000***	*0.000***	*0.000***
FMLEVEL	*P < 0.05*	*P < 0.05*	*P < 0.05*	*P < 0.05*
ENZYME	*P < 0.05*	*NS*	*NS*	*P < 0.05*
*FML*ENZ*	*NS*	*NS*	*P < 0.05*	*NS*

PER – Protein efficiency ratio; EE – Energy efficiency
Mean values ± SEM of four replicates*;* Means in the same column with similar letters are not significantly different (P>0.05)*;* ^{NS} Not significant (P>0.05); * Significant (P<0.05); ** Highly significant (P<0.01)

4.3.6 Intestinal Histomorphology

Pre-trial gut histology revealed that the lining of tilapia's proximal intestine had deep and complex folding extending into the lumen (Fig. 4.3.1). The villi often showed branching, increasing intestinal surface area. They were supported at the base by a thin *lamina propria* which merged with the *tunica muscularis* and were dispersed with goblet and lymphoid cells in a random fashion. Post-trial samples showed less folding, loss of compactness and larger lumens. Figure 4.3.2 shows average villi lengths between pre-trial and post-trial samples. There was a clear reduction in villi length irrespective of treatment. Lengths were reduced by 59.3% in the C0FM diet. Statistical analysis showed significant differences between the samples taken at the start of the experiment (parent stock) and all treatments ($P < 0.01$) suggesting dietary NSP had significant effects on villi structure. Villus length was correlated to FM level in the control (no enzyme) treatments; however, results were less

consistent for enzyme-supplemented diets. Villus lengths were significantly different between control and enzyme-supplemented diets ($P < 0.05$).

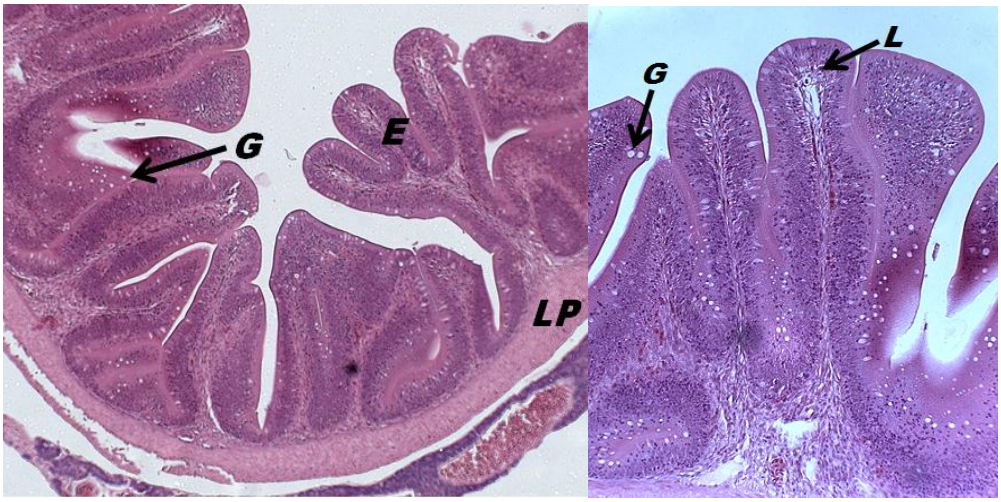
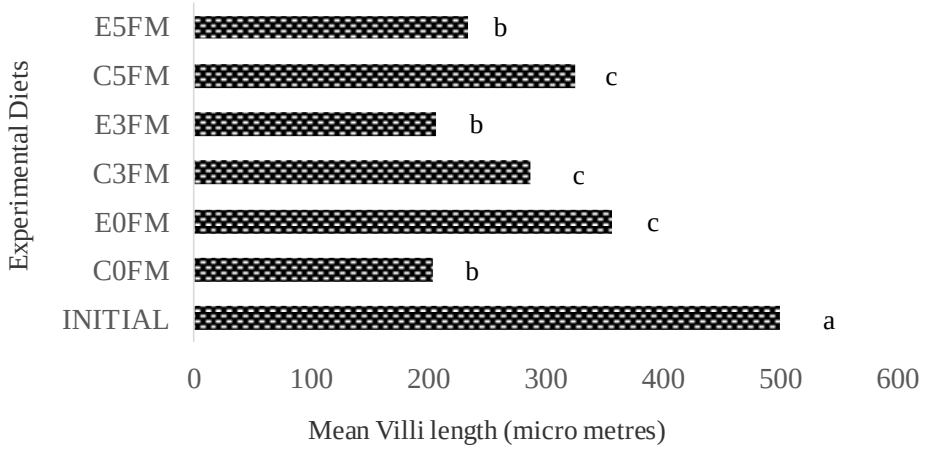


Figure 4.3.1: Section of tilapia intestinal mucosa showing normal morphology (A) (x10) and magnified portion of the same section (B) (x20). **G** – goblet cells; **E** – epithelium; **LP** – *lamina propria* and *tunica muscularis*; **L** – lymphoid cells or lymphocytes



	Initial	C0FM	E0FM	C3FM	E3FM	C5FM	E5FM	χ^2	p
Villi length (µm)	501.77	204.09	357.39	287.53	207.78	326.59	234.92	88.11	0.000**

Figure 4.3.2: Proximal intestine villi lengths of tilapia fed plant-based diets with declining FM levels with and without enzymes.

4.4 **Discussion**

4.4.1 Effects on Growth Performance

Replacing FM as the primary dietary protein source in aquafeeds remains an on-going effort based on the limitations of alternative plant-based sources (Carter & Hauler 2000; Kaushik et al. 2004). Efforts have been linked to attaining further improvements in plant nutrient bioavailability and minimising ANF impacts on digestive processes and gut integrity in fish. Commercial enzymes have been demonstrated to improve growth performance in other monogastrics (Bedford & Schulze 1998; Moreira et al. 2003; Tapingkae et al. 2008). This study evaluated the potential effects of xylanase and phytase in plant-based diets with declining FM levels on digestibility and growth in tilapia.

The present study confirmed the negative impacts of FM substitution, and showed that multi-enzymes had positive potential impacts on tilapia growth responses. Feed intake (FI) decreased from 2.1 (5% FM) to 1.74 g $fish^{-1}$ day^{-1} (0% FM) ($P < 0.05$). Contrary to the present findings, Nwanna & Schwarz (2008) reported higher FI values in common carp fed phytase diets which they attributed to a stimulation of appetence leading to direct improvement in growth. In theory, when phytase degrades phytate-protein complexes, this increases the concentration of AA which are potent olfactory and gustatory stimulators in fish (Collins et al. 2013). Sajjadi, (2004) provided supporting evidence to show increased FI and higher growth performance were directly associated with phytase inclusion (2,000 U kg^{-1}) in Atlantic salmon fed diets containing sub-optimal P (7 g kg^{-1}).

In the present study, FCR increased with declining FM and FI levels ($P < 0.05$). This, however, was not the case in the study of El-Saidy and Gaber (2005) where FCR increased with increasing FI in Nile tilapia fed all plant diets (no enzymes). FCR values (1.95 – 1.99) were highest in the 0% FM diets potentially due to lower nutrient digestibility and FM exclusion. Notwithstanding, similar efficiencies were observed in fish fed E3FM and C5FM diets ($P < 0.05$). Highest FBW was recorded in fish fed E5FM with corresponding highest AWG. C0FM and E0FM, however, showed the lowest improvement in AWG over trial duration suggesting a potential dietary deficiency. SGR decreased with lower FM inclusion ($P<0.05$) nevertheless, tilapia fed E3FM and C5FM performed on par.

4.4.2 Effects of Enzymes on Digestibility

There was an inconsistent enzyme-related effect on protein digestibility contrary to previous studies which found significant improvements in Crucian carp and rainbow trout fed practical diets supplemented with 500 and 2,000 FTU kg^{-1} phytase respectively (Kumar et al. 2012). Nevertheless, Papatryphon and Soares, (2001) and Sajjadi, (2004) also found protein digestibility was not affected by phytase in striped bass and Atlantic salmon respectively. Despite the presence of phytase, theoretically insoluble phytate-protein moieties formed under low stomach pH may persist in the small intestine impeding protein digestion. Phytate will bind more aggressively to protein including endogenous enzymes (below their isoelectric point) than minerals at low pH (Sajjadi 2004). If these binary protein-phytate complexes remain associated post-stomach, they may further bind to important nutrients (*e.g.* minerals) forming ternary complexes in the intestinal digesta (Selle et al., 2012). On the contrary, ash digestibility increased with xylanase and phytase inclusion ($P < 0.05$) suggesting dissociation of phytate-mineral complexes. Despite that, there were no improvements in P digestibility at 0% FM where the highest impact was expected. On the contrary, Tudkaew et al., (2008) reported 21% improvement in apparent P digestibility in red tilapia fed high FM (10%) diets supplemented with 750 FYT g^{-1}-phytase.

Refstie and Svihus (1999) demonstrated that NSP in plant-based ingredients, especially SBM, reduced lipid digestibility in salmon. Xylanase was therefore expected to have a pronounced effect on lipid ADC, however, this was not supported by the current findings. Digestible energy content is thought to influence FI (Lupatsch 2003) and fish have the capacity to regulate energy intake (Morales et al. 1994). Though there were no differences in FI, energy digestibility increased with higher FM levels ($P < 0.05$). Furthermore, Castillo and Gatlin, (2015) indicated that enzyme supplementation is more beneficial when DE is suboptimal, which was not the case in the present study. Theoretically, carbohydrases should increase energy utilisation by shifting the absorption of energy-yielding nutrient, such as lipid and starch, to the proximal intestine (Castillo & Gatlin 2015). The shift in nutrient utilization to the proximal intestine would decrease host–microbe competition for nutrients

and ensure availability of nutrients where absorption efficiency is greatest (Adeola & Cowieson 2011). Like energy digestibility, carbohydrate ADC had a similar trend. Other monogastric studies, however, confirmed lower synergistic responses to the use of xylanase and phytase in combination on nutrient digestibility (DM, GE, CP, P) relative to their use singularly (Kim et al. 2008).

4.4.3 Effects on Protein and Energy Utilisation

Despite no significant improvement in protein digestibility, PER increased with supplemented enzymes as seen in Tudkaew et al., (2008). In support, Adeola and Sands (2003) cited observed changes in protein retention in pigs though there were no changes in protein ADC. On the contrary, Biswas et al. (2007) reported no apparent improvements in protein retention in red sea bream fed 30% SBM diets supplemented with graded level of phytase (1000 – 4000 FTU kg^{-1}) though protein digestibility had increased. In a recent tilapia study, Krome, (2014) found no improvements in PER or protein productivity values when two types of phytases (3-phytase and 6-phytase; 2000 U kg^{-1}) were utilised. The impact of phytase on protein availability and its utilisation, nevertheless, remains debatable. On the other hand, Ai et al. (2007) demonstrated that 1000 IU g^{-1} xylanase significantly increased carcass protein levels in Japanese seabass fed a 47% CP plant diet. Similar to other monogastric research, the impacts of enzymes observed in fish are still inconsistent (Kumar et al. 2012).

4.4.4 Effects of Enzymes on N and P Retention

Environmental impacts of N and P from aquaculture effluents have become a growing concern (Biswas et al. 2007). There was no enzyme-related effect on N retention which differed from previous research using phytase only. On the other hand, other studies (Portz & Liebert 2004; Liebert & Portz 2005; Ai et al. 2007; Biswas et al. 2007; Liebert & Portz 2007; Verhlac et al. 2007) have demonstrated that phytase can lower P load in agreement with findings of this study. P retention were higher in enzyme-supplemented diets ($P<0.05$). In support, Goda (2007) demonstrated that 1500 FTU kg^{-1} phytase supplementation of SBM-based diets resulted in the highest level of P intake, ADC and retention in juvenile tilapia compared to diets supplemented with 1000 and 2000 FTU kg^{-1}. Ai et al.

(2007) also found significant improvements in carcass P, Ca and Zn levels in Japanese seabass fed diets supplemented by 200 mg (*i.e.* 2500 IU g^{-1}) phytase.

4.4.5 Effects of Enzymes on Intestinal Morphology

Though relatively undifferentiated, the proximal section accounts for three quarters of tilapia intestine and is characterized by elongated mucosal folds (Gargiulo et al. 1998). Significant digestion and absorption occurs in this region. This is supported by the findings of Jun-Sheng et al. (2006) that the highest levels of endogenous enzyme activities are present in the foregut of hybrid tilapia. Unfortunately, little information is available regarding the mode of action of exogenous enzymes in the digestive tract of tilapia, their effects on intestinal integrity and tilapia's post-digestive mechanisms. The current study produced inconsistent results in terms of enzyme-related effects on proximal intestinal morphology. Effects of the enzymes, particularly xylanase, on villi structure varied significantly between FM levels. Nevertheless, based on the control diets only, the results agreed with those of Borgeson et al., (2006) who reported that villi length decreased with declining FM levels. The signs were consistent with SBM induced enteritis in salmonids. There were losses of mucosal folds leading to reduction in epithelium absorptive capacity (Uran et al. 2008). Uran et al. (2008) also demonstrated similar effects in common carp. In their study, there was evidence that villi shrunk and appeared irregular in fish fed SBM-based diets while no negative effects were seen in the control group. This was also consistent with Heikkinen et al., (2006). Enes et al. (2012), however, presented opposing results. The authors cited no differences in intestinal morphology, height or density of intestinal villi of white sea bream fed FM diets and treatments supplemented with graded level NSP (Enes et al., 2012). On the contrary, Mathlouthi et al. (2002) found that xylanase/β-glucanase cocktail counteracted NSP effects of a rye-based diet and increased small intestine villi length of broilers. They, however, used higher concentrations (560 and 2,800 IU respectively) of the carbohydrases.

4.4.6 Conclusions

Studies have shown that combined inclusions of xylanase and phytase have beneficial effects in the diets of broilers and to some extent swine. Although the size of the effects on growth and nutrient utilisation in tilapia were relatively modest, this study demonstrated that xylanase and phytase show potential. Furthermore, the ES 3% FM diet performed comparably to the 5% FM control diet with no significant differences in growth and nutrient utilisation. In the present context, this was indicative that xylanase and phytase could at least justify a 2% FM reduction in tilapia diets. Enzyme supplementation improved P uptake but had no effect on N retention and intestinal morphology. Differences between this study and other research may be due to dietary composition, enzyme source, dosages, and notably the age of experimental animals which significantly impacts enzyme efficiency.

Chapter 5

Effects of Xylanase and Phytase on Grow-out and Fattening of Adult Tilapia Fed Declining FM Diets

5.1 **Introduction**

Thailand produced the 5th largest volume of global aquaculture products in 2012 (FAO 2012c) and was ranked 6th largest tilapia producer (FAO, 2015). Tilapia production is still largely traditional with fish cultured primarily in earthen ponds and fed a variety of compound feeds and agro-industrial by-products. Most tilapia grow-out diets in Thailand still contain high FM inclusion (≥ 10%) (Henriksson et al. 2014). As aquaculture intensifies, sustainable formulations will require much lower FM levels. Legumes, oilseed and root tubers have great value for the aquaculture industry. Agricultural commodities such as RB, CM, MA and SBM are potential FM/protein-sparing alternatives. In Thailand, RB is the primary by-product of the milling process, abundant in supply and largely used as supplemental feed. Cassava is an important economic cash crop. Higher inclusion of cassava in animal feed has been linked to the demand and price of maize. 94.7% of Thailand's annual maize production is consumed locally. On the other hand, domestic SBM production is low supplying only 10% of local demand and so much is imported. The increase use and demand of these plant-based ingredients is driven by their lower prices over other feed substitutes. CM, RB, MA and SBM cost 10, 12.5, 13 and 22 THB respectively compared to 34 THB for local FM. Figure 5.1.1 illustrates the 16 year price trends (A) and demands for (B) these ingredients in Thailand (Thai Feed Mill Association 2013; Santella & Prasertsri 2014).

Commercial enzymes have encouraged further changes in formulation and the use of higher plant-based ingredients inclusion for aquafeed. Enzymes have also promoted the use of lower cost and less-processed materials yielding similar performance to more expensive ingredients, thereby increasing the choice and flexibility of feed manufacturers (Deguara et al. 1999).

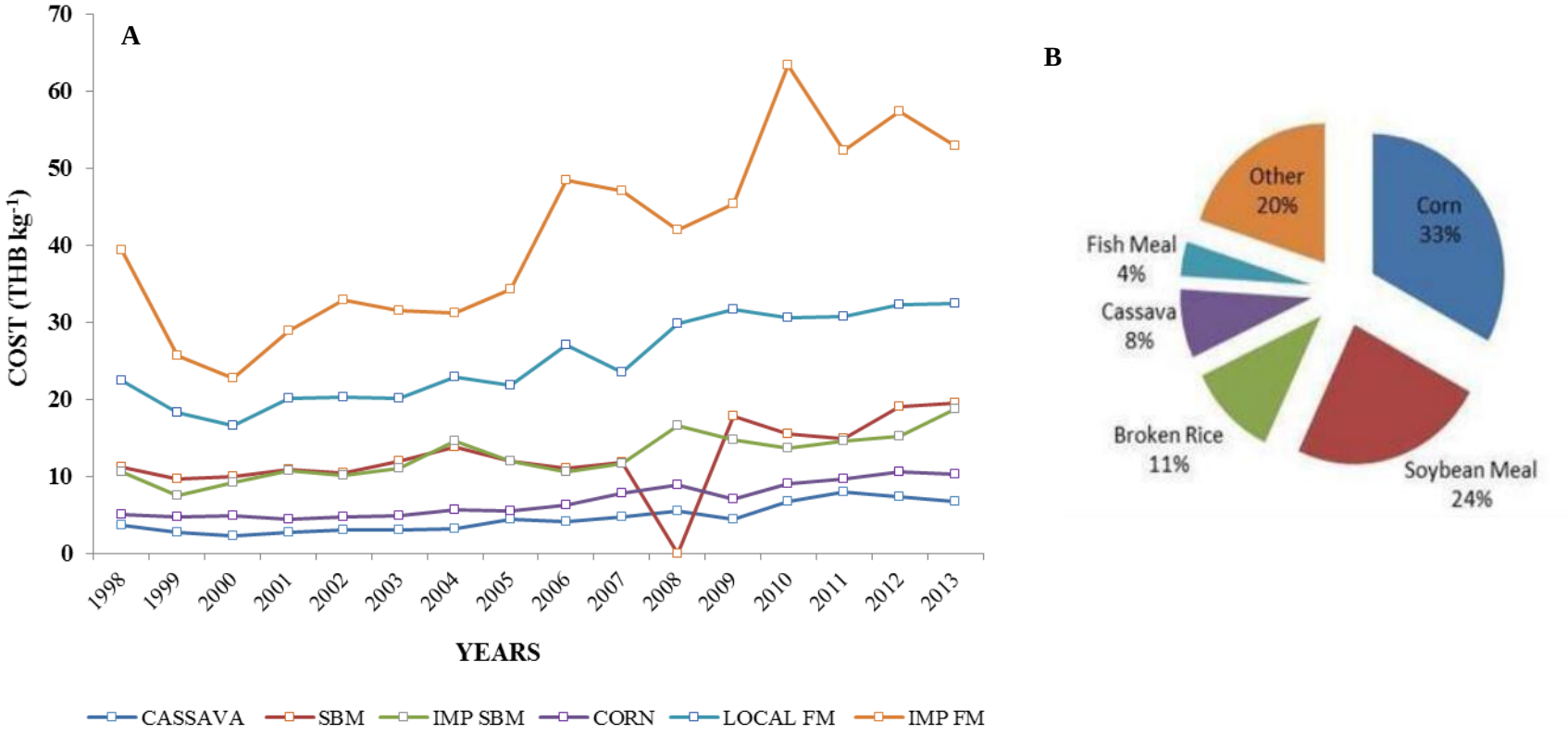


Figure 5.1.1: (A) Average feed ingredient price trend (1998-2013) and (B) demand for ingredients (%) in Thailand. 1US$ = 31.91 Thai baht (THB). IMP SBM – imported soybean meal; IMP FM – imported fish meal. Sources: Thai Feed Mill Association (2013) and Santella and Prasertsri, (2014)

Phytase is used to hydrolyse phytate liberating bound nutrients for growth (Kumar et al. 2012) while carbohydrases (*i.e.* xylanase) are used to hydrolyse insoluble carbohydrates to generate lower molecular weight, digestible monosaccharides (Castillo & Gatlin 2015). Based on advances in poultry and swine research, phytase has now become a common yet important feed additive (Chesson 1993; Choct 2006; Simbaya et al. 1996). There has also been rapid development in its use in aquatic diets (Dalsgaard et al. 2012) with positive research results in different fish species (Hussain et al., 2011; Kumar et al., 2012; Yan et al., 2002). The interactive effect of enzymes, particularly phytase and xylanase, require further elucidation in tilapia, though previous studies have demonstrated the potential benefits of other multi-enzyme cocktails (Ogunkoya et al. 2006; Lin et al. 2007; Khalafalla et al. 2010)

Enzyme supplementation also should demonstrate substantial improvements in formulation cost, animal health and growth performance, *i.e.* a reduction in cost to produce one unit of animal protein (Chesson 1993). Economic benefits of using exogenous phytase is more straightforward compared to xylanase because it delivers a direct cost benefit by replacing inorganic phosphate (Bedford 2000). The present study evaluated the feasibility of using high inclusions of local plant-based feedstuffs in Thailand to substitute FM at four levels 0%, 3%, 5% and 10% with or without enzyme supplementation for grow-out and fattening of adult tilapia. The trial was also designed to simulate an actual on-farm tilapia production system and assess the economic efficiencies of each diet.

5.2 **Material and Methods**

5.2.1 Experimental Diets

Lower-cost plant-based ingredients were sourced and used to formulate four basal diets containing 250 g kg^{-1} CP and marginal Av-P (0.4 %). SBM and MA were adjusted to substitute FM at four levels – 0%, 3%, 5% and 10%. The first three FM levels were divided and half coated with xylanase (0.385 g kg^{-1}) and phytase (0.075 g kg^{-1}) to form a total of seven treatments (Table 5.2.1).

Table 5.2.1: Formulation and proximate composition of experimental diets

	C0FM	E0FM	C3FM	E3FM	C5FM	E5FM	C10FM
Ingredients (g kg^{-1} as fed basis)							
SBM	497.5	497.5	455.0	455.0	425.0	425.0	355.0
MA	102.5	102.5	115.0	115.0	125.0	125.0	150.0
FM	0.0	0.0	30.0	30.0	50.0	50.0	100.0
CM	150.0	150.0	150.0	150.0	150.0	150.0	150.0
RB	200.0	200.0	200.0	200.0	200.0	200.0	200.0
Palm oil	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Vitamin/Mineral mix	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Dicalcium phosphate	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Lysine	4.9	4.9	4.9	4.9	4.9	4.9	4.9
Methionine	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Phytase	-	0.075	-	0.075	-	0.075	-
Xylanase	-	0.385	-	0.385	-	0.385	-
Proximate analysis (g kg^{-1} as fed)							
DM	945.0	907.3	938.0	923.7	946.2	915.3	923.7
CP	257.6	242.9	252.9	246.9	276.5	263.5	251.7
Lipid (EE)	44.1	45.9	34.8	41.8	26.2	39.8	29.2
Carbohydrate	570.0	548.4	571.7	557.5	562.7	533.8	559.6
CF	38.8	36.3	38.8	36.6	39.9	35.7	33.1
NFE	531.3	512.1	532.9	520.9	522.8	498.2	526.5
Ash	73.4	70.2	78.7	77.7	80.9	78.3	83.3
Total-P	8.2	8.0	8.7	8.6	9.0	9.2	10.9
Ca	6.6	5.2	7.3	8.3	8.9	8.0	14.0
Zn	0.510	0.542	0.679	0.582	0.517	0.521	0.633
Fe	0.025	0.036	0.048	0.048	0.030	0.039	0.068
GE (MJ kg^{-1})	20.8	22.1	19.7	19.2	19.9	19.2	21.7
Enzyme Recovery (g kg^{-1})							
Phytase	NT	0.104	NT	0.076	NT	0.087	NT
Xylanase	NT	0.447	NT	0.550	NT	0.557	NT

Av-P – Available phosphorus; NT – Not tested

The dry ingredients were mixed on farm and extruded at Kasetsart University. Feeds were stored at ambient temperature under normal farm conditions. Enzyme diets were coated weekly and stored at 4^{o}C until fed (Section 2.2.7)

5.2.2 Chemical Analysis

Proximate analyses of raw ingredients and experimental diets were performed using standards methods (AOAC 1990; AOAC 2005) (Section 2.2.4). GE was determined using analytical methods for oxygen bomb calorimeters. Carbohydrate and NFE were calculated. Phytate-P levels were calculated from proximate analysis and arabinoxylan content quantitated indirectly from the D-xylose content of plant-based ingredients using spectrometry (Megazyme 2012). Final xylanase and phytase activities of the diets were determined by Biopract, GmbH, Germany.

5.2.3 Experimental Conditions

The grow-out (fattening) trial was conducted using a hapa-in-pond, unfertilized green water system (Section 2.2.8). 28 5m^3 hapas were stocked with 20 tilapia each (223.86 g ± 46.9 g; mean ± STD) and fish acclimated for one week on a commercial diet (30% CP). The treatments were randomly assigned to four replicates each and fish fed a percentage of their cumulative body mass daily for 8 weeks (60 days). Individual weights and lengths were taken biweekly. Performance data was evaluated for FCR, SGR, ADG, PER, condition factor, hepatosomatic index (HSI), villi length and profitability. Five (5) fish were taken at the start and end of the trial (per treatment) for liver and intestine samples. Liver weights were used for HSI calculation. Intestine samples were stored in 10% formalin until processed for histomorphology.

5.2.4 Histomorphology

Five (5) mm section of fixed proximal gut samples were embedded using paraffin and 5 µm sections stained using H&E (Section 2.2.12). Imaging and measurements were done using light microscopy and AxioVision Imaging software. Mean villus height of ten well-oriented villi per section were calculated after Borgeson et al. (2006). Each treatment were compared with samples taken from the starter population at the beginning of the trial.

5.2.5 Calculations

Changes in unit profit were calculated according to the following equations (Section 2.2.13):

$$\Delta U_{\text{Feed efficiency (FE)}} = -\Delta FCR * \alpha^{1}P$$

$$\Delta U_{\text{Growth (G)}} = (1-(W_f/((W_f - W_i) * (1 + \Delta G) + W_i))) * P * \alpha$$

5.2.6 Statistical Analysis

The trial tested the null hypothesis that there were no differences in growth, economic performance and gut structure of tilapia fed declining FM diets supplemented with and without exogenous phytase and xylanase. Test for normality and homogeneity of variance were performed using K-S and Levene tests respectively. Based on significant values of both test statistics, non-parametric KW or parametric ANOVA was conducted. *Post hoc* analyses were performed using DMRT for ANOVA and Mann Whitney for KW. Statistical significance was reported at P = 0.05. Statistical analyses were performed using SPSS *v* 19 and 21. Treatment means are presented ± STD or SEM.

5.3 **Results**

5.3.1 Water Quality

Water quality was stable throughout the trial, average temperature DO and pH were 32.76 ± 0.76 ^{o}C, 6.12 ± 0.67 mg L^{-1} and 8.33 ± 0.52 (Mean ± STD) respectively. Mean NH_3, NO_2 and alkalinity were <0.03, 0.03 and 136 mg L^{-1} respectively.

5.3.2 Growth Performance

Table 5.3.1 shows the body weights, lengths, and survival of adult tilapia grown for 8 weeks. Final weights doubled initial measurements for all treatment. There were, however, high variations in starting fish weights and lengths due to on-farm batch weighing technique at the start of the experiment (trial limitation). This significantly affected the derived performance data. Analysis of growth performance showed no treatment related effects on AWG but the C10FM and C0FM fed fish

had the highest and lowest values of 254.5 g and 211.9 g respectively. Average weight increased with higher FM inclusion but the reverse trend was observed for enzyme-supplemented diets ($P > 0.05$) (Figure 5.3.1). The enzyme supplemented diets resulted in higher absolute values except at the 5% FM level.

Table 5.3.1: Body weight and length for tilapia fed various levels of FM with and without enzymes

Exp. Diets	Body weight (g)		Body length (cm)		Survival[§]
	Initial	Final	Initial	Final	(%)
C0FM	196.2 ± 43.9	408.6 ± 95.1	21.94 ± 1.82	28.01 ± 1.84	95.0 ± 0.0
E0FM	233.4 ± 44.9	474.4 ± 83.4	23.58 ± 1.71	29.24 ± 1.68	95.0 ± 2.0
C3FM	226.2 ± 45.6	440.2 ± 84.1	23.05 ± 1.78	28.57 ± 2.00	92.5 ± 4.3
E3FM	229.5 ± 44.1	456.1 ± 86.0	23.39 ± 1.37	28.88 ± 1.71	93.8 ± 1.3
C5FM	233.7 ± 38.1	474.1 ± 83.2	23.48 ± 1.29	29.58 ± 1.54	90.0 ± 4.6
E5FM	227.6 ± 97.8	460.4 ± 97.8	22.96 ± 1.91	29.13 ± 2.08	87.5 ± 9.2
C10FM	222.1 ± 49.2	476.5 ± 84.2	22.95 ± 1.75	29.21 ± 1.88	91.3 ± 4.3
Pooled SEM	5.16	6.78	*1.66*	*0.06*	1.62
(P)					0.989[NS]

Mean ± STD; Means in the same column with similar letters are not significantly different ($P > 0.05$)
[NS] Not significant; * Significant; ** Highly significant ($P < 0.001$); [§]Kruskal Wallis

Feed intake was highest in fish fed the E5FM which recorded the poorest FCR. Overall, FCR ranged from 1.66 – 2.07 with tilapia fed 10% FM diet converting feed to flesh most efficiently ($P > 0.05$). Surprisingly fish fed the E0FM diet had a comparable conversion ratio of 1.67. No treatment effects were observed in SGR, ADG and PER (Table 5.3.2).

