FANNY AYUMI YASUMARU

In vitro digestion of protein and phosphorus with species-specific digestive enzymes: potential for method development and application in the aquaculture of fish species

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Thesis submitted to the Oceanographic Institute of the University of São Paulo, in fulfilment of the requirements for the degree of Doctor of Sciences

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習うのは一生

Learning is a lifetime.

Japanese proverb

RESUMO

YASUMARU, F. A. **Digestão *in vitro* de proteína e fósforo utilizando enzimas digestivas espécie-específicas: potencial de desenvolvimento e aplicação para a aquicultura de espécies de peixes**. 2014. 124 f. Tese (Doutorado) – Instituto Oceanográfico, Universidade de São Paulo, São Paulo, 2014.

Esta tese teve como objetivo o desenvolvimento de método *in vitro* pH-stat com enzimas espécie-específicas, avaliando a digestão de proteína (PB) e fósforo (P) em peixes, para prever a digestibilidade *in vivo*. As espécies utilizadas como modelos experimentais foram a truta arco-íris, *Oncorhynchus mykiss*, o bijupirá, *Rachycentron canadum* e a tilápia-do-Nilo, *Oreochromis niloticus*. Extratos enzimáticos foram obtidos de estômago, cecos pilóricos (truta e bijupirá) ou intestino (tilápia) de indivíduos de diferentes tamanhos e estado alimentar. A capacidade hidrolítica das enzimas foi padronizada utilizando substratos protéicos de grau analítico por meio da determinação do grau de hidrólise protéica (DH) em ensaio pH-stat. Ingredientes práticos foram hidrolisados com extrato enzimático de (i) estômago, (ii) cecos pilóricos (truta e bijupirá)/intestino (tilápia) ou (iii) estômago seguido de cecos pilóricos/intestino (dupla hidrólise) para determinar os valores de DH. O método de determinação de DH apresenta baixo coeficiente de variação, e pode ser uma ferramenta útil no ranqueamento e no controle de qualidade de ingredientes práticos. O P solúvel liberado de amostras de nove rações comerciais para tilápia, com níveis de garantia similares (32% proteína bruta, 4-6 mm) submetidos à digestão *in vitro* com extrato de estômago, intestino ou dupla hidrólise foram avaliados. Os extratos enzimáticos foram obtidos de tilápias cultivadas em condições comerciais em tanque-rede. Liberação de P solúvel foi determinada em amostras incubadas somente em água destilada (pH 6.4 ± 0.4), em água destilada a pH 2.0, pH 8.0, e sequencialmente em pH 2.0 e 8.0. Amostras também foram incubadas nesses valores de pH e incluindo os extratos enzimáticos de estômago e intestino separada e sequencialmente (dupla hidrólise). A liberação de P solúvel após digestão do estômago foi maior do que após digestão do intestino ou após dupla hidrólise. A digestibilidade do P parece estar relacionada mais ao pH do meio do que à hidrólise enzimática. Correlações significativas ($P<0,05$) foram observadas entre P total das rações e o P solúvel liberado em água destilada, e entre P total da ração e P liberado após digestão intestinal. O método *in vitro* apresentou baixo coeficiente de variação (< 5% c.v.). Coeficientes de digestibilidade aparente (CDA) de PB e P de nove rações comerciais para tilápia foram avaliados *in vivo* utilizando cinzas insolúveis em ácido como marcador interno. O ensaio foi feito em laboratório com tilápias em fase de crescimento simulando condições comerciais de estocagem. Foram avaliados também o crescimento e correlações entre CDA *in vivo* e DH *in vitro* de PB e CDA P *in vivo* e liberação *in vitro* de P solúvel. Não houve diferença no ganho de peso entre as rações ($P>0,05$). Valores de DH não apresentaram correlação significativa ($P>0,05$) com CDA PB, mas foi possível discriminar entre os maiores e menores valores de CDA PB. A liberação *in vitro* de P solúvel em água destilada e após digestão intestinal demostraram correlação significativa ($P<0,05$) com o teor de P total da ração e com o P disponível. Mais estudos são necessários para aumentar a precisão analítica dos teores de cinzas insolúveis em ácido, bem como aprimorar os métodos para correlacionar valores de digestibilidade *in vivo* com dados de métodos *in vitro* para proteína e fósforo.

Palavras-Chave: digestão, digestibilidade, fósforo, indicador, *in vitro*, previsão, proteína, solubilidade.

ABSTRACT

YASUMARU, F. A. ***In vitro*** **digestion of protein and phosphorus with species-specific digestive enzymes: potential for method development and application in the aquaculture of fish species**. 2014. 124 f. Tese (Doutorado) – Instituto Oceanográfico, Universidade de São Paulo, São Paulo, 2014.

