Improving water stability of extrudate feed for *C. porosus* using Sodium alginate

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Dedication

This thesis is dedicated to my late dad Feriwangui Wimo who died during the crises in Bougainville in PNG during the 1980s; to the women behind my success, my beloved mum, Tep Kambong; and in loving memory of my only aunty Boniningke Kambong.

Declaration

I, Magdalene Francis declare that this thesis/dissertation, which I hereby submit for the degree of Master of Science (Animal Science) at Massey University is my own work that is not previously been submitted or published; independently carried out, and only with the cited sources, literatures and other professional sources.

Signature: …….

Date: …….

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Abbreviation, Acronyms and Units

AA	Amino acid
AIA	Acid insoluble ash
BW	Body weight
$CaCl_2$	Calcium chloride
$CaCO_3$	Calcium carbonate
Cpm	Cycle per minute
DE	Digestible Energy
DM	Dry matter
DMR	Dry matter retention/Dry matter retained
IUCN	International Union for the Conservation of Nature
LBW	Live body weight
MC	Moisture content
MHL	Mainland Holdings Ltd
MHCF	Mainland Holdings Crocodile Farm
N	Nitrogen
N HCL	Normality of hydrochloric acid
NH_2	Amine group consisting of nitrogen bonded to two hydrogen group
OM	Organic matter
Rpm	Revolution per minute

Abstract

The extrudate sausage-pellet feed that is used currently to feed farmed *C. porosus* disintegrates on contact with water, which leads to leaching of nutrients and results in approximately 50% of feed being wasted. The objective of this study was to ascertain if Na alginate could be used to improve feed stability in water and then examine its effects on animal digestibility. The approach first looked at in vitro laboratory experiments to examine optimal conditions for Na alginate use. The second phase then applied those findings on-farm in a digestibility trial to measure if Na alginate affected digestion. Acceptance of feed containing Na alginate by crocodiles was also observed.

In the laboratory, a new diet was replicated from the 'MHCF 2012/2 Fine' feed formulation obtained from Mainland Holdings Crocodile Farm (MHCF) and had three sodium alginate products (Protanal XP 3639, Manucol DM and Kimica) added separately at 1.7 and 3.3% (as-fed). Each of these was cross-linked with either $CaCaO_3$ or $CaCl_2$ at 1.9% w/w rate. Feed was subjected to sensory evaluation and water stability measurements. For sensory evaluation, the diets were assessed by physical observation of their texture by sight and by feel and scores were given for traits including cohesiveness, viscosity, adhesiveness, and wetness. For water stability, a sample of feed for each alginate product, alginate level, and Ca source (15-30g) was collected and subjected to water submersion for 18-24 hours and then was oven dried. Its dry matter recovery (DMR) was calculated as a proportion of the DM remaining after water immersion to the initial DM added to the water.

Reaction with $CaCO_3$ resulted in greater DMR (80.68% DMR; $P < 0.05$) compared with $CaCl_2$ (16.08% DMR). There was a greater than 10-fold increase in DMR when Na alginate was used with $CaCO_3$ compared to the control (86.7% vs. 6.2% DMR), however there were no differences in DMR among all Na alginates and inclusion levels with $CaCO_3$. Therefore, the least expensive Na alginate product at the lower inclusion level with $CaCO_3$ was recommended for use on-farm and in the digestibility experiment.

For the on-farm digestibility experiment, ten juvenile crocodiles (2.2-2.4 years of age, 1.2-1.9 kg BW) were chosen from farm raised stocks and fed the extrudate chicken by-product-based diets with and without 1.4% sodium alginate and 1.9% $CaCO_3$ added. The percentage composition of sodium alginate was reduced due to increase in component of other ingredients (except sodium alginate) to meet tha capacity of the processing machine. Animals were fed 2% BW for 12 d, with faeces collected the last 5 d. Animals were then slaughtered and digesta sampled from the ileum. Acid insoluble ash was used an internal marker.

There were no effects of alginate on faecal digestibility of DM (0.350 vs 0.664, SE = 0.101) and N (0.249 vs 0.556, SE = 0.15) as well as ileal digestibilities for AA, DM, OM and N between the two diets (DM = 0.360 vs 0.410, SE = 0.146; OM = 0.396 vs 0.468, SE = 0.128; N= 0.558 vs 0.650, SE=0.122), except OM and energy faecal digestibilities were greater with Na alginate (OM = 0.392 vs 0.698, SE = 0.091; energy = 0.444 vs 0.722, SE = 0.083) These results indicate there were no deleterious effects of alginate on digestibility of nutrients in *C. porosus*.

In conclusion, this study showed that Na alginate had the potential to effectively reduce feed wastage and cost by preventing the feed from disintegrating and dissolving on contact with water. Furthermore, Na alginate did not interfere with feed digestion in *C. porosus.*

Chapter 1: Introduction

Crocodile eggs and young crocodiles were traditionally hunted for food in wetland areas of Papua New Guinea (PNG) until the 1940s when demand for highly priced leather products in the developed world triggered large scale hunting and buying of crocodile skin. The uncontrolled shooting and killing of crocodiles escalated during the 1960s and caused a drastic decline in the crocodile population (Kohun, 2009). In response, the government of PNG developed its first legislation in 1966 to protect the trade of crocodiles. This was followed by the Crocodile Trade (Protection) Act of 1974, while did not ban the killing of crocodiles *per se*, banned the possession, sale, and export of skins smaller than 18 cm wide and wider than 50 cm. This protected the breeding-sized animals while it allowed for the harvest of juveniles. Traders and hunters were also required to have licenses (traders' licence, buyers' licence, and exporters' licence) in order to buy, sell, and export crocodile skin overseas (Kohun, 2009). The legislation also stated that crocodiles of any size could be killed by people only when in danger of attack, but the skin could not be sold if too large.

In 1975, the United Nation's International Union for the Conservation of Nature (IUCN) observed that the crocodile population was on the verge of being annihilated in countries around Africa and northern South America, whereas PNG was the only country left with a viable crocodile population (Kohun, 2009). The IUCN then pledged K1.2 million to the PNG government for the development and implementation of a crocodile management program. A wildlife management program was established and PNG became a member of the IUCN, adhering to the Union's standard in its operations in the crocodile industry. PNG was also barred from exporting its raw crocodile skins to fellow Union members, in particular France and Japan. The wildlife crocodile management programme established a network of village-based rearing farms and holding pens for the live crocodile trade; government farms for research, demonstration, and holding; and large commercial farms to buffer the entire farming scheme (Kohun, 2009).

Today, economic reasons are the primary motivators for building farms to breed and raise crocodiles. Production of leather, meat, and other products like teeth, and in some cases tourist attractions are the primary benefits from commercial crocodile farming. In addition to the production of leather and meat, breeding crocodiles in captivity is a suitable method for protecting these large reptiles from extinction or being threatened in their natural environment. The two strategies used in large-scale crocodile production and rearing include ranching and farming.

> In ranching, eggs, young animals, and in rare cases medium to large adults, are taken from the wild. The ranching facilities incubate the eggs and raise the young until they are large enough to harvest. In farming, sexually mature crocodiles reproduce under captive conditions and the eggs are incubated; hatchlings are raised until they are large enough to be harvested. (Kohun, 2009, p. 48)

Mainland Holdings Crocodile Farm (MHCF) is a commercial entity that combines both ranching and farming strategies in its operation. This is achieved by (i) having breeding crocodiles under captive conditions to produce eggs, and (ii) purchasing wild eggs and juvenile crocodiles (as well as wild skins) from different wetland areas of PNG. Strict adherence to national and international regulations has placed MHCF and the PNG crocodile industry very high on the world stage. The MHCF is one of the biggest and most successful commercial farms in the southern hemisphere, stocking approximately 20,000 – 40,000 crocodiles of different sizes/ages, annually. Strategies developed and used in the management and sustainable utilisation of the crocodile resource in PNG have been hailed as a benchmark in crocodilian conservation and has received much international recognition and praise (Kohun, 2009).

However, as a business enterprise, MHCF is faced with several obstacles. Since crocodiles are carnivorous and depend on protein, especially from animal flesh, to supply much of their nutritional requirements, there is lack of consistent and adequate supply of animal meat to meet their demand. Chicken carcasses are the main source of animal meat available in good supply and this is supplied by Table Birds Pty Ltd, a division of Mainland Holdings Ltd (MHL). MHCF depends mainly on chicken carcasses and its by-products (such as chicken head and feet) as the main source of animal meat for its crocodiles. However, poultry and its by-products fed to grower animals tend to produce, "over fat-shaped square" skin (Peucker & Jack, 2006, p.22) which is not desired by tanners and leather manufacturers whose preference is for narrow-shaped skins with small scale patterns (Peucker & Jack, 2006, p.22). The use of animal meat as the main ingredient in diets involves high transportation costs, high electricity usage for cold storage (freezer), and is labour intensive in terms of handling and preparing animal carcasses and ensuring there is correct size and quantity for distribution and consumption. Furthermore, the very humid tropical climate triggers bacterial growth which spoils the meat and lead to other potential disease problems (Isberg, 2007).

The development and introduction of extrudate feed as an alternative to raw animal carcasses has proven to be accepted by the crocodiles and allowed a more consistent product to be fed. Those that

were fed this diet have shown growth rates as good as those fed solely animal meat (Peucker & Jack, 2006). The extrudate feed has been created and introduced to minimise problems associated with raw animal meat as mentioned above. By using extrudate feed, there is opportunity to manipulate the ingredients of the diets to meet the animals' nutritional requirement for optimum growth while reducing the cost of the diet (Peucker & Jack, 2006). Furthermore, it encourages the prevention of selective feeding of more palatable feed ingredients over those that are required for optimum growth, and increases utilisation of feed components, thus increasing conversion rate (Smith, 1995).

However, despite the advantages of extrudate feed, MHCF could not afford the highly-priced feed processing machines for processing quality pellet feed; hence they produce a moist extrudate which is similar to sausages without the casing using a Thompson Frozen Meat Machinery 4000 series mincer mixer bought from Australia. The Thompson Frozen Meat Machinery 4000 series is simply a mincer which mixes the ingredients and forces them through the spiral extruder (without heat). The machine pushes the mixed feed against a die plate with a hole size specific to different diets for different age/size animals (ranging from 13 to 19 mm in diameter) to produce long "sausages" from each hole. These sausages were manually broken down into shorter pieces (called sausage-pellets/extrudate) depending on the size required per age/size group of crocodiles. However, this extrudate feed lacked firmness and the pellets disintegrated when coming into contact with water and nutrient leaching ensued. Also, it's easily squashed when crocodiles crawl over them during feeding, thus resulting in approximately 50% feed loss. This rate of feed wastage contributes to inefficient food conversion; high water pollution loads and is costly to the company.

The following research questions were developed to guide the investigation into improving extrudate feed for *Crocodylus porosus* (salt water crocodiles) by enhancing water stability while maintaining palatability using low-cost machinery.

1) *To what extent does the addition of sodium alginate in crocodile feed as a binding agent improve pellet integrity during feed processing and feeding and reduce ingredients leaching during feeding?*

2) *Will sodium alginate addition to crocodile feed increase feed stability and enhance nutrient uptake and utilization while still being economical?*

Sodium alginate was chosen as the feed binder based on its ability to form strong ionic bonds when cross-linking with certain divalent or multivalent cations, particularly Ca^{2+} ions, to form a high

viscosity gel (Gacesa, 1988). Also, alginate has the ability to stabilize aqueous mixture dispersion and emulsion in the presence of Ca^{2+} ions (Donati & Paoletti, 2009).

The research hypothesis developed for the investigation was:
The effect of sodium alginate on the integrity of pellet feed in water is influenced by its inclusion rate as well as the source of calcium used for cross linking its molecules; and that sodium alginate will not affect nutrient availability for digestion, absorption, and utilisation in crocodiles.

The research is based on the current problem experienced by MHCF in Papua New Guinea. An experimental research strategy divided into laboratory and on-farm experiments was conducted to test the research hypothesis. A new 'fine' diet (called 'R-MHCF Fine' for replicate of Mainland Holdings Crocodile Farm Fine diet) was designed based on feed formulation (2012/2 fine) obtained from MHCF. The diet was made in the laboratory and subjected to three different sodium alginate products (Protanal XP 3639, Manucol DM & Kimica Sodium Alginate) sourced from three different companies in New Zealand and included at 1.7 and 3.3% levels; and further mixed with 1.9% $CaCO_3$ and $CaCl_2$ separately to cross-link the molecules. Straight after the feed came out of the machine, a sample (approx. 15g) was sent directly to the lab for oven drying while another sample (20-40g) was placed in an excess of water, allowed to soak for 18 - 24 hours, removed from the water and then oven dried in order to determine the percentage of dry matter retained (DMR). The percentage DMR was calculated using the following formula by Obaldo et al., (2002):

$$\frac{\text{Dry matter of feed sample after being subjected to water stability test}}{\text{Dry matter of the original feed sample}} \times 100$$

Each diet was assessed based on DMR as well as the cost (PGK) of retaining per unit dry matter to identify the least cost diet with optimum DMR.

A digestibility experiment was done on-farm for measurement of both faecal and ileal digestibility of dry matter (DM), organic matter (OM), energy, nitrogen (N) and amino acids (AA) to compare the availability of nutrients for absorption and utilisation between the MHCF 2012/2 and new formulated diet (R-MHCF) Fine diets.

This research aims at identifying the most cost effective level of sodium alginate to use in the production of crocodile feed. The binder should ensure that feed does not disintegrate during processing and that it maintains its integrity when it touches water, while at the same time retains feed palatability and quality for optimum growth. The binder with optimum DMR and minimum cost identified from this research will be recommended for use in the manufacturing of crocodile feed in PNG. It can also be used in other countries that are facing the same problem with production

and usage of wet extrudate sausage-pellet feed, with similar industry requirements as experienced in PNG. Although sodium alginate may result in an increase in the per ton cost of feed, drastic reduction in feed waste will result in a more efficient production. The findings from this research will also contribute to the body of knowledge in crocodile farming as a new emerging industry.

Chapter 2: Literature Review

2.1 Background

The feed formulation of the extrudate sausage-pellet feed for crocodiles at MHCF was developed in the early 1990s by Dr Mark Staton who did his PhD research project on alligator nutrition. In the mid-90s, the MHCF management endeavoured to improve the nutritional value of these extrudate feed used by the company and invited Dr Mark Read to undertake his PhD research project titled: 'Aspect of Protein Utilisation and Metabolism by Post-hatchling Estuarine Crocodiles (*Crocodylus porosus*)' at the farm. For his trials, Read kept the crocodiles in small individual tubs and force-fed them with a homemade syringe. These crocodiles had no choice but to ingest the pellets, even if they did not like them. They also did not interact or compete with each other for food. After Read had completed his trials and analysed the results, the best performing feed formulation was selected and tested "in situ." Based on his findings, he recommended that animals should be fed a diet at 2% live body weight (LBW).

Eric Langelet (current farm manager) then re-formulated Read's recommendations by making slight adjustments to the diets based on the availability of raw ingredients. In 2000, company management contracted Dr Robert van Barneveld to reformulate the feed aimed at improving the binding properties of the pellets as it was observed that they tended to disintegrate almost instantly when coming into contact with water. They were also easily squashed when the crocodiles crawled over them during feeding. Both of these factors combined resulted in approximately 50% of the feed being lost which impacted on the growth of the animals and also incurred a significant cost to the company.

Dr Robert van Barneveld used wheat gluten as the binding agent in his feed formulation. The feed as formulated by van Barneveld (called the 'RvB' pellets, after his name) was stickier and stuck together like plasticine. These RvB pellets were made using the Thomson Frozen Meat Machinery mincer mixer – model TMM 4200. A type of extrusion barrel was installed to compress the extrudate feed and was fitted with a cutting knife to make pellets of the required size (diameter and length). This device was not used for the original formulated wet feed, as its product was too soft and also tended to form a messy pulp.

The feed mixing and processing procedure involved setting up of the mincer mixer with the correct plate dies (13-19mm wide specific for the different diet types, namely Hatch, Fine and Coarse for

different age group of animals) and compression barrel (if needed). Once the machine was set, all ingredients (wet and dry) for the specific diet were weighed and placed into the mixer where they were mixed until they were thoroughly blended. The feed mixture was then pushed through the mincing blades by the worm screw against the die plate, forming long sausages (without casing) which were manually broken into shorter length sausage-pellets suitable for ingestion by different age/size group animals.

Unfortunately, and despite all the efforts made, the crocodiles did not accept the new RvB formulated extrudate feed. They ate them for a short while and then completely lost interest. Management suggested that the loss of interest in RvB extrudate feed might have been due to the textural property of the feed, being too sticky like plasticine. Later, van Barneveld made slight changes in the reformulation of the hatchling diet to make it more attractive by adding poultry/fishy flavouring; however, this still did not improve acceptance by the animals. Hence, management completely gave up on using RvB feed and returned to the use of the 'original' extrudate feed.

2.2 Crocodile evolutionary adaptation

Kohun (2009) provided a summary on the evolution of crocodiles drawn from a collection of literature. He stated that the first reptilian appeared around 320 million years ago and they were more successful than other vertebrates, especially amphibians, due to the structure of their eggs. Unlike the amphibians whose unfertilized eggs were normally deposited outside their body in only limited areas containing water or at least in very moist places, reptilian eggs were normally fertilized within the female's body before being deposited outside. Moreover, the embryo within the egg is protected by a hard shell, which controls dehydration and protects it from desiccation.

Between 320 – 220 million years ago, there was a boom in the population of reptiles and during this period reptiles of different forms (large, small, aquatic, and terrestrial) appeared and flourished for 155 million years until 65 million years ago when they suffered mass extinction (Kohun, 2009). Just before this mass extinction, the *Orthosuchus* terrestrial crocodilian appeared and lived for 20 million years before invading seas, lakes, and swamps, giving rise to *mesosuchians* (Kohun, 2009). The *mesosuchians* were both of terrestrial and marine origins. Even though most of the crocodilian species became extinct, a few of the *Mesosuchians* managed to survive in Australia until about 1 million years ago (Kohun, 2009).

The phylogeny of crocodiles can be traced back to 200 million years ago when they were the rulers of the earth for millions of years (Kohun, 2009). Kohun further stated that due to enormous changes

in biodiversity, the only surviving crocodiles belonged to the Eusuchians; a conservative group which evolved around 130 million years ago. This includes the four sub-families namely Alligatorinae, Crocodylinae, Tomistominae, and Gavialinae, which are found in Africa, Asia, the Americas and Oceania (Kohun, 2009).

2.2.1 Crocodile general anatomy and physiology

Though crocodiles have features dating back to prehistoric times, their life study is complex compared to other reptiles (Kohun, 2009). Paleontological evidence has shown that their ancestors were active and endothermic. This is supported by the physical and anatomical features of a crocodile's heart which is four chambered like the heart of mammals and birds (Huchzermeyer, 2003). Their external morphology signifies their semi-aquatic and predatory lifestyle. They have a streamlined body that enables them to swim fast with the aid of the tail which provides the main thrust (Kohun, 2009). Crocodiles develop three modes of locomotion on land: crawling on the belly, walking with body raised above the ground, and galloping. Their front feet bear sharp claws for traction and catching prey while the hind feet are webbed and purposely designed to improve balance while walking on land, enable them to make fast moves while in water, and for use in initial swimming (Kohun, 2009). The features of the crocodiles' eyes and nostrils also signify their semi-aquatic lifestyle. The two nostrils used for breathing are comprised of the external nostril located at the tip of the jaw and the secondary bony palate, which is grown back in the roof of the mouth. When the animal is under water with minimum exposure of the nostrils and eyes above water surface, the palate valve separating the mouth from the throat and oesophagus pushes the internal nostril above the back of the throat allowing air passage for breathing (Kohun, 2009). When they are on land, they bask with open mouth, open palatal valve, and open throat allowing air to flow through. A crocodile's eyes have two eyelids: the semi-transparent eyelid, which slides across the eye when the animal is submerged with open eyes, and the conventional eyelid with thick skin which completely covers the eye. Unlike other reptiles, crocodiles have well-developed hearing. Their ears are protected by flexible flaps comprised of hollow spaces which assist in pressure equalisation during diving (Kohun, 2009).

All modern crocodilians share the same basic features. Their anatomical and physiological features classify them as carnivorous. They prefer animal protein to meet their bodies' metabolic demands. The metabolism of crocodiles is a subject of vast discussion and studies. Most work undertaken on crocodilian gastrointestinal tract (GIT) function has focused only on morphology, including the gross morphology, histology, and cytology (Diefenbach, 1975).

2.2.2 Digestive mechanistic adaptations

Crocodiles and birds are believed to be the descendants of archosaurs (Manley, 1990), a non-avian dinosaur. They both possess basically similar GIT features including a short and efficient digestive tract, inflexible tongue, and a stomach comprising of two chambers. While chickens possess a proventriculus and ventriculus, crocodiles have a muscular gizzard (main stomach) and pylorus, which perform similar functions in both animals. Changes in the environment over time have caused them to develop certain features and different mechanisms suitable for ingesting and digesting food within their ecological niche, and for utilizing and partitioning nutrients from these foods to other body depots.

Crocodiles developed a low fasting metabolic rate through both metabolic and behavioural modifications (Garnett, 1986a). For example, they depend on anaerobic metabolism (Bennett, et.al., 1985), and select habitats with an ambient temperature conducive for reduced energetic cost (Wright, 1986) and for achieving fast growth rate under conditions of minimal disturbance (Garnett & Murray, 1986b). They are poikilothermic, indiscriminate opportunistic carnivores whose diet consist mainly of protein and fat (Coulson & Hernandez, 1965), and their metabolic rate is commensurate with their body temperature (Isberg, 2007).

The amount and size of food crocodiles consume changes in size and quantity as they grow. The diet of hatchling and adolescent crocodiles in the wild comprise of insects, crustaceans, and small fish (Webb & Manolis, 1989). As they grow to sub-adult stage their diet changes to mainly vertebrates such as fish, tortoise, mud crabs, and other marine creatures (Taylor, 1978). Each crocodile species has a preference for certain prey. For example, Alligatorinae have strong broad jaws ideal for crushing snails, mussels, and turtle shells, while Gavialinae have a slender snout with needle-sharp teeth and jaw that sweeps effortlessly through the water to snare slippery fish (Kohun, 2009).

2.2.3 GIT morphology

Research on crocodiles' digestive physiology is very limited**.** The carnivorous feeding behaviour of crocodiles has led to the development of a shorter and less complex gut (Richardson, et.al., 2002) with a very acidic stomach (pH 1.5 – 2) (Isberg, 2007) for enhancing digestion of protein. The mouth comprises of strong powerful jaws and teeth (designed purposely for grasping and crushing), and a large fleshy tongue rooted to the floor of the mouth along its entire length (Trutnau & Sommerlad, 2006). A crocodile's teeth are conically shaped with a cylindrical base and pointed tip

with a thick crown wall (Edmund, 1969, as cited in Read, 2000). The mouth has an extensive nervous system which triggers the jaw to clamp shut with enormous power (Kohun, 2009). The mouth is widely open and can close at a fast snap with the aid of the jaw muscles attaching to the posterior edge of the upper and lower jaws (Trutnau & Sommerlad, 2006). However, the crocodile's jaw cannot be moved sideways to allow chewing, instead the rim of the tongue moves freely to aid in swallowing of whole small prey (Trutnau & Sommerlad, 2006). Small prey can be swallowed whole without cutting, while large prey are crushed and broken up using their powerful teeth and jaws before swallowing.

There is no salivary gland in the tongue of crocodiles (Taplin & Grigg, 1981). A palatal valve at the back of the mouth separates the mouth from the oesophagus, which can be opened and closed allowing the food to pass into the oesophagus. The oesophagus is relatively straight (Trutnau & Sommerlad, 2006), long and narrow with a folded smooth surface within (Parsons & Cameron, 1977, as cited in Read, 2000). It is connected to the left side of the stomach via the cardiac sphincter (Van der Merwe & Kotze, 1993). A crocodile's sac-like stomach comprises of two chambers (muscular gizzard/main stomach and pylorus) that is covered in mucosal glands (Huchzermeyer, 2003). The aponeurosis on each side of the muscular chamber comprises of a zigzag fold with epithelium comprising of tall, prismatic mucus producing cells, in between which are gland ducts for gastric juices that opens into the stomach during digestion (Trutnau & Sommerlad, 2006). The muscular chamber (main stomach) is mainly involved in the chemical and mechanical breakdown of food. It is the main site of protein digestion. Hydrochloric acid and pepsin are released (Coulson, et.al., 1989) during digestion, giving rise to a highly concentrated acid (pH 1.5-2) which aids proteolytic digestion (Isberg, 2007).