Table 5.3.2: Growth matrices for adult tilapia fed various levels of FM with and without enzymes

Exp. Diets	FI[§] (g fish^{-1})	FCR	SGR (% day^{-1})	ADG (g day^{-1})	PER
C0FM	401.17 ± 0.29	1.90 ± 0.07	1.23 ± 0.08	3.53 ± 0.12	2.05 ± 0.07
E0FM	401.31 ± 8.70	1.67 ± 0.05	1.19 ± 0.05	4.01 ± 0.14	2.47 ± 0.07
C3FM	414.74 ± 21.59	1.97 ± 0.22	1.12 ± 0.10	3.57 ± 0.23	2.07 ± 0.21
E3FM	407.04 ± 6.10	1.82 ± 0.12	1.14 ± 0.07	3.78 ± 0.27	2.25 ± 0.15
C5FM	427.30 ± 21.50	1.80 ± 0.15	1.17 ± 0.06	3.99 ± 0.21	2.05 ± 0.18
E5FM	454.45 ± 60.45	2.07 ± 0.17	1.12 ± 0.08	3.62 ± 0.18	1.87 ± 0.14
C10FM	420.11 ± 20.58	1.66 ± 0.10	1.28 ± 0.10	4.24 ± 0.16	2.42 ± 0.16
Pooled SEM	9.62	0.05	0.03	0.08	0.06
ANOVA (F)		1.210	0.573	1.942	2.298
(P)	0.856^{NS}	0.340^{NS}	0.747^{NS}	0.121^{NS}	0.073^{NS}

Mean values ± SEM of four replicate; Means in the same column with similar letters are not significantly different (P>0.05); [§]Kruskal Wallis; FI – Average feed intake; FCR – feed conversion ratio; SGR – Specific growth rate ADG - average daily gain; PER – protein efficiency ratio; [NS] Not significant; * Significant; ** Highly significant (P < 0.001)

5.3.3 Histomorphology

Condition factor remained relatively consistent with initial measurements. HSI values were lower for enzyme diets compared to their respective non-enzyme diets (P > 0.05) (Table 5.3.3). Vacuolation was more pronounced for diets without enzyme supplementation (Figure 5.3.2). There was also noticeable thickening of the *lamina propria* and increased number of goblet cells in samples taken from tilapia fed control diets. Nevertheless, there were no clear treatment effects/trend observed in villi length or mucosal folding depth in relation to enzyme supplementation.

Table 5.3.3: Condition factor, hepatosomatic indices and villi length of tilapia fed declining FM diets with and without enzymes

Experimental Diets	Condition factor	HSI	Villi Length (µm)[§]
Control			
Initial	1.97 ± 0.22	1.24 ± 0.27	325.86 ± 12.36^{ab}
C0FM	1.77 ± 0.12	1.20 ± 0.22	369.57 ± 16.88^{bc}
C3FM	2.03 ± 0.48	1.35 ± 0.50	338.83 ± 17.90^{b}
C5FM	1.84 ± 0.17	1.29 ± 0.36	387.48 ± 11.91^{c}
C10FM	1.99 ± 0.61	1.09 ± 0.44	287.51 ± 10.45^{a}
Enzyme			
E0FM	1.96 ± 0.36	1.13 ± 0.13	364.57 ± 16.30^{bc}
E3FM	2.15 ± 0.47	0.96 ± 0.19	350.78 ± 12.08^{bc}
E5FM	1.88 ± 0.18	1.55 ± 0.27	347.37 ± 12.37^{bc}
Pooled SEM	0.62	0.59	5.12
F value	0.556	1.759	
P value	0.761^{NS}	0.144^{NS}	0.000**

n = 5, Mean ± STD. [§]Kruskal Wallis; CFr - Condition Factor; HSI - Hepatosomatic index

5.3.4 Economic Analysis

From a formulation perspective, diets supplemented with xylanase and phytase cost an additional 0.003 US$ kg^{-1} compared to their respective controls (Table 5.3.4). On the other hand, the enzyme-supplemented diets proved less costly in terms of converting feed to fish protein with the exception of tilapia fed E5FM which had a high FCR linked to high mortality levels. Comparison of the declining FM diets (supplemented and un-supplemented) to the commercial-like 10% FM control, from production perspective and assuming all other costs were equal, showed poorer economic efficiencies in terms of conversion cost, profit index and changes in unit profit. Nevertheless, when the enzyme supplemented diets were compared with the controls, unit profit, (based on feed efficiency and growth) improved by 0.098 and 0.054 US$ kg^{-1} at the 0% and 3% FM levels respectively. No improvements were seen at the 5% FM level due to mortality reasons.

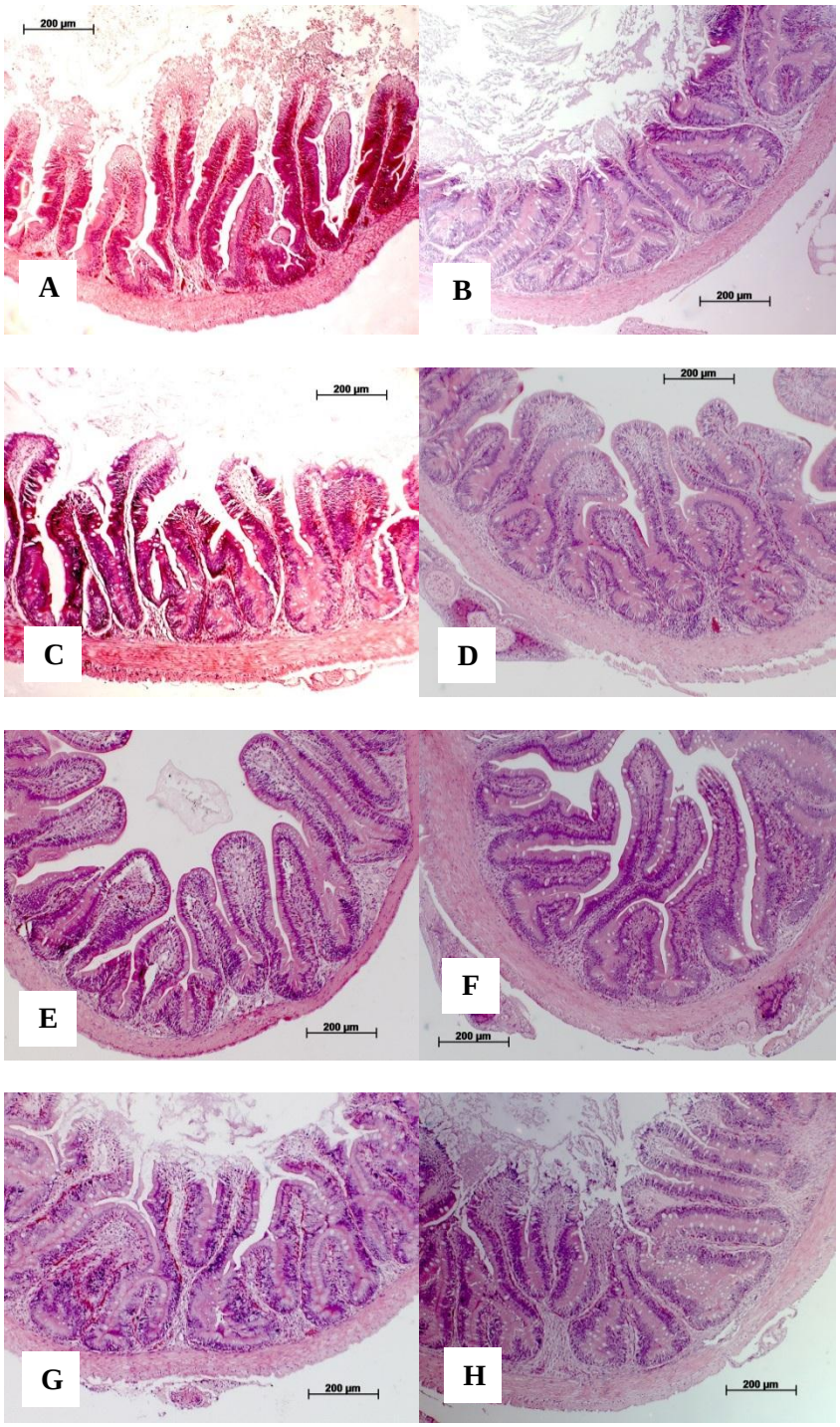


Figure 5.3.1: Intestinal morphology of control sample taken before trial (x 10) (**A**); Villi length of fish fed control diet containing 10% FM (**B**); Proximal intestines taken from tilapia fed 0% FM with enzymes (**C**) and without enzymes (**D**); Comparison of villi sampled from fish cultured with 3% FM diets containing enzymes (**E**) and those without (**F**). Changes in villi structure in fish fed 5% FM diets with supplemented with phytase and xylanase (**G**) and no enzymes (**H**).

Table 5.3.4: Economic analysis of adult tilapia production fed declining FM diets with and without xylanase and phytase

Exp. Diets	Feed Cost (US$ kg^{-1})	FCR	Conversion Cost (US$ kg kg^{-1})	Total FI (kg)	TBI (kg)	Profit Index	ΔU_{FE} (US$ kg^{-1})	ΔU_{Growth} (US$ kg^{-1})	$\Delta U_{FE + G}$† (US$ kg^{-1})
C0FM	0.631	1.89	1.19	7.62	4.03	1.58	(0.092)	(0.035)	-
E0FM	0.633	1.67	1.06	7.61	4.57	1.78	(0.008)	(0.010)	0.098
C3FM	0.638	1.92	1.23	7.62	3.99	1.54	(0.104)	(0.031)	-
E3FM	0.641	1.79	1.15	7.63	4.25	1.63	(0.054)	(0.022)	0.054
C5FM	0.643	1.78	1.14	7.63	4.32	1.66	(0.051)	(0.011)	-
E5FM	0.646	2.09	1.35	7.62	3.74	1.43	(0.172)	(0.017)	(0.119)
C10FM	0.658	1.65	1.09	7.61	4.65	1.77	-	-	-

Conversion rate US$1 = THB 31.91; THB – Thai baht
Total FI – Total feed intake; TBI – Total biomass increase
Exchange rate: US$1 = 31.91 THB (Thai Baht); Average price per kg fish = US$1.25
Conversion cost is the cost per kg weight gain in US$ = Feed cost * FCR
Profit Index (PI) = Value of fish stock/ cost of feed consumed (El-Sayed 1998)
Δ Unit Profit $_{\text{Feed efficiency}} = - \Delta FCR * \alpha^{1} P$ and Δ Unit Profit $_{\text{Growth (size)}} = (1 - (Wf/((Wf - Wi)*(1+\Delta G) + Wi)))*P*\alpha$ (Kankainen et al., 2012)
† Comparison of enzyme supplemented diets against respective controls

5.4 **Discussion**

5.4.1 Effects on Growth Performance

A feed is said to be only as good as its ingredients (Glencross et al. 2007) and in this context, as good as the experimental design. Studies replacing FM with plant-based alternatives have been conducted for a number of aquatic species (Gallagher 1994;Gomes et al. 1995; McGoogan & Gatlin 1997; El-Sayed 1998; Satoh et al. 2003; Koumi et al. 2009), however, the current focus lies with improving diet efficiencies. The objective of the study was to examine the effects of xylanase and phytase on growth in adult tilapia fed plant-based diets containing 0 – 10% FM. Although the effectiveness of phytase and xylanase are mediated by their dietary substrate levels (Selle et al., 2003), in diets supplemented with both enzymes, xylanase should theoretically improve the access of phytase to its substrate (phytate) thereby liberating bound nutrients for growth (Cowieson & Adeola 2005).

Most growth trials generally use juvenile fish due to their fast growth rates, often ignoring the potential dietary effects in the final stages of grow-out and production. Unlike juveniles, adult fish have more developed digestive systems. It is believed that tilapiine species possess the capability of degrading phytate based on localized phytase activity found within their intestinal brush border (Kumar et al. 2012), this, however, requires more research. In support, Lavorgna et al., (2003) showed that tilapia may possess endogenous phytase, however, phytate concentration above 3.5 mM (2.3 g kg^{-1}) inhibited its effect *in vitro*. More recently, it has been found that a particular strain of gut-associated yeast isolated from Nile tilapia also has the ability to produce xylanase (Banejee and Ghosh, 2014).

In the present study, adult tilapia fed diets containing the enzyme cocktail had higher AWG than the un-supplemented feed except at the 5% FM level ($P > 0.05$). The highest ADG value was however observed in tilapia fed C10FM. Unlike the current study, Dias et al., (2012b) found that 200 FXU kg^{-1} (0.308 g kg^{-1}) xylanase significantly improve body weight of Nile tilapia fed plant-protein rich 5% FM diet in line with levels of the control group fed a 10% FM diet. Similarly, Liebert and

Portz, (2005) cited significant effects of phytase supplementation on growth performances in Nile tilapia.. FCR was the lowest (1.66) in the C10FM fed tilapia, potentially due to higher nutrient digestibility (Chapter 4). This was similar to FCR reported in red tilapia fed basal diets containing 10% FM, SBM, RB and cassava by Tudkaew et al. (2008). Surprisingly, fish fed E0FM recorded a comparatively low FCR of 1.67. The enzyme supplemented diets had better FCRs compared to the controls except E5FM. Nevertheless, there were no significant treatment effects on FCR, ADG and SGR. C3FM and E5FM fed fish recorded the lowest SGR of 1.12 % day^{-1}. In support, Moreira et al. (2003) reported no effects on FI, FCR and weight gain in swine fed diets supplemented with 253 – 1748 U phytase. On the contrary, Kim et al., (2008) found combined supplementation of xylanase and phytase significantly improved FCR, phytase only improved ADG while xylanase only had no effect.

Despite inconsistencies in the mode of action of xylanase (Bedford & Schulze 1998), positive improvements in animal performance have been reported by Selle et al., (2003), Silversides et al., (2006), Tapingkae et al., (2008) and Selle et al., (2009). PER ranged from 1.87 – 2.47 with no treatment related improvements. These were lower than values reported by Dias et al., (2012b) who found significant improvements in PER in Nile tilapia (25.9g) fed 5% and 10% FM diets (32,4% CP; *dry weight basis)* supplemented with 100 – 200 FXU kg^{-1} xylanase. Phytase and xylanase have been incorporated in aquafeeds but very few studies have focused on the synergy of the enzymes in terms of growth performance. Since the interactive benefits of these enzymes on growth were not statistically clear from this experiment, further research is needed to validate their potential in tilapia for grow-out and fattening.

5.4.2 Effects of Enzymes on Intestinal Morphology

Sinha et al. (2011) noted specific research gaps related to the effects of NSPs in fish. Very little information is present on arabinoxylans in omnivorous diets and the potential counteractive effects of xylanase on this ANF. It is acknowledged that measuring villi length is subject to some degree of error (Heikkinen et al. 2006) nevertheless, the current findings suggest that there were no dietary

trend related to FM levels or enzyme supplementation, contrary to Borgeson et al., (2006). It is theorized that adult tilapia may be more tolerant of NSP compared to juvenile tilapia. It has been proposed that tilapia's intestinal bacteria has the ability to ferment carbohydrates (Ng & Romano 2013), however, it was evident that tilapia may still be sensitive to NSP. Overall histological changes of the proximal intestine appeared similar within groups, however, fish fed the control diets had slightly thicker *lamina propria*, more vacuoles and goblet cells; characteristics of salmonid enteritis (Evans et al. 2005; Heikkinen et al. 2006; Uran et al. 2008).

5.4.3 Cost Benefits of Multi-enzyme Supplementation

The cost of feed decreased with declining FM, as the marine commodity was substituted by higher inclusion of lower cost plant-based ingredients. FM cost at least 0.376 US$ kg^{-1} more than SBM which was the most expensive plant-based ingredient used. The economic benefits of substitution were immediately observed. On the contrary, supplementation of phytase and xylanase added to the cost per unit feed. Assuming all other feed production-related cost (electricity *etc.*) were similar, the cost per kg of feed was higher due to enzyme inclusion. Nevertheless, the returns on investment per kg whole fish produced were higher compared to control diets. In the animal production industry like aquaculture where profit margins are often quite narrow, even minor improvements as those conferred by these enzymes can be substantial (Wilson 2014).

Iyayi and Davies (2005) reported the cost of feed per kg weight gain was lower in broilers fed enzyme supplemented diets at the starter phase but no benefits were realised at the finisher phase. This was potentially due to less developed digestive systems and lower endogenous enzyme activities in younger animals. In support, Novak et al. (2008) using a similar enzyme cocktail reported significant cost savings in pullets after 126 days compared to a commercial control. Conversion of biological efficiencies to economic benefits in this study support the potential and importance of enzymes in aquatic diets. While highlighting possible gain to farmers who would be the potential end-users of low FM sustainable feeds.

5.4.4 Conclusions

The study confirmed to some extent the economic potential of utilising commercial phytase and xylanase in sustainable aquafeeds formulated from lower-cost plant-based ingredients. Inconsistent yet incremental increases were observed in growth performance variables but there were no significant effects of the enzymes on gut morphology. Economic benefits coupled with biological gains may support the use of enzyme supplementation and the potential of lower-cost plant-based ingredients in grow-out diets for adult tilapia. However, additional research is warranted.

Chapter 6

Effects of Combining Protease with Xylanse and Phytase on Digestibility and Growth in Tilapia Fed 2% FM Diets

6.1 **Introduction**

Aquaculture's dependency on feed inputs from limited fisheries sources, FM and FO, is still a major sustainability challenge particularly as the industry intensifies (Tacon & Metian 2008). Early FM replacement studies initially scoped single ingredients as partial replacers but then these studies developed further as multiple-ingredient mixtures, primarily of plant origin, became popular (Appler & Jauncey 1983; Shiau et al. 1987; Olvera et al. 1988; El-Sayed 1998; Shiau et al. 1990; Gomes et al. 1995; Borgeson et al. 2006; Madalla 2008; Khan et al. 2013). The consistent and repeated conclusions of these studies were the negative effects on growth and feeding due to reduced FI and poorer digestibility. These outcomes were due primarily to comparatively lower protein quality of plant-based ingredients and associated ANFs. Highly specific commercial enzymes can be useful in breaking down complex feed matrices, improving digestibility and neutralizing ANF effects (Munir & Maqsood 2012). This becomes advantageous when applied to low FM diets if production efficiencies are to be maintained in order to meet the increasing global seafood demand.

Though commercial enzyme application in animal feeds has been practiced for over 20 years (Walk 2009; Choct 2006), the momentum has been particularly slow in terms of their adaption for aquafeed formulation. In the last decade, xylanase and phytase have been successfully applied to improve productive value and efficiency of monogastric feeds. When the two enzymes are used in combination, xylanase is thought to increase the access of phytase to its substrate hereby improving their synergistic effects on nutrient digestibility, particularly P, and also increase dietary energy availability (Cowieson & Adeola 2005). However, their secondary mode of action on protein and lipid digestibility remain slightly inconsistent and highly debated (Bregendahl 2007).

In 2012, despite protease accounting for 60% of global enzyme production used for industrial purposes, for the animal feed industry it merely represented for 5% of the feed enzyme market (Smith 2014). Exogenous proteases were initially designed to counteract PIs. On the other hand, the protease used for this experiment was designed to complement the animal's own endogenous supply improving the efficiency of protein hydrolysis over a longer section of the GI tract (Angel et al. 2010). Most protease studies have investigated the effects on PI, particularly trypsin, but rarely have they targeted its extra-proteinaceous effects when combined with other exogenous enzymes in fish. In addition to that, most studies involving animal feed protease generally make it difficult to assess the direct impact of protease on animal growth due to the complexity of commercial enzyme cocktails (Iyayi & Davies 2005; Ogunkoya et al. 2006; Novak et al. 2008; Ayhan et al. 2008; Angel et al. 2010; Dalsgaard et al. 2012). Nevertheless, some studies have demonstrated the potential of protease when applied alone or in defined combinations (Walk 2009). Other authors have acknowledged, however, that more research is needed to determine the ideal protease dosage for growth, particularly in fish, and the cooperativity of proteases with phytase and other carbohydrases such as xylanase (Dalsgaard et al. 2012; Simbaya et al. 1996).

In view of higher dietary inclusion of plant-based proteins driven by the volatility/supply of global FM supply and higher demand for aquaculture products, mean FM inclusion for tilapia diets is projected to be fall to 2% in 2015 and be further reduced to 1% by 2020 (Tacon & Metian 2008). On the contrary, for example FM inclusions are still above 10% in Thailand and Mexico, two of the world's leading tilapia producers (González-Félix et al. 2010; Henriksson et al. 2014). The present FM replacement/enzyme supplementation study was therefore designed to evaluate the additive, semi-additive or non-additive effect of protease in combination with xylanase and phytase on digestibility, growth and economic performance in tilapia fed a 2% FM diet.

6.2 **Material and Methods**

6.2.1 Experiment 1 – Growth

Two isonitrogenous (280 g kg^{-1} CP) and isoenergetic (18 MJ kg^{-1}) basal control diets were formulated to contained FM at two inclusion levels (2% and 10%). The dry ingredients were premixed on-farm then transferred to Kasertsart University for homogenizing and pelleting. The 2% FM diet was supplemented with xylanase (0.385 g kg^{-1}) and phytase (0.075 g kg^{-1}) at fixed dosages without and in combination with protease at two levels (0.2 g kg^{-1} and 0.4 g kg^{-1}) to form the enzyme supplemented diets (Table 6.2.1). Proximate dietary composition and targeted ANFs (arabinoxylan and phytate-P) content are presented in Table 6.2.2. 600 sex-reversed red tilapia (71.1 ± 10.9 g STD) were randomly stocked in 30 $1m^3$ nylon cages (1.42 kg m^{-2}) suspended within an unfertilised pond and fed the five dietary treatments twice daily to apparent satiation for 12 weeks. Each treatment was randomly assigned six replicates.

The fish were anaesthetised, weighed and measured individually at the start of the experiment and every 28 days (Section 2.2.9). Proximal intestine samples (5 fish) were collected from the starter population and fixed in 10% formalin, then from six fish per treatment at the end of the trial to assess changes in villi structure. Liver samples were also collected and fixed until processed for histomorphology (Section 2.2.12). Water quality parameters was monitored weekly (Section 2.2.10)

Table 6.2.1**:** Formulation of experimental tilapia grow-out diets (as fed basis)

Treatments	2% FM NC	2% FM NOPRO	2% FM LOPRO	2% FM HIPRO	10% FM PC
Ingredients (g kg^{-1})					
SBM (45% CP)	499.0	499.0	499.0	499.0	370.0
MA (7.8% CP)	82.9	82.9	82.9	82.9	127.5
FM (56% CP)	20.0	20.0	20.0	20.0	100.0
CM (1.8% CP)	150.0	150.0	150.0	150.0	150.0
RB (7% CP)	200.0	200.0	200.0	200.0	200.0
Vegetable oil[1]	20.0	20.0	20.0	20.0	30.0
Dicalcium phosphate	10.0	10.0	10.0	10.0	5.0
Vitamin premix[2]	10.0	10.0	10.0	10.0	10.0
Lysine[3]	5.4	5.4	5.4	5.4	5.6
Methionine[3]	2.7	2.7	2.7	2.7	1.9
Protease[4]	-	-	0.200	0.400	-
Xylanase[4]	-	0.385	0.385	0.385	-
Phytase[4]	-	0.075	0.075	0.075	-
Enzyme Recovered (g kg^{-1})*					
Protease	NT	-	0.168	0.348	NT
Xylanase	NT	0.311	0.363	0.356	NT
Phytase	NT	0.085	0.098	0.109	NT

[1] Palm Oil – 1 ml contains 0.06 g Linolenic acid (Omega 3), 0.53g Linoleic acid (Omega 6) and 0.23 g Oleic acid (Omega 9)
[2] Vitamin/Mineral Mix (per kg) – Cholecalciferol (D3) 9000 IU; Tocopherol (E5) 187 mg; Menadione (K3) 19 mg; Thiamine (B1) 52 mg; Niacin 130 mg; Pantothenic acid 93 mg; Pyroxidine (B6) 46 mg; Biotin 450 mcg; Folic acid 10 mg; Cobalamin (B12) 600 mcg; Riboflavin (B2) 97 mg; Retinol (A) 36,000 IU; Inositol 225 mg; Ascorbic acid (C) 69,800 mg; Mn 105 mg; Zn 90 mg; Fe 90 mg; Cu 9 mg; Co 450 mcg; Na 117 mg; Ca 219 mg,; I 1.8 mg, K 3,600 mg; Mg 1900 mg.
[4] Royal DSM France, RONOZYME; * Enzyme recovery; Biopract GmbH
NC – negative control, NOPRO – no protease inclusion, LOPRO – low protease inclusion, HIPRO – high protease inclusion, PC – positive control; NT – not tested;

Table 6.2.2: Proximate composition of basal diets based on chemical analysis (as fed basis)

Proximate Composition ($g\ kg^{-1}$)	2% FM NC	10% FM PC
DM	951.8	956.5
CP	279.8	291.6
Lipid (EE)	19.0	32.5
CF	106.3	104.6
Carbohydrate	580.1	553.3
NFE	473.8	448.7
Ash	72.9	79.2
Total-P	8.1	13.1
GE ($MJ\ kg^{-1}$)	18.7	19.2
Antinutrients ($g\ kg^{-1}$)		
Phytate-P	2.4	2.1
Arabinoxylan	0.58	0.53

6.2.2 Experiment 2 – Digestibility and Nutrient Utilisation

This experiment was conducted to evaluate the digestibility of the diets owing to limitation of faecal collection from a pond-based system (Experiment 1). Basal formulations were adjusted by adding chromic oxide as an inert marker for determination of apparent digestibility coefficients (ADC; Table 6.2.3). Fifteen 650L (water volume) indoor static tanks (1.22 x 1.01 x 0.80 m) were each stocked with 10 adult red tilapia (113.2 ± 7.3 g STD), stocking density 1.74 $g\ L^{-1}$. The five dietary treatments were assigned to triplicate groups and fed three times daily by hand to apparent satiation for eight weeks. They were acclimated for one week to tank conditions on a commercial diet followed by two weeks on the experimental diets prior to faecal collection. Floating faeces were collected manually one hour following the 12 noon feeding daily (between weeks 2 and 8). Samples were pooled per tank and frozen at -14^{o}C until analysed. Water quality was monitored every other day for temperature and DO, and weekly for pH, NH_3, NO_2, NO_3 and alkalinity (Section 2.2.10). Tanks

were exposed to natural photoperiod (~12 hr light and 12 hr darkness) and water exchange rates averaged 50% $tank^{-1}$ $week^{-1}$. 10 fish were sacrificed at the start of Experiment 2 for carcass composition and proximate analysis and five fish per treatment at the end. Carcasses were pooled per treatment for analysis.

Table 6.2.3**:** Diet formulation for digestibility experiment

	2% FM	10% FM
Ingredients (g kg^{-1})		
SBM	499.0	370.0
MA	77.9	122.5
FM	20.0	100.0
CM	150.0	150.0
RB	200.0	200.0
Vegetable oil	20.0	30.0
Dicalcium phosphate	10.0	5.0
Vitamin premix	10.0	10.0
Chromic oxide	5.0	5.0
Lysine	5.4	5.6
Methionine	2.7	1.9
Proximate Composition (g kg^{-1} as fed)		
DM	958.8	954.0
CP	249.4	281.9
Lipid (EE)	15.3	36.8
CF	99.5	88.8
Carbohydrate	620.9	557.2
NFE	521.4	468.4
Ash	73.3	78.2
Total-P	8.0	14.1
Chromic oxide	5.2	3.6
GE (MJ kg^{-1})	18.8	19.2

6.2.3 Chemical Analysis

Proximate analyses of the raw ingredients, experimental diets, fish carcass and faecal material were performed using standard methods (AOAC 1990; AOAC 2005) (Section 2.2.3). GE was determined using the analytical methods for oxygen bomb calorimeters. Chromic oxide levels were analysed using photometry according to Bolin et al., (1952). Dietary arabinoxylan (NSP) was quantitated indirectly from D-xylose content of the plant ingredients using spectrometry.

6.2.4 Histomorphology

Fixed tissue samples were processed and thin sections (5µm) of rehydrated blocks were mounted and stained using H&E (Section 2.2.12). 10 well oriented villi from five sections per treatment were measured and mean calculated after Borgeson et al., (2006). Imaging and measurements were done using light microscopy and AxioVision software.

6.2.5 Calculations

Apparent digestibility coefficient (ADC) and Unit profit (U) were calculated using the following equations**.** The bio-economic profit model was adapted from Kankainen et al. (2012).

$$ADC = [1 - ((Cr_2O_{3diet}/Cr_2O_{3faeces}) * (\text{Conc. or \% Nutrient}_{faeces}/\text{Conc. or \% Nutrient}_{diet}))]*100$$

$$\Delta U_{\text{Feed efficiency}} = -\Delta FCR * \alpha^1 P$$

$$\Delta U_{Growth(size)} = (1- (W_f/((W_f - W_i) * (1 + \Delta G) + W_i))) * P * \alpha \text{ (See Section 2.2.13)}$$

6.2.6 Statistical Analysis

AWG, TGC, SGR, FI, FCR, PER and survival were used as performance indicators. Data were assessed for normality and homogeneity of variance using K-S and Levene tests. Treatment means were subjected to orthogonal comparison using one-way ANOVA and significant differences further analysed using DMRT. Survival data were transformed using arc-sine calculations prior to analysis. Weight and survival data did not conform to parametric assumptions and were analysed using KW and Mann-Whitney *post hoc*. Mean values are reported ± STD or SEM. Significance levels are

reported at $P < 0.05$. All statistical tests were performed using SPSS® version 19 and 21. Note: Although the experimental design was semi-structured, polynomial regression was not used because the trial was not a typical dose-response experiment.