The aim of this study was to develop a species-specific *in vitro* pH-stat method to assess protein and phosphorus digestion in fish. The fish species used as models to assess protein digestion of feed ingredients were rainbow trout, *Oncorhynchus mykiss*, cobia, *Rachycentron canadum*, and Nile tilapia, *Oreochromis niloticus*. Crude digestive enzyme extracts were recovered from stomach and pyloric caeca or intestine of different groups of individuals. The hydrolytic capacity of the enzyme extracts was standardized on analytical grade protein substrates and measured as degree of protein hydrolysis (DH) in the pH-stat assay. Feed ingredients were assessed for DH and hydrolyzed with fish (i) stomach extract, (ii) pyloric caeca/intestine extract or (iii) stomach enzymes followed by pyloric caeca/intestine extract (two-stage). The DH determination has shown to be a precise method that may be a useful tool to rank feed ingredients, and also an accessory method in the quality control of feedstuffs. The amount of soluble phosphorus (P) released from feeds submitted to *in vitro* digestion with stomach, intestine, and stomach followed by intestine (two-stage) enzyme extracts was evaluated. Nine commercial feeds for Nile tilapia from different manufacturers with similar crude protein content (32%, label value) and pellet size (4-6 mm) were tested. Digestive enzyme extracts were obtained from growing Nile tilapia, *Oreochromis niloticus*, commercially farmed in cages. Release of soluble P (%) was determined after feed samples were incubated in distilled water (pH 6.4 ± 0.4), in distilled water at pH 2.0, in distilled water at pH 8.0, and after feed digestion with stomach and intestine extracts separately and sequentially (two-stage digestion). The amounts of enzyme extracts tested were 50, 100 and 200 µL. In general, released soluble P with stomach digestion was higher compared to intestine digestion or two-stage digestion. Solubility of P appeared to be more related to medium pH rather than enzymatic hydrolysis. Significant correlations ($P<0.05$) were observed between feed total P and soluble P released in distilled water and between soluble P released in distilled water and soluble P released in intestinal digestion. This *in vitro* method was found reproducible as variation was low (< 5% c.v.). Crude protein and phosphorus digestibility coefficients of commercial feeds simulating commercial stocking density, using AIA as internal marker, further to evaluate fish growth and the potential correlation of *in vivo* apparent digestibility data with *in vitro* protein digestion and released soluble phosphorus from commercial feeds for Nile tilapia. Growth performance between feeds were not significantly different ($P>0.05$). The *in vitro* DH did not correlate significantly ($P>0.05$) with ADC of CP, but it was possible to discriminate between the highest and lowest ADC of CP. *In vitro* release of soluble P in distilled water and after digestion with intestine extract correlated significantly ($P<0.05$) with total feed P and available P. Further studies are necessary to increase analytical precision of the AIA determination and also to improve methods to predict *in vivo* digestibility values with *in vitro* methods for protein and phosphorus.

Keywords**:** digestion, digestibility, indicator, *in vitro*, phosphorus, prediction, protein, solubility

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GENERAL INTRODUCTION

Global aquaculture production has been growing at a rate of 7.5% per year and providing approximately half of total global fisheries production, which correspond to 66.4 million metric tonnes (FAO, 2014) and for the first time overcoming beef production in 2012 (63 million metric tonnes in 2012) (FAOSTAT, 2014). In Brazil, aquaculture production has grown almost three times in the last decade, from 247 thousand metric tonnes in 2002 to 706 thousand metric tonnes in 2012 (FAO, 2014). It is a fast-growing industry to provide a growing global population with high quality protein sources with increasing medical evidence of health benefits (SUGIURA et al., 2006; TACON; METIAN, 2013). In many cases aquaculture has depended on marine capture fisheries to produce feeds; however, it is a resource that is becoming scarce and increasing prices. Aquaculture is then faced with the challenge of reducing the use of fish meal (and fish oil) in feeds and alleviating the pressure on wild fisheries (NAYLOR et al., 2009; TACON; METIAN, 2008). Various by-products of animal and plant origin are alternate and available ingredients to replace fish meal use, but the nutritive value of such alternatives should be assessed (MOUGHAN, 1999). Further to chemical and nutrient compositional analyses, other measurements *in vivo* such as nutrient digestibility are taken in research laboratories (BUREAU et al., 2002; NRC, 2011; SUGIURA, 2000). However, in general, the apparent digestibility coefficient is a measure of the rate of disappearance of a nutrient, i.e., nutrients analyzed to be present in feeds but not in feces are assumed as absorbed and utilized by the fish, and does not take into account endogenous losses, leaching or if the nutrient present in the feed is metabolically available to the fish (BUREAU, 2008; NRC, 2011). Although *in vivo* digestibility trials are important, it may be laborious and time consuming and not adequate for application at industrial level (LEMOS; TACON, 2011), which motivated the development of faster *in vitro* methods based on the digestion of small sample amounts.