The chyme exits the stomach through the pyloric sphincter into the duodenum, which arises at a point adjacent and ventrally to the oesophagus (Chiasson, 1962; Guard, 1980, as cited in Read, 2000). Similar to the gizzard of the chicken, the intestinal walls of crocodiles do not contain villi, but comprise of zigzag, ridge-like folds (Huchermeyer, 2003). The intestine ends with a short colon which opens into the cloaca (Isberg, 2007). The cloaca is partitioned into 3 sections: the first compartment acts as a bladder storing urine; the mid-compartment is the junction where ureters, reproductive tract, and alimentary canal are joined; and the final compartment is where all wastes and reproductive materials exit the body (Read, 2000).

2.2.4 GIT function in digestion

Crocodiles are unable to masticate effectively. They depend on the mechanical and chemical breakdown of food along their digestive tract (Huchzermeyer, 2003). Their inflexible tongue enables them to swallow food whole or in big portions and their muscular stomach is involved in the mechanical breakdown of food. No digestion takes place in the mouth due to the absence of salivary glands (Taplin & Grigg, 1981).

Shortly after a meal, the oxynticopeptic cells in the stomach secrete gastric juice which sets in immediately to aid digestion (Coulson et al., 1989). The chloride ions originating from NaCl in plasma enter the stomach via the blood stream (Trutnau & Sommerlad, 2006), producing hydrochloric acid (HCL) in vast quantity, giving rise to highly concentrated acid (pH 1-2) (Trutnau & Sommerlad, 2006). This aids the proteolytic digestion and denaturing of whole ingested protein leaving behind protein derivatives of low molecular weights (Hawk et al., 1954 as cited in Read, 2000).

There is little knowledge on the chemical breakdown, enzymatic degradation, and absorption of chyme when it enters the duodenum (Read, 2000). Isberg (2007) suggested that when the chyme enters the duodenum, the highly concentrated acid of the chyme coming from the stomach triggers the secretion of sodium bicarbonate ($NaHCO_3$) from the liver and pancreas to neutralize the acid released from the stomach. However, since the metabolic rate of the pancreas and liver are not sufficient to supply an adequate quantity of $NaHCO_3$ to neutralise the acid, it is believed that $NaHCO_3$ originally comes from blood plasma instead (Coulson, et al., 1989). When highly concentrated acid from the stomach enters the duodenum, remaining Na^+ ions (from NaCl in the plasma) react with carbonic acid (H_2CO_3) partially released from CO_2 and H_2O, forming sodium bicarbonate ($NaHCO_3$) in excessive amounts, which then neutralises the acid and increases alkalinity (pH 7-8) (Trutnau & Sommerlad, 2006). It is thought that the combined action of bile and pancreatic secretion with the aid of functional proteolytic enzymes of the duodenal and pancreatic tissues such as trypsin, chymotrypsin, carboxypeptidases, and aminopeptidase further digest protein and fat in the upper intestines (Huchzermeyer, 2003; Read, 2000).

2.2.5 Dietary absorption and utilization

Like all other crocodiles, *Crocodylus porosus* (*C. porosus*) have nutritional requirements for protein and amino acids (AA), but they can also utilise fat and carbohydrates. Their ability to utilise nutrients is not yet fully understood (Garnett, 1985). They have developed an ability to depend on

anaerobic metabolism (Bennett et al., 1985), hence can go without food for long periods with low metabolism. Their feed intake, digestion, absorption, and growth are determined by environmental temperatures (Trutnau & Sommerlad, 2006). A rise in temperature increases the biochemical processes within the GIT, stomach contraction, and proteolytic activities (Trutnau & Sommerlad, 2006), hence increases feed intake, digestion, absorption, and growth. For instance, the American alligator prefers an ambient temperature of range 1-3^{o}C higher and less variable than their normal body temperature following feeding (Isberg, 2007). This is purposely for facilitating digestion and absorption while reducing transit time. At 28-30^{o}C, the American alligator eats daily and consumes food at 20% LBW in a week. However, when the temperature drops to 10-20^{o}C, daily feed intake is reduced (Coulson et al., 1973; Joansen & McNeese, 1976 as cited in Trutnau & Sommerlad, 2006). On the other hand, *C. porosus* prefers an environmental temperature of 32^{o}C for optimum growth and survival (Turton et al., 1997; Meyer, 1998, as cited in Isberg, 2007).

The knowledge on the site of absorption and utilization of protein, fat, glycogen, and starch by crocodiles is very limited. Generally in the wild, crocodiles achieve good growth rate when fed high protein diets with fat and fairly high ash content (Webb et al., 1991), indicating that crocodiles can achieve optimum growth on high protein diets with supplementary fats and ash. In captivity, the absolute dietary requirement for singular AA has not been quantified. However, threonine, valine, leucine, isoleucine, methionine, lysine, tryptophan and phenylalanine were recommended as essential amino acids (Coulson & Hernandez, 1974). Arachidonic acid was well utilised by *Alligator mississippiensis* (Staton et al., 1990b) while docosahexaenoic fatty acid was recommended as essential for *C. porosus* (Garnett, 1985). Crocodiles fed protein diets supplemented with carbohydrates have shown improved growth (Coulson et al., 1976; Read, 2000; Staton et al., 1990b; Smith & Coulson, 1992, as cited in Isberg, 2007). When Coulson (1976) carried out carbon utilisation studies on *Crocodylus caiman*, he observed that glucose was the only carbohydrate that was absorbed and utilised compared to other monosaccharides, disaccharides, and polysaccharides like maltose, lactose, sucrose and starch.

The area of nutrition for crocodiles' vitamin and mineral requirements, absorption and utilisation still requires further research. Vitamins A, D and E have been recommended in some literature while in others calcium and phosphorous are considered essential for bone formation and good growth performance (Huchzermeyer, 2003).

2.2.6 Metabolic adaptation

Cats, like crocodiles, are obligate carnivores that depend solely on animal flesh to derive their energy and nutrient requirements. Cats have an increased need for taurine, arginine, methionine, and cysteine because they lack synthetic pathways for these AA and cannot synthesize these AA. Their high requirement for methionine and cysteine is due to the fact that methionine and cysteine are gluconeogenic AA that are catabolized to pyruvate and then subsequently oxidized to provide energy, and also they are typical precursors for taurine whose synthesis is very low in cats (Zoran, 2002). Cysteine is also a component of the antioxidant glutathione which is important in carnivores. The requirement for arginine is because cats are unable to synthesize sufficient amounts of ornithine or citrulline for conversion to arginine (Zoran, 2002). Unlike cats, there is no published literature on crocodiles' metabolic adaptations to justify their higher requirement for protein from animal flesh. Several researchers have studied levels of AA in the plasma, urine, and faeces after a meal to determine AA requirements for different species, but to date there is no published literature on metabolic adaptation for the specific AA that determine the carnivorous feeding behaviour of crocodiles and their high requirement for animal flesh.

Garnett (1988) reported that the higher requirement for protein in *C. porosus* was in preference to fat storage, and the preference for the storage of fat may be an adaptation mechanism to survive long periods of starvation (Garnett, 1986a). Garnett (1986a) further reported that food containing more fat results in greater storage of fat at the expense of somatic growth, without replacing protein as an energy source. Coulson et al., (1987) contradicted that provision of less fat in a diet limits maximum rate of growth by forcing alligators to rely on protein for all the energy needs and gluconeogenesis. Crocodiles probably have a basic need for lipid as a source of glycerol and FA (Staton et al., 1986). Since crocodiles have a small brain and low metabolic rate, the glycerol in fat provides the nervous system with adequate amount of glucose without the need for gluconeogenic amino acids (Coulson et al., 1987).

When fish was fed to crocodiles, the total free AA content of the plasma increased several fold, but the composition of plasma was not the same as the content in the protein fed (Herbert & Coulson, 1975). The non-essential AA glycine, alanine, and glutamine made up approximately 90% of the total plasma increase, while essential AA only increased slightly (Herbert & Coulson, 1975). Coulson et al. (1987) observed an initial rise in these three AA while reaching their maximal level several hours later after the meal and suggested that they were probably being absorbed by the intestines but the prolonged elevation could have been derived from metabolism of other amino acids.

On the other hand, when Coulson et al. (1987) measured the free AA in the plasma of A.*mississippiensis* after a meal to quantify the type of AA that were present, they reported that the plasma AA peak level for methionine and lysine were very low (Table 2.1). Therefore, they suggested that these two AA might be limiting in crocodiles just as they were in other monogastric animals like pigs and poultry. Lysine is the first limiting amino acid for pigs and the second limiting amino acid for poultry, while methionine is the first limiting amino acid in poultry. Since crocodiles and chickens originated from the same ancestor (archosaurs) and having shared a common digestive tract physiology, they may have developed similar requirements for lysine and methionine.

However, since crocodiles are carnivorous and have higher requirement for protein from animal flesh, they may have a similar requirement for specific AA like cats as discussed above. There is no detailed information available on the role of metabolic adaptation for protein and AA in crocodiles and of crocodiles having a higher requirement for protein and fat as energy sources, which is a similar requirement to cats (Coulson et al., 1987; Garnett et al., 1988; Zoran, 2002). Therefore, it can be suggested that the low plasma peak level of methionine and lysine as observed in *C. porosus* (Coulson et al., (1987) could suggest a similar requirement for specific AA like taurine (as in cats) or, for the need of gluconeogenic AA to be catabolized to pyruvate and then subsequently oxidized to provide energy.

Table 2.1 Free amino acid plasma level in A.*mississippiensis* after a single meal

Plasma AA level	Observation
Casein	Slow to digest but nutritionally complete with exception of arginine and glycine. Was well tolerated and only a little portion appear in faeces
Edestin	Was slowly digested
Gelatin	Poorest protein available. Proves quite easy to digest but lacks several AA and those present are in improper ratio as to render body protein synthesis from its component AA impossible. Failure to remove the absorbed AA by incorporating them into protein forces the alligator to resort to the slower catabolic routes for their disposal
Alanine	Rise slowly but adequate amount was available for protein synthesis
Glutamine	Digest well in alligator but the absorbed AA remain unchanged
Glycine	Peak concentration in plasma reflects its content in the diet. Similar to glutamine and alanine, synthesis from other AA was responsible for its prolong elevation. However the plasma AA concentration was higher than protein content AA
Glutamate	Like aspartate, it was never a major constitute in the plasma. Level in plasma was related to level in protein content. Glutamine was not a substituting glutamate
Methionine & Lysine	Have very low peak value in plasma. Modest deficit in feed always lead to major shortage in plasma

Note: Information in this table is a direct quote from Coulson et al., 1987.

2.3 Dietary nutrients recommended for *Crocodylus porosus*

There is very limited knowledge on specific nutrient requirements for different age groups of *C. porosus*. Few nutritional studies have focused on understanding their basic nutrient requirements by comparing their performance responses to different diets fed. This is because the animals are farmed in groups and their daily intake varies greatly between individual animals. Also, feed intake and feed conversion efficiency are directly affected by environmental temperature, hence resulting in variation of metabolic rate among individuals (Van Barneveld, et al., 2004). Despite this, a few researchers have made progress and their findings are discussed in the next section below.

2.3.1. Protein

Feed contains nutrients such as protein, carbohydrates, and lipids that contribute the energy that the animals need for their life processes: growth, maintenance, and reproduction (Read, 2000). Protein is made up of 20 AA which include the following: alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. These AA form the basic building blocks from which an animal's body is composed. Some of these AA are synthesized by the animal and are regarded as non-essential. The others cannot be synthesized, but are needed by the animal to carry out its essential life processes. These are regarded as essential AA.

There is no quantified dietary requirement for a single AA in crocodiles. However, threonine, valine, leucine, isoleucine, methionine, lysine, tryptophan, and phenylalanine were recommended as essential (Coulson & Hernandez, 1974). Coulson et al. (1986) argued that all AA except alanine and glutamine are "essential" for maximal growth rates in a small alligator.

The digestibility of protein was dependent on protein source with higher digestibility from animal protein based diets (90%) compared to plant protein based diets (10%) (Coulson et al., 1989). According to Read (2000), protein fed at 237.5, 507 and 588.8 g/kg DM showed a positive correlation when fed at 2% LBW. He recommended that the diet of hatchling *C. porosus* should contain 595g/kg DM crude protein for improved growth rate.

2.3.2 Lipid

Lipid provides glycerol and fatty acids (FA) such as linoleic acid, arachidonic acid, and docosahexaenoic acid, amongst others. Arachidonic acid is essential for maximum growth in

Alligator mississippiensis (Staton et al., 1990b). *C. porosus* are more efficient at digesting saturated FA than longer chained unsaturated fatty acid with exception of $C_{20:5}$ (eicosapentaenoic acid, EPA) and $C_{22:6}$ (docosahexaenoic acid, DHA), indicating that these two polyunsaturated FA are essential for *C. porosus* (Garnett, 1985).

Coulson and Hernandez (1983) reported that dietary fat was readily digested by alligators whose growth were improved on diets ranging from 14 to 24% fat. Garnett (1985) observed an apparent higher digestibility of docosahexaenoic acid compared to apparent digestibility of saturated FA. Similarly, Morpurgo et al., (1993) observed higher percentage of unsaturated FA, particular Ω-3 unsaturated FA in the plasma of wild Nile crocodiles compared to their captive counterpart with significantly higher ratio of Ω-3/Ω-6 unsaturated FA.

The FA composition of the heart, liver, muscle, skin, and adipose tissue lipids of *Alligator mississippiensis* was influenced by dietary fat composition (Staton et al., 1990a). Major FA found in the body of *C. porosus* include oleic (C18:w9) and palmitic (C16:0) acids (Mitchell et al., 1995). Other FA found in significant concentrations include stearic (C18:0), linoleic (C18:2w6), and palmitoleic (C16:1w7) acids (Mitchell et al., 1995). Staton et al., (1990a) suggested that a dietary source of arachidonic acid may be required to support maximum growth while 1% linoleic acid and 0.5-1.0% of both arachidonic and docosahexaenoic acids were recommended for diet formulation of young *C. porosus* (Staton & Vernon, 1989 as cited in Read, 2000).

2.3.3 Carbohydrate

Crocodiles do not have a specific requirement for carbohydrates. Staton et al. (1990b) studied the relationship of utilization of protein with carbohydrates in American alligators and found that supplementation of protein with carbohydrates resulted in improved growth rates. Similarly, Peucker (2005) found an increased growth rate when lupin was fed to *C. porosus*; Read (2000) confirmed an improved growth rate when soybeans were included in *C. porosus* diets at 95-140g/DM; and, *C. niloticus* showed maximum growth rates when fed 22.3% cooked maize supplemented with 450g crude protein/kg DM (Smith & Coulson, 1992, as cited in Isberg, 2007). These researchers did not clearly justify reasons why lupin, soybeans, and cooked maize improved growth rate of crocodiles. However, it maybe suggested that glucose from these carbohydrates might have supplied energy for these crocodiles rather than them having to rely on oxidation of AAs either directly or via gluconeogenesis, hence allowing the AAs to be used for other processes, thus improving growth.

2.3.4 Vitamin and mineral

This area of crocodile nutrition has received little attention; hence, different supplement formulators supply them in different quantities. However, overdosing of vitamins is as damaging as their lack in the case of fat soluble vitamins such as A, D and E (Trutnau & Sommerlad, 2006). Amounts of 1 and 0.5% (dry weight) calcium and phosphorus, respectively, were recommended for alligators (Staton & Edwards, 1987).

Based on these few studies, crocodile farmers are producing extrudate-pellet feed using ingredients from both plant and animal sources as an alternative feed to pure animal meat. However, like pellet feed for other aquatic animals, the extrudate or pelleted feed has experienced problems associated with lack of firmness and disintegration, thus leaching of nutrients ensues when the feed comes into contact with water during feeding.

2.4 Pellet feed for aquaculture animals

In any aquaculture farming business, feed is an important farm input estimated to cost about 60% of the total operational cost (Falayi et al., 2004). Water stability of feed is important in aquaculture. Feed stability is crucial in the ability of feed to withstand leaching of nutrients. Unstable feed is prone to leach quickly, hence affecting the uptake of nutrients for the aquatic animals (Falayi et al., 2004; Ruscoe et al., 2005; Pearce et al., 2002), thus impacting negatively on the animal growth and the company's operations.

When formulating feed for aquatic animals including crocodiles, it is important to take into consideration nutritional and non-nutritional factors such as palatability, physical properties, and water stability (Luis et al., 1996). Water stability should be an important parameter of consideration as it contributes to the cost of feed. The more the feed is stable in water, the less cost is involved in processing more feed to compensate for wastage through disintegration in water (Lim & Cuzon, 1994), and the more feed will be consumed, thus increasing feed conversion efficiency of the animals.

2.5 Field measurement of water stability for aquatic feed

Methods that have been used in measuring water stability include immersion of pellets in static water and use of mechanical agitation or water flow to stimulate pellet movement in water (Obaldo, Divakaran, & Tacon, 2002). In these systems, initial weight of feed samples were recorded and

pellets were either placed into a pouch made of nylon or inert fiberglass mosquito netting (Knauer et al., 1993), a PVC pipe covered at the bottom with mesh to facilitate water circulation (Ruscoe et al., 2005), or placed onto on a sieve (Fagbenro & Jauncey, 1995). These were left in the water for several hours and then oven dried. Final weight of the remaining dry matter was recorded and this was subtracted from the initial weight to calculate the amount of dry matter (DM) loss. This was then multiplied by 100 to calculate the percentage DM loss.

2.6 Laboratory measurement of water stability for aquatic feed

Three methods used by Obaldo et al., (2002) for measuring water stability and leaching of shrimp feed included horizontal shaking methods, static water methods, and vertical shaking methods. In the horizontal shaking methods, feed samples were placed in a 250ml flask containing 100ml of water and placed in a shaker tray water bath (of either 15, 25 or 35^{o}C) with a shaker, which shakes at a speed of 1 to 300 cycles per minute (cpm) in a straight horizontal back and forth motion. After shaking, the sample and solvent were filtered through a Buchner filtration apparatus. The recovered solid and an original feed sample were oven dried at 105^{o}C. The DM was calculated according to AOAC (1990, as cited in Obaldo et al., 2002). In static methods, there is no water agitation or shaking. The vertical shaking method is similar to the horizontal shaking method, but uses the Van Kel disintegrating testing system which moves the pellets in a vertical position at a 5.5cm stroke distance with a fixed speed of 30cpm. However, the rate of pellet disintegration was affected by these different methods. The static method retained more DM than the other two (Obaldo et al., 2002).

Other general methods used in determining DM retention or water stability of aquatic pellet feed involved weighing feed samples and placing them into a sieve or pouch of 5mm or less and placing these into beakers containing water (either from the tap or sample water from culture ponds). The feed is then allowed to remain in water for a specified time interval, after which the undissolved solid is separated from the water using filter paper or sieves of different hole sizes (1-5mm). The undissolved, unleached solid is then oven dried to constant weight. The loss in weight of pellets is calculated by the difference in weight before and after immersion (Ali, 2011; Falayi et al., 2004; Ighwela et al., 2013).

2.7 Type of binders and their effects on water stability of pellet feed for aquatic animals

Feed binders have been used in processing feed for aquatic animals to enhance the physical and nutritional properties of feed in situations where usual ingredient selection and processing

conditions cannot produce pellets of satisfactory characteristics (Dominy et al., 2004). Binders improve the stability of feed by sticking feed particles together, exerting chemical attraction between ingredients and altering the nature of feed, hence resulting in the production of more durable pellets (Ruscoe et al,, 2005).

Binders such as agar, gelatine, guar gum, sodium alginate, corn starch, carrageenan, and carboxymethylcellulose (CMC) have long been used in the aquaculture industry to enhance the stability of pelleted feed for fish and other aquatic animals (Pearce et al., 2002; Ruscoe et al., 2005). The role of these binders in the overall stability of feed pellets was tested by subjecting pellets of different binder percentages in aerated and agitated water to determine the percentage of dry matter remaining (Ruscoe et al., 2005).

It has been reported that the influence of binders on feed nutrient retention while immersed in water (Falayi et al., 2004) and the stability of the pellets (Pearce et al., 2002) is determined by the percentage of the binders in the diet formulation and the type of binder used. Ruscoe et al. (2005) found that a diet formulation containing 5% or more of the binders including agar, alginate, gelatine and carrageenan retained more dry matter in the feed than feed containing 3% of these binders. In their study, Ruscoe and colleagues found that carrageenan was the best in maintaining the integrity of the feed in water compared to agar, alginate, and gelatine. In another study conducted by Dominy et al. (2004) where physical and biological properties of commercially available binders were compared, it was observed that there was great variation among the binders in terms of their ability to enhance pellet stability. Ruscoe et al. (2005) reported that the stability of feed is not only influenced by the type of binder used and the percentage in the formulation, it is also affected by the moisture content of the diets and the processing conditions the feed is subjected to during pelleting (Ruscoe et al., 2005).

2.7.1 Cost of binders in feed for aquatic animals

Since feed accounts for about 60% of the total operating cost of aquaculture farms (Falayi et al., 2004), feed processing and production should focus on reducing feed cost whilst increasing animal production. The use of binders in feed adds to the cost of feed, hence the selection of binders should be based on the binder's potential to retain the optimum amount of feed in water at a low cost. Falayi et al. (2004) made economic evaluations of seven fish diets made from several binders (including wheat flour, cassava, maize offal, paddy rice, sorghum grains, yellow maize grain, and CMC) and found there was no significant difference in the cost of feed produced from these binders except CMC, which was more costly than the others. In another similar study comparing guar gum

and gelatine, guar gum was seven times less expensive than gelatine, but seven times more was required to retain the same amount of feed as retained by gelatine (Pearce et al., 2002).

Despite numerous studies that have been conducted to understand the role of binders in pelleted feed for fish and other aquatic animals, there is not much scientific information available on the role of binders in improving water stabilising properties of wet extrudate feed for *C. porosus*.

In this research, sodium alginate was used to improve water stability of wet extrudate sausage-pellet feed for *C. porosus* due to the following factors:

1. It forms a 3-dimensional gel that is able to capture all the other raw materials in the formulation, including the added wet ingredients;
2. It produces this gel network in a very short time compared to other gelling agents.
3. It forms a stable gel that is able to resist shear stresses and thus handling and most importantly not break up when dropped in water.
4. It forms this stable gel at room temperatures.
5. It requires little in the way of extra equipment.
6. Finally, because of its simplicity it is considered to be an appropriate technology for this particular operation.

2.7.2 Application of sodium alginate

The gel-forming and viscous properties of alginate together with its ability to stabilize an aqueous mixture, dispersion or emulsion in the presence of Ca^{2+} ions has enabled it to be used in many food applications (Donati & Paoletti, 2009) as a thickening agent, gelling agent, dispersion stabilizer, texturizer, and a filament or coating material (Krasaekoopt et al., 2006). Some of its practical applications include thickeners in yogurt and mayonnaise; water dessert gels; milk pudding and ice-cream stabilizers; fish and meat preservatives and sausage casings; as well as in bakery toppings, beverages, and salad dressings (Ren, 2010).

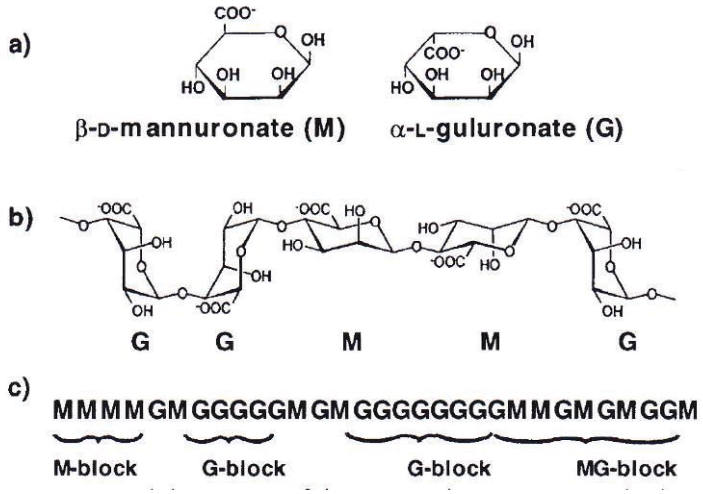
2.7.3 Alginate

Alginate is the term used to describe alginic acid and its various inorganic salt forms which are derived from brown seaweeds (McHugh, 1987). Alginate is a linear copolymer monovalent salt (McHugh, 1987) extracted from naturally occurring polysaccharides of brown marine algae including *Laminaria hyperborean, L. digitata, L. japonica, Lessonia nigrescence, Mycrocystis* and

Duvillea (Smidsrod & Draget, 1997) commonly found in the cold temperate waters of Northern Europe, west coast of America, the southern part of Australia and Tasmania, and around Japan and China (Ren, 2010).

The combination of alginate with calcium, magnesium, potassium, and sodium salts from sea water form alginic acid, an insoluble mixture of jelly bodies found in the intercellular mucilage and algal cell wall which constitutes approximately 40% dry weight of the brown algae (Haug et al., 1967). Alginates are comprised of two monomeric units of unbranched binary polymer: 1-4-linked β-D mannuronic acid (M) and α-L-guluronic acid (G) (Donati & Paoletti, 2009a). These units form the fundamental structure of the alginic acid (Draget et al., 2006). These structures are stable in their conformation: $^4C_1$ for M and $^1C_4$ for G (Whistler & BeMiller, 1997) and contain all four possible glycosidic linkages: diequatorial (MM), diaxial (GG), equatorial-axial (MG), and axial-equatorial (GM) (Figure 2.1) configurations (Draget et al., 2005) that determine their physical properties, especially the gelling capability and strength.