6.3 **Results**

6.3.1 Experiment 1

6.3.1.1 Water Quality

Average water temperature (pond) was 31.35 ± 0.74 ^{o}C, slightly above optimal growth range. Average DO reading taken at dawn (6 am) was 3.13 ± 0.30 mg L^{-1} and average pH was 7.66 ± 0.24. Average values for NH_3, NO_2, NO_3 and alkalinity were 0.90 ± 0.18, 0.06 ± 0.08, 0.0 and 99.8 ± 12.9 mg L^{-1} respectively. Water quality parameters were stable throughout the trials and did not significantly affect growth.

6.3.1.2 Growth Performance

Final body weight and length were significantly different between treatments (Table 6.3.1; $P < 0.05$). Treatments were equally affected in terms of mortality (5.0 – 7.5%) with no treatment-related effects due to enzyme supplementation ($P > 0.05$). Fish fed the 10% FM PC diet showed the highest percentage weight gain (410% or 288.2 g) followed the by 2% FM diet with low protease inclusion (LOPRO; 337% or 240.3 g) (Figure 6.3.1). Though enzyme supplementation had a positive effect on the performance of the 2% FM diet, there were no differences between diets supplemented with xylanase-phytase only (NOPRO) and those including protease (LOPRO and HIPRO) ($P > 0.05$).

TGC and SGR both improved with enzyme addition over the NC ($P < 0.05$) but no differences were found between the enzyme supplemented treatments ($P > 0.05$). The enzymes had a positive impact on feed intake though protease addition to the cocktail had no significant effect at either level. Nevertheless fish fed the LOPRO diet had a similar intake to the PC ($P < 0.05$). On the contrary, the feeding efficiency for the PC diet was similar to both NOPRO and HIPRO diets though feed intakes of the latter diets were lower ($P < 0.05$). Additionally, FCR trends showed the HIPRO diet having the

best conversion after 4 weeks (1.49) (Figure 6.3.1). PERs were not significantly different between treatments (Table 6.3.2).

Table 6.3.1: Mean body indices and survival of tilapia fed low FM diets after 12 weeks.

Experimental Diets	Body Weight (g)		Body Length (cm)		Survival (%)
	Initial	Final	Initial	Final	
NC	71.18 ± 1.10	261.45 ± 4.35[a]	16.18 ± 0.08	23.84 ± 0.12[a]	95.0 ± 1.80
NOPRO	71.52 ± 0.92	306.69 ± 5.87[b]	16.10 ± 0.07	24.72 ± 0.16[b]	92.5 ± 2.50
LOPRO	71.13 ± 1.03	311.45 ± 5.81[b]	16.19 ± 0.08	24.88 ± 0.15[b]	93.3 ± 2.10
HIPRO	71.35 ± 1.04	308.49 ± 5.34[b]	16.17 ± 0.08	24.82 ± 0.15[b]	92.5 ± 2.10
PC	70.20 ± 0.89	358.40 ± 5.40[c]	16.02 ± 0.07	25.98 ± 0.14[c]	93.3 ± 2.80
MSE	0.44	2.73	0.04	0.71	1.48
F or χ^2 *value*	1.002[†]	130.059[†]	0.793[§]	27.394[§]	0.798[†]
P-value	0.939 [NS]	0.00**	0.530 [NS]	0.00**	0.939[NS]

Mean values ± SEM of six replicates; MSE – Pooled standard error of the means
Mean with the different letters are significantly different (P < 0.05); NS – Not significant, * Significant, ** Highly significant; [§]ANOVA – *F* value; [†] Kruskal Wallis – χ^2 value

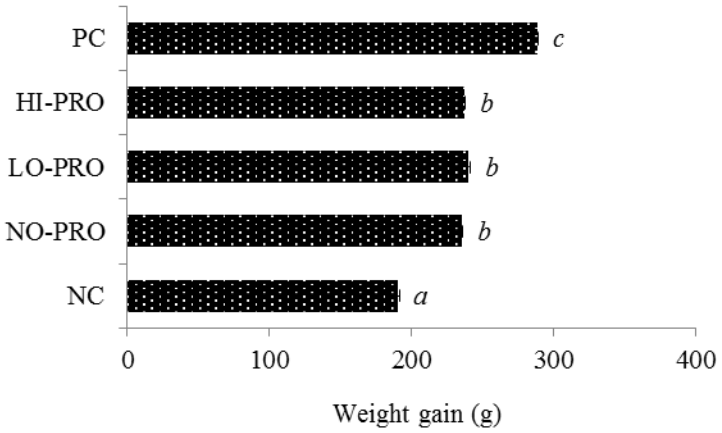


Figure 6.3.1: Average weight gain comparison of tilapia fed low FM diets after 12 weeks (Mean values ± SEM of six replicates)

Table 6.3.2**:** Performance comparison of tilapia fed 2% FM diets with and without enzymes to 10% FM industry control

Experimental Diets	Average Daily Gain (g $fish^{-1}$)	Thermal Growth Coefficient	Specific Growth Rate (% day^{-1})	Average Daily Feed Intake (g day^{-1})	Feed Conversion Ratio	Protein Efficiency Ratio
NC	2.24 ± 0.12^{a}	0.085 ± 0.003^{a}	1.53 ± 0.04^{a}	4.69 ± 0.19^{a}	2.09 ± 0.07^{a}	1.63 ± 0.05
NOPRO	2.77 ± 0.10^{b}	0.098 ± 0.002^{b}	1.71 ± 0.03^{b}	5.37 ± 0.12^{b}	1.94 ± 0.05^{ab}	1.75 ± 0.05
LOPRO	2.83 ± 0.11^{b}	0.100 ± 0.003^{b}	1.73 ± 0.04^{b}	5.58 ± 0.14^{bc}	1.97 ± 0.10^{a}	1.73 ± 0.09
HIPRO	2.79 ± 0.07^{b}	0.099 ± 0.002^{b}	1.72 ± 0.02^{b}	5.26 ± 0.17^{b}	1.88 ± 0.07^{ab}	1.81 ± 0.06
PC	3.39 ± 0.08^{c}	0.113 ± 0.002^{c}	1.92 ± 0.02^{c}	5.89 ± 0.15^{c}	1.73 ± 0.06^{b}	1.89 ± 0.06
MSE	0.08	0.004	0.03	0.09	0.04	0.03
ANOVA						
F-value	17.34	19.69	21.14	8.28	3.47	2.34
P-value	0.00**	0.00**	0.00**	0.00**	0.02*	0.08^{NS}

Mean values ± SEM of six replicates; MSE – Pooled standard error of the means
Mean with the different letters are significantly different ($P < 0.05$); NS – Not significant, * Significant, ** Highly significant

6.3.1.3 Bio-economics

Assuming all production costs were similar, enzyme supplementation had positive impacts on profitability of fish production (Table 6.3.3). Compared to the NC, it cost 0.002, 0.005 and 0.011 US$ more to produce 1 kg of the NOPRO, LOPRO and HIPRO feeds respectively. However based on FCRs, it cost 0.09, 0.06, 0.11 US$ less to produce 1 kg of live fish respectively. Changes in unit profit due to improvements to feeding efficiencies and growth (in weight gain) increased with protease inclusion. Benchmarked against the NC, the HIPRO treatment (of the three enzyme supplemented diets) produced the highest level of change in net profit due to gains in feeding efficiency (0.066 US$ kg^{-1}), however, the LOPRO treatment showed better improvements in terms of growth (0.063 US$ kg^{-1}). Benchmarked against the PC, profitability decreased even with enzyme supplementation. Feeding an un-supplemented 2% FM diet (NC) was US$0.258 less profitable per kg of tilapia produced compared to the industry-like 10% FM standard.

6.3.1.4 Histomorphology – Intestine and Liver

Villi length increased with the addition of exogenous enzymes compared to the NC (χ^2 = 95.375, df = 5, P = 0.00**) (Figure 6.3.2). However, when the treatments were compared to samples taken from the starter population at the beginning of the experiment, there was no difference between the fish fed LOPRO diet only ($P < 0.05$). Villi length improved in PC fed tilapia. There were also no differences between the villi lengths of fish that consumed PC and those fed LOPRO diets ($P < 0.05$). Physical examination of the gut cavity showed higher fat accumulation around the intestines of fish fed both control diets (without enzymes) particularly that of the 10% FM diet.

Table 6.3.3**:** Cost benefit analysis of feed and tilapia production without and without exogenous enzymes

Exp. Diets	Feed Cost (US$ kg-^{1})	FCR (kg kg^{-1})	Conversion Cost (US$)	Total Feed (kg)	Total Biomass Gain (kg)	NC ΔU_{FE} (US$ kg^{-1})	NC ΔU_{G} (US$ kg^{-1})	PC ΔU_{FE} (US$ kg^{-1})	PC ΔU_{G} (US$ kg^{-1})
NC	0.642	2.09	1.34	45.35	29.81	-	-	(0.134)	(0.124)
NOPRO	0.644	1.94	1.25	50.68	34.09	0.046	0.058	(0.078)	(0.062)
LOPRO	0.649	1.97	1.28	52.99	34.93	0.037	0.063	(0.090)	(0.055)
HIPRO	0.653	1.88	1.23	49.51	34.24	0.066	0.060	(0.057)	(0.059)
PC	0.660	1.73	1.14	55.93	40.17	0.114	0.110	-	-

Unit scaled up to kg to offer ease of comparison to a commercial operation.
Exchange rate: US$1 = 31.91 THB (Thai Baht); Average price per kg fish = US$1.25
Conversion cost is the cost per kg weight gain in US$ = Feed cost * FCR

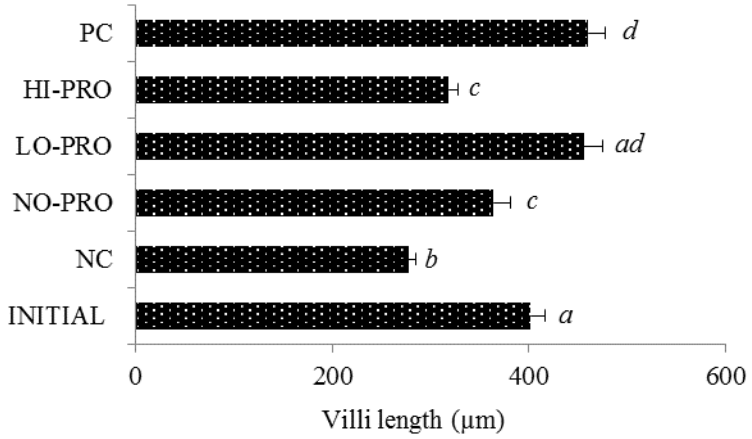


Figure 6.3.2: Villi length comparison of tilapia from the starter population and five treatments after 12 weeks (Mean values ± SEM of six replicates)

Light photomicrographs highlight changes in villi length and gut structure of cultured tilapia (Figure 6.3.3). Compared to villi from the parent stock (Figure 6.3.3 A), Figure 6.3.3 B showed distinct shortening of intestinal villi taken from fish fed NC diet. The LOPRO diet was the only ES diet where fish showed significant elongation of the proximal villi. Figure 6.3.3 F also illustrated increased lipid vacuolation of the PC sample. There were no significant histological changes observed between livers from the five treatments (Figure 6.3.4 A-E).

6.3.2 Experiment 2

6.3.2.1 Performance and Fish Composition

Experiment 2 was used to assess the digestibility of the five dietary treatments**.** General growth performances of the cultured tilapia is presented in Table 6.3.4, though less important in this experiment. Average water temperature, DO and pH were 27.31 ± 1.35 ^{o}C, 7.78 ± 0.99 mg L^{-1}, 7.82 ± 0.50 respectively. Average values for NH_3, NO_2, NO_3 and alkalinity measurements were 0.75 ± 1.01, 2.64 ± 4.97, 26.71 ± 19.88 and 73 ± 16.53 mg L^{-1} respectively. All parameters remained within acceptable ranges for tilapia production (DeLong et al. 2009). Nutrient profiles of carcass are detailed in Table 6.3.5.

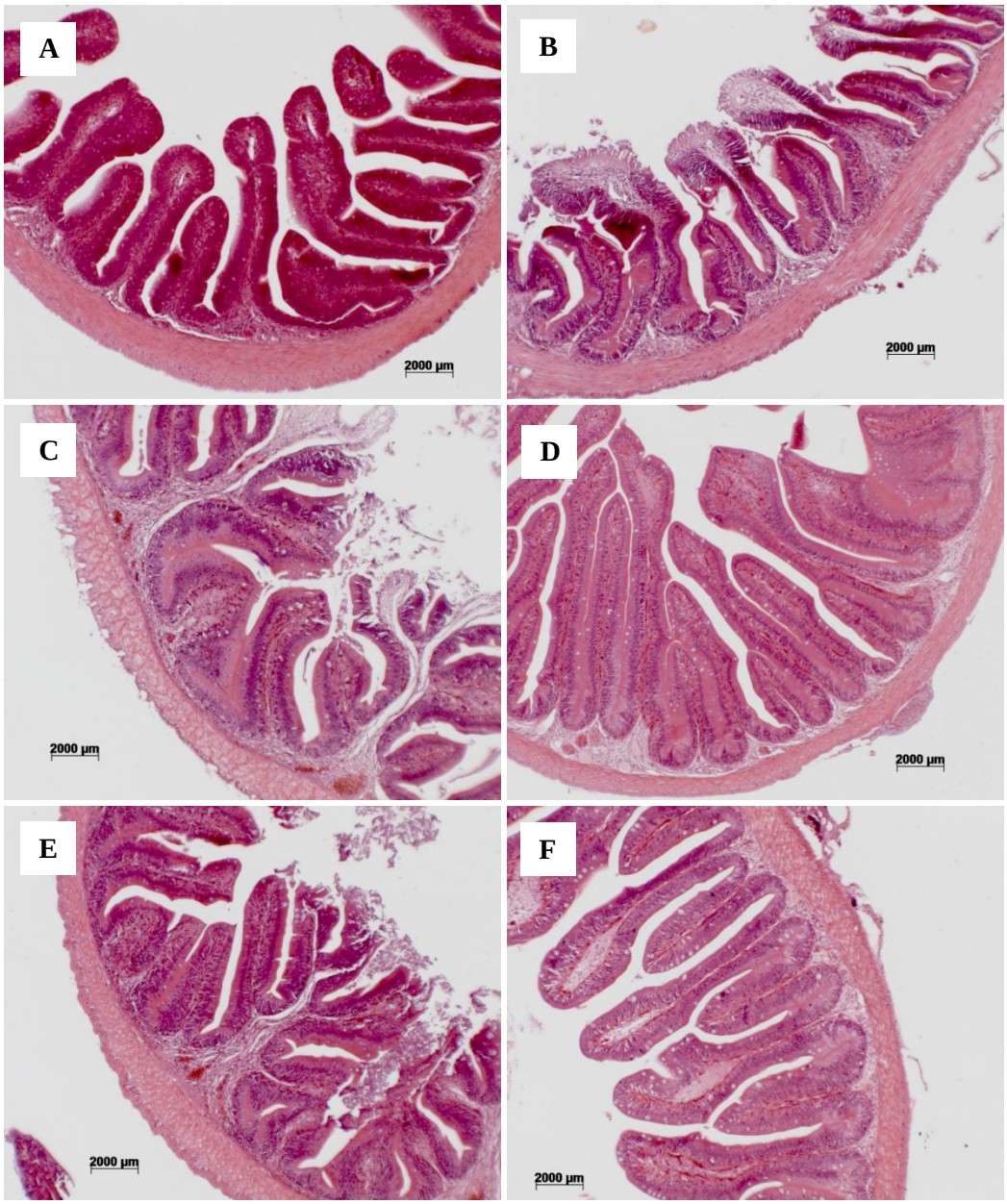


Figure 6.3.3: Photomicrographs of the proximal intestine of red tilapia fed low FM diets with and without exogenous enzymes (protease, xylanase and phytase). **(A)** Control samples taken prior to experiment 1. **(B)** NC – negative control 2% FM without enzymes. **(C)** NOPRO – 2% FM with XYL/PHY. **(D)** LOPRO – 2% FM with XYL/PHY and low protease inclusion (0.2 g kg^{-1}). **(E)** HIPRO – 2% FM with XYL/PHY and high protease inclusion (0.4 g kg^{-1}). **(F)** PC – positive control 10% FM without enzymes. (x10)

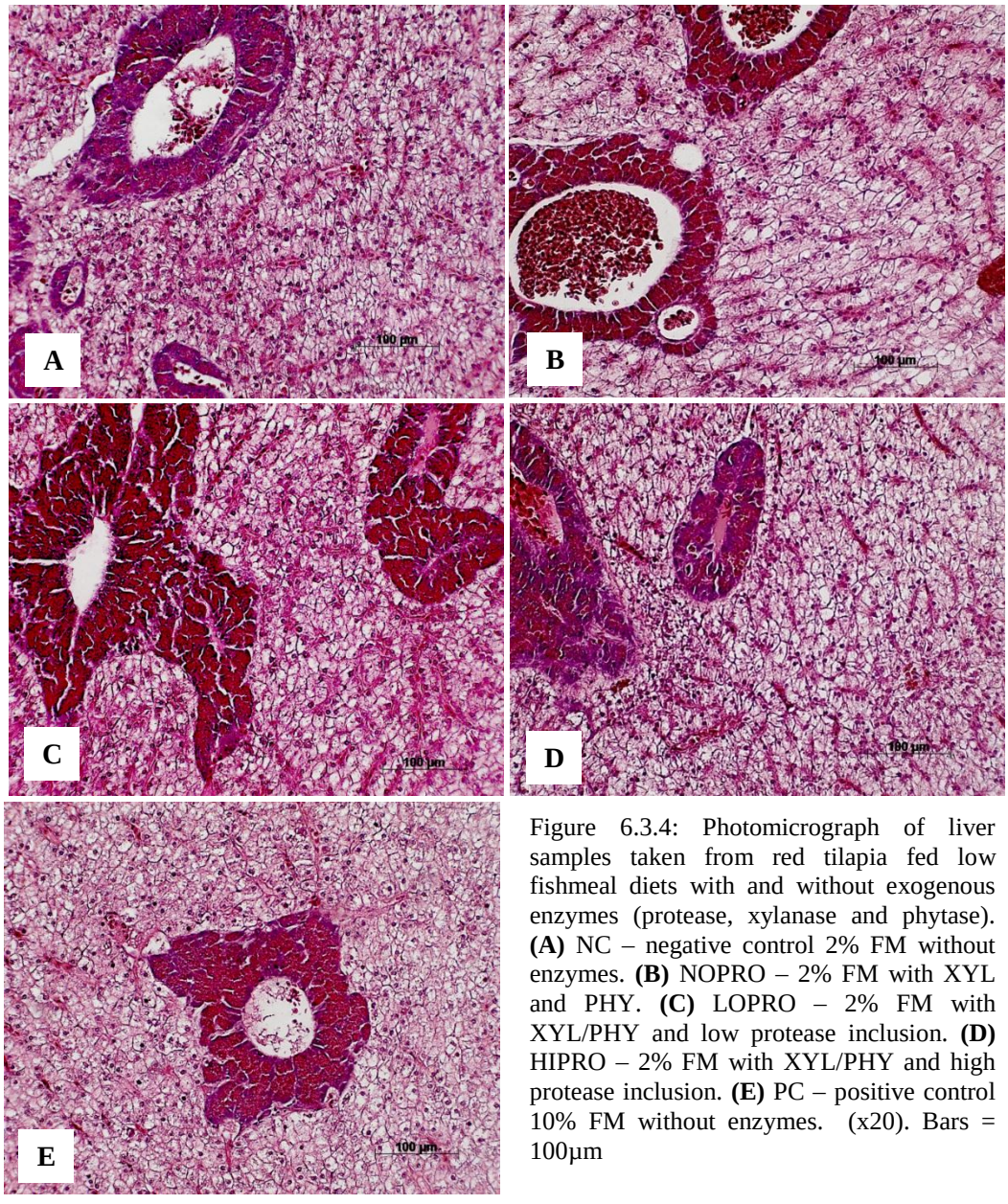


Figure 6.3.4: Photomicrograph of liver samples taken from red tilapia fed low fishmeal diets with and without exogenous enzymes (protease, xylanase and phytase). **(A)** NC – negative control 2% FM without enzymes. **(B)** NOPRO – 2% FM with XYL and PHY. **(C)** LOPRO – 2% FM with XYL/PHY and low protease inclusion. **(D)** HIPRO – 2% FM with XYL/PHY and high protease inclusion. **(E)** PC – positive control 10% FM without enzymes. (x20). Bars = 100µm

Table 6.3.4: Growth performance of tilapia fed experimental diets containing graded level protease

Exp. Diets	**IBW** (g)	**FBW** (g)	**FCR**	**Protein In** (g fish^{-1})	**PER**	**Survival** (%)
NC	112.83 ± 1.21	206.28 ± 3.99	1.89 ± 0.10	43.94 ± 4.24	2.13 ± 0.10	86.7 ± 8.8
NOPRO	113.37 ± 1.08	230.43 ± 10.19	1.78 ± 0.07	51.87 ± 5.42	2.26 ± 0.09	80.0 ± 5.8
LOPRO	113.20 ± 1.26	208.64 ± 7.91	1.91 ± 0.28	45.31 ± 2.37	2.11 ± 0.29	76.7 ± 8.8
HIPRO	113.07 ± 1.34	217.61 ± 6.57	1.82 ± 0.12	47.38 ± 2.56	2.21 ± 0.15	80.0 ± 10.0
PC	113.87 ± 1.77	253.39 ± 8.76	1.52 ± 0.01	59.76 ± 3.81	2.33 ± 0.02	86.7 ± 6.7
MSE	*0.17*	*8.64*	*0.07*	*2.86*	*0.04*	*2.00*

Mean values ± SEM of three replicates; MSE – Pooled standard error of the means
IBW – Initial body weight; FBW – Final body weight; FCR – Feed conversion ratio; Protein In is based on average feed intake per fish; PER = Protein Efficiency Ratio

Table 6.3.5**:** Proximate composition of fish carcass (g kg^{-1} wet weight basis)

Treatments	**DM**	**CP**	**CL**	**Ash**	**P**	**Energy***
Initial	351.7 ± 2.2	194.5 ± 1.5	88.6 ± 0.0	55.5 ± 0.3	8.5 ± 0.0	7.83 ± 0.03
NC	338.5 ± 2.6	189.1 ± 1.9	102.0 ± 0.6	38.5 ± 0.2	5.7 ± 0.3	8.16 ± 0.08
NOPRO	282.6 ± 2.6	142.4 ± 1.0	86.3 ± 1.2	36.0 ± 0.8	5.3 ± 0.2	6.50 ± 0.04
LOPRO	332.8 ± 0.5	176.7 ± 0.8	102.3 ± 0.6	44.1 ± 0.1	6.6 ± 0.0	8.10 ± 0.05
HIPRO	332.9 ± 0.5	180.4 ± 0.8	102.3 ± 0.1	43.7 ± 0.0	5.9 ± 0.0	7.96 ± 0.02
PC	282.9 ± 1.9	154.0 ± 1.0	77.5 ± 0.6	38.6 ± 0.2	4.8 ± 0.4	6.70 ± 0.05

* Energy given as MJ kg^{-1}; DM – Dry matter; CP – Crude protein, CL – Crude lipid (ether extract); CF – Crude fibre; P – Phosphorus

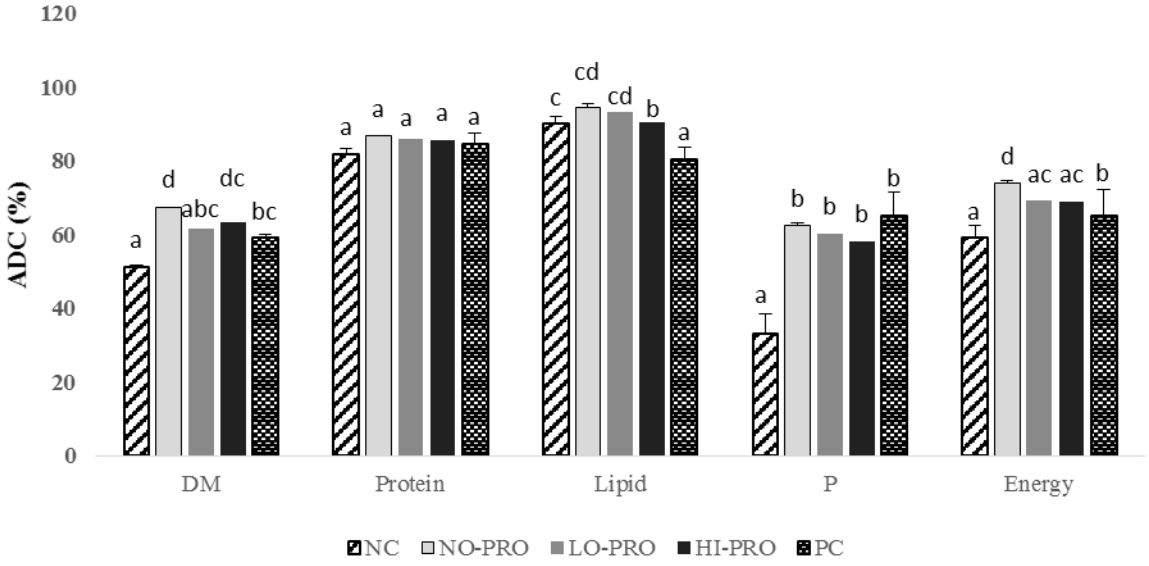
6.3.2.2 Apparent Digestibility

Table 6.3.6 shows the proximate composition of the faecal material by treatment. Effects of enzyme supplementation on digestibility showed overall improvements in ADC values when compared with both NC and PC diets (Figure 6.3.5). Nevertheless, there was no marked improvement in protein digestibility due to protease; differences in DM digestibility were, however, significant. Compared to NC and PC, lipid and energy digestibility improved with exogenous xylanase and phytase, tilapia fed the NOPRO diet had the highest ADC values of 94.4% and 73.9% respectively, suggesting no additive effect of protease on these coefficients. P digestibility increased with the addition of enzyme cocktail, NOPRO P digestibility improved by 29.3% ($P > 0.05$).

Table 6.3.6 Proximate composition of faecal material (g kg^{-1} dry matter basis)

Exp. Diets	CP	CL	CF	P	Cr_2O_3	Energy
NC	97.2 ± 0.3	3.3 ± 0.9	252.8 ± 0.5	11.4 ± 0.5	13.3 ± 1.1	15.75 ± 0.11
NOPRO	104.8 ± 0.5	4.0 ± 0.6	248.5 ± 2.1	8.9 ± 0.1	16.6 ± 0.1	15.25 ± 0.40
LOPRO	95.0 ± 0.2	4.4 ± 0.7	260.6 ± 2.4	8.7 ± 0.3	15.5 ± 0.2	15.85 ± 0.06
HIPRO	102.0 ± 0.7	6.8 ± 0.1	293.4 ± 1.1	9.0 ± 0.1	15.1 ± 0.1	16.0 ± 0.07
PC	111.2 ± 0.1	18.3 ± 1.1	279.8 ± 1.2	12.6 ± 0.3	9.2 ± 0.1	16.31 ± 0.10

Mean values of 3 replicates; MSE – Pooled standard error of the means; Mean in the same column with the different letters are significantly different (P < 0.05); NS – Not significant, * Significant, ** Highly significant



KW	DM	Protein	Lipid	P	Energy
χ^2 value	6.779	9.733	7.084	10.133	9.743
P value	*P < 0.05*	*P > 0.05*	*P < 0.05*	*P < 0.05*	*P < 0.05*

Figure 6.3.5: Apparent digestibility coefficients (ADC %) of dry matter (DM), protein, lipid, phosphorus (P) and energy of experimental diets (Mean of three replicates).

6.4 **Discussion**

6.4.1 Effects of Protease on Growth and Digestibility

Experimental studies are limited for feed protease in comparison to that of xylanase and phytase (Walk 2009). The present study investigated the additive, semi-additive or non-additive effects of protease in combination with xylanase and phytase on growth and digestibility in tilapia fed low FM diets. Growth performance improved overall with enzyme supplementation compared to the negative control 2% FM diet (NC; $P < 0.05$). The study further found no differences in FI of tilapia fed LOPRO diet and those fed the positive (industry-like) 10% FM control (PC; $P < 0.05$). This suggested that the enzymes may have stimulated an increase in appetence. This differed, however, from the findings of Lin et al., (2007) who cited a decline in FI in Nile tilapia fed plant-based diets (7% FM, 30% CP) supplemented with a neutral protease and two carbohydrases, but who surmised that an accumulation of digestive products was the cause. The present findings also opposes Walk, (2009) who found no improvements in broiler FI and weight gain over the NC or PC diets when the NC diet was supplemented with a combination of 8,000 U kg^{-1} PRO + 1,200 U kg^{-1} XYL + 1,000 U kg^{-1} PHY or individual enzymes.

In the present study, FCR values were lowered by the inclusion of protease over 12 weeks however there were no differences compared to both the NC and PC diets. In addition to that, the HIPRO diet (high protease inclusion) had a significantly better performance compared to the NC (2% FM) diet after four weeks. This suggested greater effects of the protease in younger/smaller animals. In support, it is believed that adaptive changes in digestive processes (endogenous enzyme secretion and activity) and intestinal microbial community may occur over time improving overall digesta degradation while decreasing the impact of exogenous enzymes (Amirkolaie and Schrama, 2015; Lin et al., 2010; Sklan et al., 2004).

In a poultry study, a cocktail of protease and carbohydrases (E1) or single protease (E2) were used to boost growth in broilers fed maize-SBM diets (Yu et al. 2007). The cocktail demonstrated better weight gain but the single protease (E2) achieved a lower feed conversion compared to E1 and

the controls. On the contrary, supplementation of pea-based diets with protease and α-galactosidase resulted in poorer growth and FCR in growing pigs, however, supplementation with protease alone showed nutritional benefits of improved FCR (O'Doherty & Forde 2013). Deguara et al. (1999) also found mixed effects on growth performance in sea bream (Pagrus major) when protease and α-galactosidase were applied to their diets in combination. The present findings and other studies indicates that there might be a semi-additive effect of protease with other exogenous enzymes on feeding efficiency and weight gain. However, the level of effectiveness is clearly diet-dependent and may be more evident in younger animals having under-developed digestive tracts and limited endogenous enzymes (Bedford & Walk n.d.; Walk 2009). Further research is therefore needed to validate size and age-specific effect of protease in tilapia which was outside the scope of this study.