In vitro digestion methods have been developed for terrestrial animals (ruminants and monogastrics) and humans, and methods for aquatic organisms are largely based on such studies (MOYANO et al., 2014). For aquatic animals, the focus has been on protein hydrolysis, as this nutrient may be more relevant in the metabolism and requirement than carbohydrates and lipids. The *in vitro* methods may include open or closed vessels, measure different products, e.g., released amino acids, degree of peptide bond hydrolysis, use commercially available enzyme sources or from the target species (ALARCÓN et al., 2002;

BASSOMPIERRE, et al., 1997a; DIMES; HAARD 1994; EZQUERRA et al., 1998; HAMDAN, et al., 2009; LAZO et al., 1998; MORALES; MOYANO, 2010; SHIPTON; BRITZ, 2002; TIBBETTS et al., 2011a; TONHEIM et al., 2007). Substrate and enzymes may be incubated in aqueous solution under controlled conditions and final products measured after a given time (BASSOMPIERRE et al., 1998; DIVAKARAN et al., 2004; LAN; PAN, 1993); membrane reactors in which an inner reaction chamber is separated from the outer chamber by a dialysis membrane of variable molecular weight cut off (1000 – 3500 Da) through which passes amino acids released during hydrolysis and are continuously removed from the outer chamber and quantified (HAMDAN et al., 2009; MOYANO; SAVOIE, 2001; MORALES; MOYANO, 2010; SAVOIE; GAUTHIER, 1986); substrate and enzymes may be incubated in aqueous solution under controlled conditions and the course of protein hydrolysis be evaluated by changes in pH, either by recording the change in pH (pH-drop or pH-shift) (LAZO et al., 1998) or by keeping the pH constant with continuous addition of titrant and measuring consumption (pH-stat) (CÓRDOVA-MURUETA; GARCÍA-CARREÑO, 2002; DIMES et al., 1994a; EL-MOWAFI et al., 2000; EZQUERRA et al., 1998; LEMOS et al., 2009; LEMOS; YASUMARU, 2010; TIBBETTS et al., 2011b). The pH-stat method is based on the principle that during protein hydrolysis protons (H^+) are released at alkaline pH, decreasing medium pH, which in turn is automatically corrected by titrant addition and the amount of peptide bonds cleaved is proportional to the volume of titrant consumed (ADLER-NISSEN, 1986). Protein hydrolysis in acidic medium increases pH, which is corrected by titrant addition and volume consumed is also equivalent to peptide bonds cleaved (DIERMAYR; DEHNE, 1990). Results are given as degree of protein hydrolysis (DH, %). This method has been used in *in vitro* digestion studies with aquatic animals (MOYANO et al., 2014). Moreover, some of the advantages of the pH-stat technique may be the non-use of buffer solutions, the possibility to quickly calculate the degree of protein hydrolysis by the automatically plotted titration curve (PEDERSEN; EGGUM, 1983), and tests different ingredients in small amounts (GRABNER, 1985; LEMOS et al., 2009). *In vitro* digestion methods may also work as tools to assist in the prediction of *in vivo* results (PEDERSEN; EGGUM, 1983; GRABNER, 1985; EZQUERRA et al., 1998; LAZO et al., 1998; LEMOS et al., 2004). The use of species-specific enzyme extracts are recommended in *in vitro* digestion studies so as to better predict the apparent digestibility in the target species (DIMES et al., 1994b; EZQUERRA et al., 1997; 1998). The *in vitro* pH-stat method to determine protein digestion with species specific enzymes may predict performance of salmonid fish (DIMES et al., 1994a) and marine shrimp (LEMOS; NUNES, 2008). *In vitro* studies to assess protein

digestion in fish feeds have been mostly carried out for some salmonid (DIMES et al., 1994a, b; GRABNER, 1985), gadoid fish (TIBBETTS et al., 2011a, b), and marine shrimp (EZQUERRA et al.1997; 1998; LAZO et al, 1998; LEMOS et al., 2009).

Another challenge faced by the aquaculture industry is to improve effluent water quality while maintaining high levels of fish production (GREEN et al., 2002; SUGIURA et al., 2006). In intensive aquaculture systems, compound feeds may be a source of nutrients and also of pollution, as nutrients may leach from feces and uneaten feed and also as soluble waste through urine and gills after absorption, specially nitrogen and phosphorus, which stimulate eutrophication (SUGIURA et al., 1998). Phosphorus in freshwater and nitrogen in sea water are the nutrients of high concern, as they are limiting to plants and algae in those environments (MANAHAN, 2001). Compound feeds should be nutritionally and economically adequate, i.e., nutrients should not be in excess and at the same time without causing any clinical deficiencies, and consequently reducing excretion into effluent water (EZQUERRA et al., 1998; SUGIURA et al., 1998). Further to feedstuff protein quality, phosphorus content and digestibility should be explored since higher availability may mean lower excretion and, consequently, reduce the impact caused by aquaculture (KETOLA; HARLAND, 1993; SUGIURA et al., 1998; CHO; BUREAU, 2001). Environmental burden caused by nitrogen and phosphorus excretion may be managed by improvements in feed formulation and feeding strategies (CHO; BUREAU, 2001; GODA, 2007; HLAVÁČ et al., 2014; KETOLA; HARLAND, 1993; SUGIURA et. al., 1998). *In vitro* methods can be useful tools in predicting nutrient digestibility and also variation in digestibility of feed ingredients and may vary from simple solubility tests to simulation of the digestive process (VAN DER KLIS; PEDERSEN, 2010). *In vitro* studies to assess phosphorus digestibility in aquatic animals are scarce and only studies with salmonid fish have been done so far (MORALES; MOYANO, 2010; MORALES et al., 2011; WEERASINGHE et al., 2001).