Fig 2.1 Structural characteristics of alginate: (a) alginate monomers, (b) chain conformation, (c) block distribution.

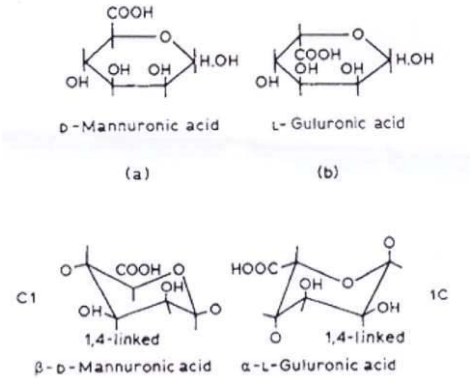


Source: Draget *et al*. (2005)

The chemical structure of alginate is further explained here in reference to carbohydrates. Most carbohydrates consist of glucose polymers formed predominantly by α-1, 4 linkages, meaning, the residues are joined by a linkage between the α-anomeric form of C-1 on one sugar and the hydroxyl oxygen atom on C-4 of the adjacent (Feng et al., 2001). Carbohydrates contain five or six carbons denoted as pentoses and hexoses, respectively, in a cyclic form from which the carbonyl carbon becomes sterogenic and form two configurations (α or β) (Zaccheus, 2012) referred to as anomers, based on their stereochemical relationship. The ring of monosaccharides also exists in two isomeric

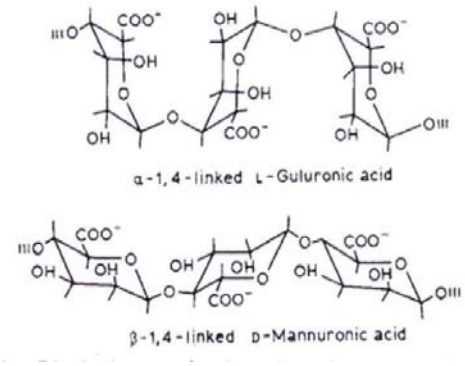
chair conformations, which are specified as $^{1}C_{4}$ and $^{4}C_{1}$, respectively, where the letter C stands for 'chair' and the superscript and subscript numbers denotes carbon atoms located above or below the reference plane of the chair respectively, made up by C–2, C–3, C–5 and the ring oxygen (Zaccheus, 2012). "Monosaccharides can adopt two absolute configurations, D or L, where the descriptor is related to the optical chirality of glyceraldehyde and determined by the configuration of the highest numbered chiral carbon in the chain" (Zaccheus, 2012, p2) (See Figure 2.2 and 2.3 for illustration).

Figure 2.2 Chemical structure of mannuronic (M) and guluronic (G) acid



Source: (Sime, 1990)

Figure 2.3 Chemical structure of M-M and G-G bonding of mannuronic and guluronic acids



Source: (Sime, 1990)

2.7.4 Calcium ions

Calcium sources that are commonly used with alginate to form calcium alginate gels include calcium sulphate (usually as dehydrase), dicalcium phosphate (calcium hydrogen orthophosphate) (Ren, 2010) and calcium carbonate. The rate at which calcium ions are released from these salts and become available for the alginate molecule depends on the pH of the solution as well as the particle size of the salts and their intrinsic solubility characteristics (Ren, 2010). The pH of the solution is

the main determining factor that exerts a significant influence on the solubility of different calcium salts. For example at pH 7, anhydrous dicalcium phosphate is insoluble and, therefore, cannot react with alginate to form alginate gel, whereas dicalcium phosphate dehydrate is highly soluble and can give rise to premature gelling, while calcium sulphate is more soluble than the other two (ISP, 2007).

2.7.5 Alginate solution

Since all alginate products come in powdered form, they need to be dissolved in water for gel formation. However, because of their affinity with water they quickly form lumps once they come in contact with water (Ren, 2010). Draget et al., (2006) reported that a tiny portion of pure alginate powder placed into water and subjected to high speed mixing would still form a gel with lumps. This problem can be avoided by mixing the alginate with sugar and vegetable oil or glycerol to disperse it prior to mixing with water and subjecting the mixture to a very high shear mixer to break down the lumps (Nussinovitch, 1997). Another possible way of achieving full functionality is to fully hydrate the alginate solution at 70°C to ensure its structure is open to allow water molecules to enter and fully hydrate the active sites.

The formation of alginate solution and the solubility of alginate are influenced by ionic strength, pH and heat. Ren (2010) explained that at higher ionic strength, solubility of the alginate is low and precipitation can result due to the presence of a high concentration of inorganic salts such as potassium chloride (Ren, 2010). Draget et al., (2006) recommended less than 0.1M salt concentration, but if more salts are required, the polymer should be fully hydrated before salt is added. Different alginates react differently to pH (Ren, 2010). For instance, solutions of sodium alginate become unstable above pH 10, alginate precipitates at pH of 3.5 or lower due to the dominated COOH functional group within its chemical structure (Ren, 2010), and dissociation of mannuronic and guluronic acids of the alginate become constant at pH of 3.38 and 3.65, respectively (Nussinovitch, 1997). A sudden decline from neutral pH will cause the alginate solution to precipitate while a slow decline from the pH 7 leads to the formation of alginate acid gel (Ren, 2010). Alginate gels can be depolymerised by heat (Ren, 2010). The depolymerisation rate and stability to heat varies between mannuronic and guluronic acid composition of the alginate. For instance, alginate with high level of mannuronic acid is less stable than those having high levels of guluronic acid (Oates & Ledward, 1990).

2.7.6 Alginate gel formation with calcium ions

Alginates form gels in the presence of cations, especially Ca^{2+} (Gacesa, 1988). The physical property of the alginate-calcium gel depends on the way the mannuronic and guluronic acids are grouped along the alginate chain (Penman & Sanderson, 1972; Reece, 1972, as cited in Gecesa, 1988). The L-guluronic rich alginate regions (blocks) must have at least 10 guluronic acids in a continuous sequence to form gels. Alginates with >10 guluronic molecules form a strong but brittle gel, while alginates rich in M-M blocks form weaker gels and are mainly utilised as thickeners (Storebakken & Austreng, 1987).

2.7.7 Calcium-alginate gel

Alginate reacts with polyvalent metallic ions such as calcium ions to form gels or solutions with very high viscosity. During the reaction, a strong ionic bond is formed resulting in the solution becoming thickened and forming a gel (Meyers, et al., 1972). The reaction between the alginate solution and Ca^{2+} ions is very fast and normally results in lump formation. This can be avoided by using either the dialysis method or the internal gelation method during the reaction process. In the dialysis method, an aqueous solution of sodium alginate is dripped into a solution containing calcium ions (Draget et al., 2006), thus forming ionic bonding between G-blocks, resulting in the formation of a 3-D (dimensional) network (Roussaeu et al., 2004) as shown in Figure 2.4.

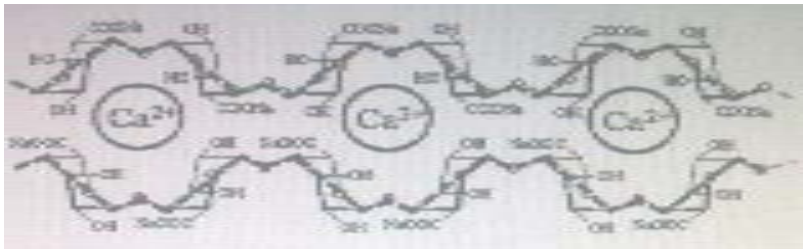


Figure 2.4. Egg box model of sodium alginate gelation with calcium ions
(Donati & Paoletti, 2009)

In the internal gelation method, a sequestering agent or an insoluble salt such as sodium hexametaphosphate is mixed with the alginate to interfere with the chemical reaction between alginate and calcium sources, forming an inactive form of cross-link between the ion and slowing down the chemical reaction. Gel that is formed by this method is prone to syneresis (Draget et al., 2006).

Other factors contributing to the final characteristics of the calcium alginate gel include pH, sequestrant, water hardness, addition of hydrocolloids, and water intake, with pH having the most significant effect on gel formation (Ren, 2010). The optimum pH for gel formation is between 2.8 – 4.0 (A. King, 1983). At low pH with a high level of soluble salt, a small amount of calcium is needed for gel formation, however, when there is high level of calcium, formation of gel starts at pH of 5 (ISP, 2007, as cited in Ren, 2010).

The properties of gels produced can be affected by the acidification rate of the alginate solution. Weak and brittle gels can be caused by rapid acidification and can also result in values below the polydisparity index (the measure of the distribution of molecular mass in a polymer sample) (Ren, 2010).

During the mixing of calcium with alginates, calcium that appears naturally in the water may cause premature gel formation. To avoid this, a calcium sequestrant such as sodium hexametaphosphate (SHMP), tetrasodium pyrophosphate, or sodium citrate is used during mixing in order that they may react with the calcium in the water and slow down gel formation (ISP, 2006, as cited in Ren, 2010). Sequestrant also aids in preventing polyvalent ion contamination through its reaction with these ions (that may come from water, chemicals and pigments), thus removing them and forming a good quality gel (Ren, 2010). Also, the reaction of sequestrants with polyvalent ions of minerals in hard water aids in minimizing any impact that could be caused on the formed gel (Ren, 2010).

2.7.8 Use of sodium alginate as binding agent in pelleted feed for aquatic animals

Sodium alginate has long been used as a binding agent in several research studies on pelleted feed for aquatic animals such as fish, shrimps, crustaceans, and others (Kneuer et al., 1993; Pearce et al., 2002; Ruscoe et al., 2005; Storebakken, 1985; Storebakken & Eustreng, 1987). Meyers et al., (1972) reported that sodium alginate and wheat gluten included at 50g/kg were the best in maintaining water stability, and minimising water absorption and protein leaching for *Litopenaeus vannamei* brood stock. Similarly, 24 hour stability was observed in both dry and moist pellet diets for crustaceans when agar and sodium alginate was included in the diet (Heinen, 1981). When Ruscoe et al., (2005) compared water stability of alginate with other binders in pelleted feed for crayfish, he found that 10% moisture alginate-bound crayfish pelleted feed was more water stable than other binders. When agar, polyvinyl alcohol, and sodium alginate were included at 2% in the diet of prawns, all three binders retained 76% of the pelleted feed after 8 hours compared to gelatine with 72%, and cassava with 52% (Ali, 2011).

Despite having greater potential in maintaining the integrity of pelleted feed in water for several aquatic animals, sodium alginate has been shown to reduce digestibility of certain nutrients (Cuzon et al., 1994). Argüello-Guevara and Molina-Poveda (2012) reported that sodium alginate has prevented the release of amino acids when included in the diet of *Litopenaeus vannamei* brood stock, and octopus showed negative growth rate and some died when fed crab meat with 10g/kg alginate added (Rosas et al., 2008). Storebakken (1985) reported lower ingestion and apparent protein digestibility, and reduced dry matter in the faeces when sodium alginate was included in the diet of rainbow trout.

These studies show that although sodium alginate is capable of maintaining the integrity of pelleted feed, it has the potential to reduce digestibility of certain nutrients in some aquatic animals.

2.8 Methods of measuring nutrient digestibility

The feed that is liked and ingested by the animal will be of no value if not digested to meet the animal's requirement for optimum growth, maintenance and reproduction. Since feed constitutes the major cost of production in any intensive animal production system, it is important to know the animals' nutritional requirement for growth, maintenance, and reproduction in order to formulate and feed them the right quantity of feed with adequate nutrient components to maintain productivity and performance (Noblet et al., 1994).

The measurement of these nutrients released from food during digestion and becoming readily available for absorption and utilization as retained nutrients necessary for growth, maintenance, and reproduction is referred to as digestibility (Goddard & McLean, 2001). The knowledge of digestibility, absorption, and utilisation of these nutrients is necessary for determining how well a feed will meet an animal's nutrient requirements. This is necessary to optimise animal growth and performance and minimise feed cost and wastage.

Digestibility can be measured by feeding the animal a known amount of feed and collecting its total faeces for analysis or by feeding the animal a diet containing a known concentration of an indicator marker (Atkinson et al., 1984). In the latter method, only a sample of the animal's faeces and feed is collected for analysis, and digestibility is measured based on the relationship between the nutrient content in the feed/faeces with that of the inert marker. In an aquatic environment, collection and measurement of all animal faeces is difficult, hence the use of an inert maker is commonly used for estimation of nutrient digestibility (Tacon & Rodrigues, 1984).

2.8.1 Markers use in digestibility Studies

For the marker to be effective, it must be indigestible, not absorbed, and remain homogenously mixed with the digesta during its passage through the gut, and also have no effect on the digestive metabolism of the animal (Schneider & Flatt, 1975). Furthermore, it must be non-toxic, completely inert, and should move through the gut at the same rate as the digested food (De Silva & Anderson, 1994).

Markers are categorized under two sources: internal and external. Internal markers are those that exist as part of the diet (Atkinson et al., 1984), such as crude fibre, hydrolysis-resistant organic matter and acid insoluble ash (AIA) (Goddard & McLean, 2001). External markers are those that are added to the diet, such as chromic oxide (Vandenberg & De la Noüe, 2001), polyethylene (Tacon & Rodrigues, 1984), and oxides of yttrium, etc. (Storebakken et al., 1998). Chromic oxide is the most widely used external marker in nutritional studies of aquatic animals. However, recent studies have doubted its suitability due to its analytical variability and poor recovery (Riche, et al., 1995), differential intestinal transit time (Leavitt, 1985), and digestive and metabolic effects (Shiae & Chan, 1993, as cited in Vandenberg & De La Noue, 2001).

Research has proven that AIA, particularly silica, has been successfully used in mammals, poultry, penaeid shrimps, tilapia, cichlids, and rainbow trout (Van Keuben & Young, 1997; Vogtanan, 1975; Derring et al., 1996, as cited in Goddard & McLean, 2001). This can be supplemented with external sources like celite, acid-washed diatomaceous silica powder, and acid-washed sand which is present in a low concentration in the diet (Tacon & Rodrigues, 1984).

The calculation of digestibility can be made using the ratio of the marker in the food and faeces to the specific nutrient (Goddard & McLean, 2001). That is, the relative changes in marker concentration in the faeces relative to the dietary marker concentration and the nutrient to be monitored (Maynard & Loosli, 1956).

2.8.2 Digestibility measurement system

Carbohydrate and fat primarily serve as the energy source (Ravindran & Bryden, 1999) supplying energy needed by the animal. Protein provides energy through oxidation and gluconeogenesis, but primarily supplies AA (Ravindran & Bryden, 1999). These two main functions of food in animals give rise to two digestibility measurement systems: the energy evaluation systems, and AA digestibility systems.

2.8.2.1 Energy systems

The three different energy systems (see Figure 2.5) used in determining the energy requirements of an animal include digestible energy (DE), metabolisable energy (ME), and net energy (NE). These all use gross energy as the basis of their calculation.

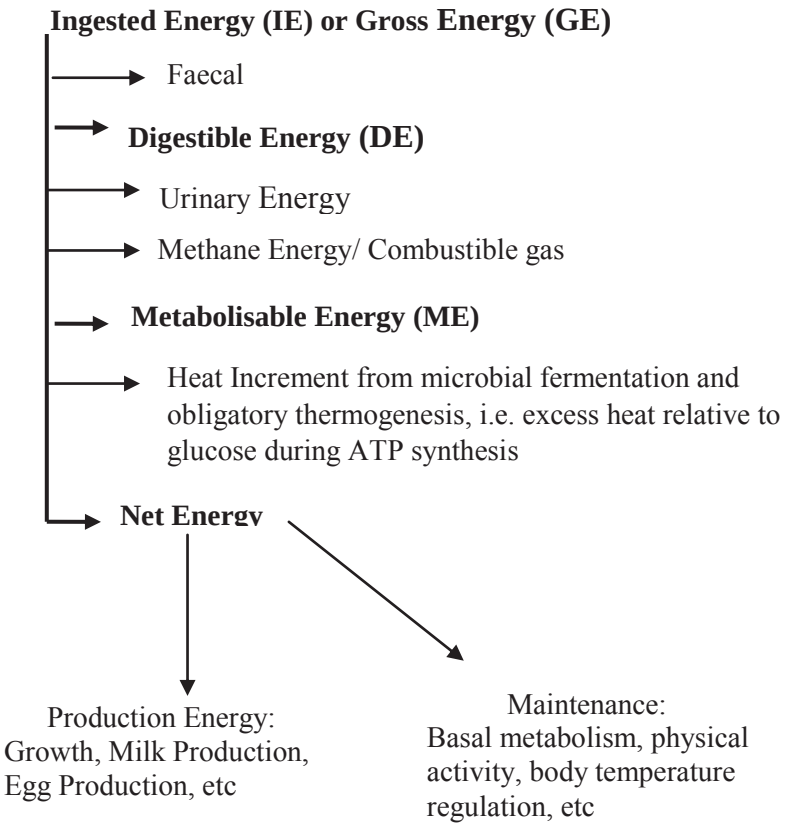
Gross energy (GE) is the energy content of the feed, normally determined by measuring the heat of combustion in a bomb calorimeter when a feed sample is completely oxidised. It forms the basis from which digestible energy is calculated. DE is the amount of energy that disappears when an animal eats the feed. That is, the GE of feed minus the GE of faeces. It assumes that the energy that disappears when ingested is digested, absorbed and available for use by the animal (McDonald et al., 2011). It is determined by recording total feed intake and total faecal output, measuring their GE content, and subtracting faecal energy from intake energy, that is: DE = IE – FE (Noblet & Henry, 1993). An alternative method is the inclusion of indigestible markers (Boisen & Fernandez, 1997). DE is the simplest method of measuring digestibility, but it fails to take into consideration energy losses through urine and combustible gasses during feed metabolism. ME is calculated by subtracting all the energy lost as waste in the excretion of urine and production of combustible gasses from DE (Pond et al., 1995). The energy lost through urine is measured by collecting urine and analysing it for energy content. Energy lost through combustible gasses is measured by keeping the animal in a respiration chamber where the amount of gasses produced is collected. The values obtained are used to determine ME using the following formula (Boisen & Fernandez, 1997; McDonald et al., 2011; Noblet & Henry, 1993).

$$ME = DE - (\text{urinary energy} + \text{energy in combustible gases})$$

This method of measuring digestibility gives a better estimate of energy available to the animal as it corrects the DE for efficiency of protein use, however; it involves more procedures than DE.
NE describes the energy that is actually used by the animal and is obtained from subtracting heat increment produced during digestion of feed, metabolism of nutrients, and excretion of waste from ME. It is the amount of energy actually used for maintenance and production (growth, lactation and gestation) and is calculated using the following equation (McDonald et al., 2011; Pond et al., 1995):

$$NE = ME - \text{heat increment energy}$$

Figure 2.5 Three Energy Evaluation Systems.



Source: Leeson & Summer, 2001.

The amount of energy produced during heat loss can be obtained from adiabatic or gradient layer calorimetry. In adiabatic calorimetric studies, the animal is fed and placed in a shield chamber filled with a weighed volume of water and a bomb calorimeter. The chamber is shielded with an adiabatic shield that serves as a heat insulator. The heat of combustion produced from the animal's body during digestion and metabolism of feed causes an increase in the level of water. This increase in water level over time is measured by the adiabatic bomb calorimeter, which indicates the amount of heat produced. In gradient layer calorimetry, heat produced passes through the chamber wall and is measured by the flow of heat from it. An alternative method includes calculation of oxygen consumption and excretion of NH_4, CO_2 and N using the following equation proposed by several researchers (Boisen & Fernandez, 1997; McDonald et al., 2011; Noblet & Henry, 1993).

$$\text{Heat Production} = 3.866 \times O_2 + 1.2 \times CO_2 - 0.518 \times NH_4 - 1.431 \times N$$

It is the most accurate and unbiased way of characterising energy content of feed. However, it is much more difficult to determine, as it is more complex than DE and ME.

2.8.2.2. Methods of measuring amino acid digestibility

Energy systems indicate the distribution and absorption of energy from feed. Protein and AA are other factors to be considered in formulating feed for the animals. The accurate measurement of these values in feed and their digestibility in the animal is necessary for maintaining optimum growth and performance. These values are determined using three methods of measurement including apparent faecal digestibility, apparent ileal digestibility, and true ileal digestibility.

Apparent faecal digestibility (AFD) is the procedure used in evaluating digestibility of dietary protein based on the difference between the amount ingested and the amount appearing in faeces. Apparent protein digestibility can be determined as the difference between the amount of N ingested and excreted, expressed as a proportion of N ingested. AFD can also be used to determine digestibility of a specific AA when the concentration of that AA in feed and faeces are measured. It is calculated as: AA digestibility = AA in feed – AA in faeces / AA in feed. AA in faeces is the total AA from undigested diet residues and endogenous secretion; hence, it underestimates the value of digestible AA (McDonald et al., 2011). The amount of AA in feed is measured before feeding, and all faeces are collected and analysed after egestion for AA content (Low, 1982).

Apparent ileal digestibility (AID) is determined by measuring the quantity of AA remaining at the end of the small intestine or ileum. This provides a much more accurate indication of protein digestibility and availability by the animal because the problems associated with microbial protein degradation in the large intestine and caecum does not arise. McDonald et al., (2011) suggest that microbial activity in the small intestine may possibly affect the accuracy of apparent ileal digestibility values, however, it seems reasonable to assume that any affect would be minimal compared with that observed in the large intestine (McDonald et al., 2011). AID is calculated using the following equation:

$$\text{AID\%} = \frac{\text{AA intake} - \text{ileal AA outflow}}{\text{AA intake}} \times 100$$

There are several methods available to collect ileal digesta for measuring apparent ileal digestibility. Sampling from the terminal ileum after euthanasia is common in both rats and pigs, whereas other methods, such as cannulation and bypass of the large intestine by ileorectal anastomy or ileostomy, are more commonly performed on pigs. Each of these methods have their own advantages and disadvantages involved (Moughan, et al., 2000).

True ileal digestibility (TID) measures the proportion of dietary AA that disappears from the digestive tract before the distal ileum and does not include endogenous AA. It is a fundamental

property of the food that is not affected by the dietary conditions under which the food is fed to the animal (e.g., the protein content of the test diet). The apparent digestibility measure, however, is affected by the assay conditions and is, therefore, variable and subject to error. True digestibility is a superior measure for determining the AA that are absorbed from the gut, and therefore, gives a better representation of protein quality than apparent digestibility (Stein et al., 2007). It is calculated using the following equation:

$$\text{TID \%} = \frac{\text{AA intake} - (\text{ileal AA outflow} - \text{total IAA end})}{\text{AA intake}} \times 100$$

It is determined by subtracting the amount of AA in ileal digesta from the amount of AA that were ingested, to obtain an "apparent" digestibility coefficient. However, ileal digesta also contains a significant proportion of non-dietary AA, from sources such as mucus, cells, digestive enzymes, and bile known as endogenous amino acids loss (EAAL) (Moughan et al., 2000). These EAAL can be quantified using several methods. One method is by feeding a protein-free diet to the animal with the protein remaining at the end of the small intestine assumed to be the basal EAAL. The provision of a diet devoid of protein, however, leads to negative body nitrogen balance in the recipient animal (Moughan et al., 2000). Another method is to feed the test animal an enzyme hydrolysed protein (usually enzyme hydrolysed casein) diet with ultrafiltration of the ileal digesta collected to remove any unabsorbed dietary AA. It is assumed any protein at the terminal ileum is from endogenous sources. When the basal EAAL is determined using techniques such as the protein-free method or the enzyme hydrolysed protein method, apparent AA digestibility is corrected in the calculation of digestibility, and the "true" digestibility coefficient is obtained (Moughan et al., 2000).

2.8.3 Nutrient digestibility measurement in crocodiles

In order to develop a low-cost diet for crocodiles, one's knowledge on digestibility, metabolisability, and the efficiency of utilising the ME are important considerations (Staton et al.,1990a). Several digestibility studies have been done on different species of crocodiles.

Coulson et al., (1987) studied AA and protein requirements of alligators by placing the animals (0.5–2kg) in a controlled temperature chamber (28±0.3), gavage-feeding them and, collecting their urine and blood samples for analysis. Urine was collected by inserting a fire-polished glass tube (6mm) into the cloaca from which the urine was drained through into a flask containing 1ml 1N HCL to trap the NH_2. Following urine collection, the animals were fed and their blood was collected from the tip of their tail using a Pasteur pipette. Once the blood was collected, the pipette was placed inside micro-centrifuge tubes and subjected to 13,000 rpm centrifugation for 20 sec.

After which, 0.2ml plasma was removed and placed into 3.8ml of 95% ethanol for precipitation of protein. This was then subjected to centrifugation at 2500rpm for 5min. The clear colourless supernatant formed was collected and stored at -20^{o}C until required for AA analysis (Coulson et al., 1987).