There were no differences observed in protein digestibility between the five treatments ($P > 0.05$). Considering FM and soybean meal, sources of highly digestible protein (NRC 2011), made up 52% of the 2% FM diet (or >86% of the protein content), it is likely that protease may have a higher responsiveness in diets with lower digestible protein levels. Furthermore, Selle et al., (2012) surmised that phytates have a higher propensity to bind to proteins in soya but does not complex with the protein of maize or RB. This possibly made the proteins in the 2% FM diet more responsive to phytase than to protease. Consequently, the NOPRO had the highest ADC protein values (86.8%) which suggested that phytase and xylanase had contributed to protein digestibility theoretically as a secondary benefit of phytate and NSP hydrolyses (Bao et al. 2013). Some of the benefits of protease were evidently lost in the presence of xylanase and phytase in tilapia particularly at the higher concentration. Unlike the present study, Dias et al., (2012a) reported significant gains in protein and DM ADC in Nile tilapia fed diets containing 2 – 8% FM at three levels of CP and protease (only) inclusion. Protein digestibility improved in a dose dependent manner but it was more evident in fish fed the lowest CP (2% FM, 26% CP). An experiment involving Coho salmon, Atlantic salmon and rainbow trout fed 15% and 35% FM diets showed positive results in protein digestibility for Atlantic salmon only when the salmonid feed (15% FM, 45% CP) was treated with 0.175 g kg^{-1} protease only

(Dantagnan et al. 2014). Drew et al (2005) also reported that the addition of 0.25 g kg^{-1} of protease significantly increased the coefficient of total tract digestibility in rainbow trout fed a canola/pea diet but no effect was seen when fish were fed a coextruded flax/pea diet. The authors concluded that the benefits of protease were highly dependent on ingredient choice as also concluded by Lopez et al (1999). The previous studies confirmed that greater impacts may be seen with protease-only supplementation and in a more protein-deficient diet.

In vitro analysis of corn/SBM-based diets with protease, carbohydrase and phytase suggest that protease may digest the other enzymes, however, this appears inconsistent with *in vivo* results (Walk 2009). Considering the enzymes were recovered in adequate amounts prior to feeding, it is suspected that there may have been an antagonistic reaction of the enzymes with each other or other components (endogenous enzymes, mucosal secretion etc.) of the luminal content under digestive conditions. This, however, requires additional research for elucidation.

6.4.2 Effects of Xylanase and Phytase on Digestibility

Though there were no additive effects of protease observed on DM, P, lipid and energy digestibility, there were overall improvements with enzyme supplementation particularly due to xylanase and phytase inclusions compared to the NC diet ($P < 0.05$). P ADC values for PC diet was the highest based on FM level in the diet, P digestibility nevertheless improved by 29.3% (NOPRO) over the NC diet ($P < 0.05$). Lipid and energy digestibility were higher for diets supplemented with xylanase and phytase only (NOPRO) compared to both NC and PC diets. This further indicated that xylanase and phytase had compensated for the effects of viscosity caused by the soluble NSP fractions in the diet which are known to cause a decline in nutrient ADCs and slower growth in tilapia (Amirkolaie et al. 2005). This also supports other studies in Nile tilapia, channel catfish and rohu carp (Yan et al. 2002; Tudkaew et al. 2008; S. Hussain et al. 2011). The cooperativity of enzymes, particularly carbohydrases, phytase and protease, and their impact on digestibility, however, remains slightly inconsistent, but more so between monogastric groups (Cowieson & Adeola 2005; Bedford &

Cowieson n.d.). It confirms also the need for further investigation into the synergy of enzymes and their mode of action in cocktails.

6.4.3 Effects on Intestinal Morphology

Histology results showed villi length increased with enzyme supplementation when compared with the NC 2% FM diet. Yet, only the samples taken from fish fed LOPRO treatment had similar villi length to the starter population sampled prior to the start of the experiments and those fed the PC industry-like 10% FM diet. In support, Guajarado et al., (2014) reported linear increase in villi length in rainbow trout fed diets containing up to 250 ppm protease (0.25 g kg^{-1}). On the contrary, in the current study there was no further improvement in villi length at 0.4 g kg^{-1} protease level as HIPRO-fed tilapia had similar measurements to those fed the NOPRO diet. The NC group was negatively impacted the most confirming earlier experiments. Villi length decreased due to reduction in dietary FM inclusion levels as seen in Borgeson et al., (2006), however, there were also associated loss of branching and disintegration of villi tips. Furthermore NSP is also known to disrupt digestive processes by interfering with enzyme-substrate interactions at the point of contact with the intestinal walls where absorption mainly occurs (Bregendahl 2007). Despite the omnivorous feeding habit, it is clear that tilapia is still sensitive to ANFs in terrestrial plant-based ingredients but very little information is still available demonstrating direct linkages between NSP and gut integrity in tilapia (Sinha et al. 2011). The current findings suggest that there may also be a secondary correlation between villi length as an indicator of gut health and that of FI (or appetence) in tilapia. Fish fed LOPRO and PC diets had similar villi lengths and FIs which were correspondingly the longest villi measurements and highest apparent FI of all the treatments ($P < 0.05$).

6.4.4 Economic Efficiency of Multi-Enzyme Supplementation

Since dietary protein is the most expensive component of feeds (Coyle et al. 2004), it is important from an economic perspective to improve the efficiency with which protein sources are utilised by animals, especially those from less digestible plant origins. A standard commercial tilapia grow-out diet (20 – 25% CP) in Thailand costs approximately 0.626 – 0.783 US$ kg^{-1} demonstrating

to some extent the formulation competitiveness of the experimental enzyme diets (0.642 – 0.660 US$ kg^{-1}) on a cost per kg basis (ingredients only). The cost per kg weight gain decreased with enzyme supplementation and with higher protease dosage. While most enzyme studies acknowledge similar formula cost savings of supplementation, rarely are these figures published for reference. Nevertheless, Cowieson et. al (2013) reported that the value of xylanase, in the absence of phytase, may be 4.50 US$ $tonne^{-1}$ based on a reduction in added lipid in poultry diets. Where as in the presence of phytase this is reduced to 3.50 US$ $tonne^{-1}$ as the energy digestibility effects are not fully additive.

When a comparison to the PC 10% FM diet (representing an average commercial diet) was made in the present study, the formula cost saving diminished from 15.36 US$ $tonne^{-1}$ to 6.58 US$ $tonne^{-1}$ as the number of enzymes and dose increased. Considering the performance of the LOPRO diet was comparable to the PC in some performance parameters (FI, AWG, villi length), a formula cost saving of 10.97 US$ $tonne^{-1}$ is nonetheless very encouraging from a general production standpoint. Cowieson et al., (n.d.) argued, however, that the efficacy of an exogenous enzyme will always reduce the impact of others and even if several combinations are explored, it is unlikely that the cost saving will be greater than 20 – 25 US$ $tonne^{-1}$. This is, however, debatable if additional improvements in biological productivity at the farm level are considered. Economic analysis of unit profit due to changes in performance parameters (AWG and FCR) in the present study supports this conclusion. Using the NC diet as the basis for comparison, improvements in productivity based on feeding efficiency showed HIPRO achieving the highest profit margin (0.066 US$ kg^{-1}) yet simultaneous evaluation of profit from a growth perspective demonstrated the LOPRO achieved greater net profit (0.063 US$ kg^{-1}). The economic difference in performance between the NC 2% FM and the PC 10% FM is the theoretical cost of sustainability or FM replacement and closing this gap should be a research priority. Nevertheless, despite the best improvements due to inclusion of protease, xylanase and phytase, net profit still diminished by 0.116 US$ kg^{-1} compared to the PC.

6.4.5 Conclusions

Ternary combination of protease with xylanase and phytase has potential and can be applied with some success to tilapia production. The study validated the semi-additive effects of protease on apparent FI, FCR and villi length. In terms of overall growth performance and economic efficiencies, a 2% FM tilapia diet supplemented with low protease inclusion (0.2 g kg^{-1}) in combination with 0.0385 g kg^{-1} xylanase and 0.075 g kg^{-1} phytase has potential in tilapia diets, however, performances and efficiencies were still below that of a 10% FM diet. Agreeing with Bedford and Walk, (n.d.) and Cowieson et al., (n.d.), it is, however, critical to understand the composition of indigestible nutrients (substrates) as well as the digestibility of the control diets in order to select the appropriate enzymes and their dosages. Age-specific considerations may also be warranted to determine which stage of the culture cycle is likely to gain the most from enzyme supplemented diets.

Chapter 7

Comparative Life Cycle Assessment of Enzyme Supplemented Low FM Diets and Commercial Feeds Associated With Tilapia Production In Thailand

7.1 **Introduction**

Aquaculture provides food security and economic opportunities for a large portion of Thailand's population (Pongpat & Tongpool 2013). Domestic aquatic food consumption is increasing and local production continues to expand (Belton 2006). As the industry grows globally, it is likely to place greater demands on finite natural resources (water, raw ingredients, land *etc*.) which provide goods and services. In fact, the livestock sector currently consumes ~35% of total cropland and ~20% of water for feed production (FAO 2015a). It follows therefore that with greater demands for resources, greater negative environmental impacts may arise. Prior to the 1990's, aquaculture impacts were narrowly viewed from a *two*-dimensionally perspective, direct inputs (fish and feed) and outputs (uneaten feed and faeces). Fortunately the days when agricultural systems, their consumption and waste flows are viewed in isolation are over (Pelletier 2006; Kautsky et al. 1997). Though aquaculture now has far-reaching impacts on a global scale, its impacts are still largely dietary in origin (Preetha et al. 2012). Feeds are major sources of aquaculture waste and their manufacturing processes contribute to several environmental impacts categories (Pelletier 2006; Papatryphon et al. 2004; Mungkung et al. 2013). It is logical therefore that future aquaculture management strategies should prioritise feed ingredient choices, diet composition and on-farm feeding practices (Amirkolaie et al. 2005; Samuel-Fitwi et al. 2013). This becomes even more important as FM is increasingly replaced by less digestible plant-based ingredients for aquafeed production.

The first LCA was done in 1969 and through series of methodological developments over 40^{+} years; it is now widely applied to food production including aquaculture (Pelletier 2006; Caffrey & Veal 2013). Despite the existence of an international standard, certain inconsistency in methodology

remains a challenge (ISO 2006; Pelletier 2006; Caffrey & Veal 2013). These includes the selection of appropriate impact categories and specific guidelines for allocating environmental burdens. LCA generally uses one of two broad approaches, attributional and consequential allocation and categorises midpoint indicators. Impact categories are critical points in the cause and effect chain where characterization factors can be calculated to reflect the impact on an endpoint category, *e.g.* ecosystem damage (Bare et al. 2000). While endpoint categories represent greater relevance, midpoint categories represent greater levels of reliability (Bare et al. 2000). Midpoint categories include, but are not limited to, eutrophication potential, global warming potential, acidification potential *etc*. (Lindeijer 2000; Mattsson et al. 2000). Regardless of variation in methodologies, the main benefit of LCA is to identify "hotspots" within production process which may potentially contribute to significant global impacts.

Aquaculture production impacts have been increasingly reviewed since the first published study by Papatryphon et al. (2004). There are now a number of system and feed-related LCA studies (Ellingsen and Aanondsen, 2006; Pelletier et al., 2007; d'Orbcastel et al., 2009; Henriksson et al., 2011; Mungkung et al., 2013; Wilfart et al., 2013; Avadí et al., 2015; Dekamin et al., 2015). This, however, was the first study designed to 1. evaluate the combined environmental impacts of multi-exogenous enzyme supplementation (protease, xylanase and phytase) on tilapia feed production (to inform feed development strategies) and 2. comparatively assess enzyme supplemented low FM diets (containing alternative feedstuffs) and commercial feeds associated with tilapia production in Thailand. The work was intended to provide insights for tilapia feed manufacturers regarding implications of ingredient choices and potential cradle-to-farm-gate impacts on tilapia value chain products.

7.2 **Methods**

LCA methodology was customized from Henriksson et al., (2011) which focused primarily on Asian aquaculture systems, Pelletier, (2006) and Avadí et al., (2015) which targeted feed use, and

three studies on enzyme application in monogastric animal production (Nielsen & Wenzel 2006; Nielsen et al. 2007; Oxenboll et al. 2011). The structure of the study, however, followed the ISO (LCA) 14040 and 14044 framework (ISO 2006). This comprises four phases; Definition of Goal and Scope, Life Cycle Inventory, Life Cycle Impact Assessment and Interpretation.

7.2.1 Definition of Goal and Scope

7.2.1.1 Goal and Scope

The study assessed the life cycle impacts of five tilapia feed formulations. These included an average Thai commercial tilapia diet according to Henriksson et al., (2014), two control diets (5% and 10% FM) and two enzyme-supplemented (ES) diets (2% and 3% FM). The study compared the impacts of two sets of feeding scenarios based on actual and reported FCRs. The first set included a comparison of low FM diets (2%, 3% and 5%) with and without enzyme supplementation. The second included the first five formulations for intensive tilapia pond production in Thailand.

7.2.1.2 Systems and Feeding Scenarios

In order to evaluate the direct impacts of enzymes on feed production (and in turn tilapia production), the first set of feeding scenarios compared the basal 2%, 3% and 5% FM formulations with their respective ES formulation. The 2% FM diet was supplemented with phytase, xylanase and protease while the 3% and 5% FM diets were supplemented with phytase and xylanase only. FCR values were obtained from growth experiments (Sections 4.3.2 and 6.3.2.1). The first scenario assessed feed improvement factors (FCR and growth) due to supplementation on selected impact categories to inform future aquafeed development strategies using exogenous feed-grade enzymes. In most studies, an attributional approach is taken, however, Samuel-fitwi, (2012) argued that system expansion was most appropriate for evaluating aquafeeds impacts because attributional LCA underestimated environmental impacts. This study therefore utilised a *simplified* version of system expansion by considering feed enzyme application as an extra, post-pelleting process in the feed production chain, as opposed to complete substitution of existing ingredients *e.g.* phytase for inorganic phosphate (as a source of P) and xylanase for maize (as a source of energy).

The second set of feeding scenarios were based on recent findings of field surveys (Chapter 3; n = 199 farms) and on-farm pilot scale growth trials (Sections 5.3.2 and 6.3.1.2). The average Thai tilapia feed (Henriksson et al. 2014) was used as a benchmark for comparison. From Phase 1 experiments, the 3% FM diet with phytase and xylanase and the control 5% FM diet had similar performances; the latter used in this context as the global average in 2012 according to Tacon and Metian (2008). From Phase 2 experiments, the 2% FM diet supplemented with phytase, xylanase and protease was selected and compared to the control 10% FM diets; the latter used in this context as the standard Thai tilapia feed (Table 7.2.1). The ingredient and proximate compositions are given in Table 7.2.2.. Though feeding practices of tilapia farmers in Thailand were mainly semi-intensive, for the purpose of this model intensive feeding as means of fattening was considered. Nevertheless, the study attempted a simple semi-intensive scenario using the average Thai tilapia feed composition in order to account for normative feeding practices. Therefore for the purpose of this analysis, data from monoculture was used for simplicity and robustness. Finally this comparison was intended to inform which feeding scenario had the least environmental impacts, where along the production value chain were the major "hotspots" and what were the relative effects of replacing FM.

Table 7.2.1: System and feeding scenarios

Feeding Intensity	**% CP**	**% FM and Enzyme**	**FCR**	**Relevance**
Intensive	30	10	1.38/1.70*	Thai Industry Average (SEAT)
Intensive	28	10	1.73	Standard tilapia feed (from experiments)
Intensive	25	5	1.80	2012 global average
Intensive	25	3 with PHY+XYL	1.82	Performed similar to 5% FM control
Intensive	28	2 with PHY+XYL +PROT	1.97	2015 global target

CP – Crude protein; FCR – Food conversion ratio; PHY – Phytase; XLY – xylanase; PROT – Protease.
*FCR – average reported feed conversion for semi-intensive and intensive systems based on Henriksson et al 2014,

Table 7.2.2: Ingredient and chemical composition of enzyme supplemented diets and commercial feeds associated with tilapia production in Thailand.

	Commercial feed*	**C10FM**	**C5FM**	**E3FM**	**E2FM**
Ingredient composition (g kg^{-1})					
SBM (IMP)	481.6	370.0	425.0	455.0	499.0
MA (IMP)	100.0	127.5	125.0	115.0	82.9
By-product Tuna FM (LOC)	93.5	100.0	50.0	30.0	20.0
CM (LOC)	98.3	150.0	150.0	150.0	150.0
RB (LOC)	180.7	200.0	200.0	200.0	200.0
Fish or Vegetable oil[1] (IMP)	31.3	30.0	30.0	30.0	20.0
DCP (IMP)	-	5.0	5.0	5.0	10.0
Vitamin/Mineral premix (IMP)	14.6	10.0	10.0	10.0	10.0
Lysine (IMP)	-	5.6	4.9	4.9	5.4
Methionine (IMP)	-	1.9	1.0	1.0	2.7
Protease (IMP)	0.0	0.0	0.0	0.0	0.200
Xylanase (IMP)	0.0	0.0	0.0	0.385	0.385
Phytase (IMP)	0.0	0.0	0.0	0.075	0.075
Proximate Analysis (g kg^{-1} as fed)					
DM	939	957	946	924	944
CP	316	292	277	247	242
Lipid (EE)	38	33	26	42	24
Carbohydrate	507	553	563	558	608
CF	38	105	40	37	90
NFE	470	449	523	521	518
Moisture	61	43	54	76	56
Ash	78	79	81	78	71
P	10	13	9	9	8
GE *(MJ kg^{-1})*	19.8	19.2	20.0	19.2	19.6

*Ingredient composition according to Henriksson et al 2014, average of six feed millers. Domestic and tuna fishmeal were summed. Inclusions for rice bran, corn and plan bran were summed. Fish oil and salmon fish oil was added to rice bran oil. And soya bean meal/cake was composted with other protein/carbohydrate sources. However for the purpose of the actual model, each ingredient will be assessed individually.
Proximate analysis based on commercial feed used for acclimation in on-farm growth trials.
IMP – imported; LOC – locally produced

7.2.1.3 Temporal Scale and Geographic Scope

Data on feed production and feeding practices of tilapia farmers in Thailand were collected between 2010 – 2014 as part of the SEAT project (Henriksson et al. 2014). Tilapia production data were generated from pilot-scale growth trials conducted between 2013 – 2014. Enzyme and amino acid (AA) production data were collated from studies conducted in Europe between 2006 – 2011 (Nielsen & Wenzel 2006; Nielsen et al. 2007; Marinussen & Kool 2010; Mosnier et al. 2011; Oxenboll et al. 2011). Ingredients sourced from overseas were linked to the feed production model using appropriate life cycle inventory (LCI) transportation data (Henriksson et al. 2014).

7.2.1.4 System Boundary

The system boundaries were in keeping with research objectives and incorporated all material inputs (fisheries and agriculture), transportation, energy consumption, major material outputs and emissions related to Thailand's tilapia production. The model included (where appropriate) enzyme production processes, their incorporation into tilapia feed production and finally, the on-farm production of market-ready tilapia. Microingredients (feed-grade AAs, dicalcium phosphate, vitamins/mineral) were also considered. The process flow interactions for the integrated enzyme, feed and fish production systems are illustrated in Figure 7.2.1. Postharvest processing, marketing, consumer activities and other downstream processes (*i.e.* waste management) were ignored.

7.2.1.5 Functional Unit

The functional unit was defined as one tonne of market-ready tilapia (live weight) produced by an undetermined amount of feed with and without enzyme supplementation. (Model assumption - Final products were of similar nutrition quality, edible yield and value).

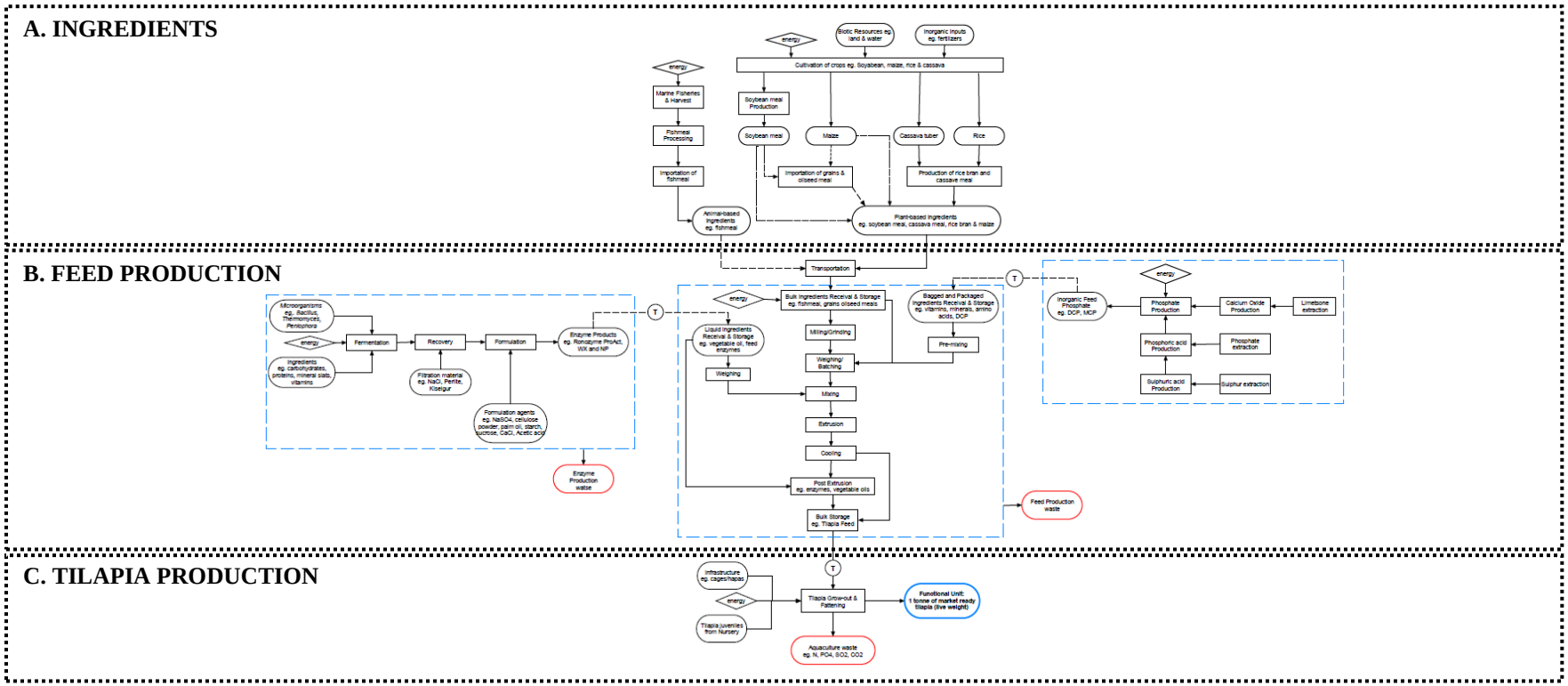


Figure 7.2.1: LCA boundary for ingredient processing (A), feed production (B) including enzyme and inorganic phosphate production (B2 and B3) and tilapia production (C).

7.2.2 Life Cycle Inventory

7.2.2.1 Data Collection and Inventory

Ayer and Tyedmers, (2009) defined life cycle inventory as the process involving collection of data required to quantify material inputs and outputs associated with the production of a given functional unit, *i.e.* 1 tonne of market-ready tilapia at farm-gate. The model included raw materials, resource use and ingredients (fisheries products, terrestrial crops, inorganic additives) as well as energy consumption and transportation. Descriptions of inputs were addressed based on source of commodity (*i.e.* local or imported). Data for the major ingredients listed in Table 7.2.2 are given in the following sections. Each ingredient was assessed individually from source (locally and overseas) to feed mill/farm (Thailand).

7.2.2.2 Local Ingredients, Feed and Fish Production

Four of the macro-ingredients were local, FM, maize, RB and cassava. FM is produced locally from three sources; local by-catch, surimi factories and tuna processing by-products (Henriksson et al. 2014). However, high quality Peruvian FM is also imported for high valued export feed/fisheries production *e.g.* shrimp. Denmark and Chile also accounts for 9.5% and 4.2% of FM imports respectively. Based on proximate composition it was assumed that the FM used came from local sources (Grade 2, tuna fisheries by-product). Figure 7.2.2.A shows the FM production pathway and contribution from the three local sources (Thongrod 2005).

90% of local maize production enters the domestic market (4.75 million tonnes). Total grain production (4.18 tonnes ha^{-1}) utilises 1.1 million hectares and general fertilizer application of 20-25-0 kg $N\text{-}P_2O_5\text{-}K_2O$ ha^{-1} (Yodkhum & Sampattagul 2014). High import tariffs (20 – 73%) encourages greater reliance on local supply (Thongrod 2007). Feed mills, however, use maize from two sources, local production and imported grains from the United States (US) (Henriksson et al. 2014; Santella & Prasertsri 2014), the model therefore relied upon a composite of local and US maize production (Figure 7.2.2. B). Dry milling produces 60 – 70 g kg^{-1} of maize bran and ground maize. The latter is further processed to maize flour or to ethanol/DDGS by-product mix. Environmental inflows and

emissions are given in Henriksson et al., (2014). Figure 7.2.2. C details the production pathway of rice milling, inputs, outputs and processes. In Thailand, the process produces 50 – 60% rice (polished/white), 10 – 20% broken rice, 6 – 10% RB, 20 – 25% rice husks and 1% waste per tonne of paddy rice (Sethanan 2009; Sriroth 2001). Thailand produced 34 million tonnes of rice per year on average (2006 – 2012) from 12,65 million hectares (Henriksson et al. 2014; Yodkhum & Sampattagul 2014). Of which it is estimated that 10% produces RB destined for animal production and feed industries (Sethanan 2009). Each tonne of RB can produced 800 kg of defatted RB, 160 kg of rice oil and 40 kg of by-products (fatty acids and wax) (Henriksson et al. 2014). It is also estimated that 50 – 100 m^3 of natural gas is required per tonne of RB produced. Economic and environmental inputs and outflows are also described in Henriksson et al., (2014).

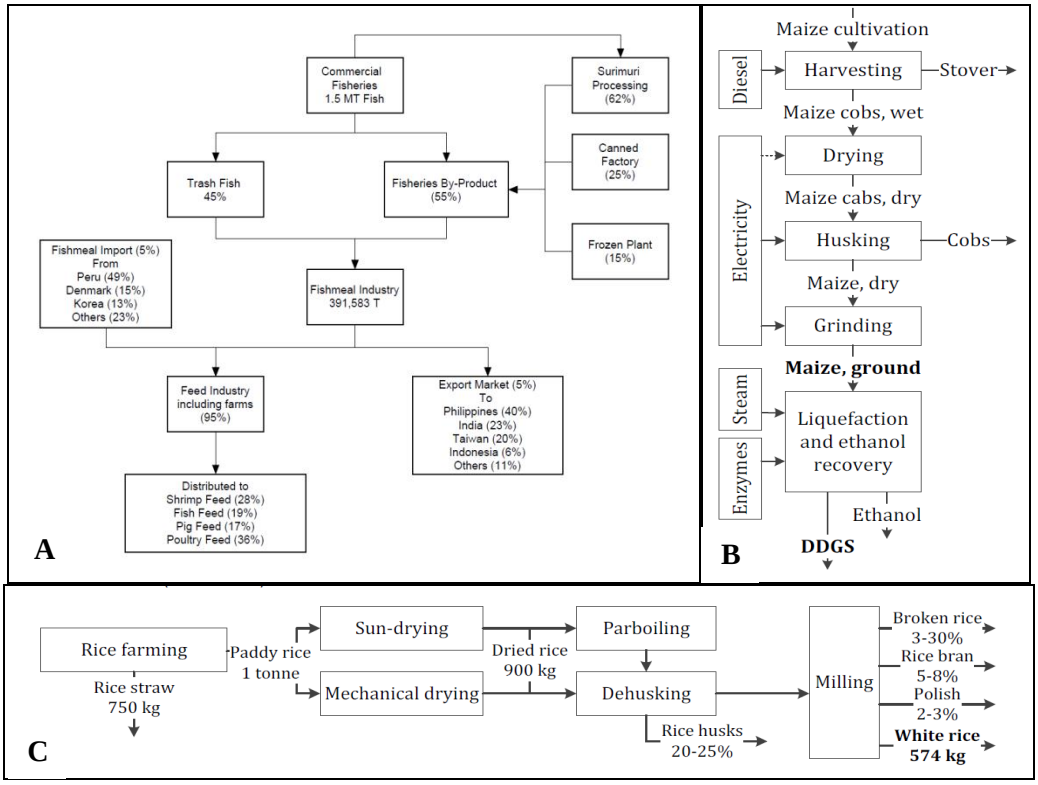


Figure 7.2.2: Production pathway of feed ingredients (Thailand): Fish meal (A), Maize (B) and Rice (C).

Cassava is used as a common feed additive in tilapia feeds and is becoming more competitive in light of global ingredient demands and prices. In 2008/09, Thailand produced ~25 million tonnes of cassava and by 2014/15 this figure had increased to 31 million tonnes (Thai Tapicoa Starch Association 2015), 55% of which is destined for chip production and 45% for tapioca starch production (Figure 7.2.3). It requires an estimated 4.75 tonnes of fresh root to produce a tonne of starch (Sriroth et al. 2001). 30.09 million tonnes (22.67 tonne ha^{-1}) of fresh root required 1.33 million hectares using general fertilization rate of 50-25-25 kg $N\text{-}P_2O_5\text{-}K_2O$ ha^{-1} (Yodkhum & Sampattagul 2014).