Therefore, the main objective of this thesis was to develop species-specific *in vitro* methods for the digestion of protein and phosphorus using the pH-stat technique to predict *in vivo* apparent digestibility of protein and phosphorus, respectively. In **Chapter 1**, *in vitro* protein digestion was tested with practical feed ingredients. In **Chapter 2,** *in vitro* digestion of phosphorus was tested with compound feeds. And in **Chapter 3** *in vitro* digestion of protein and phosphorus of compound feeds were assessed to predict *in vivo* apparent digestibility of protein and phosphorus. Specifically, **Chapter 1** describes the recovery of

crude enzyme extracts from fish digestive tract (different fish species with different feeding habits and from different habitats), enzyme extract standardization method, use of standardized species-specific enzyme extracts to test *in vitro* pH-stat technique to determine protein degree of hydrolysis (DH) of some practical ingredients commonly used in the industry; **Chapter 2** evaluates the amount of soluble P released after *in vitro* (pH-stat) digestion procedures by incubating commercial feeds for Nile tilapia with digestive enzyme extracts recovered from Nile tilapia, i.e., stomach and intestine enzyme extracts, separately and sequentially (two-stage) at 25 °C; compared the values with feed total phosphorus content, and also between the feeds tested and between the digestion procedures; and **Chapter 3** assesses *in vivo* protein and phosphorus digestibility coefficients of commercial feeds simulating commercial stocking density, using an internal marker (acid-insoluble ash), evaluates fish growth and the correlation of *in vivo* apparent digestibility data of protein and phosphorus with *in vitro* protein digestion and released soluble phosphorus. Results are discussed in each chapter. As illustrative material, photographs of fish sampling sites, experimental procedures, equipment and facilities used for the studies are presented in the **Appendixes** section.

This thesis has been written following the guidelines for thesis and dissertations of the University of São Paulo based on Brazilian Standards (ABNT) described in: “Diretrizes para apresentação de dissertações e teses da USP: documento eletrônico ou impresso – Parte I (ABNT – Associação Brasileira de Normas Técnicas)”.

CHAPTER 1

Species specific *in vitro* protein digestion (pH-stat) for fish: method development and application for juvenile rainbow trout (*Oncorhynchus mykiss*), cobia (*Rachycentron canadum*), and Nile tilapia (*Oreochromis niloticus*)

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Abstract

Aqua feed manufacture requires flexible formulations and effective methods to screen suitable feed ingredients. *In vitro* digestion may assist in the characterization and quality control of protein in feedstuffs for fish species once standardized species-specific digestive enzyme extracts are available. This study aimed to develop a species-specific *in vitro* enzymatic method to assess protein digestion in fish under the pH-stat concept. Two carnivorous (rainbow trout, *Oncorhynchus mykiss*, and cobia, *Rachycentron canadum*) and one omnivorous (Nile tilapia, *Oreochromis niloticus*) fish species were used as models. Crude digestive enzyme extracts were recovered from stomach and pyloric ceca or intestine of individuals of different weight groups, feeding status, and farming systems. The hydrolytic capacity of the species-specific enzyme extracts was standardized on analytical grade protein substrates and measured as degree of protein hydrolysis (DH) in the pH-stat assay. A group of twenty-four feed ingredients, including fish meals and by-products of plant and animal origin, were assessed for DH using the recovered enzymes from stomach and pyloric ceca/intestine. Ingredients were hydrolyzed with fish (i) stomach extract, (ii) pyloric ceca/intestine extract or (iii) stomach enzymes followed by pyloric ceca/intestine extract. Among plant by-products, cotton seed meal presented the highest DH with stomach plus pyloric ceca/intestine enzymes, followed by soy protein concentrate and soybean meals. Blood meals were the land animal by-product with higher DH outputs compared to poultry by-product meals and feather meals. No significant difference was observed among the DH of fish meals tested. The significance of measuring the DH with stomach enzyme extract is still not well understood but, overall, the pre-hydrolysis of feedstuffs with stomach enzymes increased pyloric ceca/intestine DH value. For cage and pond farmed Nile tilapia, ingredient DHs followed the same trend, describing a significant correlation and a high determination coefficient regression. Routine use of the method may yet depend on the prompt availability of more practical sources of enzymes. The determination of the degree of protein hydrolysis by the *in vitro* pH-stat with species-specific enzymes has shown to be a precise method that may be a useful tool to rank feed ingredients, and also an accessory method in the quality control of feedstuffs.