Based on their observation, Coulson and colleagues concluded that all AA, except alanine and glutamine were incorporated into the body at a faster rate than their gain in the blood, indicating them as essential AA, while glycine reaches its peak several hours later due to absorption by the intestines during an initial rise and then by synthesis from other AA. While the method by Coulson et al., (1987) is better for understanding how AA becomes available after feeding as well as its rate of incorporation into the body, it is not a relevant method for determining AA requirements. AA requirements are best determined by digestibility experiments where the digestibility coefficient of an AA for the type of feed fed is determined from digesta samples collected from the ileum and calculated based on standard formulas described in section 2.8.2.2

Garnett (1988) measured digestion of lean and fatty pork in 8 juvenile *C. porosus* (257-522g) by placing them individually into tilted plastic tubs of 30 x 60 cm (filled with water at the lower end) in a temperature controlled room (30^{o}C for both air and water) with artificial illumination from 6am to 6pm. He gavage-fed the animals with a meal of 2% LBW daily at 6pm for 5 days by placing the feed behind their palatal valve to ensure all feed was ingested. Twenty four hours after each feeding, voided faeces and urine with water were collected together and homogenised using a blender and then dried at 80^{o}C. All dry matter collected over 5 days were pooled together for analysis.

The protein, organic matter, fat and energy digestibility were calculated using the following formulas he created and those he obtained from several researchers as shown next to their formula:

Protein metabolisable energy (PME) = protein intake x % protein apparently digested - (6.25 x free NH_4^+ and uric acid recovered in waste water).
Fat metabolisable energy (FME) = % fat apparently digested.
Organic matter metabolisable energy (OME) = PME + FME
Gross Energy = measured in feed
Faecal Energy = (energy in faeces+ urine) – urinary energy
Digestible Energy = Gross energy – faecal energy

Metabolisable Energy = Digestible energy – urinary energy

Urinary Energy = M x N x O x P, where;

M= µmol AA excreted by the animal/g live weight /day

= 9.0 x 10^{-3} for fasting animals and 6.10 x 10^{-2} for fed animals (Coulson & Hernandez, 1967 and 1970b as cited in Garnett, 1988)

N= kJ/gPM (PM = protein metabolized) = 22.186 (Coulson & Hernandez, 1979)

O= g PM/µmolAA = 1.15 x 10-4

P= live weight (g)

The method used by Garnett (1988) was one which could best determine protein, fat, and OM digestibility from analysis of the feed and faecal samples. However, protein determination may be susceptible to errors associated with volatilisation of N as a result of oven drying samples at high temperature (80^{o}C). For energy digestibility, there may be errors associated with how urinary energy was calculated as it is difficult to differentiate faecal energy from urinary energy since both faeces and urine were excreted together into the water bath. The formula used in calculating urinary energy is unique compared to how urinary energy was calculated for other animals in any digestibility experiment where urine is collected separately from the faeces and its energy was measured differently. For instance, in ruminants, a volume of urine can be collected separately from the faeces and subjected to combustion in a bomb adiabatic calorimeter to determine the value of urinary energy. In fish, urine may be collected by the use of urethra or bladder catheter and combusted for analysis. Since urinary energy formed the basis of calculation for faecal energy, digestible energy and metabolisable energy in Garnett's research, the energy digestibility values obtained by Garnett (1988) may have underestimated the actual value if urine was collected separately using the method used by Coulson et al., (1987) and combusted. Despite the likely errors, the apparent energy digestibility for *C. porosus* obtained by Garnett (1988) was found to be similar to the results for the animals presented in Table 2.3. Fat digestibility of the lean and fatty pork meat were 86.4% and 65.1% compared to 86% and 90% in pig and chicken respectively, while apparent protein digestibility of 92.6% lean pork was similar to salmon fish (Smith et al., 1980) and poultry (Krogdahl & Dalsgard, 1981).

Table 2.2 Apparent energy digestibility for immature *C. porosus* and other animals

	Immature *C. porosus*	Mature *C. porosus*	Lizard *Anolis lineatopus*	Carnivorous lizard	Natrix snake	Rainbow trout
Digestible energy	65.1 - 86.4	78.8 - 85.1	63-88	54-93	80	78 - 80

Source: Lawrence and Loveridge (1988)

Lawrence and Loveridge (1988) studied digestibility, metabilisability, and efficiency of utilisation of energy (EUME) in young *C. niloticus* by examining the carbon, nitrogen and energy balances. They placed two crocodiles (a 10yr old male weighing 10kg and a 3-4yr old female weighing 4.25kg) on a mesh wire platform inside a respiration chamber at 30 ±1^{o}C with a 12L: 12D photoperiod. Ventilation was provided by an aquarium pump and an apparatus was used for measuring gas exchange. Feed was placed in front of the animals overnight and orts were removed the following morning and weighed. Water was provided for only 3 hours daily (1100 – 1400) and removed. Liquid urine was obtained from the chamber via an attached tube while faeces and solid urine were scraped from the chamber and stored at -10^{o}C until analysed. The crocodiles occasionally urinated into the water bath, but since the main excretory product in urine is ammonia, its presence in the water bath was detected using Nesslers reagent (Vogel, 1961 as cited in Lawrence & Loveridge, 1988). The crocodiles excreted faeces in frequent and erratic intervals, and these were pooled and the dry faecal production was assumed to be proportional to dry feed intake. Likewise, solid and liquid urine and water were pooled. The overall digestibility, metabolisability, and net energy values of ox heart fed to the crocodiles were calculated using the following equations:

$$\text{Digestibility (\%)} = 1 - \frac{\text{faecal energy output}}{\text{energy input}} \times 100$$

$$\text{Metabolisability (\%)} = 1 - \frac{\text{output of energy in faeces and uric acid}}{\text{energy input}} \times 100$$

$$\text{Net energy value (\%)} = 1 - \frac{\text{energy output, uric acid and heat increment}}{\text{energy input}} \times 100$$

The methods used by Lawrence and Loveridge (1988) may have underestimated the values of metablisability and net energy as they did not take into consideration energy of liquid urine, except for uric acid combined with faeces as seen in the formula above. However, it could be best used in determining faecal digestibiltiy of nutrients if the faeces were not exposed to pen water, otherwise, error could have occurred due to leaching of nutrients from the faeces into the water.

Staton et al., (1990a) compared the digestibility of protein, fat, and carbohydrate by placing four alligators (377- 857g) into 0.6m deep fibreglass pens (0.5m^2) with 1:2 ratio of dry:water, respectively, and fed them thrice daily for 15 weeks. Tanks were washed and filled with warm water after every feeding session. The animals were kept in total darkness, except during feeding and cleaning. Dry matter of the feed consumed was calculated by subtracting orts from feed intake and adjusting for the moisture. Alligators normally excrete semi dry faeces into the water. This was

collected 1-2 hours after tank cleaning, stored frozen, then freeze dried before analysing. Chromic oxide was included at 0.1% in the diet to determine digestibly of nutrients in the faeces and feed. Crude protein (CP) was determined by using the Kjeldahl Nitrogen Method (AOAC, 1970) while gross energy (GE) was measured in adiabatic bomb calorimeters. Protein and energy digestibility were calculated using CP and GE analysed values by their respective digestive coefficient using the following equation:

$$\text{Percent Nutrient Digestibility} = 100 - 100 \times \frac{(\%\ Cr_2O_3 \text{ in diet})(\%\ \text{nutrient in faeces})}{(\%\ Cr_2O_3 \text{ in faeces})(\%\ \text{nutrient in diet})}$$

This method may underestimate the nutrient digestibility values due to the likely occurrence of error associated with leaching of solubles from the faeces into the water. Similarly, Beyeler (2011) examined protein utilisation in 5-6 months old *C. niloticus* by placing 205 crocodiles into pens (5.2m x 5.2m x 1.5m) comprising of 2/3 dry:1/3 water. Twelve pens were divided into 4 treatments and 3 replicates. Crocs were fed ad-lib over 8 hours after which, orts were subtracted from feed intake to estimate the amount of feed consumed. The total feed consumed was divided by the number of crocs per pen to estimate the amount of feed consumed per crocodile per pen. Two samples of feed and faeces were collected per pen and chemically analysed. The digestibility of CP, energy and dry matter (DM) was determined using 0.1% chromium oxide as an indicator and their digestibility coefficients were calculated using the standard equations by Staton et al., (1990a). In Beyeler's research, 5 or more replicates and faeces samples would have given a better indication of digestibility for a good spread in the curve when data analysis is of concern. The digestibility values he obtained may be underestimated due to soluble leaching from the faeces into pen water. Since he used pen as the experimental unit, there may be differences associated with pen and animal variation when compared with results from other researchers who have used animal as the experimental unit.

Read (2000) studied protein utilization in post-hatchling *C. porosus* by placing crocodiles into tubs (60 x 40 x 12cm) in a temperature-controlled room and gavage-fed them using a syringe feeder. Immediately following feeding, most water was drained leaving only a little portion for drinking and hydration. The crocodiles remained in this condition until the following feeding period. Chromium oxide was used as the inert indigestible indicator. Faecal samples were collected by both a total collection technique and a partial faecal collection technique. In total collection technique, he collected all faeces, urine and water together. In partial faecal collection technique, he collected faeces only from the tubs prior to pan cleaning or within two hours when water was added. This was done in order to minimise nutrient leaching. The samples collected were stored in a freezer until required for proximate analysis. All samples per treatment were pooled together and freeze

dried to remove moisture, and then oven dried again at 65^{o}C for 24 hours to remove other residual moisture before undergoing chemical analysis. The nutrients analysed from Read's work may be underestimated due to error associated with leaching. Since the faecal samples undergo two drying methods for the purpose of removing excess moisture, heat induced changes may have affected the nutrient component of the samples (Abascal et al., 2005; Burritt et al., 1988; Dzowela et al., 1995; Ozcan et al., 2005; Yang & Atallah, 1985), thus affecting the result. Despite this, the results obtained by Read (2000) were similar to those observed by Staton et al., (1990a) on hatchling alligators (see Table 10.2 and Table 10.3 in Annexure).

Despite several studies on nutrient digestibility in crocodiles, quantitative collection of faeces in an aquatic environment is tedious and quite difficult. The likely method to estimate nutrient digestibility should best be the indirect approach involving the use of dietary inert markers to follow the process of digestion (Tacon et al., 1984) and to determine digestibility coefficients (Atkinson et al., 1984). For crocodiles, there is no best recommended method for the collection techniques used in collecting faeces and urine for measurement and determination of digestibility for the nutrients consumed. Researchers have adopted methods that have been used in measuring digestibility in warm blooded terrestrial animals and fish. Based on the accuracy of the methods used by researchers as described above, the suggested method for the determination of digestible and metabolisable energy should be the one used by Coulson et al (1987) method for collection of urine to determine urinary energy. However, since this method requires strict supervision of operators and lengthy training to become skilful, an alternative method could be used by placing animals in a mesh netted platform with a tub underneath so that when it removes waste, urine would pass through the mesh net into the tub where it will be collected while faeces can be collected from the mesh netted platform. When determining net energy, firstly, urine should be collected using Coulson et al.'s (1989) method or both faeces and urine can be collected together first by the later method. After duration of faecal or urine collection, the animals can be subjected to the method developed by Lawrence and Loveridge (1988) for determining heat increment and gaseous exchanges.

For determining nutrient digestibility, faeces collected without the presence of water would best measure the nutrient composition of the faeces as the presence of water contributes to leaching of nutrients, thus underestimating digestibility. However since faecal samples contain both exogenous and endogenous nutrients, digestibility based on faecal sampling may be misleading. Digesta collected from the ileum should best describe nutrients absorption in the upper intestines as digesta from the ileum is largely free from microbial degradation and fermentation.

Apart from improving the water stability of feed for aquatic animals and understanding digestibility of nutrients in feed for aquatic animals, the feed will not be consumed if not palatable, desired or liked by the animals. Texture also plays an important role in the palatability and acceptability of feed by aquatic animals.

2.9 Methods of Measuring Textural Properties of Feed

Texture can be described from the collective definition as:

1. A sensory property *and, thus, only a human being (or an animal in the case of animal food) can perceive and describe it. The so-called texture testing instruments can detect and quantify only certain physical parameters which then must be interpreted in terms of sensory perception*; 2. It is a multi-parameter attribute, *not just tenderness or chewiness, but a gamut of characteristics*; 3. It derives from the structure *of the food (molecular, microscopic or macroscopic);* and 4. It is detected by several senses*, the most important ones being the senses of touch and pressure.* (Szcnesniak, 2002, p 1-2)

Texture can be assessed either by using physical sensory measurements such as sight, smell, and feel or by using instruments specialised for testing different textural parameters. For physical sensory measurements, the foods is assessed by sight, taste, and feel (by hands) and categorised by textural classification system as described in Table 2.2.

Table 2.3 Classification of textural characteristics

Mechanical Characteristics		
Primary Parameters	*Secondary Parameters*	*Popular terms*
Hardness		Soft, Firm, Hard
Cohesiveness	Brittleness	Crumbly, Crunchy, Brittle
	Chewiness	Tender, Chewy, Tough
	Gumminess	Soft, Mealy, Pasty, Gummy
Viscosity		Thin, Viscous
Springiness		Plastic, Elastic
Adhesiveness		Sticky, Tacky, Gooey
Geometrical Characteristics		
Class		*Examples*
Particle size and shape		Gritty, Grainy, Coarse, etc
Particle shape and orientation		Fibrous, Cellular, Crystalline, etc
Other characteristics		
Primary Parameters	*Secondary Parameters*	*Popular terms*
Moisture Content		Dry, moist, wet, watery
Fat Content	Oiliness	Oily
	Greasiness	Greasy

Source: Szczesniak, 2002

Conclusion and remarks

Wet extruded manufactured pelleted feed has been proven acceptable by crocodiles (Peuker et al., 2006) as an alternative feed to raw animal meat. Despite successful pellets made for alligators and Nile crocodiles, the pelleted feed developed for salt-water crocodiles in Papua New Guinea has been less successful in terms of palatability and water stability. The fast rate of pellet disintegration once the pellet comes into contact with water leads to rapid loss of ingredients, insufficient food intake, deficiency in nutrient levels, and deterioration of water quality. The ability of the feed to remain stable in water is influenced by the type of binders used, the processing conditions, types of ingredients and composition of diet, and coating materials (Dominy et al., 2004).

Feed binders have been used in fish feed processing to enhance the physical and nutritional properties of feed in situations where available ingredients and processing conditions cannot produce pellets of satisfactory quality (Dominy et al., 2004). Binders such as wheat gluten, lignin, corn gluten, agar, gelatine, carrageenan, CMC, and alginates have long been used in the aquaculture industry to enhance the stability of pelleted feed for fish and other aquatic animals. Despite numerous studies conducted to understand the role of binders in pelleted feeds for fish, shrimp, crustaceans, and other aquatic animals (Dominy et al., 2004; Falayi et al., 2004; Pearce et al., 2002; Ruscoe et al., 2005; Obaldo et al, 2002), there is not much scientific information available on the role of binders in improving water stability of wet extruded pelleted feed for crocodiles.

The ability of crocodiles to utilize nutrients is not well understood (Garnett, 1985). Research and knowledge of crocodiles' digestive physiology, chemical breakdown, enzymatic degradation, site of absorption and utilization of nutrients is very limited (Diefenbach, 1975; Read, 2000).

Chapter 3 Materials and Methods

The research approached use in this study was an experimental design, divided into laboratory and on-farm experiments. In laboratory experiment, the optimal condition for sodium alginate use was examined. The on-farm experiment was done following laboratory findings to measure digestibility of nutrients in *C.porosus* to evaluate effects of sodium alginate on nutrient digestibility.

3.1 Laboratory feed processing

The measurement of water stability, dry matter retention (DMR) and sensory textural evaluation were done on laboratory diets prior to the execution of a digestibility experiment and observation of water stability of feed on-farm. Sodium alginate was used as a binder in the feed formulation to improve its water stability because of its 3-dimensional gel formation at room temperature which captures all raw materials in the formulation in a very short time compared to other gelling agents. Apart from that, the gel formed is capable of resisting shear stresses; and the procedure involved is simple and appropriate for this particular operation.

3.1.1 Ingredients used

For the laboratory experiment, all wet and dry ingredients, including minced chicken carcass, chicken blood, pulped (shell-less) eggs and millrun were obtained from Massey Poultry and Feed Processing Unit. Calcium carbonate and calcium chloride were obtained from the Massey University Nutrition Laboratory. The three sodium alginate products including Protanal XP3639, Manucol DM and Kimica Sodium Alginate were respectively supplied by Hawkins Watts Pty Ltd, Tari International Pty Ltd, and an agent for Kimica Chile Ltd in New Zealand. Supplement ingredients for the crocodile feed were imported from MHCF in PNG.

Initially, in a preliminary feed processing trial, a replicate of the MHCF 2012/2 Fine diet was made in the laboratory in reduced amount (see Table 3.1, Control 1). The amount of feed was reduced to minimise wastage of ingredients. The mixing and processing procedures used in the laboratory were similar to that used at MHCF (i.e., ingredients were first mixed in a Kenwood bowl mixer and then extruded through dies of a Kenwood mincer).

The extrudate feed from this formulation crumbled on exit without forming continuous ribbons of extrudate. The surface of the feed was rough, and the feed retained 4.3% of dry matter (DM) after being submerged in water for 18-24 hours (see Appendix 1). Since inclusion of millrun contributes

to an increase in the DM content of the feed and makes it moderately dry and easy to break apart when existing out of the machine, another formulation was used without millrun as a Control 2 diet (See Table 3.1). The resulting extrudate feed from this formulation was smooth, stuck together, and retained 6.2% DM. Based on the percentage of DMR, as well as the textural quality of the Control 2 diet, it was recommended for use as the basis for all the laboratory diets made with alginate-calcium reaction.

Table 3.1 Reduced ingredient components of MHCF 2012/2 Fine as Control 1 and Control 2 Diet

Control 1			Control 2	
Ingredients	*Composition (%)*		*Ingredients*	*Composition (%)*
Minced chicken carcass	35		Minced chicken carcass	41
Chicken blood	15		Chicken blood	18
Millrun	15		Poultry offal meal	6
Poultry offal meal	5		Pulped chicken eggs	6
Pulped chicken eggs	5		Supplements	29
Supplements	25			

Another twelve laboratory diets comprising of the main ingredient components of the Control 2 diet were made with inclusion of the alginate products and the calcium sources respectively (see Table 3.2).

Table 3.2 Ingredient composition of laboratory diets (%)

Feed Ingredients												
Main Constituents:	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12
Poultry offal meal	5.7	5.7	5.6	5.6	5.7	5.7	5.6	5.6	5.7	5.7	5.6	5.6
Supplement	28.3	28.3	27.8	27.8	28.3	28.3	27.8	27.8	28.3	28.3	27.8	27.8
Chicken carcass mince	39.6	39.6	38.9	38.9	39.6	39.6	38.9	38.9	39.6	39.6	38.9	38.9
Chicken blood	17.0	17.0	16.7	16.7	17.0	17.0	16.7	16.7	17.0	17.0	16.7	16.7
Chicken pulped eggs	5.7	5.7	5.6	5.6	5.7	5.7	5.6	5.6	5.7	5.7	5.6	5.6
Sodium Alginates:												
1.7% Protanal XP3639	1.7	1.7										
3.3% Protanal XP3639			3.3	3.3								
1.7% Manucol DM					1.7	1.7						
3.3% Manucol DM							3.3	3.3				
1.7% Kimica									1.7	1.7		
3.3% Kimica											3.3	3.3
Calcium sources:												
1.9% $CaCO_3$	2.1		2.1		2.1		2.1		2.1		2.1	
1.9% $CaCl_2$		2.1		2.1		2.1		2.1		2.1		2.1
Total (%)	100	100	100	100	100	100	100	100	100	100	100	100

3.1.2 Feed mixing and processing

Prior to feed mixing and processing, each ingredient was weighed, bagged, and stored at 4°C until required. Before manufacturing each diet, all the dry ingredients for that diet were mixed and placed in a plastic bag. The respective amounts of the appropriate sodium alginate and calcium source ($CaCO_3$ or $CaCl_2$) were weighed into their own separate bags.

The mixing and processing procedure involved: placing the wet ingredients including chicken carcass mince, pulped chicken eggs and chicken blood into a Kenwood mixer bowl (Figure 3.1) and mixing the wet ingredients until blended. Then, add the calcium source (e.g., $CaCl_2$) into the mixture and mixed for 2 minutes. After which, the dry ingredients with appropriate level of alginate and calcium source were added and mixed for another 2 minutes until thoroughly blended. The blended mix was then placed on a shallow tray above the mini Kenwood sausage making machine and was manually forced down into the extrusion barrel using a polyethene rod (see Figure 3.2). Once the extrusion barrel was filled with the feed mixture, the machine was switched on and the extrusion screw forced the feed through the 19-mm dies onto the mini trays where they. These were then evaluated for sensory textural traits. Following sensory evaluation, two samples (approx. 15-30g) were collected: one sample was subjected to oven drying directly to determine the initial DM while the other was immersed in water for 18 - 24 hours for stability testing before the undissolved solids were recovered and subjected to oven drying for determination of the final DM.

Figure 3.1 Kenwood mixer

Figure 3.2 Kenwood sausage maker

3.1.3 Sensory textural evaluation.

The textural sensory evaluation of each diet was performed through physical sensory measurement in accordance with an evaluation guide by Szczesniak (2002). The textural characteristics of each feed sample was categorised by scores ranging from 1-10 as described in Table 3.3 with the least desirable characteristic represented by a score of 1 and the most favourable trait being given a score of 10.

Table 3.3 Textural traits and their score range

Traits		Sensory Description	Sensory Evaluation Scores	Quality Category
Cohesiveness	Crumbly	▪ Loose and rough ▪ Breaks instantly into very small pieces	1	Not recommended
	Firm	▪ Smooth and tough ▪ Form smooth continuous sausage without breakage	10	Recommended
Viscosity	Thick viscous	▪ Feed covered with jelly-like material	1	Not recommended
	Thin Viscous	▪ No sign of jelly-like material on feed	10	Recommended
Adhesiveness	Paste-like	▪ Soft, loose and sticky	1	Not recommended
	Gooey-like	▪ Soft, tight and sticky	10	Recommended
Wetness	Wet	▪ Damp wet	1	Not recommended
	Moist	▪ Moderately wet	10	Recommended

3.1.4 Water stability test

The efficacy of the processing methods was tested by subjecting the resulting feed to water submersion. One sample per diet was weighed (initial weight) immediately after the feed was processed and was subjected to oven drying for 24 hours at 106^{o}C for determination of initial DM (AOAC, 2005). Another sample was weighed and placed inside a vial containing 100ml warm water (26-31°C). This was manually shaken vertically by hand for 30 seconds and left overnight (18-24 hours) at ambient temperature (29-34^{o}C). On the following morning, the water together with the feed sample was poured through a 0.5mm sieve. The leached or dissolved solubles were removed with the water while the intact feed was subjected to oven drying for calculation of final DM (final weight after immersion). DMR was calculated using the formula by Obaldo et al., (2002):

$$\% \text{ DMR} = \frac{\text{Dry matter of feed sample after being subjected to water}}{\text{Dry matter of the original feed sample}} \times 100$$

Once the percentage of DMR and the sensory evaluation of all laboratory diets were determined, the cost per unit of DMR per diet was calculated. The diet that retained optimum DM compared to the control diet at a minimum cost was selected for use on-farm in the digestibility experiment. Based on observations of the percentage of DMR per diet as well as the textural quality and cost involved, Diet 1 in Table 3.2 was recommended as the best replicate of MHCF 2012/2 Fine (without millrun) to be used on farm as the R-MHCF Fine diet.

Since the laboratory diets were evaluated at 1.7 and 3.3% only, and that diet 1 with Protanal XP3639 alginate at 1.7% was recommended for use on-farm; another four diets were made with Protanal XP3639 alginate at lower concentrations (0.3%, 0.7%, 1.0% and 1.4%) to compare against the 1.7% to evaluate if alginate included at any level lower than 1.7% would retain more DM at a lower cost.

3.2 Feed processing and feeding on-farm

On-farm, the observation on the textural properties of the MHCF 2012/2 Fine and R-MHCF Fine diets were done thrice on-farm: straight after manufacture of the sausage-extrudate, during feeding of the general stock 2-3 hours after the feed was processed, and after the feed was stored in refrigeration overnight and fed the following afternoon. The observation on the reaction of the feed when it came into contact with water was done during feeding of the general stock 2-3 hours after the feed was processed, and after the feed was stored in refrigeration overnight and fed the following afternoon.