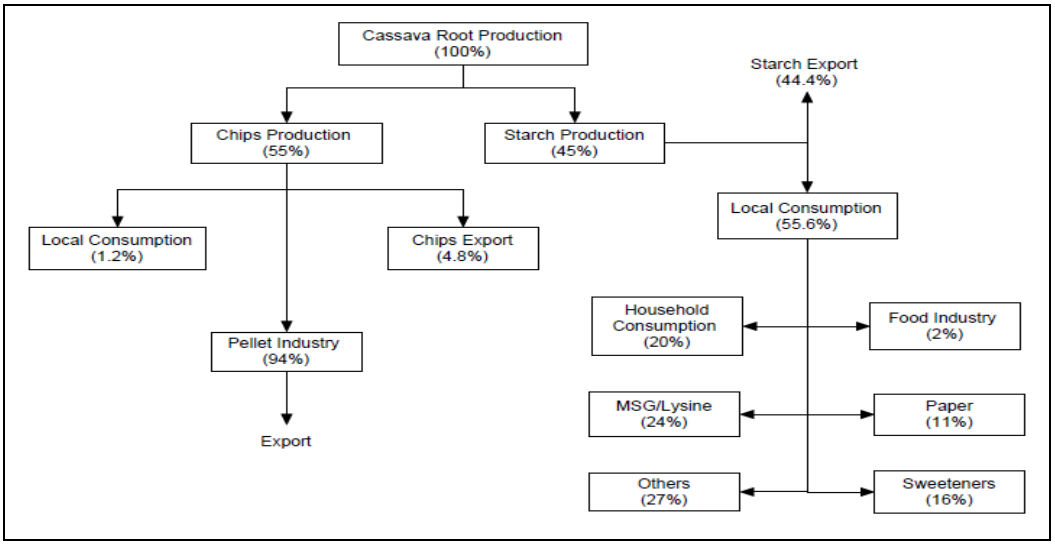


Figure 7.2.3: Process flow of cassava starch (flour)

Feed Production

Detailed information on feed production in Thailand was gathered through face-to-face interviews with local feed manufacturers. Data including feed composition was collected from six feed millers and averaged for confidentiality and sensitivity reasons (Henriksson et al. 2014).

Tilapia Production

Most of the primary inventory data for tilapia production was collected by the SEAT project through a series on farm surveys (Henriksson et al. 2014) and current growth experiments. There are two main system types used for tilapia production in Thailand, cages and pond. However, for the purpose of this model, only pond culture was considered for comparability to growth trials. Feeds affect almost all impact categories, and so much effort was placed on the collection of feed-related data. Feed conversions were estimated from total reported feed used and final market yields. Nevertheless, the limitations included a low response rate (21% of surveyed farmers) and evidence of inadequate record keeping. Other noted discrepancies on FCR values were polyculture systems/yields and mortalities. Most pond culture systems for tilapia farming utilised manure for fertilisation, but for the purpose of this model this was ignored as the contribution of these inputs to live production cannot be accurately computed. This would inflate uncertainty values.

To facilitate comparative analysis, data generated from the two phases of on-farm growth experiments was used for feed formulation (Table 7.2.2), nutrient budgeting for outputs from current tilapia system was calculated using **Equation 1** based on chemical analysis of feed, carcass and faecal material. Results were cross referenced using Avadi et al. (2015), Pelletier et al. (2010) and Boyd et al, (2001). Average harvest size of Thai tilapia was 524 g ± 257 STD, each animal is assumed to contain 2.6% N and 0.82% P (Henriksson et al. 2014). Repositories of excess nutrients from ponds are mainly sediment and water. Sediment N and P (from effluents and other organic waste) were 16.5% and 84.4% while run-off N and P were 83.5% and 15.6% respectively. 36% of pond water was pumped directly into public canals, 56% to pond dykes and 2% dumped unto agricultural field (Henriksson et al. 2014).

$$\mathbf{P_e = (P_f \times FCR) - P_a} \quad \textbf{(Equation 1)}$$

Where P_e = environmental P load (kg tonne fish^{-1}), P_f = P concentration in the feed (kg tonne feed^{-1}), FCR = feed conversion ratio and P_a = concentration of P in the harvested fish (kg tonne fish^{-1})

Energy Consumption and Transportation

Thailand produces its own crude oil (13.9 million tonnes in 2012), however, they import 70% of crude oil consumed locally (46.8 million tonnes). Oil import data were assumed to be of Middle East origin due to regional market dominance and data availability (Henriksson et al. 2014). Electricity, however, is produced from a mixture of 0.5% crude oil, 19.9% coal, 4.8% hydro, 4.0% biomass and 70.7% natural gas. Characterisation and emission flows from the production of different energy sources are given in Henriksson et al., (2014). Natural gas is also used for industry (8%) and transportation (4%). Transportation data was modified slightly to reflect imports into Thailand. Within the country, diesel trucks (lorries) were assumed to account for 89% of transportation and compressed natural gas accounted for the rest. Electricity (natural gas) and diesel oil were used to run paddle wheels on farm.

7.2.2.3 Imported Commodities

SBM is the most widely used FM replacer by the animal feed industry. Global cultivated area has tripled from 1975 (38 million hectares) to 2005 (91 million hectares) and its production is projected to rise to 66.2 million tonnes by 2020 (Dalgaard et al. 2008; Samuel-Fitwi et al. 2013). SBM and its oil are co-produced. Every 2.3 tonnes ha^{-1} of soya bean produces 1.8 and 0.4 tonnes ha^{-1} of SBM and oil respectively (Marinussen & Kool 2010). Though Thailand produces soya bean (187,783 tonnes, 2011), it is insufficient to meet domestic consumption (1.7 million tonnes) (Henriksson et al. 2014). 65% of SBM was imported from Brazil (USDA 2015). US and Argentina accounted for remaining 33% of Thailand SBM imports (Henriksson et al. 2014). The model adopted a SBM mixture based on percentage contribution from each country.

Though Thailand also produces its own palm oil (Chavalparit et al. 2006), domestic consumption (and exports) outweighs production leading to importation (Termmahawong 2014). Malaysia (and Indonesia) accounts for 85% of global production and 24.1% of global trade. Oil palm produces ~25% palm oil and palm kernel oil (Reijnders & Huijbregts 2008; Pleanjai & Gheewala 2009) (Table 7.2.3). It also produces 3.6% of oil meal. Production equally generates significant

amount of process waste, empty fruit bunches (9.0×10^5 tonnes $year^{-1}$), fibres (6.0×10^5 tonnes $year^{-1}$) and shells (2.0×10^5 tonnes $year^{-1}$) and 2.5 million m^3 wastewater (Chavalparit et al. 2006). Dalgaard et al., (2008) presented energy inputs and emissions from Malaysian palm oil production.

Table 7.2.3: Production of palm oil in Malaysia and Thailand

Country	**Malaysia**	**Thailand**
Reference	(Reijnders & Huijbregts 2008)	(Pleanjai & Gheewala 2009; Chavalparit et al. 2006)
Total cultivated area (million ha)		0.3
Planting cycle	25 years	25 years
Tonnes per fruit bunch (per ha)	19.1 – 19.6	17
Extractable oil (%)	20 – 29% (25% avg.)	16 – 17%
Oil Production (tonnes ha^{-1} yr^{-1})	4.9	2.89
Crude palm oil yield (tonne)	4.22	2.54
Crude palm kernel oil (tonne)	0.68	0.35
Oil meal (tonne ha^{-1} yr^{-1})	0.70	0.62

Micro-additives

There was no vitamin and mineral premix production information available for Thailand therefore data from Europe was adopted for the model. Information for commercial AAs and calcium phosphate was taken from Mosnier et al., (2011) and Marinussen and Kool, (2010). The production of 1 kg lysine was assumed to require 1 kg of sugar, 0.5 kg of maize starch, 0.5 kg of wheat starch 0.3 kg of liquid ammonia and 30 MJ of energy (50:50 electricity and natural gas). While the production of 1 kg of methionine required 0.43 kg of propylene, 0.27 kg of hydrogen sulphide, 0.39 kg of methanol, 0.21 kg of hydrogen cyanide and 7.4 MJ of energy (50:50 electricity and natural gas) (Mosnier et al. 2011). Supporting data for L-lysine and DL-methionine production including emissions were taken from Marinussen and Kool, (2010). Information for dicalcium phosphate (DCP; $CaHPO_4$) production was also unavailable, therefore inputs for monocalcium phosphate (MCP; $Ca(H_2PO_4)_2$) were used. MCP is produced through sulphuric acid digestion from chalk/limestone, and

phosphoric acid from phosphate rock (Nielsen & Wenzel 2006). The production of enzymes by Novozyme is covered by Nielsen et al., (2006). Secondary data for phytase, xylanase and protease productions were taken from Nielsen and Wenzel, (2006); Nielsen et al., (2007); Oxenboll et al., (2011). In addition, unpublished data from internal reports was supplied by Novozyme.

7.2.2.4 Allocation Procedure

The study applied a mixed method approach. Economic allocation was used for the standard feed manufacturing and fish production processes. Economic allocation is useful because it summarizes complex systems that often cannot be easily measured by physical criteria (Ardente & Cellura 2012). Where appropriate, the model was expanded using consequential analysis to compare feed production having post extrusion enzyme application. The latter was useful in providing information that could justify the use of exogenous enzymes for aquafeed formulation in response to FM replacement with higher levels of plant-based ingredients. Environmental burdens were expressed for single end-products.

7.2.3 Life Cycle Assessment

Life cycle impact assessment involves calculating potential burdens associated with different aspects of product life cycle processes or activities in terms of their contribution to one or more impact categories (Pelletier 2006). CMLCA v 5.2 (University of Leiden) software was used for comparative modelling and Ecoinvent v 2.2 database of life cycle inventory materials and processes.

7.2.3.1 Impact Categories

An impact category is a class representing environmental issues of concern to which the life cycle inventory analysis results may be assigned (Souza et al. 2014). The impact categories listed in Table 7.2.4 were selected based on their relevance to the industries being investigated within this study - enzyme, feed and tilapia. A review of impact categories common to seafood production and aquaculture has been addressed by Pelletier et al., (2007) and Henriksson et al., (2011).

Table 7.2.4: Midpoint impact categories and relevance to the study

Impact Categories	**Unit**	**Justification**	**References**
Global warming potential **(GWP)**	kg CO_2 eq.	Impact of processes on global climate conditions and change	Papatryphon et al. (2004): Nielsen and Wenzel (2006); Nielsen et al. (2008); Oxenboll et al. (2011); Henriksson et al. (2011)
Eutrophication potential **(EP)**	kg PO_4 eq.	Aquaculture waste water is released back into public water ways in Thailand	Papatryphon et al. (2004); Nielsen and Wenzel (2006); Nielsen et al. (2008); Oxenboll et al. (2011); Henriksson et al. (2011)
Acidification potential **(AP)**	kg SO_2 eq.	Impacts of feed ingredients; also an issue with many farmers	Papatryphon et al. (2004); Nielsen and Wenzel (2006); Nielsen et al. (2008); Oxenboll et al. (2011); Henriksson et al. (2011)
Energy use **(EU)**	MJ	Feed and enzyme productions are energy intensive processes	Papatryphon et al. (2004); Nielsen and Wenzel (2006); Nielsen et al. (2008);
Agricultural land use **(ALU)**	m^2 $year^{-1}$	FM replacement with terrestrial plant based ingredients. Substrate for enzyme production is derived from agriculture	Nielsen and Wenzel (2006); Nielsen et al. (2008); Henriksson et al. (2011)
Photochemical smog formation **(PSF)**	kg C_2H_4 eq.	Significant environmental impact for micro-additives including enzymes	Nielsen and Wenzel (2006); Nielsen et al. (2008); Oxenboll et al. (2011); Mosnier et al. (2011)
Ecotoxicity Potential **(ETP)**	kg 1-4-DCB eq.	Significant impact categories for feed ingredients	Henriksson et al. (2011)

DCB – dichlorobenzene: Photochemical smog formation can be used interchangeably with photochemical ozone formation

Global warming potential (GWP) is defined as calculated emissions from a production process which contributes to atmospheric heat radiation (Papatryphon et al. 2004). Units of this category are given in kg CO_2 *eq.* Eutrophication potential (EP) includes environmental impacts from macronutrients particularly N and P. This impact category is measured in kg PO_4 *eq.* (Papatryphon et al. 2004). Acidification potential (AP) is the possible impact from acidifying pollutants on soil, groundwater, ecosystems *etc*. This impact category is measured in kg SO_2 *eq.* (Papatryphon et al. 2004). Energy use is defined as depletion of non-renewable energy and is quoted in mega-joules (MJ). Agricultural land use is defined as the area of land used to produce a certain output over time (Lindeijer 2000). This impact category is slightly contentious (occupation versus transformation), however, it is believed to be relevant due to higher inclusion of plant-based resources for feed formulation. Nevertheless, it was only applied where there was sufficient data to do so. Photochemical smog formation (in kg C_2H_4 *eq.*) and ecotoxicity (kg 1-4-DCB *eq.*) were also considered.

7.2.4 Interpretation

Interpretation is defined as the systematic evaluation of LCIA results in order to determine disproportional contributions of processes within a life cycle chain based on the chosen functional unit (Pelletier 2006). Data analysis and robustness of results were assessed using uncertainty and sensitivity analyses. Sensitivity analysis measures the extent to which LCI results, characterisation models, allocation approach *etc*. influence indicator categories (Pelletier 2006). In other words, the objective is to establish a required degree of confidence in the results by identifying the parameter(s) which have the largest influence on final results, then change these parameters (according to different data sources) and compare the outcomes (Dekamin et al. 2015). Data uncertainty is related to the lack of knowledge regarding an accurate value and is often represented by a log-normal probability distribution (Dekamin et al. 2015). Uncertainty analysis therefore measures the spread of variability factoring inherent uncertainties (data accuracy) and unrepresentativeness, see **Equation 2**.. The latter is facilitated through Monte Carlo analyses of multiple variation (Henriksson et al. 2011).

$$\sigma^{o}_{cv} = \sqrt{(\sigma^{i}_{cv})^2 + (\sigma^{s}_{cv})^2 + (\sigma^{r}_{cv})^2} \quad \textbf{(Equation 2)}$$

Where o_{cv} = overall dispersion of the coefficient of variation, i = inherent uncertainty related to inaccuracy of measurements or model averaging; s = spread of variability around the mean; and r = uncertainty resulting from level of representativeness.

7.3 **Results**

7.3.1 Life Cycle Inventory Results

7.3.1.1 Enzymes and Micro-additives

While the importance of micro-additives in nutritionally balanced diets is undebatable, some feed-related LCA studies have included their impact analysis (Moe et al. 2012; Avadí et al. 2015; Mungkung et al. 2013; Papatryphon et al. 2004; Samuel-fitwi 2012) and others have not (Pelletier 2006; Pelletier & Tyedmers 2010). Notwithstanding, the latter studies adequately covered the impact potentials of commonly used macro-ingredients utilised for tilapia and salmonid feeds. In the present study, the impact potentials of 1 kg of each micro-additive including enzymes are given in Table 7.3.1. Calcium phosphate (MCP) had the highest ETP and TEP values of 3.9 x 10^{-2} kg PO_4 *eq*. and 0.4 kg 1-4-DCB *eq*. respectively. Lysine equally had the highest ALU and PSF values of 4.0 m^2 $year^{-1}$ and 1.6 x 10^{-2} kg C_2H_4 *eq*. respectively. Protease had the highest impacts potential on GWP and EU with values of 6.5 kg CO_2 *eq*. and 80 MJ (fossil energy) respectively. However, AP was impacted the most by xylanase (0.1 kg SO_2 *eq*.). The cumulative environmental impacts per 1 tonne of tilapia feed based on the enzyme inclusions rates used for experimental formulations were calculated and are illustrated in Figure 7.3.1. Inclusions rates were 0.075, 0.385 and 0.200 g kg^{-1} of phytase, xylanase and protease respectively. Impact analysis showed that enzyme production process may contribute significantly to one main mid-point categories along the tilapia value chain, energy use.

Table 7.3.1: Potential environmental impacts of 1 kg of each micro feed additive (Mean ± STD)

Micro-additive	**GWP** (kg CO_2 eq.)	**EP** (kg PO_4 eq.)	**AP** (g SO_2 eq.)	**ALU** (m^2 $year^{-1}$)	**EU** (MJ)	**PSF** (kg C_2H_4 eq.)	**TEP** (kg 1.4-DCB eq.)
Enzymes							
Phytase[1,2,4]	2.02 ± 0.11	0.0031 ± 0.0008	0.0063 ± 0.0021	0.42 ± 0.38	24.67 ± 1.15	0.00057	-
Xylanase[1]	3.00	0.0038	0.1050	0.60	38	0.00145	-
Protease[1]	6.50	0.0030	0.0200	1.20	80	0.00200	-
Free AA							
Lysine[2,3]	4.90 ± 0.85	0.0050 ± 0.0040	0.0209 ± 0.0106	4.00 ± 2.44	59.98 ± 84.74	0.01622	0.0226
Methionine[2,3]	5.45 ± 3.61	0.0012 ± 0.0003	0.0118 ± 0.0070	0.04 ± 0.04	44.69 ± 63.10	0.00596	0.0027
Other Micro-additives							
MCP[1,2,4]	1.15 ± 0.05	0.0393 ± 0.0211	0.0246 ± 0.0088	0.32	15.87 ± 2.34	0.00041	0.4182 ± 0.5790
PX	0.40	0.00	0.0002	0.00	0.90		0.0014

GWP – global warming potential, EP – eutrophication potential, AP – acidification potential, ALU – agricultural land use, EU – energy use, PSF – photochemical smog formation, ETP – terrestrial ecotoxicity potential; AA – amino acids; PX – vitamins and mineral premix; STD – standard deviation

[1] Nielsen and Wenzel, (2006); Nielsen et al., (2007), (2006); Oxenboll et al., (2011) *Per Nielsen, personal communication*

[2] Mosnier et al., (2011);

[3] Marinussen and Kool, (2010)

[4] Nagaraju and Nielsen, (2011)

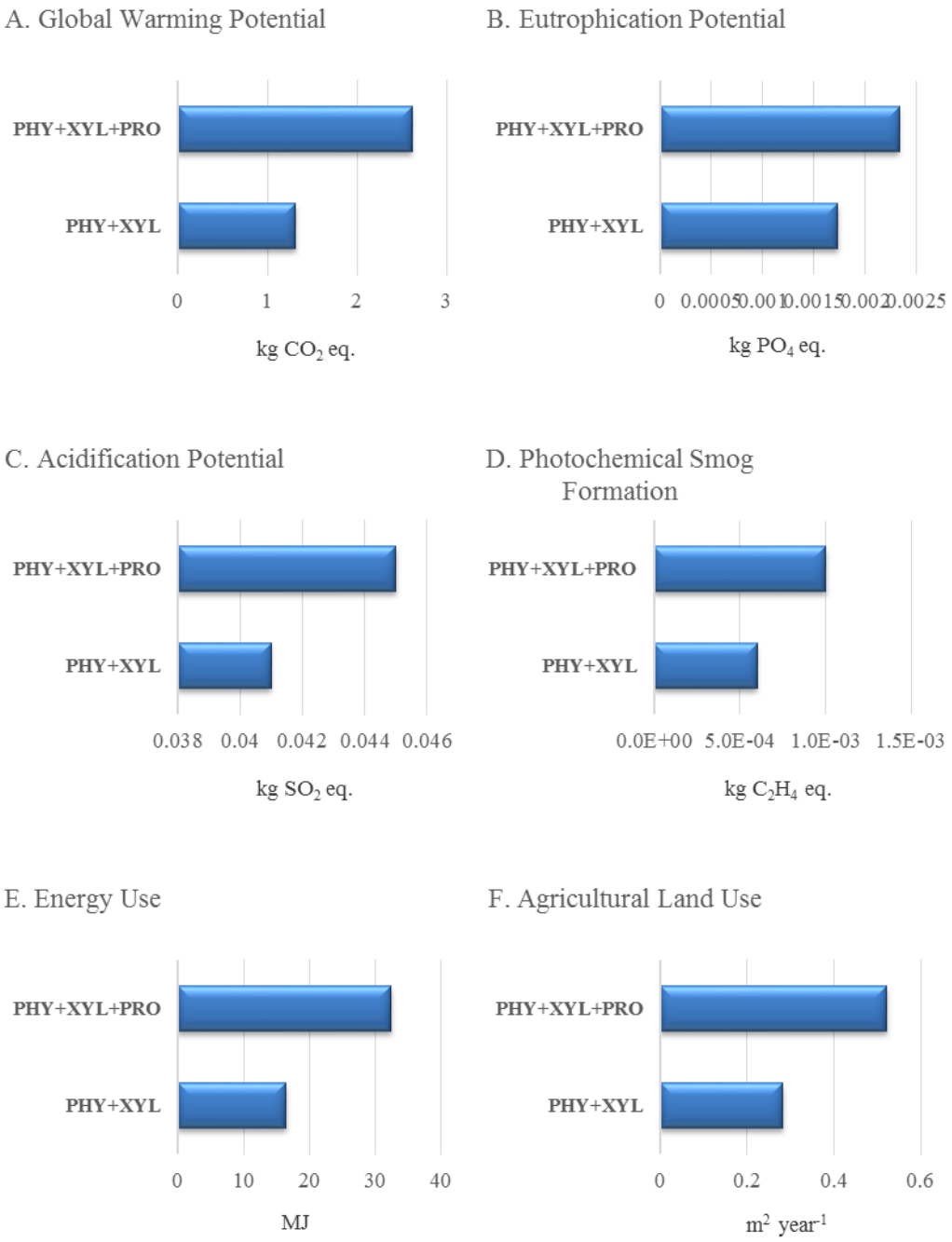


Figure 7.3.1: Relative impact potentials of six midpoint categories per 1000 kg of low FM ES tilapia feed.

7.3.1.2 Macro-ingredients

Protein sources (>40 CP%) are the most expensive components of manufactured feed. FM and SBM accounted for ~50% of each formulation (Table 7.2.2). Carbohydrates sources (*e.g.* RB, CM and MA) were used as low cost protein-sparing and energy sources. Analysis showed that 1 tonne of FM had the highest GWP (3,070 kg CO_2 *eq.*), EP (18.7 kg PO_4 *eq.*), AP (20.9 kg SO_2 *eq.*) and energy (4.17 x 10^4 MJ) values (Figure 7.3.2). The energy required to process FM was at least 74% higher than all the plant-based ingredients including palm oil. On the other hand, palm oil impacted PSF (4.53 kg C_2H_4 *eq.*) and ETP (986 kg 1-4-DCB *eq.*) the most. Notwithstanding this, palm oil also generated relatively high impacts on the other four categories considered. Overall, SBM and the other carbohydrate sources generated less potential impacts than FM. Due to cost effectiveness (0.25 US$ kg^{-1}), RB is generally used in commercial feeds and is the most widely used ABP for tilapia farming in Thailand. Interestingly, however, its production resulted in higher impact potentials than SBM in all categories except ecotoxicity. CM had the least overall impacts of the macro-ingredients.

7.3.2 Life Cycle Impact Assessment Results

7.3.2.1 Effects of FM Replacement and Enzymes in Tilapia Feeds

The first set of feeding scenarios assessed the effects of enzyme supplementation in declining FM diets. The results of following impact assessment were used to understand the effects of feed choices and options for improvements in feeding strategies for tilapia. Though FM had the highest impacts per tonne, replacement of the fisheries commodity with higher inclusions of the plant-based mix did not improve overall impact potentials but rather had the opposite effect (Figure 7.3.3).. Nevertheless, there were overall positive improvements in potential impacts with application of multi-enzymes to the respective control diets (Table 7.3.2). Improvements were more pronounced at the 2% and 5% FM levels because the enzymes had contributed to 6.3% and 6.1% reduction in FCRs, respectively.

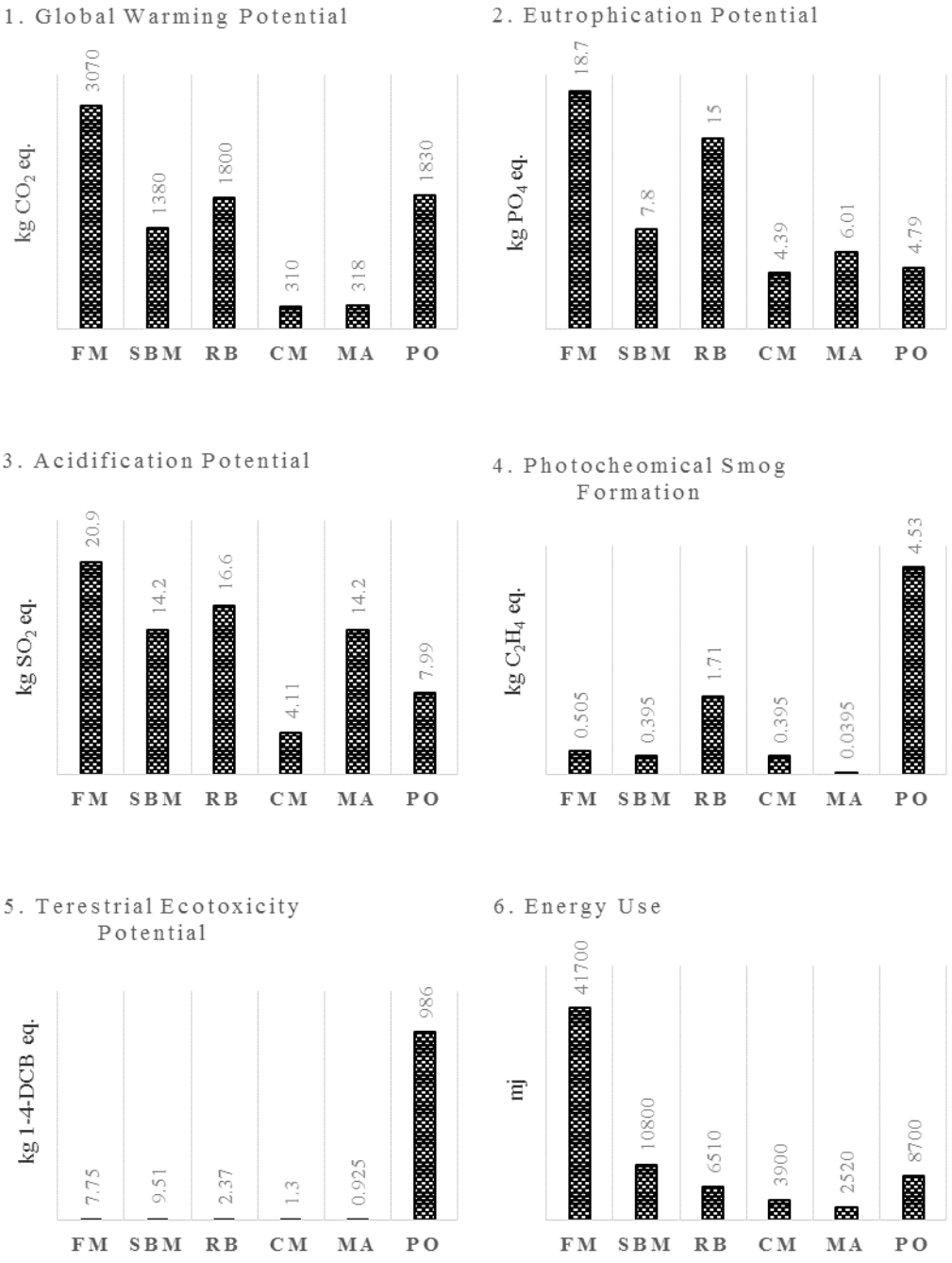


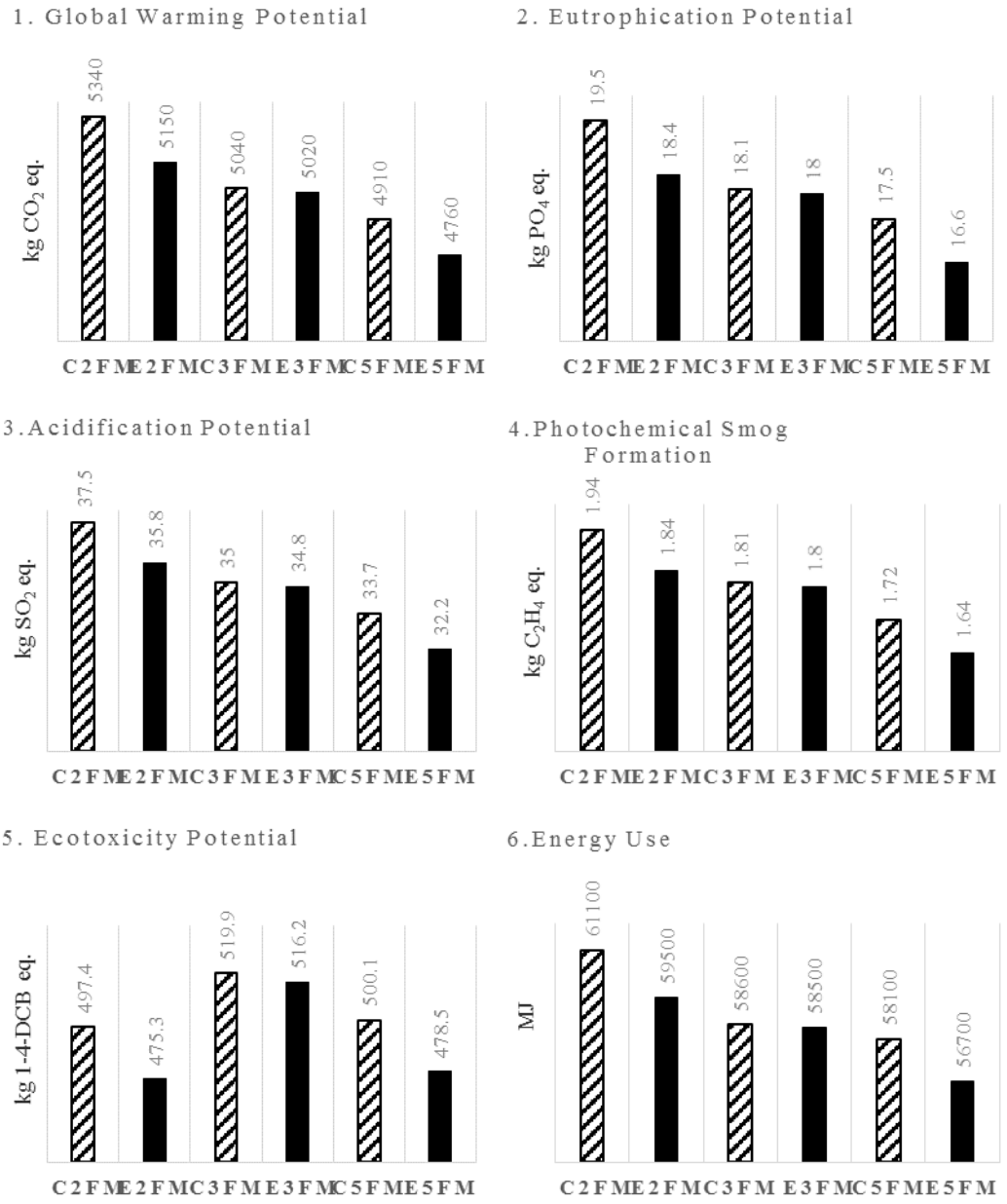
Figure 7.3.2: Potential environmental impacts of 1 tonne (1000 kg) of each ingredient at feed mill in Thailand. FM – tuna by-product fish meal (local), SBM – soybean meal (imported), RB – rice bran (local), CM – cassava meal (local), MA – maize meal (imported), PO – palm oil (imported)