1. 1. Introduction

The aquaculture feed industry has been faced with the challenge to find nutritionally suitable and economically feasible protein ingredients to formulate compound diets. Aqua feed manufacture requires flexible formulations based on nutrient delivery from feed ingredients and additives to replace scarce raw materials like fish meals, as well as other sources subject to fluctuation in cost and availability (TACON et al., 2011). In this context, searching for novel ingredients, monitoring potential nutritional variability and nutrient availability to the target species are required in the routine of the feed manufacturer. Consistent methods of nutritional evaluation are needed to assist in the characterization and quality control at the industrial level (MOUGHAN, 1999). Nutrient digestibility and availability has been determined *in vivo* in research laboratories and accepted for feedstuff assessment (BUREAU et al., 2002; NRC, 2011; SUGIURA, 2000). On the other hand, these trials may be laborious, complex, time consuming and expensive, and possibly not adequate for application at industrial level (LEMOS; TACON, 2011). This motivated the development of *in vitro* methods based on the digestion of small amounts of feedstuff samples. Reported *in vitro* protein digestion methods include the use of digestive enzymes that may be available from commercial sources (LAZO et al., 1998; SHIPTON; BRITZ, 2002; TONHEIM et al., 2007) or recovered from the target species (ALARCÓN et al., 2002; DIMES; HAARD, 1994; EZQUERRA et al., 1998; TIBBETTS et al., 2011a). Nevertheless, different results in the *in vitro* digestion have been found depending on the enzyme origin, suggesting a species-specific feature of *in vitro* protein digestion (ALARCÓN et al., 2002; EZQUERRA et al., 1998; LEMOS et al., 2004; LEMOS; NUNES, 2008; MOYANO; SAVOIE, 2001).

The *in vitro* pH-stat determination of the degree of protein hydrolysis (DH) with species-specific enzymes has been investigated in some fish and crustacean species with significant potential to distinguish feedstuff quality and also to predict protein digestibility in the live animal (CÓRDOVA-MURUETA; GARCÍA-CARREÑO, 2002; DIMES et al., 1994a; EL-MOWAFI et al., 2000; EZQUERRA et al., 1998; LEMOS et al., 2009; LEMOS; YASUMARU, 2010; TIBBETTS et al., 2011b). The principle of the pH-stat method consists in the potential shift in medium pH related to protein hydrolysis at certain pH levels. In the course of a reaction set for a constant pH, variation derived from hydrolysis may be automatically corrected by titration and, thus, the amount of peptide bonds cleaved is proportional to the volume of titrant consumed (ADLER-NISSEN, 1986). Some of the

advantages of the pH-stat technique may be the non-use of buffer solutions while maintaining constant pH during reaction and the possibility to quickly calculate the degree of protein hydrolysis by the automatically plotted titration curve (PEDERSEN; EGGUM, 1983). Besides being simple, rapid, precise, and safe, it does not require complex equipment, provides specific response (standardized species enzymes), stable reaction conditions, and tests different ingredients in small amounts (GRABNER, 1985; LEMOS et al., 2009).

The assessment of the species-specific degree of protein hydrolysis for distinct species would require the development of consistent analytical procedures considering the status of the donor individuals and standardization of the recovered digestive enzyme extracts. The present study describes a pH-stat method to determine the *in vitro* protein digestion for stomached fish species (rainbow trout, *Oncorhynchus mykiss*, cobia, *Rachycentron canadum*, and Nile tilapia, *Oreochromis niloticus*) with different characteristics in terms of domestication level, feeding habit and farming system. Digestive enzyme extracts of the species were recovered at different fish weight and feeding status, and standardized according to digestive potential as degree of hydrolysis of standard substrates. Standardized species-specific enzyme extracts were additionally used to determine the degree of protein hydrolysis of some practical ingredients commonly used in aqua feeds.

1.2. Materials and Methods

1.2.1. Fish sampling

Individuals of the three species tested (rainbow trout, *Oncorhynchus mykiss*, cobia, *Rachycentron canadum* and Nile tilapia, *Oreochromis niloticus*) of different weight groups (three groups for rainbow trout and Nile tilapia and two for cobia), feeding status (fed commercial diet or unfed –15 h fasting for Nile tilapia or 24 h for rainbow trout and cobia), and commercial farming systems were sampled for the preparation of digestive enzyme extracts. Rainbow trout were farmed in freshwater raceways in Campos do Jordão, São Paulo; Nile tilapia in cages in a commercial farm in a freshwater reservoir of the Paranapanema river, Ipaussu, São Paulo; Nile tilapia in fertilized ponds in Joinville, Santa Catarina; and cobia in circular concrete tanks with flow-through seawater in a commercial farm in Ipojuca, Pernambuco. Ten healthy fish were sampled per species, weight group, feeding status and farming system. Fish were killed by rapid cephalic concussion then measurements were taken (body weight, body total length, individual digestive organ pH, weight and length) (Table 1.1.). Mean individual body weight ± s.d. of groups evaluated were: rainbow trout, 165.2 ± 38.9 , 262.1 ± 22.5 and 393.1 ± 35.8 g; cobia, 550.5 ± 135.9 and 1052.6 ± 273.3 g; cage farmed Nile tilapia, 124.5 ± 23.2, 372.3 ± 68.1 and 598.3 ± 110.1 g; and pond farmed Nile tilapia, 116.1 ± 18.5, 332.3 ± 38.2, and 669.7 ± 82.6 g. Prior to organ excision, pH was measured in the stomach, pyloric ceca (absent in Nile tilapia) and intestine of each individual with a combined glass electrode connected to a pHmeter (7 mm immersion depth and 3 mm electrode tip diameter – Biotrode 744 pHmeter, Metrohm AG, Switzerland). After excision, the digestive tract of fasted fish was found empty, whereas of fed fish it had to be thoroughly cleansed with distilled water. Stomach, pyloric ceca and intestine were cleaned of visceral fatty tissue, dissected, measured (length, weight) (Table 1.1), pooled in plastic bags according to fish weight group and feeding status, and frozen at -20 °C. Samples were transported on dry ice to the laboratory and stored at -20 °C until further processing.