3.2.1 Ingredients used

All ingredients used on-farm were obtained from MHL feed mill and Table Birds Pty Ltd. The ingredient component of R-MHCF Fine formulated in the lab was scaled up to meet the capacity of the processing machine on-farm. This formulation, however, did not form into good shaped sausage-extrudate, instead it formed a damp wet (44% DM) sticky paste (see row 4 in Table 4.5 and Appendix 5, Figure 2). This problem may have been associated with scaling difference from the laboratory and on-farm diets; differences in ingredient characteristics between the farm and lab diets (such as the DM); and, the efficiency of the processing machine used in producing the feed.

Due to this, another diet was made with inclusion of 30 kg millrun to increase DM content of the diet. However, the feed was still damp wet and "caked up" as usually observed in the MHFC 2012/2 Fine diet (see Appendix 5, Figure 3). When the feed was manually broken into smaller pieces during feeding, the smaller portions agglomerated together forming a mass, making it hard for the juvenile crocodiles to swallow big portions of feed. Lovell (1980) reported that lower DM content of a diet resulted in the production of sticky feed that even when formed into good shaped pellets "caked up" during handling and storage, making the feed too sticky to be manually broken down into required size pellets (Royes & Chapman, 2003).

Another diet was made with inclusion of 38kg millrun purposely to improve texture by increasing the DM content of the feed (see Appendix 5, Figure 4). The diet produced was a bit sticky and moderately wet. Its ingredient composition was recommended for use on-farm for the digestibility experiment and was compared against MHCF 2012/2 Fine for water stability on farm. Since millrun was included in this formulation, the total weight of the feed was increased, hence the percentage inclusion level of each ingredients proportioned to the total feed weight was reduced and comprised of 23.26% supplement, 4.65% poultry offal meal, 1.4% Protanal XP3639, 1.86% calcium carbonate (animal grade), 32.55% minced chicken carcass, 13.95% chicken blood, and 4.65% pulped chicken eggs (Table 3.4)

Table 3.4 Ingredient composition for MHCF 2012/2 and R-MHCF Fine diets used on-farm

MHCF 2012/2 Fine	
Ingredients	*Composition (%)*
Minced chicken carcass	35.00
Chicken blood	15.00
Millrun	15.00
Poultry offal meal	5.00
Pulped chicken eggs	5.00
Fine supplements	25.00

R-MHCF Fine	
Ingredients	*Composition (%)*
Minced chicken carcass	32.55
Chicken blood	13.95
Millrun	17.67
Poultry offal meal	4.65
Pulped chicken eggs	4.65
Fine supplements	23.26
Calcium carbonate (powdered-animal grade)	1.86
Protanal XP3639	1.40

3.2.2 Feed mixing, processing and storage

Except for calcium carbonate, all dry ingredients including Protanal XP3639 alginate were weighed using Breknell Samson Electronic Scale (maximum capacity = 45 ± 0.05 kg), and then mixed together in a concrete mixer and bagged. Calcium carbonate was weighed and bagged separately. All these ingredients were stored in a shed (uncontrolled temperature) until required for processing.

Condemned chicken carcasses from Table Birds Pty Ltd were collected, eviscerated, and either stored in a refrigerator (3°C) until needed or when required immediately, were crushed into smaller pieces following evisceration using the crusher mincer (see Figure 3.3) and used immediately. Chicken blood was obtained fresh from Table Birds Pty Ltd. Pulped eggs were also obtained from Table Birds Pty Ltd and were stored in refrigeration (3^{o}C) until required. Just

before feed processing, all these wet ingredients were weighed using a bathroom scale (max. 150kg) and set aside ready for processing.

Prior to feed processing, the Thompson Frozen Meat Machinery 4000 Series Mixer Mincer (see Figure 3.4) was set up with extrusion barrel, blades, extrusion die and plate with correct die size specifically use for production of MHCF 2012/2 Fine diet. All wet ingredients were placed into the machine's bowl and mixed for 2 minutes, after which calcium carbonate was added. When the mix was homogenous, the dry ingredients comprising of a mixture of fine supplements, poultry offal meal, and Na alginate (Protanal) were added and further mixed for another 2 minutes. Millrun was finally added and the mixing continued until all ingredients were blended. Once satisfied that the mix was homogenous, the processing button was switched on and feed mixture was expelled through the dies onto trays (60 x 40 x 12 cm). These were then set aside for 2-3hrs to allow firming up of the extrudate feeds. After which, some of the feed were fed to the general stock while some were stored in a refrigerator overnight at 3^{o}C and fed the following afternoon.

Figure 3.3 Mincer

Figure 3.4 Thompson frozen meat mincer mixer machinery

The MHCF 2012/2 Fine contained a similar ingredient composition as the R-MHCF Fine, except with less millrun (30kg millrun) and no alginate and calcium carbonate included (Table 3.4). It was mixed and processed in a similar way as was the R-MHCF Fine diet, and was either fed to the general stock straight after processing or stored in refrigeration overnight and fed the following afternoon (see Appendix 2 for the figures showing ingredient preparation and feed processing).

3.2.3 Sensory textural evaluation

The textural properties of the MHCF 2012/2 Fine and R-MHCF Fine diets processed on-farm were evaluated using the same textural sensory evaluation assessment scores as used for laboratory diets.

3.2.4 Feeding of the general stock

Observation on the animal's response to the diets and integrity of both diets when coming in contact with water was done during feeding of general stock.

3.2.4.1 Housing condition

The concrete pens housing the juvenile (grower) crocodiles consist of a roof made of mesh wire and shade cloth with walls and floors of concrete. Each pen occupies a total area of approximate $300m^2$. Each pen (10 x 30 m) was divided roughly in half by an approximately 2m wide concrete walkway/feeding area. Water on each side of the walkway was approximately 0.5 to 1.5 m deep with a capacity to hold approximately $150m^3$ volume of water (Figure 3.5). The crocodiles are exposed to uncontrolled weather patterns as they would have in the wild, and were fed once daily at 1400. Each pen contains 100-300 crocodiles depending on the crocodile's size and the stocking capacity of each pen. Water is supplied from the borehole pump. Each pen is normally drained, washed, and disinfected once every week.

Figure 3.5 Pen housing the juvenile crocodiles

3.2.4.2 Existing feeding practices

A few days after hatching, the hatchlings (baby crocodiles) are normally transferred to the nursery pens and fed a hatchling diet until 6-7 months old when they undergo re-weighing.

During re-weighing, crocodiles are normally weighed and sorted into their weight groups and relocated to different pens based on their weights. For instance, crocodiles that weigh 100-299g are normally placed together while those that weigh 300-499g are put together purposely to avoid stress associated with competition for food and other basic needs. Crocodiles that weigh more than a kilogram are normally transferred to juvenile (grower) pens where they are offered MHCF 2012/2 Fine diet. Those that are less than a kilogram are usually placed back into the nursery pens and continue to be fed on the hatchling diet for another 3-6months when they are sorted again. The ingredient composition of the 'hatchling diet' is similar to the MHCF 2012/2 Fine diet, but the ratio per ingredient within each diet is different.

Once the crocodiles are moved into juvenile pens, their diet is switched to the MHCF 2012/2 Fine diet. As they get bigger in size (> 5 kg), their diet is changed to larger size extrudates (called 'coarse diet'), and then to a mixture of coarse extrudate feed and raw chicken meat pieces, head and feet, or fish as they reach more than 10 kg. From there onwards, raw chicken meat, head and feet, or fish remain their staple diet until they approach slaughtering size (> 20 kg). No growth hormones or other promotants are used in their feed to improve growth rates.

During feeding, the extrudate feed is manually broken into shorter length pellets (1-5 cm long) and placed on concrete feeding areas along the edge of the pen water from which the crocodiles normally feed (see Appendix 4). Usually during feeding, some crocodiles eat outside of water on the feeding area while others drag food into the water to eat.

3.2.4.3 Observation on water stability

A physical observation on the stability of feed in water and the acceptance of the R-MHCF Fine by the crocodiles was done during feeding of the general stock.

3.3 Digestibility experiment

A digestibility experiment was done for both MHCF 2012/2 and R-MHCF Fine diets to compare nutrient availability for digestion, absorption, and utilization.

3.3.1 Experimental animals

Ten crocodiles were obtained from wild eggs incubated and raised on MHCF. Selected crocodiles were of the same age group (2.2-2.4 years old) and of similar weight (1.2-1.9 kg).

They were picked out of the water in their respective pens by the dorsal part of their neck and their jaws were tied using an elastic rubber band to prevent them from biting the handlers. They were then placed into a 12 L empty bucket and weighed using the Brecknell Samson electronic digital scale (see Figure 3.6 below). Catching and weighing of each crocodile lasted 2-3 minutes before they were transferred to the controlled Bondor panel insulated shed, where they were placed individually into tubs (LWH= 1.55 x 0.53 x 0.13 m). Each crocodile was identified by its tag number clipped to its webbed feet.

3.3.2 Housing conditions

The experiment was conducted in an insulated room of a Bondor facility at MHCF, with controlled air temperature (31-32.4^{o}C) and humidity (98-99%) according to recommendations by Read (2000). They were allowed 9 hours of light (0700 to 1600hrs) and 15 hours (1600 to 0700hrs) of darkness. The light period was scheduled according to the workers' allocated hours of work (normally the daylight duration in PNG is from 0600hrs to 1800hrs).

Five trestle frames consisting of three horizontal and vertical layers were set up inside the Bondor shed. Ten tubs that were used to house the crocodiles were placed on the trestle frames; five on the top and another five just beneath those on top in a complete randomised design (see Figures 3.7 and 3.8). The tubs were placed in a tilted position so that the lower end of the tubs was filled with warm water (31^{o}C) to a depth of 2-4 cm prior to the introduction of the crocodiles. Water temperature was measured using a hand held digital thermometer with a flexible thermoprobe attachment. The tubs were covered with a polyethene mesh tray to prevent crocodiles from escaping.

The tubs housing the crocodiles were labelled from 1 to 10. Those on the top were labelled from 1-5 and those beneath were labelled from 6 to 10 from left to right direction (Figure 3.7 & 3.8). Crocodiles that were placed in tubs labelled 1, 3, 5, 7 and 9 were fed MHCF 2012/2 Fine while those in tubs 2, 4, 6, 8 and 10 were fed R-MHCF Fine.

A certified mercury thermometer and hygrometer was set up inside the room to monitor air temperature and humidity. The air temperature and humidity as well as the water temperature were measured thrice daily at 0700, 1100, and 1600 throughout the experimental period to ensure consistency.

Figure 3.6 Weighing of crocodile

Figures 3.7 & 3.8 Experimental setup

3.3.3 Feeding routine

Feeding commenced at 1300 daily and lasted for about 20 to 30 minutes with approximately 1-2 minutes spent on each crocodile. The time spent handling each animal was kept to a minimum purposely to reduce stress associated with handling. The first 7 days were regarded as the adaptation period where the animals were accustomed to the feeding and handling practices. During this period, animals were also gavage-fed and their pens were washed and cleaned as would be done during the actual days when faeces and digesta were collected. After the adaptation period, the next 5 days were dedicated to faecal collection, followed by digesta collection.

Crocodiles were allowed to remain in water from 0700 to 1300. Just before feeding, water from the tubs was drained and crocodiles were captured by the dorsal part of the neck and held outside of the tub for gavage feeding. After being gavage-fed, they were then placed back into their respective tubs. Following feeding, tubs were washed clean and drained. Crocodiles were allowed to remain without water from 1400 to 0700 the following morning. Faecal collection was done on the following morning at 0700. Tubs were then washed, disinfected, and refilled

with warm water. Crocodiles remained in water for the next 6 hours before feeding commenced again.

Before the adaptation period, both diets (MHCF 20/12 Fine and R-MHCF Fine) were processed and stored in the container refrigerator overnight. On the following morning, they were manually broken into small pieces and sieved through 5mm holes from a polythene mesh tray to reduce the size of the feed particles (see Appendix 3, step 1) to ease their movement through the feeding tube. The feed was then weighed according to 2% LBW per animal and was bagged separately into zip-locked bags in adequate quantities for the duration of the experiment. These were stored at 6^{o}C until required (see Appendix 3, step 1).

Prior to gavage-feeding each day, the feed was mixed with 20-40ml water to form a slurry. The slurry was placed into a 60 ml feeding syringe to which was attached a PVC tube (300 mm long x 0.5 mm wide). During gavage-feeding, crocodiles were held by the dorsal part of their neck while the tail was held between the handler's legs. An iron rod (1.8 cm wide and 23 cm long) containing a 6 mm hole in the centre was placed between the upper and lower jaw of the animal during feeding (to prevent crocodiles from biting the handlers and the feeding tube). The PVC tube was inserted through the hole in the iron rod through to the back of the crocodile's palatal valve and inserted into the oesophagus. Once the tube was in place, the slurry feed was gently pushed through the tube by the syringe plunger and ejected into the oesophagus (see Appendix 3, step 2). After gavage-feeding, the rod was removed from the crocodile's mouth and the crocodile released back into its tub. The crocodiles' tubs were then washed down with warm water (31^{o}C), drained, and left to dry until 0700 the next day. This procedure was repeated every day the crocodiles were on the experiment.

3.3.4 Faecal collection

Faecal collection was done over a 5-day period after the adaptation period. The crocodiles excreted faeces and uric acid together in a slurry. Every morning, these slurry faeces were scraped off the tub using a tablespoon and placed into zip lock plastic bags (Refer to appendix 3, step 3). The zip plastic bags were labelled for each crocodile per diet group. The same bag was used to store each animal's faeces over the duration of the faecal collection period. Faeces were stored in a freezer (-20^{o}C) until they were required for oven drying.

Even though 5 days were allocated for faecal collection, on some days, the crocodiles did not excrete at all and on other days, the crocodiles crawled over their faeces and spread it all over the tub making it hard to collect the faeces, hence collections on these days were skipped.

3.3.5 Digesta collection

Digesta collection was done a day after the faecal collection period ended. At the last day of faecal collection, the crocodiles were fed in the afternoon as usual, but faeces were not collected the following day. Instead, the tubs were washed thoroughly and disinfected with super clean disinfectant and refilled with warm water.

Two crocodiles were killed per day for digesta collection. Those that were not allocated to be killed continued to be gavage-fed normally at 1300 daily. On the day when crocodiles were killed, they were tube fed one-third of their usual ration at two hour intervals (07:45, 09:45 and 11:45) to ensure the digestive tract was full. An hour after the last feed, the crocodiles were humanely stunned and killed according to the stunning and killing technique described by Huchzermeyer (2003). Animals were humanely stunned using an electric battery-powered stunner. The stunner was placed at the dorsal part of the animal's neck and switched on for less than 10 seconds to make the animal unconscious. Once unconscious, the spinal cord was severed using a knife and the animal was held upside down to bleed for 1-2 minutes. After bleeding, the carcasses were washed down with water to remove any blood and cut open longitudinally along the ventral part from the root of the jaw down to the cloaca (see Appendix 3, step 4).

The crocodile's internal organs were pulled out of the abdominal cavity and the digestive tract was separated from the other organs. Since there is lack of information on the digestive tract of the crocodile with very few recognisable landmarks, it was difficult to differentiate between the small and large intestines. It was also difficult to distinguish between duodenum, jejunum, and ileum and to locate which part of the tract each of them starts or ends. Hence, the intestine was divided into three equal lengths measured from the end of the duodenum loop up to the part before the cloaca (referred to as the large intestine) that was larger in diameter than the small intestine. These part were separated from each other using paper clips, and cut off from each other for collection of digesta (refer to Appendix 3, step 5). Ileal digesta was collected from the segment before the large intestine (as shown in Figure 3.9).

During digesta collection, the ileum was cut from the rest of the digestive tract and one end of it was placed just inside an open zip lock plastic bag while the other end was held up and a 10 ml

syringe containing water was inserted. The water in the syringe was flushed through the ileum to push out the digesta into the zip lock bag (refer to Appendix 3, step 5). The digesta sample was labelled and stored in a freezer (-20^{o}C) until required for oven drying. This was repeated for other sections of the digestive tract

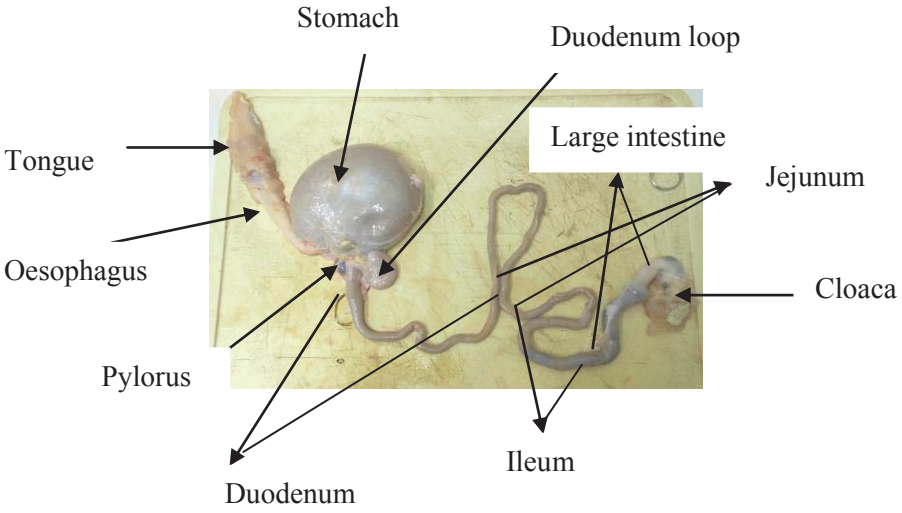


Figure 3.9 Lateral view of the digestive tract for *C. porosus*

3.3.6 Oven drying

Different muffin trays were used in holding feed samples, faecal samples and digeta samples for oven drying of the animals' faeces and digesta samples. Each muffin tray was respectively labelled with individual animal's ID number, faecal sample and names of each selected digestive tract parts from which digesta was collected as well as teh name of each feed samples. The faecal and digesta samples were placed into the muffin tray according to their respective labels and were oven dried at 60^{o}C for 24 hours. After which, the oven was switched off and allowed to cool to room temperature. The trays were removed from the oven and the dried samples were removed from the trays using a tablespoon. Samples were placed into small labelled zip lock plastic bags, sealed and stored in freezer for 2 weeks before being couriered to New Zealand to be analysed at the Nutritional Laboratory of the Institute of Food, Nutrition and Human Health at Massey University.

3.4 Dry matter and moisture content determination of feed made on-farm

A sample of approximately 100g of each feed (MHCF 2012/2 Fine and R-MHCF Fine) was weighed using the digital kitchen scale and the initial weight was recorded. The feed was then subjected to oven drying at 60^{o}C for 24 hours and weighed again for the final weight. The final dry weight was divided by the initial dry weight and multiplied by 100 to obtain the percentage

of DM. This was subtracted from 100 to get the percentage moisture content of the feed. Since the samples were dried using the oven owned by a different organisation, unfortunately there was no control over when to check the sample to ensure it had reached a constant weight before removing it from the oven. I was just given 24 hours to revisit the site and remove my samples. As a result, I did not know whether the samples had reached a constant weight before being removed from the oven or; the samples may have reached a constant weight before the 24th hour is up and may be over dried.

3.5 Laboratory analysis of nutrients

The feed samples were analysed for acid insoluble ash (AIA), DM, OM, ash, fat, crude fibre (CF), neutral detergent fibre (NDF), acid detergent fibre (ADF), total dietary fibre (TDF), gross energy (GE), N, and AA. The faecal samples were analysed for AIA, DM, ash, OM, N, and GE while digesta samples from the ileal were analysed for AIA, DM, ash, OM, N and AA.

3.5.1 Acid in soluble ash

The AIA in each sample was determine using the acid reflux, ash method (McCarthy et al., 1974).

3.5.2 Dry matter

The DM was determined using the AOAC (2005), Official Method of Analysis 930.15, 925.10. Briefly, the feed sample was put into a convection oven at 105°C. Moisture in the sample was lost by volatilisation caused by heat. The amount of material left after the removal of the moisture was defined as the dry matter.

3.5.3 Gross energy

GE was determined by bomb calorimetry according to Parr (1978). A sample of known weight was totally combusted in an oxygen rich and insulated system of known capacity. The heat that was released during oxidative combustion was determined by measuring the rise in temperature of the insulated system. The heat that was released is proportional to the calorific value of the sample.

3.5.4 Crude protein (CP)

Nitrogen was determined by Leco, total combustion method using AOAC 968.06 Dumas method and TruSpec CN to determine the nitrogen content of the feed. A sample was weighed into tin foil and loaded into the furnace where it was combusted in a stream of oxygen. The products of combustion were then passed through a secondary furnace for further oxidation and particulate removal. The moisture free gases were then swept through a heated copper catalyst under helium to remove oxygen and change NO_x to N_2, and the nitrogen content was determined by a thermal conductivity cell. The crude protein content of the sample was then obtained by multiplying total nitrogen content with 6.25.

3.5.5 Amino acid

AA were analysed using the AOAC methods 994.12 & 985.28 (AOAC, 2005). The sample was exposed to acid hydrolysis to release the free amino acids that were then separated chromatographically and quantified.

3.5.6 Crude fat

CF was determined by the Soxtec extraction AOAC 991.36 method (AOAC, 2005). In this method, the sample is repeatedly washed with petroleum-ether by refluxing in a Soxtec apparatus to dissolve the fat. The fat, oils, pigments, and other soluble substances (‘crude fat’) were then collected in the distillation flask. The increase in weight of the flask represents the dissolved fat.

3.5.7 Crude Fiber

CF was determined by gravimetric AOAC 978.10 method (AOAC, 2005). The sample was digested with 1.25% sulphuric acid and 1.25% sodium hydroxide solutions. After digestion, the remaining dried residue was lost on ignition. The amount of loss was proportion to CF. NDF, ADF and lignin were determined using methods by Robert and Van Soest (1981) and the Tecator Fibretec System (AOAC 2002.04 method) (see for example AOAC, 2005).

3.5.8 Total Dietary Fibre

TDF was determined using enzymatic gravimetric method AOAC 991.436. Duplicate samples of dried and defatted (if fat content is > 10%) material were cooked at ~ 100°C with heat stable α-amylase to gelatinisation, hydrolysis and depolymerise starch; then incubated at 60°C with protease (to solubilise and depolymerise proteins) and amyloglucosidase (to hydrolyse starch fragments to glucose); and then treated with four volumes of ethanol to precipitate soluble fibre and remove depolymerised protein and glucose (from starch). The residue is filtered; washed with 78% ethanol, 95% ethanol, and acetone; dried; and weighed. One duplicate is analysed for protein and the other is incubated at 525°C to determine ash. The TDF is the weight of the filtered and dried residue less the weight of the protein and ash.

3.5.9 Ash

Ash was determined by AOAC 942.05 method (AOAC, 2005). The sample was heated to 550^0C for 16 hours to remove all organic material. The material remaining after volatilisation at this temperature is regarded as ash.

3.5.10 Organic matter

Organic matter was determined from the difference between dry matter and inorganic matter (ash). DM was determined as described in section 3.5.2 and ash was determined described in section 3.5.9. The difference between the two is the organic matter.

3.5.11 Other analyses

Calcium/phosphorus was determined using preparation AOAC 968.08D (AOAC, 2005), followed by colorimetric analysis. Starch was determined using AOAC 996.11 method that utilises α-amylase (AOAC, 2005).

3.6 Digestibility

The digestibility coefficient or the quantity of ingested nutrients retained was determined by the analysed value of AIA concentration in the faeces and feed (Atkinson et al., 1984; Goddard & McLean, 2001) in proportion to the amount of nutrient in the feed and faeces using the standard equation:

$$\text{Digestibility coefficients} = 1 - \frac{(\%\ \text{AIA in food})\ (\%\ \text{nutrient in faeces})}{(\%\ \text{AIA in faeces})\ (\%\ \text{nutrient in feed})}$$

3.7 Statistical analysis

Data for DMR and cost per unit of DMR were statistically analysed as a factorial design using Proc GLM in SAS version 9.4. Differences were deemed significant when $P < 0.05$.

The model used is described by the following equation:

$$Y_{ijkl} = \mu + A_i + B_j + C_k + e_{ijkl}$$

Where $Y_{ijkl} = k^{th}$ observation in the i^{th} treatment group A and j^{th} treatment group B and k^{th} treatment group C

μ = a general mean

A_i = fixed effect of the i^{th} treatment group (calcium sources)

B_j = fixed effect of the j^{th} treatment group (alginate sources)

C_k = fixed effect of the k^{th} treatment group (alginate level)

e_{ijkl} = random residual error

Data on digestibility of the two diets were analysed with a linear model using Proc GLM in SAS version 9.4. Differences were deemed significant when $P < 0.05$.

The model used is described by the following equation:

$$Y_{ijk} = \mu + D_i + e_{ij}$$

Where $Y_{ij} = j^{th}$ observation in the i^{th} treatment D

μ = a general mean

D_i = fixed effect of the i^{th} treatment group (diet)

e_{ij} = random residual error

Chapter 4 Results

The research hypothesis developed for the investigation was to establish if sodium alginate could be used to improve the integrity of the feed while not affecting nutrient availability for digestion, absorption, and utilisation in *C.porosus*.