Table 7.3.2. Change in impact categories due to digestibility improvement factors as a result of enzyme supplementation (%)

Impact Categories	**Unit**	**-ΔE2FM**	**-ΔE3FM**	**-ΔE5FM**
GWP	kg CO_2 *eq.*	3.56	0.40	3.05
EP	kg PO_4 *eq.*	5.64	0.55	5.14
AP	kg SO_2 *eq.*	4.53	0.57	4.45
PSF	kg C_2H_4 *eq.*	5.15	0.55	4.65
ETP	kg 1-4-DCB *eq.*	4.44	0.71	4.32
EU	MJ	2.62	0.17	2.41

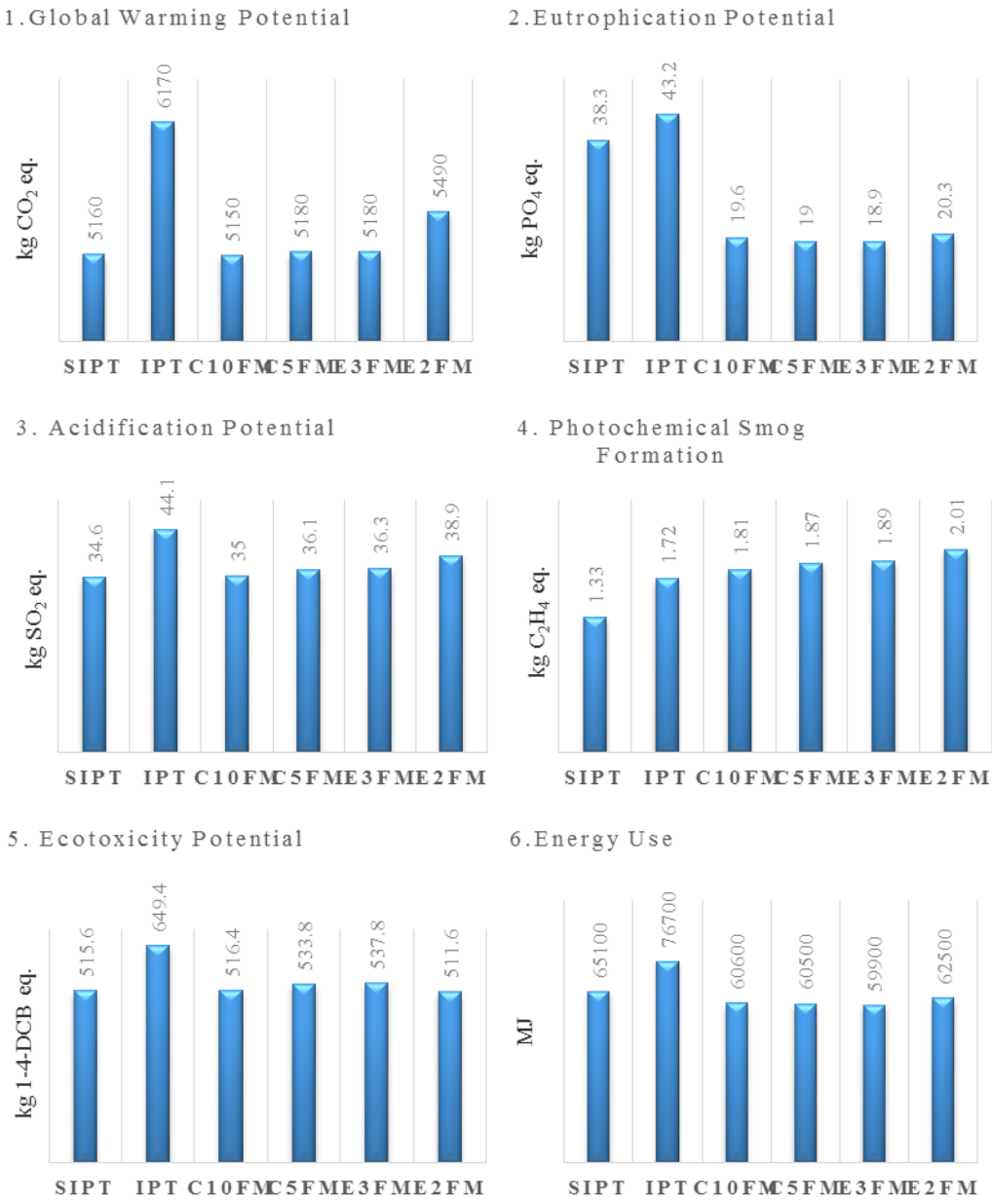
7.3.2.2 Comparison or Commercial Feed Production and Tilapia Grow-out

The second set of scenarios compared intensive tilapia farming using an average commercial feed described by Henriksson et al., (2014), two control diets containing 5% and 10% FM and two enzyme supplemented diets containing 2% and 3% FM. The scenario setting was extended to include semi-intensive farming using the average Thai tilapia diet in order to gauge the impact of conventional feeding practices by tilapia farmers in Thailand. This comparative analysis probed industry implications of using low FM enzyme supplemented diets as alternatives to current commercial feeds containing ~10% FM (Figure 7.3.4).



Exp. Diets	C2FM	E2FM	C3FM	E3FM	C5FM	E5FM
FCR	1.89	1.77	1.74	1.72	1.64	1.54

Figure 7.3.3: Comparative life cycle assessment of market ready tilapia production (1tonne) cultured using 2%, 3% and 5% FM diets supplemented with and without enzymes. E2FM contained phytase, xylanase and protease. E3FM and E5FM contained phytase and xylanase only. Ecotoxicity potential = terrestrial and freshwater ecotoxicity. C – control (no enzymes); E – enzyme supplemented, FM – fish meal



Scenarios	SIPT	IPT	C10FM	C5FM	E3FM	E2FM
FCR	1.38	1.70	1.73	1.80	1.82	1.97

Figure 7.3.4: Comparative life cycle assessment of market ready tilapia (1 tonne) cultured intensively and semi-intensively from average Thai commercial feed, 10% FM control, 5% FM control, 3% FM and 2% FM ES diets. SIPT – semi-intensive pond tilapia; Ecotoxicity potential = terrestrial and freshwater ecotoxicity; IPT – intensive pond tilapia; C – control (no enzymes); E – enzyme supplemented.

Intensive pond-raised tilapia (IPT) had highest potential impacts for GWP (6,170 kg CO_2 *eq*.), EP (43.2 kg PO_4 *eq*.), AP (44.1 kg SO_2 *eq*.), ETP (649.4 kg 1-4-DCB *eq*.) and energy use (76.7 tonnes MJ). The E2FM diet had the highest PSF value of 2.01 kg C_2H_4 *eq*.. Regardless of culture intensity, semi-intensive pond tilapia (SIPT) had equally high impact potentials as the experimental diets except for PSF. SIPT had the lowest potential impact on this category (1.33 kg C_2H_4 *eq*.). It should also be noted that though semi-intensive farming (SIPT) has potentially less impacts than intensive (IPT), the contribution of fertilizers to overall food conversion were difficult to quantity and therefore were not included in the model.

Eutrophication of the culture environment is highly debated in aquaculture (Henriksson et al. 2014). Another key implication of FM replacement is potentially higher levels of organic waste due in part to poorer apparent digestibility of plant-based ingredients. It was hypothesised that higher levels of FM would contribute to higher P outputs while higher levels of plant-based proteins/carbohydrates would increase N loss. In addition, increased nutrient utilization due to enzyme application would result in less waste excreted and reduced nutrient loading. Table 7.3.3 shows the N and P budgeting of the different feeding scenarios. N and P loading decreased overall with declining FM levels and enzyme supplementation. There were small differences between estimated figures based on previous studies and actual values.

Table 7.3.3: Estimates of nitrogen and phosphorus loading per tonne of tilapia based on different feeding scenarios

Feeding Scenario	Feed Composition (g kg^{-1} as fed)		FCR	Fish Composition[a] (g kg^{-1} wet basis)		Estimated Nutrient Loading[b] (kg tn^{-1})		Actual Nutrient Loading[c] (kg tn^{-1})		Difference in nutrient loading (kg tn^{-1})	
	N	**P**		**N**	**P**	**N**	**P**	**N**	**P**	**N**	**P**
SIPT	50.6	10.0	1.38	26.0	8.2	48.65	7.33	43.77	5.60	4.88	1.73
IPT	50.6	10.0	1.70	26.0	8.2	59.93	9.03	59.95	8.80	-0.02	0.23
C10FM	46.7	13.0	1.73	24.6	4.8	56.36	11.94	56.19	17.69	0.17	-5.75
C5FM	44.3	9.0	1.80	25.2	6.0	55.62	8.60	54.58	10.20	1.05	-1.60
E3FM	39.5	9.0	1.82	21.1	5.2	50.15	8.70	50.82	11.18	-0.67	-2.48
E2FM	38.7	8.0	1.97	28.3	6.6	53.19	8.37	48.01	9.16	5.18	-0.79
Comparative references											
Boyd and Queiroz, (2001)	48.0	10.0	1.50	21.3	7.5	33.79	5.00				
Pelletier and Tyedmers, (2010)	NI	NI	1.65	22.5	8.0	64.00	4.60				
Avadí et al., (2015)	44.8	8.0	1.40	NI	NI	34.70	3.00				

[a] Fish whole body composition
[b] Estimated nutrient loading = total N and total P, dissolved and as suspended solids. Average N and P outputs calculated from Lupatsch (2008), and Boyd and Queiroz (2001) as a percentage of feed nutrient input. N loading = 69.73% of total feed N; P loading = 53.1% of feed P.
[c] Actual nutrient loading based on Equation 1: $P_e = (P_f \times FCR) - P_a$; FCR feed conversion ratio
NI – no information

7.3.2.3 Interpretation – Sensitivity and Uncertainty

The potential impacts of using an alternative FM source, *i.e.* imported Peruvian FM as opposed to local tuna by-product FM for Thai tilapia feed production. The sensitivity of the model to FCR *i.e.* amount of feed used was obvious based on comparative analysis of semi-intensive (SIPT) and intensive pond raised tilapia (IPT) which utilised the same feed type but different FCR values. Consequently, this was ignored. Sensitivity analysis of substituting tuna by-product meal with Peruvian FM showed better environmental performance for all impact categories except under semi-intensive conditions. The differences were more pronounced for GWP and EU (Table 7.3.4). There was no difference between EP and ETP for E3FM and E2FM using either FM source.

Table 7.3.4: Sensitivity analysis of different tilapia feeds using alterntaive FM source on global warming potential and energy use

Feeding Scenario	FM Source	GWP	EU
		kg CO_2 *eq.*	MJ
SIPT	*Tuna By-product*	5,160	65,100
	Peruvian	+220	+2,600
IPT	*Tuna By-product*	6,170	76,700
	Peruvian	-100	-1,100
C10FM	*Tuna By-product*	5,230	62,400
	Peruvian	-420	-6,100
C5FM	*Tuna By-product*	5,180	60,500
	Peruvian	-180	-2,300
E3FM	*Tuna By-product*	5,180	59,900
	Peruvian	-100	-1,400
E2FM	*Tuna By-product*	5,490	62,500
	Peruvian	-80	-1,000

Monte Carlo analysis was conducted to assess the uncertainty of the final LCA results. Uncertainty results of the feeding scenarios are given in Table 7.3.5. Coefficients of variation were

higher for the semi-intensive (SIPT) and intensive (IPT) feeding scenarios. This may be due to the greater number of feed ingredients and therefore greater levels of uncertainty.

Table 7.3.5: Uncertainty analysis of tilapia feeds under different feeding scenarios. Mean ± STD

Impact Categories	SIPT	IPT	C10FM	C5FM	E3FM	E2FM
GWP (kg CO_2 *eq.*)	5,010 ± 2,440	6,330 ± 3,060	5,330 ± 531	5,200 ± 474	5,190 ± 474	5,530 ± 556
EP (kg PO_4 *eq.*)	36.6 ± 11.5	43.5 ± 15.2	20.5 ± 3.87	19.7 ± 3.64	19.4 ± 3.33	20.7 ± 3.48
AP (kg SO_2 *eq.*)	34.1 ± 14.8	45.2 ± 22.8	37.1 ± 5.11	36.5 ± 3.51	36.5 ± 4.17	39.7 ± 4.39
EU (MJ)	65,000 ± 40,400	76,800 ± 39,800	63,200 ± 8,130	59,700 ± 6,870	59,000 ± 7,170	63,330 ± 7,590
PSF (kg C_2H_4 *eq.*)	1.33 ± 0.64	1.75 ± 0.86	1.82 ± 0.49	1.90 ± 0.59	1.80 ± 0.38	1.98 ± 0.52
ETP (kg 1-4-DCB *eq.*)	531.6 ± 380.0	612.4 ± 385.2	528.6 ± 110.9	524.2 ± 88.1	536.6 ± 117.8	535.3± 141.5

7.4 **Discussion**

7.4.1 Implications of Feed Ingredient Choices

7.4.1.1 FM Replacement

Omnivores (*e.g.* tilapia) are more flexible in terms of the type of ingredients that can used for diet formulation and therefore are less dependent on FM (Tacon & Metian 2015). Nevertheless, FM replacement remains a challenge even for tilapia aquafeed formulators. Though an ideal source of digestible protein and EAAs, the fisheries-derived product generates considerable environmental burdens (Pelletier 2006). Replacement of FM with higher inclusion levels of meals from agricultural crops such as SBM, MA, RB and CM does have potential for tilapia feed production providing diet efficiencies can be improved. Competition for crops and grains may also become an inherent problem

in the near future in light of upward trends in global biofuel, livestock production and human population growth (Nguyen & Gheewala 2008; Pleanjai & Gheewala 2009; Van Zanten et al. 2014). Nevertheless, these crops have equal potential to address issues with FM supply and cost in order to meet higher global demand for seafood and aquafeeds. Fed-aquaculture will be limited by high commercial feed cost which forces artisanal farmers, particularly in Asia, to opt for cheaper less-efficient APB or alternative inputs. Though these alternatives are cost effective, the consequences are usually higher outputs of farm organic waste.

FM had the highest impact potentials of the macro-ingredients used for feed formulation per tonne. Pongpat and Tongpool, (2013) indicated FM shared the largest contributions across all impact categories in a case study of Nile tilapia farming in Thailand. Comparative analyses of macro-ingredients from different case studies are given in Table 7.4.1. Similar to Pelletier, (2006) and Pelletier and Tyedmers, (2010), the current study found that FM had the highest overall environmental impact potentials per tonne including energy use. This was potentially due to the fact that the processing of FM is often more energy intensive than crop culture and further processing combined (Pelletier 2006). The values for FM in the present study were also higher than other studies in all four categories. Nevertheless, life cycle impacts of fisheries-derived products vary significantly due to different fuel inputs and energy efficiency of reduction plants (Pelletier 2006). According to Papatryphon et al., (2004), using by-product FM would have negative effects on eutrophication due to higher levels of P per kg and higher quantities required to replace a standard high-quality FM. Theoretically, since FM generally has higher impact potentials, replacement should positively affect most impact categories. Furthermore, Iribarren et al., (2012) proposed that formulations containing more SBM and wheat (less FM) would have better environmental performances particularly on GWP. On the contrary, Papatryphon et al., (2004) found that a LF (low FM; 5%) trout diet had lower GWP, EP and AP values than a NF (no FM; 0%) diet based on the production of 1 tonne of live fish. Notwithstanding the HF (high FM; 42%) trout diet in the same study, however, had the highest EP, AP and energy use values among the treatments used.

Table 7.4.1: Comparison of feed ingredients with other studies

Macro-ingredients	**GWP** (kg CO_2 eq.)	**EP** (kg PO_4 eq.)	**AP** (kg SO_2 eq.)	**EU** (MJ)	***References***
Proteins/carbohydrates					
FM	3,070	18.7	20.9	41,700	This study
FM	1.220	4.50	10.3	19,300	(Pelletier & Tyedmers 2010)
FM	1,050	3.59	6.79	15,500	(Pelletier 2006)
SBM	1,380	7.80	14.2	10,800	This study
SBM	537	1.76	9.58	7,190	(Pelletier & Tyedmers 2010)
SBM	726	0.77	3.30	-	(Dalgaard et al. 2008)
SBM	221 – 333	1.63 – 2.89	2.45 – 3.24	3,440 – 3,990	(Pelletier 2006)
SBM	541	-	-	-	(Moe et al. 2012)
MA	318	6.01	14.2	2,550	This study
CGM	725 – 960	1.78 – 2.40	10.1 – 11.1	11,400 – 12,800	(Pelletier 2006)
CGM	1,290	3.50	20.1	18,000	(Pelletier & Tyedmers 2010)
Corn	656	-	-	-	(Moe et al. 2012)
Lipid					
PO	1,830	4.76	7.99	8,700	This study
PO	3,400	10.6	9.87	4,580	(Pelletier & Tyedmers 2010)

GWP – global warming potential; EP – eutrophication potential; AP – acidification potential; EU – energy use
FM – fish meal; SBM – soybean meal, MA – maize meal; CGM – corn gluten meal; PO – palm oil

Compared to the global FM production norm for raw material inputs (75% whole fish, 25% trimming), Thailand FM is produced from 65% trimming (canned fisheries, Surimi production and other fish process) and 35% trash fish (sardines and other local/overseas fish) (OXFAM 2014). The Thai animal feed industry is a major FM consumer. This industry set buying criteria based on ingredient quality as opposed to how the raw materials for FM are sourced which encourages unsustainable bottom trawling and other fishing practices (OXFAM 2014). In response to this and as a result of a global initiative to improve sustainability of FM supply, in 2013 the DOF established and implemented a certification scheme for Thai FM production to ensure traceability of raw fisheries material inputs from fishing vessel to feed mills.

SBM is arguably one of the most ideal replacers for FM due to its high CP and lysine contents, and its abundant yet stable supply (Drew et al. 2007). SBM demonstrated better environmental performance in all impact categories compared to FM. GWP, EP, AP and energy use values for producing 1 tonne of SBM were 55%, 58%, 32% and 74% lower than FM. Nevertheless SBM alone cannot fully replace FM without negative consequences on growth and other performance efficiencies. To dilute plant ANF effects and improve nutrient profile, the use of a mixture of plant-based ingredients is often more ideal. RB is the most extensively used ABP because of its abundance in Thailand and low cost. RB, however, had comparatively high EP and AP values to FM and a high GWP value similar to palm oil. CM, though nutritionally poor (< 2.5% CP), had the lowest impact potentials across all categories.

FM and its potential replacers will perform differently depending on diet composition. In support of Papatryphon et al., (2004) findings, though FM contributed to higher impact potentials, the level of plant-based ingredient substitution and degree of further processing will also affect the level of changes to any impact category. Contrary to Pelletier, (2006), and in agreement with Papatryphon et al., (2004), it is believed that if the quality and availability of by-product FM can be improved then this represents a viable opportunity to reduce the impacts of FM production directly from marine catch. Additionally, the use of fish by-products (which would be discarded due to its low value) to

produce FM relieves the industry of environmental burdens associated with waste (Newton, 2014). Regardless, Samuel-fitwi et al., (2013) maintained that finding suitable FM alternatives will not only minimize feed-related environmental impacts but support future aquaculture growth.

7.4.1.2 Enzyme Application to Animal feeds

There are a only few enzyme-related LCAs for swine and poultry production (Nielsen et al. 2007; Nielsen & Wenzel 2006; Oxenboll et al. 2011; Nagaraju & Nielsen 2011). The application of enzymes to basal tilapia diets containing declining FM levels resulted in improvements in environmental performance at all levels. Nevertheless improvements were higher at 2% and 5% FM levels and ranged from 2.41 – 5.64% over control diets. The most significant improvements were seen in PSF, EP and AP. GWP improved by 3% when the 2% FM diet was supplemented with PHY + XYL + PROT and the 5% FM diet supplemented with PHY + XYL only. There was only a 0.4% or 20 kg reduction at the 3% FM level.

In related studies, xylanase significantly reduced impact potential in all categories largely due to improvements in feed digestibility when 0.20 kg was added to a tonne of swine feed (Nielsen, Dalgaard, et al. 2007). Bundgaard et al., (2014) also reported a 0.09 kg CO_2 *eq*. reduction in GHG emissions with application of 0.36 kg of phytase + 0.9 kg xylanase-protease cocktail (XAP) to 1.8 tonnes of corn-soybean based broiler feeds. Oxenboll et al., (2011) also investigated the life cycle impacts of broilers grown with normal and low protein diets supplemented with 0.2 g kg^{-1} protease. They found that changes in impact potentials were higher for low protein diets, nevertheless the changes in EP, AP and GWP were -0.247 kg PO_4 *eq*., -0.813 kg SO_2 *eq.* and -11 kg CO_2 *eq*, respectively per tonne for the normal protein diet when compared to the control diet without protease. The application of protease to animal feeds has also been found to increase protein digestibility, protein retention and reduce N loading (Dias et al. 2012a; Drew et al. 2005; Dantagnan et al. 2014).

In a swine study, Nielsen and Wenzel, (2006) proposed that 1 kg of phytase would displace 29 kg of MCP *i.e.* 6.7 kg of P. This significantly reduced impact potentials by >90% for GWP, EP, AP, PSF and energy use. Furthermore, a combination of phytase and MCP had lower impacts compared to

the scenario of utilising MCP alone. Similarly, Nagaraju and Nielsen, (2011) proposed that 1 kg of phytase would displace 42.3 kg of MCP per tonne of poultry feed. The reduction in inorganic phosphate reduced P emissions to the environment. This resulted in positive improvements in GWP, EP and energy use. For this reason, the use of phytase in poultry feeds in some European countries (*e.g.* Netherlands) is now a requirement by law (Smith et al. 2013). In support, the greatest level of improvements in the present study were realised on EP. Though calcium phosphate was not completely substituted, improvements were realised due to higher dietary P utilisation. E2FM diet had the lowest actual N and P loading of 49.01 kg N tonne^{-1} and 9.16 kg P tonne^{-1} respectively. Smith et al., (2013) further argue that sustainability and profitability are not mutually exclusive. Improved nutrient utilisation due to enzymes means less feed is required to grow a tonne of live animals. This in turn means less ingredients, less land, less energy to process them and therefore lower impact potentials.

7.4.2 Implications of Feeds and Feeding Practices

Several studies have assessed the life cycle performance of various commercially important species as a function of feed (Papatryphon et al. 2004; Pelletier 2006; Avadí et al. 2015) while others focused on the general implications of aquaculture farming practices (Pongpat & Tongpool 2013; Mungkung et al. 2013; Aubin et al. 2006; d'Orbcastel et al. 2009; Ayer & Tyedmers 2009). Feed predominantly influences all impact categories and is thought to be the key indicator (independent of system type) in understanding the environmental performance of the aquaculture industry (Samuel-Fitwi et al. 2013; d'Orbcastel et al. 2009). In fact, global feed-related emissions from livestock account for about 3.3 gigatonnes of CO_2 *eq.* which is half of total emission from the livestock supply chain (FAO 2015a). Additionally, Iribarren et al., (2012) showed that on-growing accounted for 40% of GWP, 30% of EP and PSF and 30% of AP, of which aquafeed contributed 1.96%, 12.57%, 13.67% and 3.26% respectively.

The life cycle impacts of one tonne of live tilapia was compared between studies in Thailand, China and Indonesia (Table 7.4.2). Environmental impact potentials (except EP) were significantly

higher for intensive pond tilapia in the present study compared to Mungkung et al., (2013) and Pongpat and Tongpool, (2013). The EP was twice as high to produce one tonne of Nile tilapia in Mungkung et al., (2013) study potentially due to higher dietary FM (20%) and FCR (2.10) reported. Feed gain ratio also has significant effects on impact potentials and is more pronounced in terms of eutrophication at the farm level than all other categories due to additional emissions (Papatryphon et al. 2004). On the contrary, Avadí et al., (2015) reported that an artisanal tilapia feed (10% FM, 1.7 FCR) performed better than a commercial diet (4% FM, 1.4 FCR) due to less feed inputs and associated high agricultural burdens. Differences between studies in Table 7.4.2 may also have been influenced by model database, scenario setting and feed composition.

Finally, the study agrees with Pelletier and Tyedmers, (2008), that LCA of aquaculture and its supporting industries (feed and ingredients) should be used to inform more holistic approaches to eco-labelling, certification processes and sustainability education in the market place. In light of fed-aquaculture intensification and projected utilisation of 91 – 102 million tonnes of aquafeeds by 2030, understanding environmental burdens (through LCA) of compound feeds is an important first step for the aquaculture industry to improve sustainability, the potential for eco-labelling, satisfy consumer quality demands (The World Bank 2013). This may also promote more sustainable export-oriented farming systems needed to increase Thailand export volumes which currently accounts for <10% of tilapia production. Since certification schemes often financially exclude small farmers (Bosma & Verdegem 2011), LCA can be used for government extension support to small-scale fish producers for the development of proper feeding strategies. Additionally, feed manufacturers could develop LCA-based modules to support linear models used for least-cost formulation. This would assist in shifting some of aquaculture's burden from small/medium scale producers to the more financial/technical resourceful aquafeed producers. This will ensure better traceability of sustainable feeds and inputs into small scale Asian aquaculture

Table 7.4.2: Comparative life cycle impacts of fish production per tonne of live tilapia

Research Studies	% FM	FCR	GWP *kg CO_2 eq.*	EP *kg PO_4 eq.*	AP *kg SO_2 eq.*	EU *MJ*
Thailand						
Present study	9.35	1.38	5,160	38.3	34.6	65,100
	9.35	1.70	6,170	43.2	44.1	76,700
	10.0	1.73	5,150	19.6	35	60,600
	5.0	1.80	5,180	19	36.1	60,500
	3.0	1.82	5,180	18.9	36.3	59,900
	2.0	1.97	5,490	20.3	38.9	62,500
(Mungkung et al. 2013)	20.0	1.70	1,253	70	9.9	20,785
	20.0	2.10	1,444	105	11.3	23,501
(Pongpat & Tongpool 2013)	NI	NI	2,960	-	40.8	-
Other countries						
(Zhang 2014)	5.9	1.72	4,350	64.2	44.3	47,200
	5.9	1.61	3,580	57.2	38.4	36,700
(Pelletier & Tyedmers 2010)	3.0	1.70	1,520	47.8	20.2	18,200
	3.0	1.64	2,100	45.7	23.8	26,500

7.4.3 Conclusions

Though the responsibilities of the aquafeed formulator are already complex, least-cost formulation must be coupled with LCA as an option going forward. Feed manufacturers must be accountable for impact potentials of commercial feed products, assuring fish producers of product quality and environmental responsibility.. The study confirmed that higher inclusion of plant-based ingredients may have both positive and negative effects on impact categories. By-product FM is also a viable option if supply and quality can be guaranteed. Finally, the study demonstrated that enzymes can have significant environmental benefits. Due to reduced N and P emissions, enzyme application will positively improve impact potentials for eutrophication and acidification. As advances are made regarding the efficiency of combining enzymes, so will environmental benefits accrue to animal production.

Chapter 8
General Discussion

8.1 **Research Review**

Tilapia is second most cultured finfish globally and Thailand is the sixth largest producer (Tacon & Metian 2015; FAO 2015b). Though semi-intensive culture systems dominate, fed-aquaculture production is rapidly increasing to maintain pace with global seafood demands (FAO 2012a). Consequently, this has forced the aquafeed industry to innovatively replace its main yet limited fisheries-derived inputs (*e.g.* FM) with more sustainable plant-based options. Although this offers a viable FM replacement solutions, production efficiencies are likely to suffer as higher inclusions of plant-based ingredients are incorporated into aquafeed formulations. Moreover, poorer feeding efficiencies contribute to higher levels of organic N and P outputs, potential source of pollution. Poor animal performances has also been linked to plant ANFs (*i.e*. phytate-P, NSP arabinoxylan) and poor ingredient digestibility (Francis et al. 2001; Hardy 2010). Limitations which can both be addressed through multi-enzymes (*i.e.* protease, xylanase, phytase) supplementation. Though their application in other monogastrics have been extensively researched, their effects are still inconsistent with fewer published studies in fish, let alone tilapia (Bedford 2000; Choct 2006; Sinha et al. 2011).