1.2.2. Crude digestive enzyme extracts

The frozen sampled organs were allowed to partially thaw at room temperature. Fish digestive crude enzyme extract was prepared based on protocol developed for marine shrimp

(GARCÍA-CARREÑO et al., 1997). For rainbow trout and cobia enzyme extracts from stomach and pyloric ceca were prepared. For fish possessing pyloric ceca it is considered as the most suitable enzyme source for protein hydrolysis assays in alkaline medium because the number of ceca or blind diverticula may account for *ca.* 70% of the total enzymatic digestion (BUDDINGTON; DIAMOND, 1986; TIBBETTS et al., 2011a). The organs were chopped into small pieces with scissors as they were difficult to homogenize. For Nile tilapia, stomach and intestine enzyme extracts were used. Each pool of sampled organs was placed in a glass beaker on ice with cold distilled water at ratios of 1:3 (w/v) for stomach and 1:1 (w/v) for pyloric ceca and intestine. For pyloric ceca of cobia, the 1:3 (w/v) ratio was employed to allow proper homogenization of the harder tissue. This comparatively higher dilution was computed in the standardization of the enzyme extracts. Tissue was homogenized (T25 digital ultra-turrax®, 18G dispersing element, IKA WORKS, Inc., Wilmington, NC, USA) in several pulses of *ca*. 20 seconds to avoid engine overheating and possible damage to the sample. The mixture was centrifuged at 16,800 × *g* for 30 min at 4 °C and the supernatant, which constituted the crude enzyme extract, was pooled in a glass beaker on ice. The pH of the crude enzyme extracts were measured (pH Meter 744, resolution 0.01, 0.1 °C, 1 mV, Metrohm, Switzerland) and adjusted to 2.0 (stomach extract) by adding HCl 0.1 N and to pH 8.0 (pyloric ceca and intestine) by adding NaOH 0.1 N, under constant agitation on ice to keep low temperature (*ca.* 4 °C) to avoid enzyme activation. Enzyme extracts were aliquoted in 2-mL polypropylene cryogenic vials and stored at -20 °C until used. A schematic sequence of enzyme extract recovery is shown in Fig. 1.1.