4.1 Sensory evaluation of textural properties for diets made in laboratory

"Pellet texture determines crocodile's acceptance of pelleted feed" (Peucker & Jack, 2006, p.22). For example, when wheat gluten was included as a binder in the extrudate feed for hatchling *C. porosus* at MHCF, the diet was sticky like plasticine. When given to the crocodiles, they ate for a while, but eventually lost interest. Hence, textural properties of the feed were identified as well in this study.

The textural traits of the diets were evaluated based on sensory evaluation by sight and feel (by hand) using the textural sensory evaluation by Szczesniak (2002) and were scored according to scores allocated for each textural characteristics described in Table 3.3. Each diet was assessed on textural traits including cohesiveness, viscosity, adhesiveness, and wetness. Scores were given for each trait and then were combined to generally describe the characteristics of each diet. For instance, the diet with 1.7% of Protanal XP3639 alginate and calcium carbonate ($CaCO_3$) was given a score of 8 for cohesiveness, 10 for viscosity, 9 for adhesiveness, and 9 for wetness (see Table 4.1). When these scores were put together, the feed was described as, "forming a solid and smooth continuous sausage without breakage, and was moderately wet" (Table 4.2). Tables 4.1 and 4.2 show the overall scores and textural traits respectively for each diet made in the laboratory.

Moisture content (MC) also plays an important role in the quality of semi-moist pellets. The optimum range should be between 35-40%. At higher MC, the feed may be susceptible to micro-organism spoilage (unless preservative measures are used); its oxygen sensitive nutrients such as ascorbic acid may deteriorate (unless frozen); and it may become too sticky (Lovell, 1980) to form good shaped pellets and will "cake up" during handling and storage. As a result, it makes the feed too sticky to be manually broken down into required size pellets (Royes & Chapman, 2003). On the other hand, if the MC is low, it would reduce the binding properties of the feed (Lovell, 1980). Table 4.3 shows the percentage moisture content for diets made in the laboratory and on-farm.

Table 4.1 Scores given for each laboratory diets

		Textural traits	Cohesiveness	Viscosity	Adhesiveness
			Crumbly → Solid	Thick viscous → Thin viscous	Pasty-like → Gooey-like
		Textural Evaluation Score Range	1 → 10	1 → 10	1 → 10
Laboratory Diets	Nil Alginate	With Millrun	3	NA	5
		Without Millrun	6	NA	6
	Protanal XP3639 Alginate	1.7% with $CaCl_2$	3	10	5
		3.3% with $CaCl_2$	6	10	4
		1.7% with $CaCO_3$	8	10	9
		3.3% with $CaCO_3$	9	10	9
	Manucol DM Alginate	1.7% with $CaCl_2$	3	10	4
		3.3% with $CaCl_2$	3	10	6
		1.7% with $CaCO_3$	8	10	9
		3.3% with $CaCO_3$	8	10	9
	Kimica Alginate	1.7% with $CaCl_2$	3	10	5
		3.3% with $CaCl_2$	6	10	5
		1.7% with $CaCO_3$	8	10	8
		3.3% with $CaCO_3$	9	10	9

The score ranges from 1 to 10 for all given textural traits above

1 represent textural trait that is not likeable or not recommended. That is: 1 for Cohesiveness = Loose and rough and breaks instantly into

1 for Viscosity = Feed covered with jelly-like material (thick viscous); 1 for Cohesiveness = Soft, loose and sticky feed (pasty-like); 1 for

10 represent textural trait that is well liked or is a recommended traits. That is: 10 for Cohesiveness = Smooth, tough and form smooth con

breakage (solid/firm); 10 for Viscosity = No sign of jelly-like material in feed (thin viscous); 10 for Adhesiveness = Soft, tight and sticky

10 for Wetness = Moderately wet (moist).

Scores that fall between 1 and 5 indicates that the textural trait of the feed closely resembles that of 1

Scores that fall between 6 and 10 shows that the textural trait of the feed closely resembles that of 10

Table 4.2 Textural characteristics of laboratory diets based on the scores given in Table 4.1 in respect to their descrip

	Diet Type		Extrudate Sausage-Pellet Te
	Alginate Products	Calcium Sources	
LABORATORY DIET	Control diet 1	No alginate-calcium reaction. Millrun included	Loose, rough and crumbly extrudate which is mode
	Control diet 2	No alginate-calcium reaction. Millrun excluded	Loose, rough and brittle extrudate that is damp wet
	1.7% Protanal XP3639	With $CaCl_2$	Loose, rough, crumbly extrudate that is moderately
		With $CaCO_3$	Solid and smooth continuous sausage extrudate (wi wet
	3.3% Protanal XP3639	With $CaCl_2$	Loose, rough and crumbly extrudate that is modera
		With $CaCO_3$	Solid and smooth continuous sausage extrudate (wi wet
	1.7% Manucol DM	With $CaCl_2$	Loose, rough, crumbly extrudate that is moderately
		With $CaCO_3$	Solid and smooth continuous sausage (without brea
	3.3% Manucol DM	With $CaCl_2$	Loose, rough, crumbly extrudate that is moderately
		With $CaCO_3$	Solid and smooth continuous sausage (without brea
	1.7% Kimica	With $CaCl_2$	Loose, rough, crumbly extrudate that is damp to mo
		With $CaCO_3$	Solid and smooth continuous sausage (without brea
	3.3% Kimica	With $CaCl_2$	Loose, rough and crumbly extrudate that is damp to
		With $CaCO_3$	Solid and smooth continuous sausage (without brea

Table 4.3 Percentage moisture content (MC, %) for diets made in the laboratory and on-farm.

	Diets	Alginate inclusion level	Calcium source	MC %	% DM
Lab diet	Control 1	0% with millrun	none	47.3	52.7
	Control 2	0% without millrun	none	52.1	47.9
	Protanal XP3639 alginate	1.70%	$CaCO_3$	51.3	48.7
			$CaCl_2$	52.3	47.7
		3.30%	$CaCO_3$	50.0	50.0
			$CaCl_2$	50.2	49.8
	Manucol DM alginate	1.70%	$CaCO_3$	50.7	49.3
			$CaCl_2$	50.5	48.1
		3.30%	$CaCO_3$	50.5	49.5
			$CaCl_2$	50.5	49.5
	Kimica alginate	1.70%	$CaCO_3$	51.0	49.0
			$CaCl_2$	52.9	47.1
		3.30%	$CaCO_3$	49.9	50.1
			$CaCl_2$	51.6	48.4
On-farm diet	MHCF 2012/2 Fine	0.0%	none	54.0	46.0
	New diet without millrun	1.7% Protanal	$CaCO_3$	56.0	44.0
	New Diet with 17.7% millrun	1.4% Protanal	$CaCO_3$	53.0	47.0

From Table 4.3, it can be seen that the percentage concentration level of alginate for diets made on-farm was reduced from 1.7% to 1.4%. This was due to inclusion of millrun which increases the total weight of the feed thus reducing percentage of ingredient composition.

Control 1 diet (with millrun) had moisture content of 47.3%, was loose and rough, moderately wet, and broke instantly into very small pieces. Control 2 diet (without millrun) had 52.1% MC, was loose and rough as Control 1, but damp wet and formed short sausages that fell apart one at a time (Table 4.2). There was a big difference (4.8%) in the MC content between Control 1 and Control 2 diets. Even though Control 2 diet had high MC, its ingredient composition was given preference to be used as the starting point from which all other laboratory alginate-calcium diets were made due to its brittleness. In addition, it retained 6.2% DM compared to 4.3% DM retained (DMR) by Control 1.

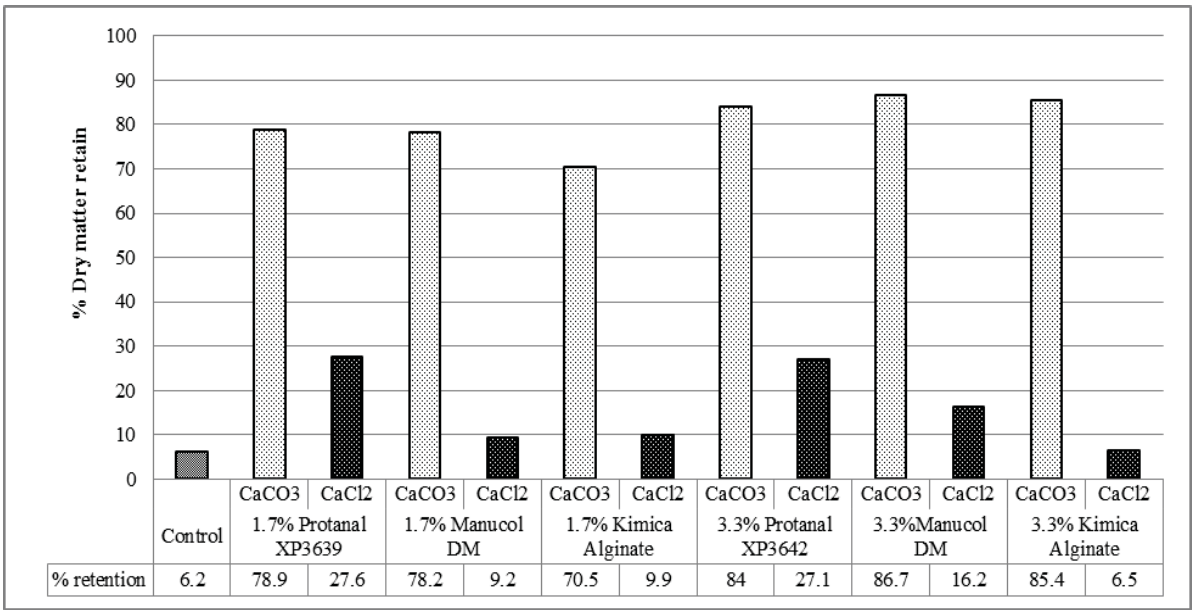
The MC value for Control 1 diet was the lowest compared to the rest of the diets made with alginate-calcium in laboratory and on-farm; while the MC value for Control 2 diet did not vary much from the rest of the diets made in laboratory. Therefore, this indicates that inclusion of millrun increases the DM content of Control 1 diet, resulting in low MC value (see Table 4.3). The texture of all alginates made with $CaCl_2$ was poor. They were soft and sticky but loose and rough, crumbly, damp to moderately wet, and did not form good continuous sausage-extrudate.

The crumbliness may be associated with a quick reaction between the $CaCl_2$ ions and the alginate ions, resulting in the formation of a premature gel within the feed particles, causing them to crumble out of the machine during processing. The texture of all alginate diets crosslinking with $CaCO_3$ were much better and formed firm soft, sticky, and smooth continuous sausages without breakage and were moderately wet (see Table 4.2).

4.2 Dry matter retention of laboratory feed and cost involved

The stability of the extrudate feed in water was evaluated based on the DMR after immersion in water. The control diet retained 6.2% of DM. All alginate products reacting with $CaCl_2$ retained less than 30% DMR, while their DMR ranged from 70.5 to 86.7% with $CaCO_3$ (see Figure 4.1).

Figure 4.1 Interaction between alginates, alginate levels and calcium sources on dry matter retention for laboratory diets



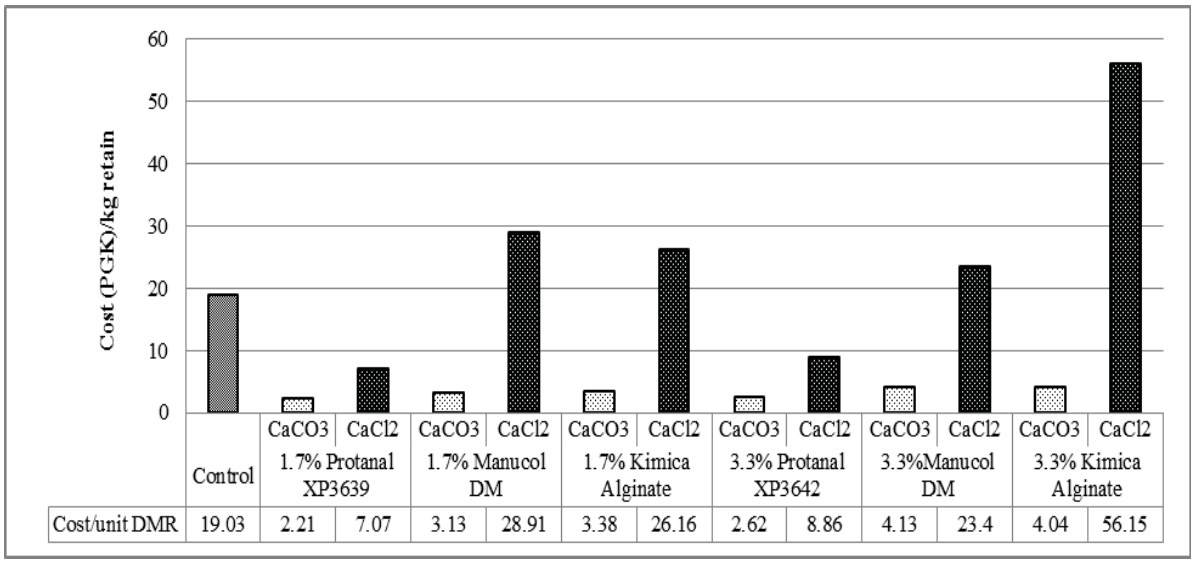
With regard to the interactions between the main effects (i.e., alginates, alginate levels, and calcium sources), Manucol DM alginate outperformed Kimica and Protanal XP3639 by retaining more DM at 3.3% inclusion level. On the other hand, at 1.7% inclusion level, Protanal XP3639 retained more DM, followed by Manucol DM and Kimica. Despite the difference in the DMR for each alginate product at both inclusion levels, these differences were not significant (See Table 4.4).

For all alginate products, the reaction with $CaCl_2$ at both inclusion levels retained less DM compared to $CaCO_3$ and the difference between these two calcium sources was significant ($P < 0.05$). In the same context with $CaCO_3$, even though more DM was retained at 3.3% level than at

1.7%, the difference was not significant (Table 4.4). It is evident that for the main effects, it did not make any difference which alginate was used, or what level it was used at, but calcium source did make a difference in determining the DMR. The inclusion of alginate cross-linked with calcium $CaCO_3$ contributed to an improvement in water stability of the extrudate feed by retaining 13 times more DM than the control diet made without alginate.

Since the DMR with $CaCO_3$ did not differ significantly within and between levels of alginate for all three alginate products, the laboratory diets were further evaluated on the cost per unit DM retained (see Figure 4.2). The costs of diets presented in this paper were solely based on the cost of the ingredients used. The total cost for all diets were calculated based on the cost per ingredients used. These were then divided by the amount of feed per diet to give the cost per kg of the feed. The cost per kg of the feed was then divided by the DMR to give total cost per kg of DM retained.

Figure 4.2 Interaction between alginates, alginate levels and calcium source on the cost per unit of dry matter retention



From Figure 4.2, it can be seen that Protanal alginate had a lower cost followed by Manucol DM, and Kimica. The cost per unit DMR did not vary much for all alginates reacting with $CaCO_3$ compared to the reaction with calcium chloride. Protanal XP3639 resulted in the lowest cost, followed by Manucol DM and Kimica. Table 4.4 shows that the cost of Protanal per unit of DMR was approximately 2 times less than Manucol DM and Kimica. The cost per unit of DMR was lower at 1.7% than at 3.3%; and the cost per unit DMR was 5-6 times less with $CaCO_3$ compared to $CaCl_2$ (Table 4.4). This indicated that $CaCO_3$ was more effective at cross-linking ingredient molecules within the feed, thus resulting in a mix with the lowest costs when it was

used as the cross-linker. Its reaction with the alginates involved a cost that is 4-8 times less than the cost of the control diet (Figure 4.2).

Since there was no big difference in the DMR and cost for all alginate products reacting with $CaCO_3$ at both inclusion levels, Protanal XP3639 at 1.7% was recommended to be used on-farm for the digestibility experiment as it cost less and can easily be imported from Australia into PNG. Prior to being used on farm, it was compared in four other diets at lower inclusion levels (i.e., 0.3%, 0.7%, 1.0% and 1.4%). The result showed that the DMR at these levels were lower than 70%, compared to 78.9% DMR at 1.7%, and the cost per unit DMR ranged from PGK3.37 to PGK7.59 while at 1.7%, it cost PGK 2.21 (see Figures 4.3 and 4.4).

Table 4.4 ANOVA values of DMR and cost per unit DMR for alginates products, alginate levels and, calcium source

			LSMeans	
		n	% DMR	Cost/kg DMR
Alginate	Protanal XP3639	4	54.265	9.27
	Manucol DM	4	47.793	26.455
	Kimica	4	43.085	32.54
	se		2.252	4.2822
% Alginate	1.70%	6	45.775	20.84
	3.30%	6	50.987	24.67
	se		1.839	3.496
Ca source	$CaCl_2$	6	16.082^a	39.713^b
	$CaCO_3$	6	80.68^b	5.797^a
	se		1.839	3.496
P-value	Alginate		0.139	0.112
	% Alginate		0.183	0.52
	Ca source		0.002	0.021

The superscript shows significant difference between values within a row

Figure 4.3 Percentage of dry matter retained (DMR) by Protanal XP3639 at lower inclusion levels

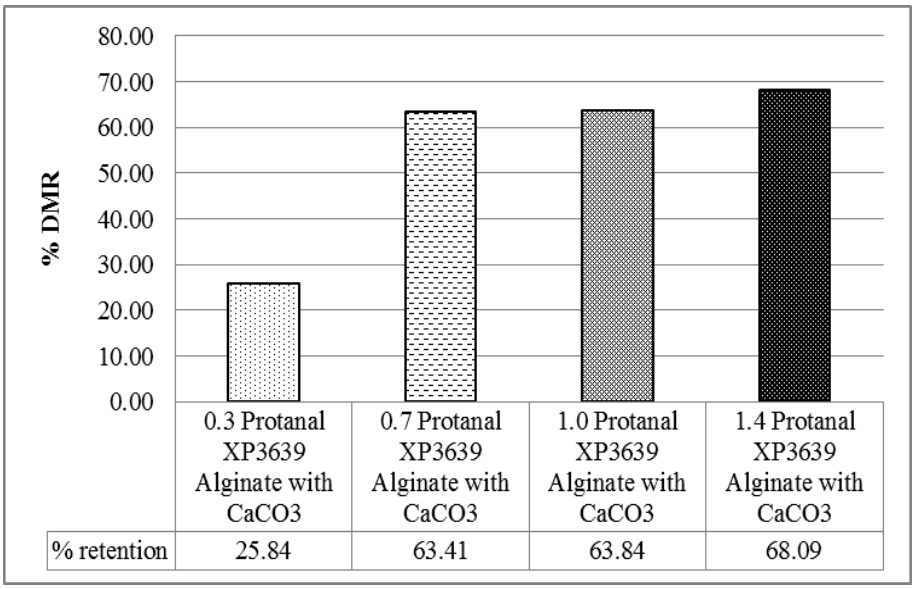
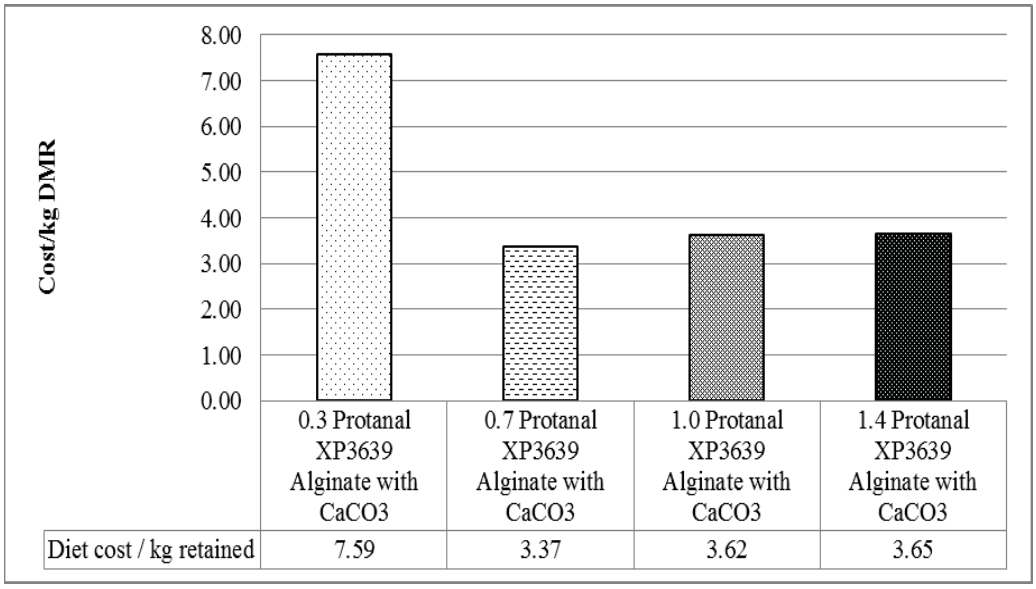


Figure 4.4 Cost per unit of dry matter retention (DMR) by Protanal XP3639 at lower inclusion levels



4.3 On-farm feed sensory and textural evaluation

In the laboratory, Protanal alginate XP3639 at 1.7% inclusion level formed a smooth continuous sausage without breaking and was moderately wet. Batch size was scaled-up for use on-farm to produce feed for the digestibility experiment, and for observation of the feed's stability in water during feeding of the general stock. However, the feed produced from this formulation was too wet (56% MC) and came out of the machine like a mud paste without forming any good sausage-shaped extrudate (see row 4 in Table 4.5 and Appendix 5, Figure 2). The difference between this

outcome and that of the diet made in the laboratory may be associated with differences in the characteristics of the ingredients available in New Zealand and diets made in the laboratory, and those available and made on-farm in Papua New Guinea. For instance, MC of the diet made on farm was approximately 4 points higher than the laboratory diets. Another factor that may have contributed to the problem was the relative mixing efficiencies of the on-farm processing machine compared to the Kenwood mixer used in the lab. Industrial equipment imparts significantly more shear than laboratory mixers.

In laboratory, there was good control over how ingredients were prepared and stored prior to mixing and processing; however, during the laboratory extrusion the mixed ingredients could not be fed easily into the extrusion barrel, hence, were made into small balls and manually pushed into the extrusion barrel from the feed holder using a polyethene rod. The mixed ingredients were pressed hard down towards the extrusion barrel until the space in the extrusion barrel and the feed holder was fully compacted with the mixed ingredients. The machine was then switched on and the extrusion screw started feeding the mixed ingredients through the die plates, where they were extruded. While the extrusion screw was expelling the feed out of the die plates, the mixed ingredients were constantly fed to the screw manually by being pressed hard down the feed holder into the extrusion barrel. This was to ensure a continuous supply of adequate compact feed being fed to the extrusion screw, thus resulting in the production of good continuous sausage-shaped extrudate of firm quality.

On farm, there was little control over how the ingredients were prepared and stored prior to feed processing. The chickens were normally obtained fresh from the poultry processing plant or sometimes obtained frozen. The fresh ones were eviscerated and washed while frozen ones were placed in a tub of water to defrost before crushing into mince. The defrosted chicken parts were normally taken straight from the water tub and placed into the crusher while fresh ones were washed straight before being placed into the crusher. Due to how the chicken carcasses/meat pieces were prepared, the mince that was produced may have contained more water than the mince used for preparation of diets in the lab. Also, during preparation of the pulped eggs and blood (mixture of liquid and clots), the prepared pulped eggs and blood were normally placed on the floor ready for processing. The floor is normally being hosed down with water for washing down any meat or blood debris, chicken feathers, and other by-products into the drainage system. During wash down of the feed preparation area, water sometimes splashed onto the prepared blood or pulped eggs, thus increasing the MC of the diet on farm, therefore, impacting on the overall quality of the feed.

Due to the poor outcome of the initial diet made on-farm, a new formulation was made with inclusion of 30kg of millrun to improve the feed's texture. This formed a soft, firm, smooth continuous sausage without breakage, which was damp wet and caked-up. The textural characteristic of the resulting feed from this formulation wase similar to MHCF 2012/2 Fine. Another diet was made with 38kg millrun to improve the textural increase DM of the feed and improve its textural property. This diet was moderately wet (53% MC) and felt a bit better in terms of cohesiveness and dryness. The latter was used in the digestibility study.

The inclusion of alginate was independent of feed compaction on-farm. The textural quality of the extrudate feed on farm depended on the characteristics of the ingredient composition as well as how the machine functioned in processing the feed. Feed texture is believed to be affected by moisture level as well as the ingredient composition of the diet. There is no standard work procedure aimed at producing a diet with a recommended level of moisture during processing prior to refrigeration or before feeding the animals. As a result, there is no control over how the ingredients have been prepared, stored, and used. More work need to be done in this area to identify the contributing factors, and to put in place a standard work protocol for maintaining a quality product. Another factor could be that the low inclusion level of sodium alginate may have resulted in insufficient ions available for reacting with $CaCO_3$ to form a strong 3-dimensional structure that could hold feed ingredients together.