The study employed a systems approach to diet development and tilapia sustainability. FM replacement options for tilapia feeds were assessed through feeding practices of tilapia farmers in central Thailand. Dietary FM levels of experimental diets were based on industry averages and projections, while replacers (SBM, RB, CM, MA) were selected from common local feed commodities. The research demonstrated the potential of exogenous multi-enzymes (phytase, xylanase, protease) to benefit nutrient utilisation, growth and sustainability in hybrid red tilapia (*O. niloticus* x *O. mossambicus*) fed low FM diets through a series of digestibility and on-farm grow-out experiments. Phytase was used in the study to target and hydrolyse native phytate-P (*myoinositol*

hexaisdihydrogen phosphate), xylanase to hydrolyse complex hemicellulose (*arabinoxylans*) and protease to improve indigestible protein bioavailability. Furthermore, experimental diets were compared to average commercial feeds and life cycle impacts assessed using CMLCA modelling.

8.2 **Feeds and Feeding Practices**

8.2.1 Feeding Practices of Tilapia Farmers

Feeding practices in Thailand (Chapter 3) are as diverse as the feed inputs used for culturing tilapia. Above 60% of the systems in the present study were semi-intensive polyculture and relied on both aquatic and terrestrial commercial feeds as well as alternative inputs (ABP, restaurant waste *etc*.). There were minimal reports of on-farm feed and trash fish use contrary to past studies (Dey et al. 2000; Thongrod 2005; Thongrod 2007). Although livestock (poultry, swine, cattle) manure was the main organic input used for pond fertilization, integrated livestock systems were also uncommon due to perceived risks associated with disease transmission (*e.g.* avian influenza) and food safety (Thongrod 2007). The study confirmed that nutrient input choices were largely driven by feed prices and market outlets (*i.e*. farm-gate price and domestic demand), however, trends in commercial feed use were linked to education ($P < 0.05$) and to a lesser extent age. Commercial feeds (<25% CP; 5 – 10% FM) were sometimes used for fattening in the final stages of production prior to harvest. Feed quality was often judged by its "fishy" smell, characteristic of high FM inclusion. This perception will require change in light of global sustainability trend towards almost complete FM replacement for tilapia feeds.

Thailand's feed industry has slowly developed since 1986 (Havanont 1993) with various commercially available feeds and inputs. Although there were less restrictions in terms of ingredient choices in Thailand, one of the main constraints to tilapia intensification and market expansion will likely be wide-spread adoption of cost-effective feeds (Thongrod 2007). Currently, while phytase is used for commercial feed production purposes. A feed mill respondent in the current study confirmed they discontinued using phytase based on poor field experiments. Xylanase and protease, on the other

hand, have only had experimental applications (Tapingkae et al. 2008). A recent feed market survey suggested, however, that fluctuating grain prices have encouraged new approaches in formulation including introduction or increased use of feed additives, such as enzymes (Roembke 2015). Approximately 49% of respondents (N = 292) adopted and increased admixture of phytase and NSPases, and 23% adopted enzymes for their benefits in environmental sustainability. Phytases were most popular globally, however, proteases ranked high in Asia Pacific (Roembke 2015). Finally, feed ingredient choices by feed manufacturers are generally based on considerations of ingredient availability, cost, nutritional quality, processing requirements, target species and market acceptability (Tacon & Metian 2015). The latter being linked to feed regulations but more importantly, food safety (Tacon & Metian 2015). The feed mill respondent also confirmed no immediate concerns with lowering FM inclusion for tilapia feeds due to a wide range of locally-available alternative ingredients, including animal by-products which are acceptable for domestic tilapia production.

8.2.2 Effects of Market and Certification

Certification schemes are driven by consumer awareness of quality assurance and food safety. These schemes, *e.g.* Global GAP, GAA-BAP and ASC, are often highly uneconomical for small-scale farmers and may cost between US$1,000 – 7,000 excluding annual fees (Thanh 2014). This was potentially one of the reasons none of tilapia farmers interviewed had third party certification. Value chain for export-driven commodities, *e.g.* shrimp, demand greater levels of traceability and environmental accountability on the part of producers and suppliers due to strict export-market requirements, particularly in Europe (EU General Food Law Regulations) and the US (Food and Drug Administration Act). This has led to improved ingredients procurement practices by several Thai feed millers (Charoen Pokphand, Thai Union *etc.*) that produce shrimp feeds (OXFAM 2014). On the other hand, Thai tilapia is still largely a domestic product so there is little impetus on the part of value chain actors to conform to international "consumer driven" standards. Additionally, third party certification do not currently make provisions for polyculture systems and the use of on-farm feed inputs common to tilapia production in Thailand (Nietes-Satapornvanit 2014).

Traceability (for certification) also relies on the ability to trace inputs and processes through proper documentation and record-keeping, another weakness of Thai tilapia production. Notwithstanding, Coff, (2006) argued that "in a society where production and consumption occurs in the same place and carried out by the same people, or where trade is dominated by face-to-face transactions where buyer and seller can verify quality, there is no need for verbalizing and formalising traceability". While this may be true, gone are days when all inputs were local, and globalization and long-distance trading of agro-commodities were rare. Today, inputs, particularly for feed production, are increasingly sourced overseas due to greater demands on local resources (*e.g.* cassava and palm oil for biofuel). Additionally, animal production value chains have now evolved and include larger numbers of intermediaries such as wholesalers, retailers, importers, exporters, shippers *etc*. Therefore, even without export-market regulations for traceability, as the aquaculture industry intensifies and the local tilapia value chain becomes more complex, tilapia production will eventually attract greater levels of scrutiny.

While there are strict legal quality standards which govern aquafeed production and distribution in Thailand (Agriculture and Cooperatives Ministry Regulation 1991, *amended* 1999), on-farm feed management and practices are still largely inadequate (Thongrod 2007). The use of commercial feeds is constrained by high prices, therefore feed millers will have to invest in even lower-cost formulations using under-utilised local inputs to promote higher commercial feed use. This will also ensure higher quality inputs for tilapia production. Improvements in tilapia farming practices will also require greater levels of support from government extension services to assist with education/training in proper record-keeping and better on-farm feed management. Zhang, (2014) argued, however, that extension support requires other incentive schemes to promote proper record-keeping among small-scale farmers because they have been largely ineffective even after a decade of effort.

8.3 **Effects of Enzymes on Nutrient Utilisation and Growth**

8.3.1 Digestibility

Compared to FM, plant-based ingredients generally have poorer nutrient contents, lower digestibility and contain ANFs (NRC 2011). Phytase has been used to hydrolyse phytate-P and improve P digestibility in commercial feeds (Kim et al. 2008; Biswas et al. 2007). Dissociation of phytate-P has also been linked to improvements in mineral, lipid and protein bioavailability (Hussain et al., 2011; Storebakken et al., 1998). Xylanase has been used successfully to minimise NSP's viscosity effects and improve nutrient assimilation (Sinha et al. 2011). While protease has been used to target PIs (trypsin *etc*.), more recently they have also been used to further improve protein digestibility in PI-denatured diets (Fru-nji et al. 2011).

Phase 1 experiment (Chapter 4) assessed the effects of phytase and xylanase in declining FM (0%, 3%, 5%) diets. Digestibility decreased with declining FM levels and with higher levels of plant-based ingredients. Ash digestibility increased with phytase and xylanase inclusion ($P < 0.05$) suggesting dissociation of phytate-mineral complexes. Based on intestinal P regulation and absorption in fish, Sajjadi, (2004) suggested that fish should be fed below their P requirement when assessing phytase efficacy. Despite P digestibility increasing by 9% at 3% and 5% FM levels, this was not statistically significant. This suggested that phytase had little effect in marginal available-P diets. No effects were seen on protein digestibility, consistent to Papatryphon and Soares, (2001) and Sajjadi, (2004) findings in salmonids. Krome, (2014) also found no effect on protein digestibility when tilapia Jatropha kernel meal-based diets were supplemented with phytase, if available-P was adequate. Theoretically, phytate-protein moieties formed under low stomach pH would not be required or hydrolysed, if dietary-P availability was sufficient to fulfil the animal's requirement. On the contrary, Hussain et al., (2011) reported improved CP digestibility in carp using phytase and Tapingkae et al., (2008) in piglets using xylanase. This unfortunately adds to the inconsistency regarding the secondary effects of phytase and xylanase on protein digestibility. Kim et al., (2008) concluded, however, that

there is a lower synergistic response in nutrient digestibility when xylanase and phytase are used in combination.

FM level for the negative control (NC) diet in Phase 2 experiment (Chapter 6) was adjusted to 2% to reflect the global projected average for 2015 (Tacon & Metian 2008). Supplementation of xylanase and phytase equally increased P digestibility (29.3%) ($P < 0.05$), however, there was no further improvement with protease. Lipid and energy digestibility also increased by 25.1% and 5% respectively over the NC diet, consistent with Simon, (2000) and Kim et al., (2008). With higher formulation levels of plant-based ingredients, protease was added to the enzyme cocktail to improve protein digestibility since there were no significant improvements with xylanase and phytase in Phase 1. Though protein digestibility improved by 6.1% over the NC, there were no significant difference between diets supplemented with protease (0.2 and 0.4 g kg^{-1} inclusion; LOPRO and HIPRO) and those having 0.385 g kg^{-1} xylanase and 0.075 g kg^{-1} phytase only. Contrary to Dantagnan et al., (2014), Dias et al., (2012a) and Drew et al., (2005), the beneficiary effects of protease-only supplementation were lost in the presence of xylanase and phytase. It also appeared that phytate-protein moieties were more responsive to phytase than protease. This was potentially attributed to that fact that ~50% of the experimental diets was composed of SBM, in which the intrinsic phytate has a higher propensity to bind to protein bodies (Selle et al. 2012).

8.3.2 Growth

Growth is the main biological measure of animal performance and an important economic variable (NRC 2011). In Phase 1 digestibility/growth experiment (Chapter 4), xylanase and phytase had marginal effects on feed efficiency and growth in terms of weight gain in juvenile tilapia. FCR decreased with higher FM levels ($P < 0.05$) but no differences were observed due to enzyme supplementation Nevertheless, in terms of FCR, AWG and SGR, the enzyme supplemented 3% FM and control 5% FM diets performed similarly ($P < 0.05$). On the contrary, the grow-out experiment (Chapter 5) showed slight inconsistency in terms of the bi-enzyme cocktail effects on growth variables (FCR, ADG, SGR *etc*.). There was less differences seen between the enzyme supplemented

diets and the respective controls in adult tilapia. Due to inherent experimental limitations (Section 8.6), no significant differences were observed between treatments. Though villi length results for the control diets were consistent with Borgeson et al., (2006), no improvements in gut morphology were also seen with enzyme inclusion. Nevertheless, under green-water pond conditions, the enzyme supplemented 0% FM diet had a comparative FCR of 1.67 to control 10% FM diet's 1.66.

The addition of protease to the cocktail (Phase 2; Chapter 6) showed significant improvements in FI, FCR and weight gain over the NC. AWG and FI increased by 26.3% and 18.9% with low inclusion of protease ($P < 0.05$). The 2% FM with 0.2 g kg^{-1} protease (plus phytase and xylanase) had a similar FI to the 10% FM control which was linked to comparative gut health (villi conditions) ($P < 0.05$). Contrary to Phase 1, villi length increased with enzyme supplementation ($P < 0.05$). There was, however, a loss in protease efficiency at high dosage (0.4 g kg^{-1}) in the presence of xylanase and phytase. FCR and PER were the only growth parameters that improved with a higher dose of protease. This suggests that protease can potentially have both semi-additive and additive effects on different variables. The level of effectiveness was clearly diet dependent and potentially more pronounced at earlier developmental stage. Furthermore, protease may be more beneficial as a micro-additive for starter diets as oppose to grow-out or 'fattening' diets.

8.3.3 Nutrient Loading

Environmental impacts of N and P are still major challenges for the aquaculture industry. Phase 1 digestibility study (Chapter 4) confirmed no significant improvements in N retention and loading due to enzyme supplementation. Cowieson and Adeola, (2005) also reported no effects of phytase on N digestibility in broilers, however, they found significant improvements with xylanase cocktail containing amylase and protease. Dias et al., (2012b) also cited xylanase-related improvements in N retention in Nile tilapia fed plant-rich diets. The current study, however, demonstrated improvements in P retention due to enzyme xylanase and phytase supplementation ($P<0.05$). Krome, (2014) also found significant improvements in P retention and loading when phytase was applied to diets containing no additional P source. Comparison of two enzyme supplemented diets (E2FM and E3FM)

with two control diets (C5FM and C10FM) (Chapter 7) confirmed the potential of enzymes to reduce nutrient (N and P) loading based on associated improvements in digestibility, lower dietary-P content and low FM levels

8.4 **Effects of Enzymes on Economic Performance**

Economic analysis confirmed that there are major differences in cost efficiencies when FM is progressly replaced by plant-based ingredients. This becomes the theoretical cost associated with sustainability and FM replacement. Nevertheless, both experimental phases confirmed the formulation benefits of higher inclusion of plant-based ingredients. Additionally while the controls diets were cheaper, tilapia production benefits of enzyme supplementation were observed in terms of reduction in cost per kg to convert feed to fish flesh. However, despite the best performances using phytase, xylanase and protease, unit profit diminished by US$ 0.018 kg^{-1} (E0FM) in Phase 1 and US$ 0.116 kg^{-1} (HIPRO) in Phase 2 compared to the industry-like 10% FM controls. To small-scale producers, fish is both a source of household income and nutrients, therefore sustainable production amd improved efficiencies would contribute to future livelihood and global food security (The World Bank 2013). Future efforts should therefore focus on reducing this gap in economic efficiency in order for aquaculture to fulfill the promise of contributing 62% of future seafood supply in 2030 (The World Bank 2013) without compromising the livelihood of farmers and industry sustainability.

8.5 **Implications for Tilapia Sustainability**

Sustainability will become even more important as the industry intensifies. This makes it even more critical for the industry, *i.e*. fish producers and feed manufacturers, to understand value chain impacts of material and energy consumption. The multiple indicator-based LCA, initially developed for the packaging industry, is increasingly becoming the standard and offers a holistic approach for assessing the environmental impacts as a new measure of the sustainability of global aquaculture

activities (Pelletier 2006; Caffrey & Veal 2013). In the wider context of sustainability, though socio-economic impacts are not adequately addressed by LCA, it transcends geographic regions linking value chain processes, *e.g.* feed manufacturing, which rely on global inputs and energy sources.

Though Thailand is almost self-sufficient in terms of local ingredient supply and availability, a significant amount of SBM (1.5 mil. tonnes) and maize (420,000 tonnes) are imported (Henriksson et al. 2014). Additionally, local FM production has been declining while consumption has increased (OXFAM 2014). Though Thailand utilises FM mainly derived from fish by-product for tilapia, this too has significant implication for life cycle assessment of compound feeds. Feeds contributed to most impact categories having pronounced impacts on GWP and EU at the production level and EP at the farm level. Based on intensive feeding scenarios, the enzyme supplemented 2% FM and 3% FM diets had lower impact potentials compared to the average Thai commercial tilapia diet described by Henriksson et al., (2014). This suggested that these diets may be environmentally superior to the average commercial feeds associated with tilapia production in Thailand.

8.6 **Research Limitations**

8.6.1 Feeding Surveys

Although the feeding practice data incorporated three independent farm surveys, there were limiting numbers of interviews conducted with feed manufacturers and suppliers to develop a more holistic perspective of Thailand's aquafeed industry. The selection of the farms for the follow-up feeding practice survey (FFPS) introduced some level of a bias because of the sample frame chosen. Nevertheless, the information was useful in supporting the data collected by the SEAT project.

8.6.2 Growth Experiments

The first phase of experiments had a few notable limitations. There was high variability in the initial fish weights due to on-farm batch weighing techniques. The digestibility experiment lacked a positive control (10% FM) due to space constraints in the warehouse where the tanks were installed. Moisture levels increased by ~3% in the enzyme diets because distilled water was used as the carrier

for enzyme coating. Crude measures of NH_3, NO_2, NO_3 and alkalinity were estimated using SERA colorimetric kits which introduced some level of subjectivity. Based on the trade-off between statistical power and effects size, the experimental design may not have been powerful enough to detect very small differences between treatments. Though non-significant results were obtained in the digestibility experiment, they were consistent with the hypothesized assumptions. This may have been due to the sample size being too small to detect true underlying effects (*i.e.* power of test). In the grow-out pond experiment, the non-significant results often had no noticeable trend which again was attributed to an issue of power and too much "noise" or variables affecting the data.

In the second phase of experiments, some of these limitations were addressed by increasing the number of replicates per treatment. Fish were weighed individually and graded twice at the start of each experiment to ensure lower variability in weights among treatments. The positive control was included as a part of the digestibility experiment and the enzyme diets were dried for longer periods post-coating to ensure consistency in moisture levels. Water quality (NH_3, NO_2, NO_3 and alkalinity) readings were done quantitatively using an electronic photometer. In regards to the treatment/handling of enzyme *versus* non-enzyme diets, all diets were stored under similar condition up to the point of coating. Due to on-farm space constraint (one refrigerator), only 5kg batches of enzyme diets were done weekly and only those small batches were able to be refrigerated in order to reduce the risk of contamination or mould formation. The enzyme diets were stored at 4^oC and used within 1-7 days of coating.

8.6.3 LCA

LCA generally relies on large data sets to develop to the underlying models. There was limited information on feed micro-additives particularly in Asia and Thailand. Model was therefore developed from data collected in Europe which inflated the uncertainty values associated with the results presented. LCA results in this study should therefore be used merely as guidelines.

8.7 **Overall Conclusions**

In light of the discussion above, the following conclusions were made from the study. A 3% FM diet supplemented with 0.385 g kg^{-1} xylanase and 0.075 g kg^{-1}phytase performed similar to the control (no enzymes) 5% FM diet in juvenile tilapia (Phase 1). Xylanase (0.385 g kg^{-1}), phytase (0.075 g kg^{-1}) and a low inclusion of protease (0.2 g kg^{-1}) can potentially improve the biological performance of a 2% FM diet for tilapia production in Thailand, however. economic efficiencies are still below that of an un-supplemented 10% FM diet under commercial-like production conditions (Phase 2). Multi-enzymes provided environmental (*e.g.* higher N and P retention) and economic benefits, however, better bio-economic efficiencies are needed based on profit analyses. As future research advances are made in aquafeed formulation and enzyme biotechnology, further improvements in tilapia performances are possible. This will, however, depend on better understanding of the cooperativity and synergy between exogenous enzymes under digestive conditions.

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Appendix A

Survey Questionnaires

For the Integrated Farm Survey (IFS) questionnaire, see Murray et al. 2013.

Table A1 Transition Farmers Survey (TFS) Questionnaire

SEAT Thailand Farmer Transition Survey – tilapia Draft5 18 Apr 13

PART 1. Telephone Survey

1. Survey Details

1.1. SurveyCD				
1.2. Interview Date				
1.3. Enumerator				
1.4. Respondent Full Name				
1.5. Farm RoleCD (manager, owner etc)				
1.6. Same Respondent? Yes/No *	**Survey 1**		**Survey 2**	
1.7. Telephone number(s)				
1.8. Gender	M:		F:	

* Survey 1 = Integrated survey Survey 2 = WP5 livelihoods

2. Farming transition status

Which of the following best describes any change in your situation since you were first interviewed (i.e. for the integrated survey)? – tick relevant box(es)

	Change Status	Tick	Month & Year
1	Farming as normal i.e. no significant change		
2	Farming as normal with some changes		
3	Temporarily stopped farming and already restarted		
4	Temporarily stopped farming with planned restart date		
5	Temporarily stopped with no planned restart date		
6	Permanently stopped farming		
7	Plan to stop temporarily in near future		
8	Plan to stop permanently in near future		

2.1. Are you planning to make any other changes to investment, production, labour use or marketing practices in the near future? Yes [ ] No[ ]

Details: _____

_____

2.2. Why did you (or do you plan to) permanently or temporarily stop farming?

Stop cause	Give details
Stock loss disease	
Stock loss other	
Seed quality	
Low sales price	
Lack operational finance	
Lack capital finance	
Have new business	
Land access	
Water access	
Regulatory burden	

2.3. If you have (or plan) to stop farming temporarily and plan to restart: i) why? _____

_____ii) when do you plan to restart? _____

2.4 If you have stopped or plan to stop farming, what are you doing now or what do you plan to do and why?

_____

3. What changes have or will you make to your farming practices? (refer to the farm-site visited for previous survey only)

Production change	First survey	Now(or planned)	Month&Yr	Details & reason(s) for change
Monoculture - Polyculture				
Species composition				Specify primary, secondary etc, if changed from mono to poly
Total culture area (ha)				
Total number of ponds				
Avg No ponds stocked/ cycle				
Avg pond area stocked/ cycle				
Pond fallow period (wks)				
No. of crops/year				
Culture period (days)				
Water exchange				
Chemical/probiotics used?				
Avg stocking density				
Stocking age of seed				
Seed source				
Supplier of feed inputs				
Type of feed inputs				
Brand of feed				
Level of feed inputs*				

Fertiliser inputs				
Sludge removal freq/cycle				
Avg Harvest size				
Integration				

4. Post harvest: What changes have been made or will you make to your marketing practices? (refer to the farm-site visited for previous surveys only)

Marketing change		**FirstSurvey**	**Now(OrPlanned)**	**Mnth&Yr**	**Details & reason(s) for change**
SalesTypeCD					Codes in IDSalesType in database
Sales to which buyers (list in order of volume; 1 = highest)	1				Can be individual not only companies
	2				
	3				
Sales contracting (use **contract code**) (list in order of volume; 1 = highest)	1				Thailand does not have this in previous survey
	2				
	3				
Farm-gate price					
Quality specification: size/wt					
Quality spec. : residue tests					
Quality spec. : specify					
% Domestic sales					

Sales contract codes (ID_SalesContract)

1. Farm is a fully owned and operated by a processor who takes all or most of its harvest (i.e. vertically integrated business)
2. Privately owned farm enterprise to which processor supplies feed (and or seed) based on agreement to sell back harvest at pre-agreed price
3. Privately owned farm to which processor supplies feed (and or seed) on credit with option to sell back at prevailing ('spot') market price
4. Privately owned farm enterprise, which independently sources feed (and or seed) inputs and sells harvest to processors at 'spot' price
5. Privately owned, feed on credit from feed company which has option to buy fish at prevailing market price

5. Investment What other changes have or will you make to your business practices? (all but first (grayed) item refer to visited farm-site)

Production change	Previous Survey	Now(or planned)	Mnth&Yr	Details & reason(s) for change
Number farms owned				
Business ownership (**code**)				
Land ownership (use **code**)				
Land area				
Labour No. full-time				Specify if HH, relatives, hired, single, couple, origin
Labour No. part-time				
Capital investment (specify)				
Certification (planned)				[ ] BAP; [ ] BMP; [ ] GlobalGAP; [ ]ThaiGap; [ ] ASC; [ ] Natural land; [ ] Others, specify

Farm ownership codes (ID_Ownership)

1	Owned by family	5	Leased from Provincial Govt.	9	Corporately owned
2	Leased from extended family	6	Lease from commune-short-term/commercial	10	Informal/ encroached
3	Leased from private owner	7	Leased from commune-long-term/domestic	11	Leased out
4	Leased from State	8	Contracted farm		

Table A2 Follow-up Feeding Practice Survey (FFPS) Questionnaires

Tilapia Farmers

1.How many rai of land do you farm?
2.Do you own the land, if rent, how much per rai per year?
3.How many full time and part-time employees do you have?
4.Do you fertilize the pond, how much of what per rai how often?
5.What tilapia stocking density do you use? What size of fish do you stock in grow-out? If nursing carried out, stocking density in nursery (pond or hapa) and culture period?
6.Does your production strategy currently include a fattening stage or system? If so explain the culture.
7.What are the FCR and growth rates (fattening ponds)?
8.What is your production volume per cycle (yield per rai)?
9.What other species, if any, and at what density do you stock in polyculture?
10.Length of culture period in grow-out?
11.What size do you harvest?
12.What is the overall cost of production per cycle and what percentage is feed/fertiliser input?
13.Do you think the commercial feed is affordable?
14.What do you think of the quality of the commercial feeds available?
15.What type of commercial feed is used? (e.g. catfish, frog etc.) And why? (If not a tilapia feed)?
16.What is the feeding method? Feeding rate?
17.Does the water or soil quality impact on the feed management? Do you treat water or soil before, during and after the production cycle?
18.What level of protein does the commercial or on-farm feed contains?
19.If on-farm diets are used, do you have any experience or knowledge of fish nutrition?
20.Do you use any agricultural or industrial by-product for feeding? In case you do, what is their origin? Are they available throughout the year or seasonally? Is its/their dietary quality similar throughout the year? Do you apply any feed additive in order to improve their quality?
21.Do you think the levels of fishmeal should be reduced in fish diets?
22.Would you be willing to use a feed without fishmeal if it had it maintain the same level of performance?
23.Do you keep actual records of feed input?
24.Do you think the waste water discharge from farm affects the environment?
25.Do you suffer from disease problems? Details.
26.Do you use any chemicals (not including lime) during culture such as probiotics, antibiotics, etc. If so estimate expenditure per rai per crop.
27.Where do you sell fish?
28.What price do you get? Seasonal variations? Prices of other species?
29.How do have tilapia prices changed over the past 5 years? Could make an estimated % increase per year.
30.Based on tilapia prices, have you ever considered the export market? And why?
31.Is muddy off-flavor an issue with regard to selling fish?
32.List the main risks to your business in order of importance – flooding, disease, lack of water, government action/policy affecting the business, theft, etc.
33.Why do you buy from Nam Sai, what are the advantages/disadvantages of buying elsewhere?
34.Are there large differences in results between batches?
35.What strain do you prefer and why?
36.Any recommendations for Nam Sai?

II. Feed Millers

1.What is the name of your Company and where is it located? (list all locations)
2.What is your name and position?

3.How long have you been employed to the Company?
4.Does your Company produce aquatic feeds? If so, in what form, pelleted or extruded?
5.Does your Company produce tilapia diets and in what feed form, sinking or floating pellets?
6.What are the CP level and percentage fishmeal inclusion? Has the level or inclusion changed over the last 10 years? Why?
7.If relevant, is this the same trend for all other aquatic feeds produced?
8.What is your Company position on the drive to reduce FM levels in aquatic feeds in the future
9.What alternative ingredients are used for fishmeal replacement and have your customers (particularly farmers) experienced any performance impacts?
10.Where does the Company source its ingredients (particularly fishmeal), locally or overseas? And why?
11.Are the raw ingredients processed before or after they are received by the Mill?
12.Is the Company/Feed Mill certified (ISO etc.) and are you involved in any sustainable certification programme (GAA, BAP etc.) to support farmers/customers?
13.What are the current production volumes per year for total feeds (both terrestrial and aquatic), total aquatic feeds and total tilapia feeds?
14.What is average cost per kg of tilapia feeds over the last 5 years?
15.Does your Company engage in any form of research and development? If yes explain? If no, how does it validate its product performances?
16.Does the Company own any fish farm(s) or have any contract farms?
17.How are the feeds marketed and who are the main customers?
18.Has the Company done any form of customer based surveys focusing particularly on product quality? If yes what were the feedbacks?
19.Has there been any noticeable trend in tilapia feeds sales? If yes explain? If no why do you think this is so?
20.What is your opinion on the state of the aquafeed industry in Thailand and what are some of the drives for expansion in the future if any?
21.Do you use enzymes in your aquatic feeds, if yes which one(s)?
22.Are they used for tilapia feeds? If no why not?
23.(if yes to Q#18 and #19) How long have you been using enzymes, have there been any positive economic feedback from your customers in terms of performance and yield, and what is the future direction?
24.What is the level of inorganic phosphate used in tilapia feeds? If applicable, has this usage changed due to enzyme (phytase) incorporation?
25.What is the average energy consumption for producing one tonne of tilapia feed?