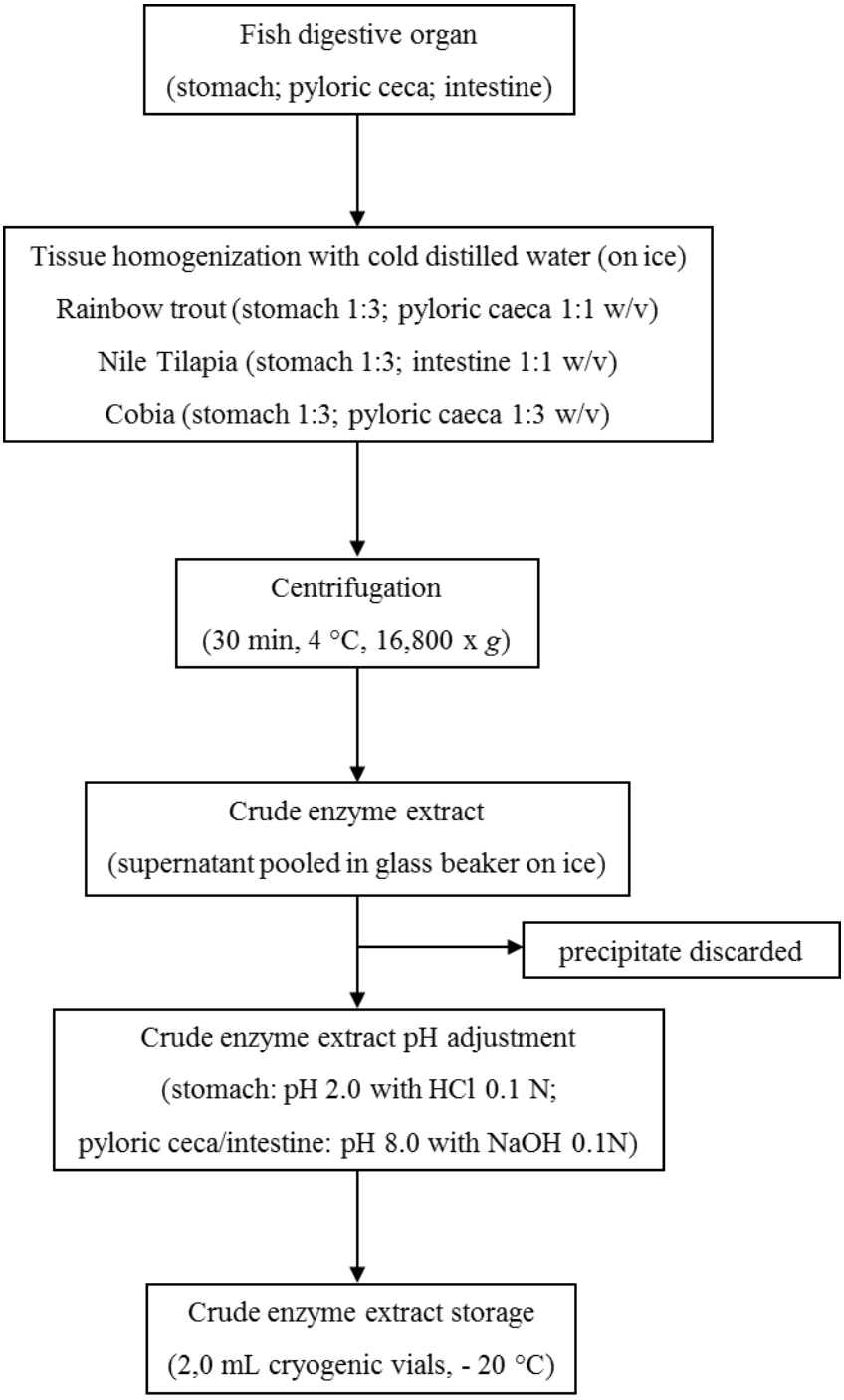

Figure 1.1 – Flow diagram of digestive enzyme extract preparation for the determination of the *in vitro* degree of protein hydrolysis (DH) in rainbow trout, *Oncorhynchus mykiss*, cobia, *Rhachycentron canadum* and Nile tilapia, *Oreochromis niloticus*. After preparation, the hydrolytic capacity of crude enzyme extracts was standardized according to DH of specific protein substrates. Frozen stored enzyme extracts (-20 °C) showed to be stable for use after several months. Further details in Materials and Methods.

1.2.3. Crude enzyme extracts standardization

The crude digestive enzyme extracts from rainbow trout, cobia and Nile tilapia were standardized according to their hydrolytic capacity using the *in vitro* pH-stat degree of protein hydrolysis (DH) to determine the volume of extract most suitable for the analysis. Briefly, in the pH-stat concept, the enzymatic hydrolysis of peptide bonds results in pH shift (increase or decrease, for acid and alkaline hydrolysis, respectively) which is automatically stabilized by the addition of the titrant (0.1 N HCl or 0.1N NaOH). The volume of titrant added is equivalent to the DH by the digestive enzyme extract, i.e., at certain pH levels the relation between equivalent peptide bonds cleaved and equivalent titrant consumed is proportional (ADLER-NISSEN, 1986; DIERMAYR; DEHNE, 1990).

Standard substrates for stomach and pyloric ceca or intestine assays were analytical grade hemoglobin from bovine blood (95% crude protein, H2625, Sigma-Aldrich, St. Louis, MO, USA) and casein from bovine milk (90% crude protein, C7078, Sigma-Aldrich, St. Louis, MO, USA), respectively. Hemoglobin was chosen because it is a rapidly digested reproducible substrate (ANSON, 1938) and casein, a highly digestible purified and standardized product. Fish stomach enzyme extracts were standardized at pH 2.0. Rainbow trout and cobia pyloric ceca and Nile tilapia intestine extracts were standardized at pH 8.0.

The assays were carried out simultaneously in three automated titrators, i.e., one single and two double measuring interfaces with burettes (718 Stat Titrino, Titrando 836, Titrando 907 – Metrohm AG, Switzerland), connected to a single controlling and data logging software (Tiamo^{TM} v. 2.2, Metrohm AG, Switzerland) operated by one person. Standard substrate samples corresponding to 80 mg protein were stirred in distilled water in the reaction tube (8.0 mL total suspension volume) and the pH adjusted to 2.0 for the stomach or 8.0 for the pyloric ceca/intestine extracts assays by the addition of HCl 0.1N or NaOH 0.1 N, respectively, and kept stable for 30 (hemoglobin) and 60 (casein) minutes. Minimum and maximum titrant addition rates were 5 μL and 0.8 mL min^{-1} (HCl) and 1.5 μL and 3.0 mL min^{-1} (NaOH). Suspension final volume was adjusted to 10 mL (considering the enzyme extract volume) by adding distilled water. If necessary, after the stabilization step the pH was automatically finely adjusted to 2.0 or 8.0. Protein hydrolysis assay started with the addition of the respective enzyme extract (from stomach or pyloric ceca/intestine) and carried out for 60 minutes. Titrant (HCl and NaOH) minimum and maximum addition rates were 3.0 μL and

5.0 mL min^{-1}, respectively. Reaction temperature was maintained at 25 ± 0.2 °C in jacketed water flow through reaction vessels connected to a heated/refrigerated constant temperature water bath (temperature uniformity ± 0.1 °C, RSWB 3222A Lindberg/BlueM, Thermo Electron Corp., MA, USA). The water was recirculated through plastic hoses from the water bath tank to the jacketed vessels with the aid of a submersible aquarium pump. During the assays, nitrogen gas was purged into the mixture and the reaction tube covered with plastic film to avoid interference of atmospheric CO_2 in the reaction pH (ADLER-NISSEN, 1986). The hydrolysis assay was carried in triplicate.