Table 4.5 Textural characteristics of feed made on-farm

	Diet Type		Extrudate Sausage-Pellet textural Trait
	Alginate Products	Calcium sources	
ON-FARM DIET	Control (MHCF 2012/2 Fine)	No alginate-calcium (with 30kg millrun)	Form soft firm smooth continuous sausage without breakage. These sausages were damp to moderately wet. Some were agglomerated, others not.
	Protanal at 1.7%	$CaCO_3$ (without millrun)	Did not form into sausages, but produced a wet, thick sticky paste
	Protanal at 1.4%	$CaCO_3$ (with 30kg millrun)	Form soft firm smooth continuous sausage without breakage. These sausages were damp wet and stuck together.
	Protanal at 1.4%	$CaCO_3$ (with 38kg millrun)	Form soft firm smooth continuous sausage without breakage. These sausages were damp to moderate wet and stuck together.

4.4 On-farm observation during feeding

The observation of the diets when they came in contact with water was done during feeding of the general stock. The diets were either fed 2-3hours after processing or were left in the container

refrigerator overnight and fed the following afternoon. When both diets were fed fresh, the MHCF 2012/2 Fine, on certain instances, did not stick together when broken manually into shorter length pellets, making the feed easy for ingestion and swallowing. In other instances, the shorter length pellets agglomerated, delaying ingestion or swallowing, because the animal tended to move the feed around in their mouths until some feed fell off, before the rest was swallowed. The feed that was normally easily broken into loose pellets were observed in diets that were moderately dry and which felt less wet. Those that were sticking together were seen in damp wet feed, indicating the effect of variation in moisture content on textural quality of the feed. The R-MHCF Fine was sticky when fed fresh and most of the shorter length pellets seemed to agglomerate together, forming small mass of feed. This indicated that there was a lack of three-dimensional bond in binding the feed molecules together. Since there was more water content in diets made on-farm, more alginate needs to be included in order to bind the water molecules to prevent stickiness in the diet.

When water was splashed onto the feed during feeding, both diets that were in mass form did not disintegrate easily, except those shorter length sausage-pellets from MHCF 2012/2 Fine that were disintegrating. When the feed was taken into water, the feed particles of MHCF 2012/2 Fine was seen falling-off from the sausage-pellet, shortly after being dragged into water whereas it took a while for feed particles to leach out from the R-MHCF Fine.

Diets that were left in the refrigerator (3^{o}C) overnight and fed the following afternoon were drier than when fed fresh. This is probably a result of drying that occurs when water evaporated from the surface because the humidity of the air in the chiller is lower than at the surface of the product and so water moves from a very humid area to a drier area. The MHCF 2012/2 Fine was a bit harder than the R-MHCF Fine. The hardness or dryness of the feed makes it difficult for the animals to swallow MHCF 2012/2 Fine on the feeding area out of the water. However, when taken into water, the feed became wet and the observation on water stability was similar to that as observed when fed fresh: the feed tended to be easily swallowed. The R-MHCF Fine was moderately dry from the surface, but was softer than the MHCF 2012/2 Fine. It was moist from within and easy to swallow. A few tended to “cake up” and stick together, but most were easily separated manually into shorter length pellets and the integrity of the feed was maintained during feeding in water or when water splashed onto it during feeding.

4.5 Digestibility

When the R-MHCF Fine and MHCF 2012/2 Fine were analysed for their nutrient composition, the percentage content of DM, OM, AIA, fat, NDF, ADF, TDF, GE, N and AA were higher in MHCF 2012/2 Fine than R-MHCF Fine, except for ash (Table 4.7). Even though the R-MHCF Fine contained more carbohydrates (17.7% millrun) than the MHCF 2012/2 Fine (15% millrun), its NDF value was lower (17.9%) compared to MHCF 2012/2 Fine (22.9%). This may be due to errors occurring during sub-sampling of feed samples, oven drying, or due to errors associated with analytical methods used in determining NDF. However, TDF analysis shows more fibre in the R-MHCF (18.6%) than the MHCF 2012/2 (15.9%) diet which fits the expected result.

Faecal digestibility was calculated for DM, OM, N, and energy while ileal digestibility was calculated for DM, OM, AA, and N. Table 4.8 shows the ANOVA for faecal digestibility coefficient of DM, OM, N and energy, and Table 4.9 shows their ileal digestibility coefficient ANOVA values. There was a significant difference in the faecal digestibility of OM and energy (P = 0.04). Faecal digestibility of DM was also significant, but at P=0.059 while faecal digestibility of N was not significant (P=0.19). R-MHCF Fine had higher faecal digestibility coefficients for OM and energy (70 and 72%, respectively) compared to the MHCF 2012/2 Fine, with only 39 and 44%, respectively.

The ileal digestibility coefficient for DM, OM and N (Table 4.9) of the two diets were not different from each other. The ileal digestibility coefficient values for DM and OM for the MHCF 2012/2 Fine were similar to their faecal values indicating that no further digestion had occurred after ileum. However, the result for R-MHCF Fine showed higher faecal digestibility for DM and OM than their ileal digestibility values. This is consistent with having more millrun in R-MHCF Fine as millrun is high in fibre, and more fibre results in higher faecal digestibility values than ileal. Ileal AA digestibility coefficients for several AA were measured (Table 4.10) for both diets. The result showed no significant difference in the digestibility of AA between the two diets. R-MHCF Fine tended to have higher values of AA digestibility compared to the MHCF 2012/2 Fine, but the difference between the digestibility of each AA for each diet was small (range from 0.02 to 0.1), indicating that the inclusion of alginate as a binder did not affect AA digestibility.

Table 4.6 Dietary ingredient composition

Ingredients	MHCF 2012/2 Fine	R-MHCF Fine
Main ingredients	%	%
Chicken Carcasses	35	32.6
Blood	15	14
Millrun	15	17.7
Poultry offal meal	5	4.7
Pulped eggs	5	4.7
Protanal XP3639 alginate		1.5
Calcium carbonate (powdered-animal grade)		1.9
Supplements		
Millrun	3.9	3.6
Expandat	11.6	10.8
Fish meal	5.3	4.9
BEC Croc Vit/Min premix	0.7	0.6
Ascorbic Acid-Vit C	0.2	0.1
Vitamin E	0.1	0
Methionine	0.2	0.1
Muriate of K	0.1	0.1
Albac zinc bacitracin (antibiotic)	0.1	0.1
Amprolium (coccidiostat)	0.1	0.1
Soybean meal	2.9	2.7
Total weight (%)	100	100

Table 4.7 Proximate nutrient analysed values for MHCF 2012/2 and R-MHCF Fine diets (%)

	MHCF 2012/2 Fine	R-MHCF Fine
Dry Matter	94.8	94.5
Ash	7.7	10.5
OM	87.1	84.0
AIA	0.4	0.2
Fat	13.2	10.8
Crude fibre	3.9	3.5
NDF	22.9	17.2
ADF	5.3	4.0
TDF	15.9	18.6
Nitrogen	6.1	5.6
GE (kJ/g)	21.0	19.4
Amino Acids Analysed Values		
Amino acids	(as received	(as received)
Aspartic Acid	3.42	2.92
Threonine	1.63	1.44
Serine	1.95	1.57
Glutamic Acid	5.46	4.76
Proline	2.18	1.72
Glycine	2.19	1.80
Alanine	2.14	1.99
Valine	2.11	1.83
Methionine	0.88	0.83
Isoleucine	1.60	1.38
Leucine	2.92	2.59
Tyrosine	1.17	0.99
Phenylalanine	1.73	1.50
Histidine	0.92	0.90
Lysine	2.06	1.95
Arginine	2.35	1.95
Taurine	0.13	0.10

Table 4.8 Fecal DM, OM, N and Energy Digestibility Coefficients for MHCF 2012/2 and R-MHCF Fine diets

	LSMeans		Standard Error	P-value
	MHCF 2012/2 Fine	R-MHCF Fine		
Dry matter	0.350	0.664	0.101	0.059
Organic matter	0.392[a]	0.698[b]	0.091	0.044
Nitrogen	0.249	0.556	0.150	0.186
Energy Digestibility	0.444[a]	0.722[b]	0.083	0.046

[a, b] Means within a row with different superscripts were different ($P < 0.05$).

Table 4.9 Ileal DM, OM and Nitrogen Digestibility Coefficients for MHCF 2012/2 and R-MHCF Fine diets

	LSMeans		Standard Error	P-value
	MHCF 2012/2 Fine	R-MHCF Fine		
Dry matter	0.360	0.410	0.146	0.814
Organic matter	0.396	0.468	0.128	0.700
Nitrogen Digestibility	0.558	0.650	0.122	0.608

Table 4.10 Ileal AA Digestibility Coefficients for MHCF 2012/2 and R-MHCF Fine diets

	LSMeans		Standard Error	P-value
	MHCF 2012/2 Fine	R-MHCF Fine		
Aspartic Acid	0.5840	0.6740	0.1055	0.5630
Threonine	0.5320	0.6280	0.1147	0.5704
Serine	0.6300	0.6960	0.0863	0.6350
Glutamic Acid	0.6880	0.7260	0.0877	0.7672
Proline	0.6120	0.6980	0.1055	0.5803
Glycine	0.5420	0.6380	0.1289	0.6126
Alanine	0.5980	0.6460	0.1127	0.7710
Valine	0.6160	0.6940	0.1087	0.6255
Methionine	0.7640	0.8000	0.0626	0.6947
Isoleucine	0.6320	0.6980	0.1039	0.6654
Leucine	0.6720	0.7380	0.0892	0.6148
Tyrosine	0.6960	0.7660	0.0764	0.5352
Phenylalanine	0.6860	0.7420	0.0863	0.6587
Histidine	0.7280	0.7540	0.0708	0.8016
Lysine	0.7160	0.7440	0.0795	0.8096
Arginine	0.7980	0.8340	0.0534	0.6465

Chapter 5: Discussion

Since feed accounts for about 60% of the total operating cost of aquaculture farms (Falayi et al., 2004), feed processing and production should focus on reducing feed cost whilst increasing production. The use of binders in feed adds to the cost of feed, hence binders have to be properly selected and must result in less feed being wasted. In several studies done on the use of sodium alginate as a binder in feed for aquatic animals, the inclusion level ranged from 1 to 5% (Ali, 2011; Rosas et al., 2008; Strokebakken, 1985; Strokebakken & Austreng, 1986) depending on the processing condition, type of feed, size of pellets, formulation of the diet, and type of animals to be fed.

Meyers et al. (1972) reported that sodium alginate and wheat gluten each included at 50g/kg were the best in maintaining water stability, while preventing water absorption and protein leaching for *Litopenaeus vannamei* brood stock. Similarly, 24 hour stability was observed in both dry and moist pellet diets for crustaceans when agar or sodium alginate were included in the diet (Heinen, 1981). When Ruscoe et al. (2005) compared water stability of alginate with other binders in pelleted feed for crayfish, he found that 10% moisture alginate-bound crayfish pelleted feed was more water stable than the other binders. When agar, polyvinyl alcohol, and sodium alginate were included at a 2% level in diet of prawns, all three binders retained 76% of the pelleted feed after 8 hours in water compared to gelatine with 72% and cassava with 52% (Ali, 2011).

Despite having the potential of maintaining the integrity of pelleted feed in water, sodium alginate has been shown to reduce digestibility of certain nutrients (Cuzon et al., 1994). Argüello-Guevara and Molina-Poveda (2012) reported that sodium alginate prevented the release of AA when included in the diet of *Litopenaeus vannamei* brood stock. In another study, octopus showed negative growth rate and some died when fed with crabmeat bound with 10 g/kg alginate (Rosas et al., 2008). Storebakken (1985) did a digestibility trial in rainbow trout by feeding a diet containing alginate at 2, 2.5, 4, 5, 8 and 10% inclusion level of dry feed stuffs with 60% added water and found that at all inclusion levels, feed intake and apparent digestibility of protein and fat were reduced, while faecal MC increased. He suggested that the increased faecal MC indicated that the water-binding properties of the alginate influences digestibility. Similarly, Strokebakken and Austreng (1986) included 5% sodium alginate and observed that apparent digestibility of N (protein), ash, fat and calcium was reduced, while the MC of faeces increased with phosphorus digestibility less affected.

Since the inclusion of alginate reduced the digestibility of certain nutrients in several aquatic animals as mentioned above, faecal and ileal digestibility measurements were performed in this study on *C. porous*. There were no significant differences in ileal digestibility of DM, OM, N, and AA as well as faecal digestibility for DM in *C. porosus.* This result indicated that inclusion of alginate did not deleteriously affect digestion in *C. porosus*. However faecal digestibility of OM and energy differed significantly between the two diets and R-MHCF Fine had higher values of faecal digestibility for OM and DM than their ileal digestibility values, indicating fermentation of these nutrients in the large intestines.

Several researchers (Baer et al., 1997; Schulze et al., 1994; Schulze et al., 1995; Souffrant, 2001) have shown reductions of apparent ileal digestibility of nutrients associated with addition of dietary fibre and dietary DM intake in humans, pigs, and poultry. Similarly, other researchers (De Goey & Ewan, 1975; Kennelly & Aherne, 1980; King & Taverner, 1975) found that a high content of fibre caused a reduction in the digestible energy concentration in rations. Connecting to this, Den Hartog et al. (1988) found a negative apparent digestibility of OM and N relating to dietary NDF. Similarly, Sandoval et al. (1987) observed a linear decrease in the digestibility of DM, CP, CF, N-free extract, OM and ADF with increasing levels of dietary fibre.

The presence of fibre also encapsulates other nutrients thus creating an impermeable barrier between the intestinal enzymes and the cell components which hinder the digestive enzymes' access to the cell content, preventing full utilization of nutrients (Boisen & Moughan, 1996; Carré & Leclercq, 1985; Knudsen et al., 1993) resulting in reduced digestibility of nutrients and energy. The reduction in the energy digestibility of the diet may also be associated with loss of energy from conversion into methane, hydrogen, carbon dioxide and other gases during fermentation in the hind gut, thus reducing the amount of dietary energy (Christensen & Thorbek, 1987). In addition, dietary fibre is less digested, and as such has low net energy value in non-ruminants (Jørgensen et al., 1996). These studies indicate that fibre decreases digestion by increasing the rate of passage of nutrient, thus decreasing the time digestive enzymes can work on the feed.

Millrun is a by-product from the milling of wheat for flour production. It consists of bran, aleurone, germ and pollard fractions. A high millrun component in a mixed diet contains more fibre and reduces nutrient digestibility (Nortey et al., 2007). In this study, millrun component of the R-MHCF Fine was higher (18.3%) than MHCF 2012/2 Fine (15%), but the laboratory analysis showed low NDF value (17.9%) and high digestibility coefficient for the different nutrients measured for the R-MHCF Fine compared to the MHCF 2012/2 Fine (with 22.9% NDF). The likely reasons for the unexpected NDF values may be due to errors occurring during sub-sampling of feed samples, oven

drying, or the method used in determining NDF. Sub-sampling error is self-explanatory, however, large differences in nutritive composition can occur from the effect of oven drying (Burritt et al., 1988; Dzowela et al., 1995). Oven drying has been shown to increase NDF and lignin concentration, and decrease the in-vivo digestibility of nutrients (Burritt et al., 1988; Papachristou & Nastis, 1994). This is thought to result from the formation of anti-lignin and non-enzymatic browning (van Soest, 1982 as cited in Dzowela et al., 1995) and formation of insoluble polymers (Dzowela et al., 1995). For instance, feed samples oven dried at 60°C are more likely to artificially increase fibre and lignin in the detergent fibre systems of analysis by producing insoluble maillard products which are normally formed between protein and carbohydrate in the presence of moisture when samples are oven dried at 60°C (Mertens, 1992), giving inconsistent NDF values. Another likely reason for the differences in the NDF value between the diets may be due to sample variations. When the NDF value of the MHCF 2012/2 Fine from this study was compared with the initial MHCF 2012/2 Fine previously analysed in a preliminary study prior to this, the NDF value for the initial MHCF 2012/2 Fine was three points lower than the one used for this research. Despite, the TDF came out as expected, with more TDF in R-MHCF (18.6%) than MHCF 2012/2 (15.9%). TDF method uses protease and amylase enzymes to digest away interfering protein and carbohydrate and prevents them from interfering with the analysis, thus give a good indication of the fibre content of the diets. This clearly illustrate why digestibility was lower in R-MHCF than MHCF 2012/2 Fine diets,

The results from this study were compared with other findings from several researchers (see Table 5.1). The ingredient composition and analysed values for the diets in Table 5.1 are found in Annexure 1. Most of the studies on nutrient digestibility in crocodiles were based on faecal digestibility. In this study, faecal digestibility was calculated for DM, OM, N and energy while DM and OM were also measured in ileal digestibility as well as N and several AA. Since digestion and absorption of AA is complete by the terminal ileum, ileal digestibility best determines digestibility of protein (Mason & Palmer, 1973; Sauer et al., 1980; Zebrowska et al., 1983). Undigested proteins leaving the ileum are metabolised by microbial action at the large intestine, which changes the AA profile of the digesta, leading to faeces containing both exogenous and endogenous proteins which may mislead determination of AA or N faecal digestibility.

In Table 5.1, data presented by Staton et al. (1990a), Read (2000), and Beyeler (2011) only represents faecal digestibility of the respective nutrients studied and not the ileal digestibility. Faecal digestibility of energy and protein obtained by Staton et al. (1990a) and Read (2000) were similar, whereas the faecal digestibility of energy and protein observed by Beyeler (2011) was lower than values obtained by Read (2000) and Staton et al. (1990). The slight variation between

Staton et al. (1990) and Read (2002) is likely to be associated with the sampling size (number of animals used). The variation in the digestibility values for Beyeler (2011) to these two studies may be associated with pen and animal variations used as observations or replicates per study. Beyeler's study involved feeding 205 crocodiles per treatment (4 treatments) on three replicate pens. He used the number of pens as his observation, rather than the animals while Staton et al., (1990) and Read (2000) used crocodiles (4 and 3 respectively) as replicate/observations in their studies.

When faecal digestible energy of diets from this study was compared with those other three studies, the energy digestibility of R-MHCF Fine was a few points lower than the values obtained by Staton et al. (1990) and Read (2000), and few points higher than energy digestibility observed by Beyeler (2011). As mentioned previously, the variation in the findings between this study and that of Staton et al. (1990) and Read (2000) may be associated with the sampling size as well. The variation between results obtained from this study with Beyeler (2011) may be due to differences between animal and pen used as observations as stated earlier. The digestible energy for MHCF 2012/2 Fine was significantly lower than the R-MHCF Fine and this value was also low compared to results observed in the other three studies. The likely reason may be due to dietary ingredient composition as indicated when it was compared against R-MHCF Fine earlier. The variation in DM digestibility between this study and that of Beyeler (2011) is likely due to sampling variation and sampling size as aforementioned.

The faecal N digestibility for both MHCF 2012/2 and R-MHCF was lower than the other three studies. In general, the variation among these studies may be associated with differences between crocodilian species, age group, the environment in which the animals were raised in these studies, and the sampling methods used in comparison to reported studies. Since crocodiles are poikilotherms, temperature plays an important role in maintaining efficient digestion. Fluctuation in temperature during execution of any digestibility experiments may impede digestion, thus affecting the digestibility of nutrients. Like any other animal, nutritional requirements vary among different age groups of animals. This may also be the likely reason for the variation in the results observed above. With regard to faecal collection techniques, Beyeler (2001) did not clearly indicate how he collected his faecal samples. Staton et al. (1990) collected the semi dry faeces after the pen was drained or cleaned. On the other hand Read (2000) collected the faeces either through total collection method where he collected all faeces, urine, and water together, and by faecal collection method where he only collected the faeces prior to pen cleaning or within two hours of cleaning and pooled the samples together. These two methods of faecal collection may underestimate digestibility due to leaching of nutrients. In this study, after feeding, the pens were washed after

feeding and no water was added. The animals were allowed to live without water for 15-16 hours until faeces were collected free of water.

Table 5.1 Apparent digestibility coefficient for some nutrients in crocodiles obtained from several researches

	Apparent Digestibility Coefficient							
Age	Diet	Faecal DM	Faecal OM	Faecal DE	Faecal Protein (Nitrogen)	Ileal DM	Ileal OM	Ilea Nitro Digesti
2.2-2.4 years old	MHLF 2012/2 Fine	0.35±0.101	0.39±0.091[a]	0.44±0.083[a]	0.249±0.150	0.36±0.146	0.40±0.128	0.56±0.
	R-MHCF Fine	0.66±0.101	0.70±0.091[b]	0.72±0.083[b]	0.556±0.150	0.41±0.146	0.47±0.128	0.65±0.
Approx. 1year old	Diet 1			0.85±0.7	0.88± 0.6			
	Diet 2			0.85±0.7	0.88±0.6			
	Diet 3			0.84±0.7	0.86±0.6			
	Diet 4			0.85±0.7	0.88±0.6			
	Diet 5			0.84±0.7	0.87±0.6			
	Diet 6			0.84±0.7	0.86±0.6			
	Diet 7			0.84±0.7	0.88±0.6			
	Diet 8			0.85±0.7	0.86±0.6			
	Diet 9			0.83±0.7	0.86±0.6			
5-6 months old	High Crude Protein Diet	0.41 ± 0.133		0.64 ± 0.084	0.72 ± 0.059[a]			
	Medium High Crude Protein Diet	0.52 ± 0.122		0.65 ± 0.107	0.72 ± 0.070[ab]			
	Medium Low Crude Protein Diet	0.46 ± 0.057		0.57 ± 0.051	0.63 ± 0.044[bc]			
	Low Crude Protein Diet	0.50 ± 0.020		0.55 ± 0.026	0.61 ± 0.021[c]			
Approx. ±11days old	Control			0.86 ± 0.96[cd]	0.77 ± 3.33[b]			
	Fish meal			0.94 ± 0.64[a]	0.88 ± 1.71[a]			
	Meat meal			0.91 ± 0.56[ab]	0.89 ± 0.32[a]			
	SMB			0.816 ± 0.24[d]	0.837 ± 1.7[ab]			
	ISP			0.867 ± 0.78[cd]	0.875 ± 0.53[a]			

[a, b, c, d] Column with different superscript per researcher differ significantly at $P < 0.05$

Chapter 6: Summary and Conclusion

The objective of this study was to determine the optimal inclusion level of Na alginate and the preferred source of Ca ions in order to increase feed stability in water and reduce feed wastage. The secondary objectives were to optimise this formulation for use on farm and explore *in vivo* digestibility of this diet in juvenile *C. porosus*.

In summary:

- There were no differences among the different types of Na alginate used.
- There was no difference in feed binding between 1.7 and 3.3% Na alginate.
- $CaCO_3$ was much more effective than $CaCl_2$ when used as a cross-linking agent with Na alginate.
- Based on *in vitro* assessment, 1.7% Na alginate (Protanal XP3639) with 1.9% $CaCO_3$ resulted in the feed cost per unit DMR at PKG2.21 compared with PKG19.03 per unit DMR when no Na alginate was used.
- *In vivo*, there were no differences in the ileal digestibility of N, DM, OM, or any AA, as well as faecal digestibility of DM and N, for a diet with 1.4% Na alginate (Protanal XP3639) with 1.86% $CaCO_3$ compared to one without.
- Faecal *in vivo* of OM and energy was approximately twice as great for a diet 1.4% Na alginate (Protanal XP3639) with 1.9% $CaCO_3$ compared to one without.

In conclusion, this study showed that Na alginate has the potential to effectively reduce feed wastage and cost by preventing the feed from disintegrating and dissolving when in contact with water. Furthermore, Na alginate did not interfere with feed digestion in *C. porosus*. In addition, there may have been some studies done on ileal digestibility in crocodiles, which were not published; however, to our knowledge, this study is the first to report data on ileal AA digestibility in a crocodilian. The use of Na alginate holds great promise for improving profitability and reducing environmental impacts of farming crocodiles in Papua New Guinea.

Chapter 7: Recommendations and Limitations

Several problems discovered while executing this study include:

- *Differences in the efficiency of the feed processing machine in lab and on-farm*

  In processing feed for the laboratory experiment, the mixture was continuously fed to the extrusion barrel by being forced manually down the feed holder. By doing so, the mixed ingredient became compacted before reaching the extrusion barrel, and once extruded, they came out thick and firm (except for those alginate-calcium chloride diets). It is, therefore, suggested that if a further research study of a similar kind is to be done, the processing machine should be one designed to automatically feed the extrusion barrel with the mixed ingredients as is normally processed by the Thompson Frozen Meat Mincer Mixer machine on-farm, rather than manually forcing the ingredients through as was done in the laboratory.

- *Lack of freeze drier and equipments for drying of feed and faecal samples on-farm*

  The moisture content for the diets made on-farm may be misleading. The samples need to reach a constant weight before they are removed from the oven in order to determine their accurate DM or MC content. Unfortunately, a convection oven from a different organisation was used and I was not allowed to enter the premises at any time to check the weight of the sample, except after 24 hours. The samples may have reached a constant weight a few hours earlier before the 24^{th} hour or may have not yet reached a constant weight. Moreover, there was no desiccator to prevent the sample from absorbing moisture associated with air when samples were removed from the oven. Samples that were over dried were likely to form maillard products that may affect the digestibility results. It is, therefore, suggested that in a future research study the samples should be freeze-dried to prevent heat-induced changes otherwise the samples should be sent to certified laboratories for convection oven drying.