Appendix B

Experimental Data and Water Quality Tables

Table B1 Data set collected in digestibility/growth experiment (Phase 1) with tilapia fed low FM diets with and without exogenous enzymes

EXP. DIET	TANK NO	Wgt Wk0	Wgt Wk2	Wgt. Wk4	Wgt. Wk6	Wgt. Wk8	Wgt. Wk10	Wgt. Wk12	Leng. Wk0	Leng. Wk2	Leng. Wk4	Leng. Wk6	Leng. Wk8	Leng. Wk10	Leng. Wk12	Total Feed[a]	No. Fish[b]
E0FM	*6*	51.40	60.30	70.75	77.95	91.75	108.30	119.65	13.50	14.47	15.20	15.71	16.31	17.53	17.87	2801	20
	13	49.67	55.70	63.55	73.25	86.10	104.40	114.50	13.29	14.36	14.73	15.67	16.26	17.40	18.05	2618	20
	8	49.07	61.35	70.00	79.00	94.30	116.20	131.00	12.82	14.62	15.44	16.20	16.66	18.08	18.56	3064	20
	21	44.67	53.50	66.80	81.20	91.95	104.16	115.83	12.82	14.00	14.95	16.13	16.31	17.32	18.11	2432	18
E3FM	16	55.79	72.75	92.45	113.60	137.15	164.35	174.05	13.41	15.06	16.55	17.55	18.16	19.83	20.34	4010	20
	9	48.53	64.35	83.95	103.60	122.20	138.35	151.65	13.16	14.85	16.14	17.32	17.94	19.01	19.49	3373	20
	10	51.03	61.55	74.65	89.10	102.40	120.90	129.00	13.40	14.45	15.68	16.55	16.96	18.17	18.45	2799	20
	14	43.30	58.85	71.60	82.95	100.40	119.20	127.10	12.67	14.63	14.95	16.21	16.93	18.14	18.56	3012	20
E5FM	4	46.28	63.70	79.45	96.45	112.95	132.68	149.11	13.35	14.82	16.10	16.95	17.72	19.00	19.60	3040	19
	2	52.03	72.25	89.65	107.15	134.65	165.65	191.50	13.80	15.39	16.49	17.41	18.78	20.49	21.27	3978	20
	5	48.83	68.70	84.75	99.25	116.45	134.10	147.10	13.69	15.41	16.14	17.15	18.11	19.14	19.77	3103	20
	7	53.59	60.30	76.25	94.55	118.55	140.00	152.40	13.70	14.76	15.68	17.28	17.91	19.36	19.81	3266	20
C0FM	15	50.77	59.55	73.00	87.05	106.56	128.33	142.44	13.45	14.41	15.54	16.34	17.36	18.71	19.12	2867	18
	20	48.03	56.50	59.40	70.30	79.55	95.70	105.65	13.17	14.31	14.61	15.38	15.55	16.79	17.33	2409	20
	11	48.17	57.65	70.47	78.42	85.68	102.50	114.76	13.33	14.33	15.22	16.02	16.19	17.41	18.01	2424	17
	3	50.60	67.95	77.10	85.60	97.85	112.00	123.42	13.77	15.05	15.14	16.28	16.91	17.82	18.37	2876	19
C3FM	22	44.47	54.25	64.30	79.10	95.95	108.55	120.20	12.72	14.14	15.07	16.07	16.51	17.62	18.20	2638	20
	19	49.44	58.45	74.95	89.50	107.95	126.65	130.75	13.43	14.36	15.65	16.72	17.45	18.62	18.90	2944	20
	18	48.07	60.75	73.55	87.65	102.60	121.00	134.40	13.00	14.44	15.35	16.54	17.00	18.25	18.78	3207	20
	12	47.32	66.90	80.50	92.45	111.05	129.70	146.15	13.29	14.67	15.71	16.50	17.35	18.72	19.17	3114	20
C5FM	24	46.07	61.35	77.00	96.60	120.35	138.95	148.25	12.82	14.58	15.63	16.84	17.86	19.08	19.47	3417	20
	17	46.86	65.40	80.35	98.10	112.80	129.50	139.25	12.86	14.78	16.06	16.98	17.45	18.65	19.06	3045	20
	1	57.79	69.15	88.30	110.55	137.50	157.75	171.90	13.97	14.86	16.52	17.67	19.03	19.86	20.65	3766	20
	23	42.79	60.35	78.80	96.95	116.90	132.60	142.55	12.66	14.30	16.03	17.19	17.61	18.91	19.43	3162	20

[a] Total feed fed (in grams) for the xperimental period; [b] Number of fish that survived. Weight in grams and length in cm

Table B2 Data set collected in grow-out experiment (Phase 1) with tilapia fed low FM diets with and without exogenous enzymes

DIET	HAPA NO	Wgt Wk0	Wgt Wk2	Wgt Wk4	Wgt Wk6	Wgt. Wk 8	Leng Wk0	Leng. Wk 2	Leng Wk 4	Leng Wk 6	Leng. Wk 8	Total Feed	No.Fish
E0FM	***9***	262.62	332.90	395.45	458.80	505.75	24.64	25.93	27.57	28.52	29.88	11343	20
	3	235.19	301.25	376.11	432.06	497.83	23.59	25.50	27.12	27.89	29.53	11346	16
	4	243.50	318.05	377.16	423.68	472.42	24.33	25.68	27.14	27.84	29.41	11317	19
	22	193.30	265.45	319.60	377.20	421.11	21.78	23.80	25.53	26.74	28.11	11331	19
E3FM	**11**	212.03	292.35	356.30	409.15	466.58	22.76	24.83	26.61	27.49	29.13	11332	19
	28	248.70	333.35	392.00	452.75	501.28	24.08	25.66	27.20	28.14	29.69	11376	17
	24	229.93	308.60	363.84	405.95	440.26	23.43	24.84	26.31	27.17	28.77	11340	18
	26	229.00	298.60	344.50	376.45	418.63	23.38	24.51	26.05	26.65	27.99	11342	17
E5FM	**27**	219.04	323.58	354.31	416.77	466.08	22.78	24.82	26.68	27.74	28.77	11344	12
	2	259.54	305.25	359.00	406.26	459.37	23.85	25.44	26.63	27.32	29.08	11332	18
	19	237.74	321.60	400.95	455.25	441.00	23.90	25.94	27.81	28.71	30.40	11349	18
	15	194.36	257.05	319.25	363.05	412.79	21.35	23.57	25.25	26.32	28.08	11329	18
C0FM	**12**	172.70	255.20	302.68	340.68	390.58	21.19	23.40	24.96	25.97	27.41	11342	19
	7	254.52	315.30	376.32	418.58	466.84	24.28	25.79	26.75	27.69	29.31	11353	18
	16	181.80	252.32	318.37	365.21	408.05	21.00	23.74	25.54	26.71	28.19	11327	19
	14	177.63	240.21	297.42	336.37	368.95	21.37	23.19	24.76	25.58	27.12	11347	18
C3FM	**10**	236.67	294.16	343.32	398.88	423.63	23.47	24.83	26.19	27.34	27.84	11366	16
	1	239.10	320.20	357.55	397.55	434.11	23.44	25.33	26.59	27.40	28.69	11331	18
	6	246.27	306.05	377.32	431.74	484.32	24.10	25.58	26.98	27.94	29.50	11329	18
	17	179.50	265.40	326.00	376.10	417.30	21.08	23.64	25.44	26.74	28.14	11321	20
C5FM	**25**	252.92	314.50	376.11	436.42	477.65	23.63	24.82	26.73	27.56	29.32	11366	16
	8	231.07	293.20	355.40	410.10	460.25	23.81	25.47	26.90	28.11	29.85	11350	20
	18	223.60	316.45	384.00	440.58	500.37	23.18	25.31	27.12	28.05	29.81	11364	19
	21	229.30	287.75	334.06	419.88	456.63	23.30	24.84	26.44	27.56	29.28	11330	15
C10FM	**20**	232.71	316.63	387.50	444.58	491.11	23.29	25.28	27.23	28.03	29.59	11329	17
	13	182.03	267.70	344.15	399.90	456.95	21.66	23.58	25.85	27.04	28.75	11339	19
	5	254.10	314.40	377.16	435.89	481.68	23.89	25.48	26.89	27.92	29.55	11329	19
	23	221.17	305.95	366.88	425.31	478.31	23.02	24.45	26.19	27.75	28.97	11341	16

Table B3 Data set collected in grow-out experiment (Phase 2) with tilapia fed low FM diets with and without exogenous enzymes

EXP. DIETS	CAGE NO.	IBW_WK0	WK4	WK8	WK12	IBL_WK0	WK4	WK8	WK12	Total Feed	No. Fish
1	**17**	74.25	153.50	228.65	298.00	16.44	20.01	22.44	24.61	8482	18
NC 2%FM	**7**	68.65	153.05	204.11	251.05	16.13	19.49	21.64	23.30	7255	20
	29	69.50	141.44	183.56	225.17	16.12	19.48	21.54	23.18	6818	18
	14	72.35	150.60	215.55	279.95	16.25	19.47	22.00	24.36	7953	20
	30	69.60	135.16	203.00	244.42	16.00	19.11	21.78	23.48	7138	19
	9	72.70	160.89	207.21	270.11	16.15	19.94	22.10	24.11	7706	19
2	**18**	71.35	149.95	228.95	301.05	16.11	19.76	22.44	24.62	8865	19
NO_PRO	**19**	69.35	149.95	212.00	274.37	15.97	19.71	22.08	24.10	7810	19
	24	73.95	168.35	254.40	345.95	16.35	20.29	23.13	25.68	9334	20
	11	70.00	160.22	224.56	301.76	15.86	19.76	22.31	24.35	7569	17
	25	71.65	162.74	230.94	308.82	16.03	20.01	22.36	24.79	7899	17
	1	72.80	169.32	258.74	308.21	16.32	20.46	23.18	24.79	9207	19
3	**6**	69.05	157.84	235.16	319.42	16.29	19.63	22.37	25.05	8725	19
LO_PRO	**10**	72.45	171.11	238.74	320.89	16.10	20.09	22.54	25.25	8819	19
	3	71.30	165.45	262.21	340.11	16.37	20.10	23.25	25.62	8574	19
	16	70.80	157.45	234.40	309.95	16.04	19.95	22.63	24.81	9584	20
	28	70.55	146.74	209.67	267.67	16.17	19.38	21.86	23.95	8277	18
	22	72.65	166.24	238.47	310.65	16.16	20.12	22.61	24.61	9006	17
4	**12**	67.40	161.63	222.89	293.05	15.90	19.64	22.08	24.31	7613	19
HI_PRO	**5**	71.95	173.05	253.79	320.22	16.36	20.26	23.09	25.23	8585	18
	20	74.45	163.16	236.21	311.00	16.31	20.20	22.61	24.79	8426	17
	4	70.20	171.37	248.79	324.47	16.18	20.27	22.92	25.53	8282	19
	27	72.60	163.50	242.25	313.15	16.27	20.29	22.71	24.78	8508	20
	21	71.50	160.37	225.84	288.50	16.00	19.87	22.28	24.30	8093	18
5	**2**	72.85	171.32	260.63	349.00	16.36	20.28	23.28	25.66	9869	19
PC 10%FM	**13**	71.30	171.10	263.35	362.70	16.17	20.34	23.50	26.43	9243	20
	26	69.80	163.58	274.26	377.63	15.98	20.30	23.90	26.72	9496	19
	23	70.15	172.44	274.76	372.35	16.06	20.46	23.85	26.48	9304	17
	8	68.10	167.00	250.11	331.71	15.83	20.14	22.88	25.16	8606	17
	15	69.00	170.65	258.45	357.00	15.75	20.13	23.10	25.43	9406	20

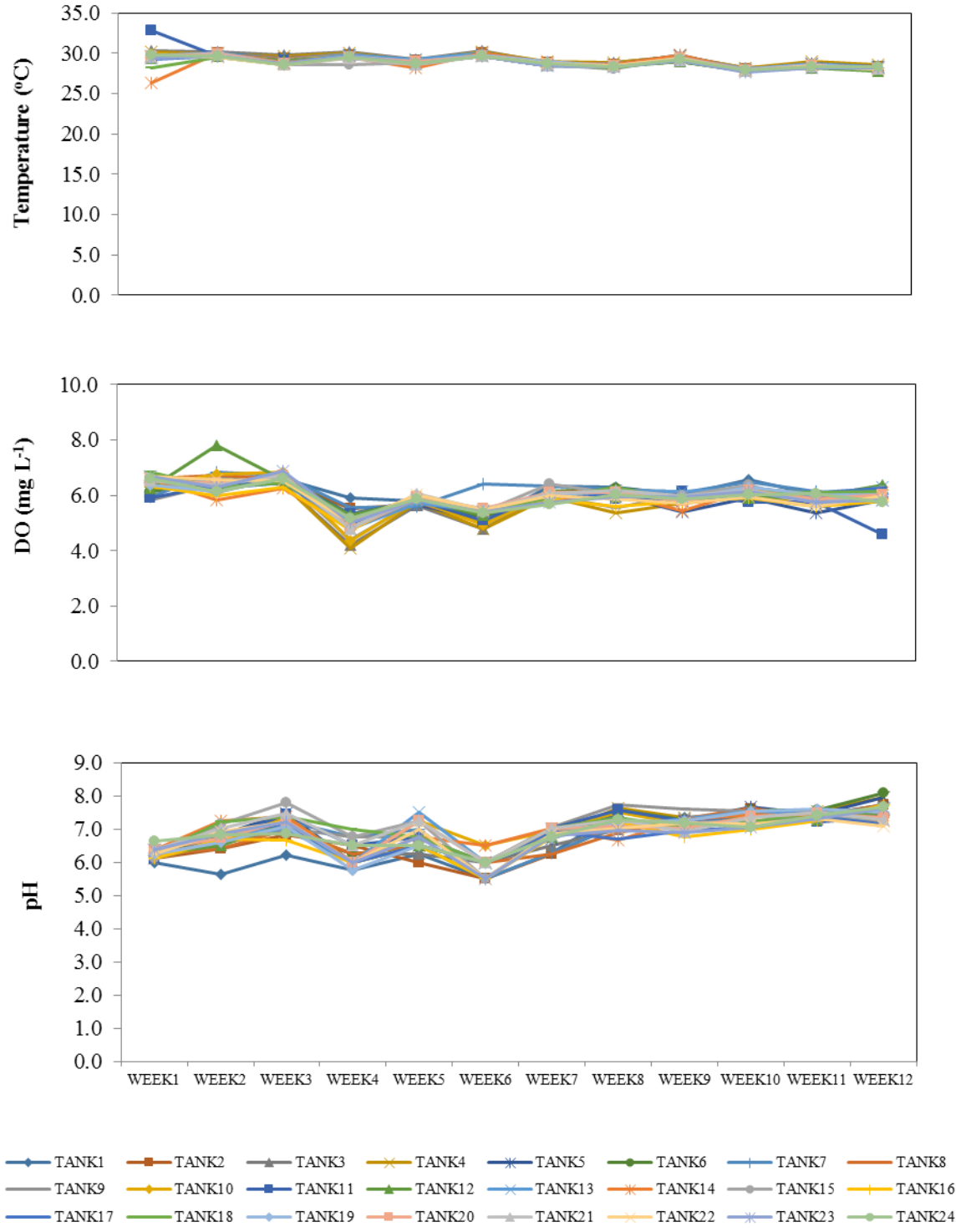
Table B4 Data set collected in digestibility experiment (Phase 2) with tilapia fed low FM diets with and without exogenous enzymes

EXP. DIETS	TANK NO.	IBW WK0	FBW WK8	IBL WK0	FBL WK8	Total Feed	No. Fish
1	**12**	113.30	201.33	18.02	21.67	1454	9
NC 2%FM	**10**	108.80	199.80	18.02	21.52	1638	10
	13	116.40	217.71	18.30	21.67	1496	*7*
2	**9**	112.40	230.71	17.83	22.31	1516	*7*
NO_PRO	**11**	113.60	201.13	18.16	21.49	1337	8
	3	114.10	259.44	18.28	23.28	2171	9
3	**8**	112.00	234.00	18.16	22.83	1699	9
LO_PRO	**4**	112.40	177.17	18.07	20.93	979	6
	15	115.20	214.75	18.57	22.13	1552	8
4	**5**	111.20	204.29	18.03	22.17	1233	*7*
HI_PRO	**2**	117.80	223.14	18.51	22.24	1472	*7*
	6	110.20	225.40	18.26	22.56	1844	10
5	**7**	109.60	261.13	18.14	23.84	1876	8
PC 10%FM	**14**	113.50	255.75	18.30	23.23	1708	8
	1	118.50	243.30	18.32	23.00	1878	10

IBW – initial body weight (g); FBW – final body weight (g); IBL – initial body length (cm); FBL – final body length (cm)
NC – Negative control; PC – Positive control

Table B5 Water Quality Data (Phase 1)

I. Tank Experiment (Temperature, DO and pH)



NB. Average colorimetric readings for ammonia, nitrate and alkalinity are given in Chapter 5.

II. Pond Trial (Temperature, DO and pH)

Weekly Average	Temperature (ºC)	DO (mg L^{-1})	pH
Week 1	32.78	6.26	8.0
Week 2	32.86	5.81	8.0
Week 3	33.27	6.31	8.0
Week 4	32.41	3.65	8.0
Week 5	33.52	5.43	7.5
Week 6	33.65	5.68	8.6
Week 7	32.86	6.97	8.9
Week 8	31.32	5.58	8.4

NB. Average colorimetric readings for ammonia, nitrate and alkalinity are given in Chapter 6.

Table B6 I – VI Water Quality Data (Phase 2)

I. Pond Trial (All Parameters)

WQ Parameters	TEMP ^{o}C	DO mg L^{-1}	pH	NH3 mg L^{-1}	NO2 mg L^{-1}	NO3 mg L^{-1}	ALK mg L^{-1}
Week 1	30.30	3.24	7.60	0.94	0.19	0.00	85.00
Week 2	30.70	3.22	7.55	0.94	0.26	0.00	100.00
Week 3	31.27	3.88	8.05	0.67	0.06	0.00	110.00
Week 4	30.22	3.35	8.15	1.01	0.11	0.00	110.00
Week 5	30.80	3.16	7.70	0.76	0.10	0.00	122.50
Week 6	32.15	2.96	7.40	0.69	0.00	0.00	97.50
Week 7	31.33	3.18	7.70	1.23	0.02	0.00	117.50
Week 8	31.09	3.10	7.55	1.02	0.03	0.00	90.00
Week 9	32.11	2.76	7.40	0.76	0.00	0.00	102.50
Week10	31.76	3.05	7.45	0.76	0.02	0.00	85.00
Week 11	32.28	2.88	7.65	1.13	0.01	0.00	87.50
Week 12	32.14	2.78	7.75	0.94	0.00	0.00	90.00

Tank Experiment – **Ammonia** (mg L^{-1})

Tank	**Week 1**	**Week 2**	**Week 3**	**Week 4**	**Week 5**	**Week 6**	**Week 7**	**Week 8**
1	0.25	0.53	0.69	0.54	0.46	0.47	0.65	0.82
2	0.35	0.45	0.43	0.44	0.50	0.48	0.59	0.58
3	4.33	0.65	0.74	0.81	0.44	0.43	0.62	0.62
4	4.81	0.39	0.51	0.34	0.37	0.42	0.47	0.48
5	5.42	0.37	0.41	0.35	0.41	0.43	0.52	0.57
6	4.47	0.50	0.52	0.64	0.34	0.58	0.59	0.67
7	4.03	0.47	0.41	0.66	0.31	0.47	0.56	0.72
8	3.58	0.62	0.66	0.60	0.47	0.42	0.53	0.54
9	6.18	0.38	0.56	0.41	0.46	0.46	0.44	0.51
10	0.35	0.55	0.43	0.51	0.42	0.40	0.60	0.59
11	0.39	0.42	0.45	0.39	0.42	0.62	0.65	0.59
12	0.51	0.46	0.00	0.43	0.22	0.47	1.03	0.59
13	0.37	0.58	0.81	0.69	0.43	0.51	0.76	0.57
14	0.35	0.60	0.55	0.29	0.29	0.68	0.45	0.51
15	0.56	0.54	0.59	0.29	0.24	0.45	0.52	0.57

III. Tank Experiment – **Nitrite** (mg L^{-1})

Tank	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
1	13.0	0.0	0.0	3.0	0.0	0.0	0.15	1.72
2	7.0	1.0	0.0	0.0	0.0	0.20	0.10	0.11
3	5.0	20.0	6.0	0.0	0.0	0.46	0.14	0.09
4	4.0	2.0	2.0	0.0	0.0	0.12	0.11	0.11
5	0.0	11.0	13.0	0.0	0.0	0.06	0.09	0.27
6	4.0	6.0	4.0	0.0	0.0	0.54	0.25	0.53
7	8.0	14.0	6.0	0.0	0.0	0.30	0.42	0.12
8	5.0	5.0	0.0	0.0	0.0	0.22	0.05	0.05
9	1.2	0.0	1.0	1.0	0.0	0.15	0.05	0.10
10	20.0	16.0	1.0	1.0	0.0	0.41	0.10	0.13
11	20.0	0.0	3.0	2.0	0.0	0.0	0.06	0.18
12	17.0	5.0	2.0	0.0	0.0	0.0	0.09	0.26
13	15.0	2.0	3.0	5.0	0.0	0.61	0.13	0.16
14	18.0	5.0	0.0	0.0	1.0	0.27	0.16	0.17
15	16.0	9.0	2.0	2.0	0.0	0.10	0.07	0.26

IV. Tank Experiment – **Nitrate** (mg L^{-1})

Tank	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
1	75.4	33.1	37.3	15.5	11.7	20.6	52.4	41.2
2	51.5	46.2	25.9	4.4	9.1	36.8	20.0	26.3
3	24.2	132.8	38.8	14.4	15.4	21.8	51.3	43.0
4	15.0	12.8	19.9	20.7	8.4	15.8	13.7	24.6
5	1.1	62.4	72.9	27.9	6.6	8.9	17.5	20.6
6	18.0	39.8	30.3	21.5	13.0	10.8	26.0	27.3
7	24.0	70.9	39.4	10.6	16.2	22.1	30.4	37.2
8	25.7	32.2	21.6	32.0	20.9	17.4	36.4	20.4
9	20.9	15.1	17.6	22.6	22.8	11.9	27.9	27.0
10	20.9	118.7	24.3	17.2	16.4	22.4	31.9	38.9
11	20.9	28.0	12.5	0.2	14.7	16.2	30.3	16.5
12	20.9	59.1	17.5	9.2	10.3	15.6	23.2	19.0
13	20.9	30.7	30.1	32.3	15.8	18.3	32.2	12.5
14	20.9	37.8	20.6	19.5	18.2	20.4	38.6	25.2
15	90.7	0.0	14.7	27.4	18.8	17.6	13.8	15.9

V. Tank Experiment – **Alkalinity** (mg L^{-1})

Tank	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
1	85	75	75	90	80	80	60	60
2	75	65	95	50	75	60	65	45
3	110	70	70	85	75	50	75	55
4	95	90	80	80	65	75	75	70
5	130	105	45	70	75	60	60	65
6	95	95	105	80	55	60	80	50
7	110	90	90	65	60	75	75	60
8	100	85	65	75	65	70	95	60
9	90	65	95	80	55	80	85	90
10	60	45	100	80	70	60	45	70
11	70	70	65	75	65	45	60	55
12	70	70	110	95	75	90	50	75
13	55	80	70	65	80	70	70	60
14	70	70	60	75	60	90	40	80
15	75	85	65	35	55	90	55	70

VI. Tank Experiment – **pH**

Tank	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
1	7.2	7.7	7.8	7.7	7.8	7.5	7.6	7.3
2	8.1	7.7	7.7	7.8	8.4	7.7	8.0	7.6
3	8.5	8.0	8.5	6.8	6.8	8.5	7.4	7.2
4	8.5	8.1	8.5	8.4	7.1	8.5	7.5	8.5
5	8.5	7.8	8.5	7.5	7.8	7.9	8.5	8.5
6	7.8	8.0	7.2	7.4	8.5	8.5	7.3	7.7
7	8.5	7.8	7.8	7.4	8.3	7.3	7.3	7.1
8	8.2	8.1	7.7	6.9	7.6	7.7	7.2	7.2
9	7.9	7.9	8.5	7.7	7.4	7.4	7.6	7.6
10	8.4	7.9	7.7	6.8	8.5	7.7	7.6	7.2
11	8.0	7.9	8.5	8.4	7.0	7.6	8.5	8.4
12	8.0	7.6	7.4	7.3	8.5	7.6	7.4	7.3
13	8.0	7.9	8.5	8.5	7.3	7.8	8.5	8.4
14	8.1	7.8	8.5	6.7	7.8	7.3	8.5	7.3
15	8.5	7.9	8.5	7.3	7.4	8.4	7.4	7.4

Chart B1. Temperature (P2 Tank Experiment)

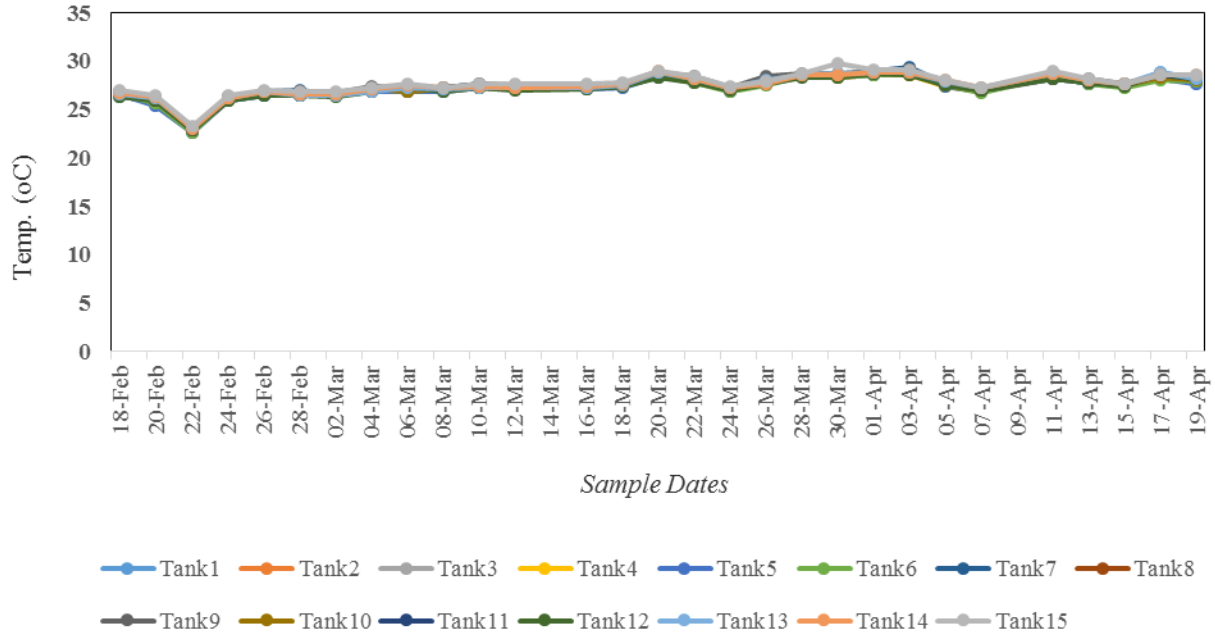
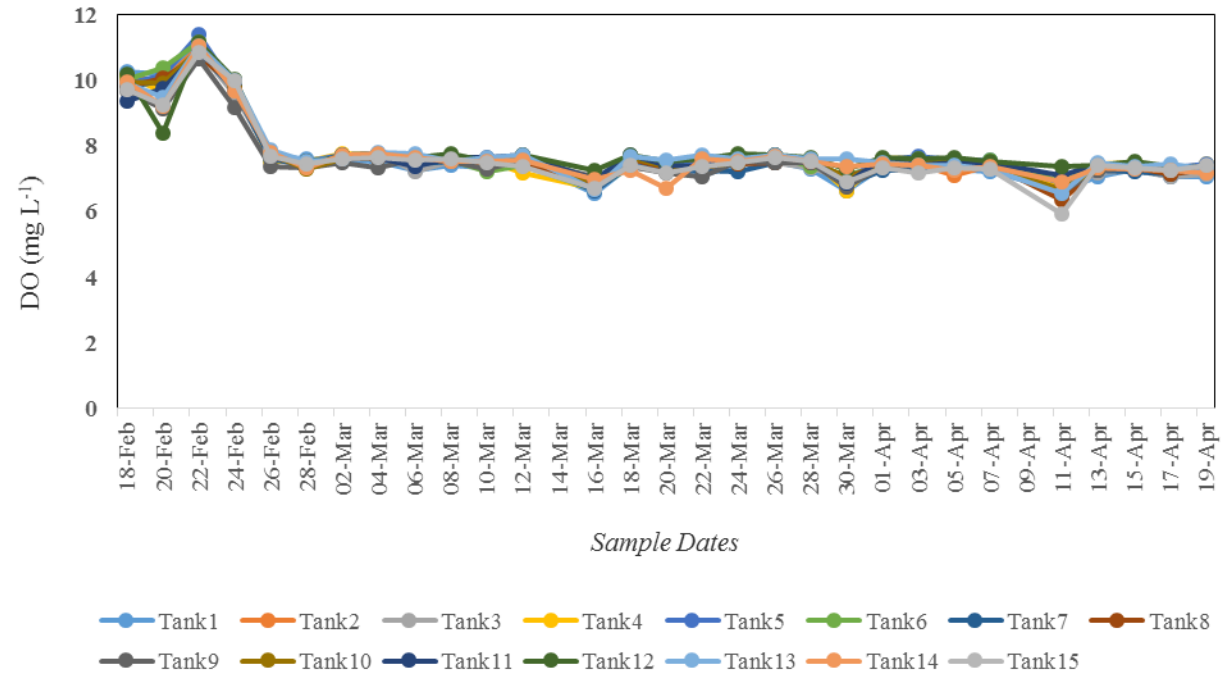


Chart B2. Dissolved Oxygen (P2 Tank Experiment)



Appendix C

Life Cycle Inventory List

Table C1 Inventory list of material inputs and energy sources using for LCA modelling

Name	Unit	Value (€)	Uncertainty
Feed Ingredients			
Fish meal from tuna by-products	kg	0.914	L(0.308)
Fish meal from bycatch	kg	0.914	L(0.308)
Fish oil from tuna by-products	kg	1.02	L(0.571)
Fish meal from Peru	kg	1.06	L(0.308)
Blood meal	kg	0.27	L(0.308)
Shrimp meal	kg	0.322	L(0.308)
Meat and bone meal	kg	0.14	L(0.308)
Mungbeans	kg	0.27	L(0.308)
Wheat flour	kg	0.3	L(0.173)
Wheat bran (world mix)	kg	0.18	L(0.173)
Maize bran	kg	0.147	L(0.173)
Soybean meal (world mix)	kg	0.346	L(0.308)
Rice bran	kg	0.253	L(0.173)
Corn gluten meal	kg	0.225	L(0.308)
Corn gluten feed	kg	0.06	L(0.173)
Cassava chips	kg	0.056	L(0.173)
Palm oil from Malaysia	kg	0.040	L(0.550)
Feed Additives			
Vitamins and minerals	kg	0.00012	L(0.071)
Lysine	kg	2.072	-
Methionine	kg	5.918	-
Calcium phosphate	kg	0.861	-
Phytase	kg	11.19	-
Xylanase	kg	4.21	-
Protease	kg	19.69	-
Energy Sources			
Electricity, medium voltage, at grid	kWh	3.874	L(0.502)
Natural gas, burned in gas motor	MJ	0	L(0.502)
Hard coal, burned in industrial furnace 1-10MW	MJ	0	L(0.503)
Heavy fuel oil, at feed mill, burned in industrial furnace	MJ	0	L(0.503)
LPG, burned in gas motor	MJ	0	L(0.502)
Wood chips, from forest, burned in furnace 300kW	MJ	0	L(0.503)
Diesel, burned at feed mill	kg	0.198	L(0.503)
Diesel at farm	kg	0.988	L(0.103)