- *No standard work procedure for on-farm ingredient mixing and feed processing*

  The difference in the textural property of the laboratory diets and diets made on-farm was likely to be affected by variation in the characteristics of their ingredients' components as well as the procedures involved in preparing the ingredients and processing the feed. A

further study is, therefore, needed to identify factors affecting the textural quality of the feed, and a standard work protocol developed and adhered to by those in the industry.

- *Lack of new literatures on nutrient digestibility for C. porosus at MHCF*

  Research done on the protein requirement for hatchling *C. porous* by Read (2000) at MHCF is more than a decade old. The current feed formulation used by MHCF has continually changed with uncontrolled changes in the availability of ingredients. New research needs to be undertaken to evaluate the digestibility of nutrients for the current feed formulations for animals of different age groups in order to provide the right amount of nutrients to meet their nutritional demand for optimum production, reduce cost of production, and minimise environmental pollution caused by excessive leaching of feed.

- *Paucity of literature on crocodile's digestive tract morphology and physiology*

  Since there was a lack of information on the digestive morphology of *C. porosus*, the location of duodenum, jejunum and ileum along the digestive tract was estimated by dividing the small intestines from the end of the duodenum loop to the part where the size of the tract was larger in diameter just before the rectum. There may be inaccuracy in the result associated in sampling part of the digesta from the first part of the large intestines. If similar research is to be done in the future, the researcher must have a clear understanding of the digestive morphology in order to collect digesta samples correctly from the ileum.

- *Did not increase concentration level of sodium alginate when millrun was included*

  Increasing the millrun content of the diet does not bind the pellets together to improve texture; it only increases the DM content of the diet. Since the diets made on-farm has high moisture content, the level of sodium alginate should have been increased as millrun increases so that adequate ions are available for formation of a 3-dimensional structure network necessary for binding feed particles together. For further research or feed processing of this particular type, the amount of alginate should be increased to 5% to ensure strong bonding is established.

This study has two main limitations.

- *Limited knowledge on the nutritional requirement for C. porosus, digestive physiology and the literature on this topic is very sparse.*

  Most published articles used in this study are more than a decade old. Beyeler (2011) suggested that new studies might have been done for different crocodilian species, but had not been published or shared publicly in order to keep information on improved performance secretive for organisations involved to maintain their competitive advantage and become leaders in the farming and trading of crocodiles. It was very difficult to access new information required to develop an in-depth understanding of crocodilian nutrition, which was crucial for writing this Thesis.

- *Strict quarantine restrictions placed on feed and samples imported from Papua New Guinea.*

  Due to strict biosecurity and quarantine in New Zealand, I was not permitted to prepare the ingredients and process them outside the certified quarantine lab. As a result, although there was a machine similar to the Thompson Frozen Meat Mincer Mixer I used on-farm in Papua New Guinea on the Massey University campus; I was not permitted to use feed and supplements imported in it. A portable Kenwood mixer and a Kenwood sausage maker were the only alternative available. It was evident that the efficiency of the sausage maker was not the same as the other mincer mixer I observed from Massey Poultry and Feed Processing Unit, but I had no option. Convenient, expedient, inexpensive, and greatly informative experiments with chicks using the exact feeds used on-farm with crocodiles in Papua New Guinea could also not be performed.

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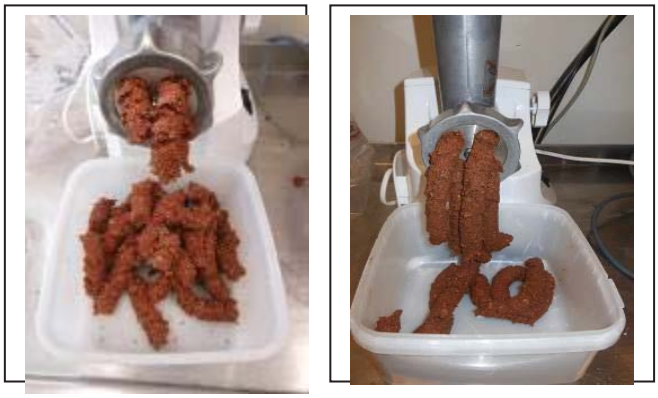
Appendices

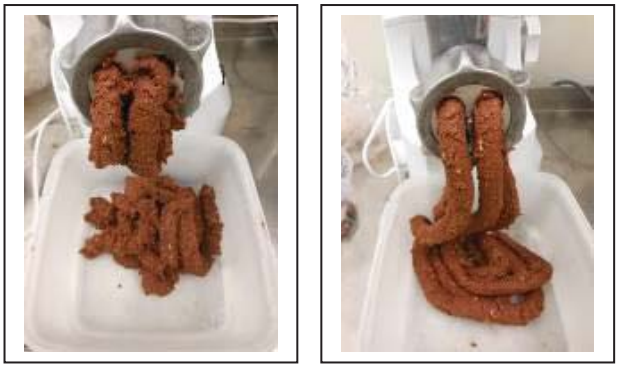
Appendix 1 Some laboratory feed

(A) Feed made with 0% alginate (left to right: 0% alginate with millrun, 0% alginate without millrun)



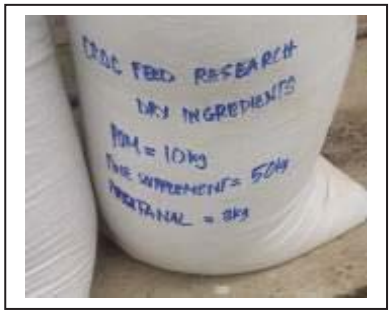
(B) Feed made of 1.7% Manucol DM alginate (from left to right: diet made with calcium chloride, diet made with calcium carbonate)

(C) Feed made of 3.3% Kimica alginate (from left to right: diet made with calcium chloride, diet made with calcium carbonate)



Appendix 2 Pictures demonstrating procedures involved in on-farm ingredient mixing and feed processing

Step 1 Dry ingredients (from left to right: ingredients bagged after weighing, and mixture of dry ingredients bagged ready for use)



Step 2 Wet ingredients (from left to right: pulped eggs, mince chicken carcass, chicken blood)

Step 3 Thomson Frozen Meat mixer mincer series 4000 (model 4200) with extrusion barrel, knife, and plates comprising of numeroud dies

Step 4 Ingredient mixing and processing (from left to rightmixture of wet ingredients and mixture of both wet and dry ingredients)


Step 5 Feed processing and storage (from left to right: sausage-extrudate expelling out of machine, feed in trays ready fed to crocodiles and feed stored in refrigerator container)



Appendix 3 Pictures demonstrating on-farm gavage-feeding, faecal collection and digesta collection

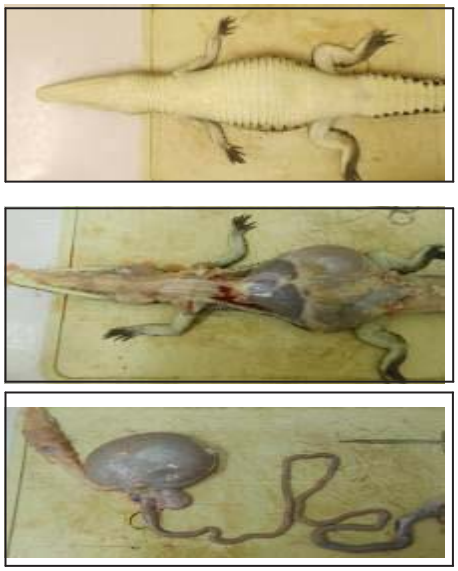
Step 1 Sieving of feed into smaller particles, weighing and packaging (from left to right & down: sausages overnight in refrigerator, manually broken into smaller size sausages, sieve through 5mm polyethene mesh tray, fine feed particles weighed and bagged in small zip lock bags)

Step 2 Slurry feed and gavage feeding (from left to right: slurry feed, feed in syringe, gavage feeding)

Step 3 Faecal Collection (from left to right: collecting of faeces, bagged faeces samples)

Step 4 Stunning and slaughtering of crocodiles (left to right: stunning and bleeding)

Step 5 Digesta Collection (down then right: ventrap part of croc, skin remove, digestive tract, sampling ieum digesta using water from a 10ml syringe)



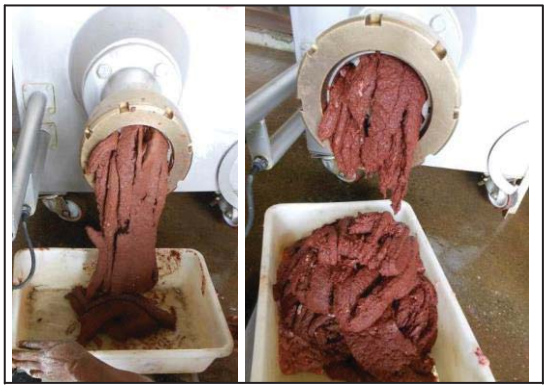
Appendix 4 On-farm procedures involved in feeding of general stock

(From left to right then down: long sausages, shorter length sausages, crocodiles feeding)

Appendix 5 Photos of feed made on farm

1. MHL fine diet

2. New diet without millrun



3. New diet with 30kg millrun

4. New diet with 38kg millrun

Annexures

Annexure 1 Ingredient composition and nutrient values of some diets previously made for the crocodiles

Table 10.1 (A) Ingredient Composition of diets for juvenile *Crocodylus niloticus* by Beyeler (2011)

Diet Composition and nutrient levels		
Ingredients	Low CP (46%) diet	High CP Diet (62%)
Fish meal	46.877	35
Chicken mince	43.996	-
Soybean oilcake meal (46%)	-	30
Full fat soya	6.361	9.778
Carcass meal	-	4.348
Maize meal	1.048	17.159
Premix	1.05	1.05
L-lysine HCl	0.069	0.754
Salmon Oil powder	0.5	0.5
DL methionine	0.099	0.288
Limestone	-	0.519
Monocalcium phosphate	-	0.604
Calculated Nutrient levels (g/kg) on a dry matter basis		
Crude protein	620	460
Crude fat	241.3	76.2
Ash	82.9	100.1
Lysine	39.5	34.3
Crude fibre	24.1	31.6
Calcium	23.2	20
Phosphorous	17.1	15
AME adult (poultry) MJ/kg	16	11.9
Methionine	15.5	12.7
Energy:Protein (kJ/g)	25.9	25.9
Ca:P	1.4	1.3

Table 10.1 (B) Analysed values of diets for juvenile *Crocodylus niloticus* by Beyeler (2011)

Analysed Nutrient Composition of the diet				
Nutrient	High CP	Medium High CP (mixture of 1/3 LCP & 2/3 HCP)	Medium Low CP (mixture of 2/3 LCP & 1/3 HCP)	Low CP
	62% CP	56.6% CP	51.3% CP	46% CP
GE (MJ/kg)	20.44	19.98	19.67	19.28
DC_{energy}[1]	0.7015	0.6415	0.5035	0.51
DE (MJ/kg)	14.34	12.82	9.9	9.83
Crude Protein	619.5	565.9	517.21	461.08
$DC_{protein}$[2]	0.7565	0.6915	0.57	0.535
Digestible Protein	468.65	381.32	294.8	246.64
Crude Fibre	61.06	54.45	61.08	46.26
Crude Fat	126.78	107.92	93.39	77.4
Calcium (Ca)	45.63	39.86	36.26	28.61
Phosphorus (P)	27.52	23.87	21.15	17.48
Ca: P	1.66	1.67	1.71	1.64
Ash	180.01	160.31	143.74	121.11
Analysed AA Composition of the diets (%)				
Amino acid	High CP	Medium High CP (mixture of 1/3 LCP & 2/3 HCP)	Medium Low CP (mixture of 2/3 LCP & 1/3 HCP)	Low CP
Aspartic Acid	56.39	52.78	46.23	42.32
Glutamic acid	89.82	84.88	76.47	70.22
Serine	24.86	23.14	20.56	19.03
Glycine	40.15	34.68	30.46	26.86
Histidine	15.61	13.01	11.35	9.8
Arginine	36.64	33.84	30.4	28.42
Threonine	25.22	22.98	19.37	16.96
Alanine	37.52	33.42	28.11	24.48
Proline	27.75	25.29	23.27	21.11
Tyrosine	20.05	18.57	15.98	14.57
Valine	30.34	27.75	23.63	20.9
Methionine	16.64	14.85	12.7	11.2
Isoleucine	26.15	24.29	20.77	18.66
Leucine	44.45	41.39	35.71	31.94
Phenylalanine	24.29	22.98	20.09	18.46
Lysine	43.47	39.35	34.09	32.98

Table 10.2 Diets for hatchling *Alligator mississippiensis* by Staton *et al* (1990a)

Nine Experimental Dietary Treatments									
	1	2	3	4	5	6	7	8	9
	Diet Composition								
Ingredient						%			
Protein mixture	72.71	83.15	57.28	82.73	64.5	45.72	71.23	45.33	55.69
Chicken liver dry matter	-	-	-	-	-	-	-	-	-
Fat mixture	0.44	2.9	2.97	8.72	8.75	8.83	14.52	14.62	17.06
Poultry oil	-	-	-	-	-	36	-	-	-
Extruded corn	18	5.4	30.6	-	18	-	5.4	30.6	10
Corn dextrin	-	-	-	-	-	2	-	-	-
Gelatin	2	2	2	2	2	2	2	2	2
Carboxymethylcellulose	2	2	2	2	2	1.5	2	2	2
Limestone	1.7	1.8	1.5	1.8	1.6	1.4	1.7	1.5	1.6
Potassium carbonate	1.4	1.4	1.4	1.4	1.4	1.2	1.4	1.4	1.4
Dicalcium phosphate	0.4	-	0.9	-	0.6	0.5	0.4	1.2	0.9
Sodium chloride	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin premix	0.5	0.5	0.5	0.5	0.5	0.2	0.5	0.5	0.5
Trace mineral premix	0.2	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.2
Chromic oxide	0.1	0.1	0.1	0.1	0.1	0.05	0.1	0.1	0.1
Selenium premix	0.05	0.05	0.05	0.05	0.05		0.05	0.05	0.05
Proximate Analysis									
Analysis (%)	1	2	3	4	5	6	7	8	9
Crude protein	61.4	68.6	50.1	67.9	54.6	41.4	59.1	40.6	47.8
Fat	4	6.4	6.4	12	12	12	17.6	17.6	20
Carbohydrate	13.1	3.9	22.2	-	13.1	26.2	3.9	22.2	13.1
Gross energy (kcal/kg)	4734	4982	4669	5283	5051	4824	5331	5085	5266
kcal/g protein	7.7	7.3	9.3	7.8	9.25	11.7	9	12.5	11

Table 10.3 Diets for hatchling *Crocodylus porosus* by Read (2000)

Composition of Experimental Diets					
Ingredients (g/kg)	Treatment Diets				
	Control	Fishmeal	Meat meal	SBM	ISP
Fishmeal	94	301	-	-	-
Poultry Offal Meal	26.6	-	-	-	-
Blood meal	146.9	-	-	-	-
Meat meal	-	-	409	-	-
SBM	-	-	-	444	-
ISP	-	-	-	-	-
Diatomaceous earth	200	166.5	58.5	23.5	230.2
Chicken heads	120	120	120	120	120
Chicken blood	115.5	115.5	115.5	115.5	115.5
Chicken liver	120	120	120	120	120
Extruded maize	132.3	132.3	132.3	132.3	132.3
Sunflower oil	22.2	22.2	22.2	22.2	22.2
Salt (sodium chloride)	0.9	0.9	0.9	0.9	0.9
Di-calcium phosphate	4	4	4	4	4
Methionine	12.2	12.2	12.2	12.2	12.2
Vitamin Premix	1.1	1.1	1.1	1.1	1.1
Coccidiostat	3.3	3.3	3.3	3.3	3.3
Chromic oxide	1	1	1	1	1
Proximate Analysis of Experimental Diets					
Nutritional Component	Treatment Diets				
	Control	Fishmeal	Meat meal	SBM	ISP
Dry matter (g/kg)	624.8 ± 1.7	654.8 ± 0.1	689.8 ± 0.3	501.0 ± 0.6	435.5 ± 1.2
Ash (g/kg DM)	351.8 ± 2.6	355.8 ± 0.4	312.4 ± 4.0	133.3 ± 10.0	370.1 ± 2.3
Gross Energy (KJ/g DM)	20.2 ± 0.4	19.1 ± 0.1	20.2 ± 0.4	26.2 ± 0.1	17.6 ± 1.0
Nitrogen (g/kg DM)	68.2 ± 0.2	64.5 ± 0.1	66.9 ± 0.3	74.2 ± 0.3	65.8 ± 0.4
Ether Extract (g/kg DM)	122.4 ± 0.7	136.3 ± 0.1	148.0 ± 1.2	101.0 ± 0.4	107.3 ± 0.4
Estimated AA profile of the experimental diets (% of dietary protein)					
Amino acid	Treatment Diets				
	Control	Fishmeal	Meat meal	SBM	ISP
Arginine	2.98	5.65	6.57	6.75	6.88
Glycine	3.43	6.35	11.37	5.43	4.68
Serine	2.58	3.82	3.79	5.16	4.91
Histidine	2.55	2.49	2.17	2.78	2.77
Isoleucine	1.55	4	2.91	4.81	4.27
Leucine	6.32	7.41	6.39	8.18	8.28
Lysine	4.94	7.01	5.5	6.6	6.28
Methionine	2.89	5.75	4.62	4.89	4.64
Cysteine	0.78	0.96	0.68	1.45	1.22
Phenylalanine	3.46	4.01	3.48	5.06	5.1
Tyrosine	1.71	2.97	1.93	2.94	3.54
Threonine	2.51	3.73	3.21	4.03	3.76
Tryptophan	0.75	1.03	0.69	1.3	1.19
Valine	1.55	5.07	4.48	5.47	5.21

Annexure 2 Certificate of analysis/Product Specification for the alginate products

(A) Protanal XP3639 Sodium Alginate

FMC BioPolymer

Know how. It works.SM

Product Specifications

Protanal® XP 3639 alginate

Product Specifications

Chemical & Physical:		Test Method
Viscosity (3%)	100 to 200 mPa·s	QCP 21702
Appearance	yellowish to white free-flowing powder almost odorless and without taste	
pH	8.0 to 11.0	QCP 10101
Particle size	minimum 75% through a US Standard Seive 150µm (Series #100)	QCP 10204
Moisture	maximum 15%	QCP 10001

Microbiology:		Test Method
Total plate count	maximum 5000 cfu/gram	QCP 40001
Mold and yeast	maximum 500 cfu/gram	QCP 40203
Salmonella	negative by test	QCP 40101
E. coli	negative by test	QCP 40302

Product Ingredients:

Calcium sulfate, sodium alginate, dextrose, sodium carbonate, tetrasodium pyrophosphate

Methods of Analysis are available on request.

02082012-1

FMC

Technical Service Centers: FMC BioPolymer

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1735 Market Street
Philadelphia, PA 19103
Phone: **1-800-526-3649**
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Peoples Republic of China
Phone: 86-21 5427-1177
Fax: 86-21 5427- 0193

Visit our website at www.fmcbiopolymer.com

Product Shelf-life / Re-evaluation Date

Two years from date of manufacture shown on product label. This applies to unopened packages stored under dry (no free moisture) and cool conditions.

Material Safety Data Sheet (MSDS) available on request.

Patents

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(B) Kimica Sodium Alginate

KIMICA corporation
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Tel: 81-3-3252-8708 Fax: 81-3-3252-8704
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Tel: 81-439-87-1131 Fax: 81-439-87-3613
E-mail: tokyo-office@kimica.jp

KIMICA

Kimica Sodium Alginate Specification

[Kimica Algin I - 3G - 120]

Items	Specification
Description	Conforms to FCC
Assay	90.8% ~ 106.0%
Loss on Drying	15.0% maximum
Viscosity (1% solution)	300 - 400 cp
pH (1% solution)	6.0 - 8.0
Particle Size 125μm pass	95% minimum
Arsenic	3 ppm maximum
Lead	5 ppm maximum
Heavy metals (as Pb)	20 ppm maximum
Coliform	Negative
Total Bacteria Count	1,000c.f.u. / g max.

(C) Manucol DM Sodium Alginate

FMC BioPolymer

Sales Specification

MANUCOL® DM - Sodium Alginate Specification No. 1040
(LIMS No.: 8132171)

DESCRIPTION
MANUCOL DM is a high viscosity, pure sodium alginate suitable for use in food products.

DETAILED REQUIREMENTS

1.	Viscosity (1% Solution)	150-300 mPa.s (cP)
2.	pH (1% solution)	5.0-7.5
3.	Loss on Drying	not greater than 13%
4.	Particle Size	at least 98% through 355 µm
		at least 80% through 250 µm
5.	(a) Appearance	cream to light brown powder
	(b) Powder Colour	not less than 48
6.	Ash (on dried solids basis)	18-27%
7.	Lead (Pb)	not greater than 5 mg/kg (ppm)
8.	Arsenic (As)	not greater than 3 mg/kg (ppm)
9.	Copper (Cu)	not greater than 10 mg/kg (ppm)
10.	Zinc (Zn)	not greater than 10 mg/kg (ppm)
11.	Mercury (Hg)	not greater than 0.5 mg/kg (ppm)
12.	Cadmium (Cd)	not greater than 0.5 mg/kg (ppm)
13.	Microbiological Limits	
	Bacteria (Total viable mesophilic aerobic count)	not greater than 5000 cfu/g
	Yeast and Mould	not greater than 300 cfu/g
	Coliform	negative by MPN
	E. coli	absent in 25 g
	Salmonella	absent in 25 g

INGREDIENT
Sodium alginate E401 CAS: 9005-38-3

REGULATORY COMPLIANCE
Complies with Purity Criteria in current EC Directives
Food Chemicals Codex
Kosher Approved
Generally recognised as safe (GRAS) in accordance with 21CFR 184.1724

QUALITY SYSTEM
MANUCOL DM is manufactured according to a Quality System registered to ISO9002

PACKAGING
MANUCOL DM is packaged in 25 kg multi-ply sacks fitted with polyethylene liner or equivalent. All packaging materials comply with relevant UK, EC and United States food contact legislation.

STORAGE
Packages should be kept sealed and stored in a cool dry place.

METHODS OF TESTING (Full details of test methods are available on request)

1. **Viscosity (1% Solution)**
Pour 450 g distilled water into a 600 ml glass beaker. Add 5.00 g product slowly while stirring the solution with an electric stirrer fitted with a propeller-type metal paddle. Adjust the weight of solution to 500 g with additional distilled water, rinsing the walls of the beaker. Stir for two hours at 800 rpm, then adjust the temperature to 20 degrees C, stirring by hand to eliminate any layering effects. Measure the viscosity immediately using an LV model of the Brookfield[1] viscometer at 60 rpm, with spindle 2, at 20 degrees C.

2. **pH (1% Solution)**
Measure the pH of a 1% solution at 20 degrees C using a pH meter.

3. **Loss on Drying**
Spread 5-10 g product evenly on a predried tared watch glass and weigh accurately. Dry in an oven at 105 ± 1 degrees C for four hours. Cool in a desiccator and re-weigh.

4. **Particle Size**
Sieve 10 g product on the specified British Standard Screens (200 mm diameter) for three minutes each screen using an Alpine[2] Air Jet Sieve. Use the finest mesh sieve first and progress to the coarsest mesh. Record the weight of product remaining on each screen and calculate the percentage which passes through each specified screen.

5. **Powder Colour**
Place powder in an optically flat Photovolt cuvette to a depth of 2 cm. Do not shake or tap. Using a green tristimulus filter, measure the powder colour on a Photovolt[3] reflectometer standardised against a white enamel standard of 75% reflectance.

6. **Ash**
Use the procedure given in the current edition of the Food Chemicals Codex.

7-12. **Lead, Arsenic, Copper, Zinc, Mercury and Cadmium**
These metals may be determined by atomic absorption techniques.

13. **Microbiological Limits**
For bacteria (TVMAC), E coli, salmonella, yeast and mould, follow the procedures as given for microbial limit tests in the current edition of the United States Pharmacopoeia. Method for coliform is available on request. For bacteria, plate out 1 ml of 1% solution and incubate for 48 hours at 30-35 degrees C. For yeast and mould plate out 1 ml of 1% solution on acidified potato dextrose agar and incubate for 5 days at 20-25 degrees C. Express results as colony forming units (c.f.u.) per gram.

SUPPLIERS OF TESTING EQUIPMENT
[1] Brookfield Engineering Laboratories, Stoughton, Massachusetts.
[2] Hosakawa Micron Ltd, Augsburg, Germany.
[3] Photovolt Corporation, Indianapolis, Indiana.