The degree of protein hydrolysis (DH) with stomach extract was calculated based on the formula proposed by Diermayr and Dehne (1990):

$$DH = [(V \times N)/E] \times (1/P) \times F_{pH} \times 100\%$$

Where:

V = volume of acid consumed in the hydrolysis reaction (mL);

N = normality of the acid;

E = mass of substrate protein (g);

P = number of peptide bonds cleaved (mol g $protein^{-1}$). For proteins which amino acid composition is not determined, P is generally suggested as 8.0.

F_{pH} = 1.08 (correction factor for pH 2.0 at 25 °C);

The DH with pyloric ceca/intestine extract was calculated according to Adler-Nissen (1986):

$$DH = B \times Nb \times (1/\alpha) \times (1/MP) \times (1/H_{tot}) \times 100\%$$

Where:

B = volume of alkali consumed (mL);

Nb = normality of the alkali;

α = average degree of dissociation of the α-NH groups ($1/\alpha$=1.50 for pH 8.0 at 25 °C);

MP = mass of substrate protein (g);

H_{tot} = total number of peptide bonds in the protein substrate [7.6-9.2 meqv g $protein^{-1}$, according to the source of protein (ADLER-NISSEN, 1986)].

To assess the proper volume of each enzyme extract batch to be used in the DH applications, the study considered the minimum enzyme volume to produce high output. The

standardization comprised the determination of the hydrolytic capacity of different volumes (50, 200, 600, 1000 μL) of stomach or pyloric ceca/intestine extracts on the same substrate amount (80 mg protein). DH values were plotted against the volumes of enzyme extracts used in the assay to check for hydrolysis performance and for the model best describing these relationships. These regressions also served to compare the possible effects of fish weight and feeding status on the hydrolytic output of digestive enzyme extracts.

1. 2.4. Test ingredients

The DH of twenty-four feed ingredients of different types and sources were assessed for the three fish species. The ingredients were obtained from feed manufacturers, ingredient manufacturers and suppliers. The set included marine and land animal (fish meals, blood meals, feather meals, and poultry by-product meals) and plant ingredients (corn grain, corn gluten meal, cotton seed meal, rapeseed meal, soybean meals, soy protein concentrate, wheat bran, wheat flour, and wheat gluten) commonly used for aqua feed manufacture. Source details corresponded to samples of blood meals, poultry by-product meals (feed-grade and pet food grade), fish meals (anchovy, herring, mackerel, menhaden, salmon by-product, miscellaneous fish by-product), and soybean meals (full-fat and solvent extracted). The proximate composition (moisture, crude protein, crude fat, crude fiber, and ash) of the feed ingredients was determined by the Bromatology and Mineral Analysis Laboratory of the Animal Science Institute (IZ, APTA, SAA) following Silva and Queiroz (2009). Moisture was determined by drying sample at 103-105 °C; protein content was determined with the micro Kjeldahl method; lipids with the Soxhlet method; crude fiber by acid and alkaline hydrolysis; ash by combustion at 550-500 °C. Nitrogen-free extract was calculated by difference [100 – (crude protein + crude fiber + crude fat + ash + moisture)]. Ingredients were processed as received from the suppliers (‘as is’) and sieved through a series of screens with mesh sizes > 1000 μm, 500 – 1000 μm, 250 – 500 μm, 125 – 250 μm, 63 – 125 μm, <63 μm to describe particle size distribution (%), as grinding of raw materials is a costly process for the feed manufacturer and *in vitro* DH may depend on particle size.

1.2.5. Species specific in vitro pH-stat determination of protein hydrolysis of feed ingredients

Following digestive enzyme extract standardization, the *in vitro* degree of protein hydrolysis of the test ingredients was determined for rainbow trout, cobia and Nile tilapia.

Ingredients were hydrolyzed in single assays with (i) stomach extract, (ii) pyloric ceca/intestine enzyme extract or (iii) stomach extract followed by pyloric ceca/intestine enzymes (two-stage hydrolysis). Assays were carried out with enzyme extracts recovered from fed fish at selected average individual weight: rainbow trout (393.1 ± 35.8 g), cobia (550.5 ± 135.9 g), and Nile tilapia (cage farmed: 598.3 ± 110.1 g or pond farmed: 669.7 ± 82.6 g). The enzyme extract volumes chosen were 250 µL from stomach and 200 µL from pyloric ceca (rainbow trout and cobia) or intestine (Nile tilapia). Four replicates of DH analysis were run per test ingredient. For the single assays with (i) stomach extract and (ii) pyloric ceca/intestine enzyme extract, samples were prepared and hydrolyzed as described in the enzyme extract standardization section (2.3) with slight modification in the titrant addition rates, i.e., in the preparation step HCl 0.1N, minimum and maximum rates were 5.0 µL min^{-1} and 0.3 mL min^{-1}, and NaOH 0.1N were minimum and maximum 1.0 µL and 1.0 mL min^{-1}, and in the hydrolysis step, minimum 1.0 µL min^{-1} and maximum 0.3 mL min^{-1}. For the two-stage hydrolysis, immediately after hydrolysis with stomach extract sample was transferred to another measuring interface with an electrode and a burette, when pH was adjusted to 8.0 with the addition of NaOH 0.1N for the hydrolysis with pyloric ceca/intestine extract. Each hydrolysis was carried out for 60 min at 25 ± 0.2 °C.

1.2.6. Statistical analysis

Data were analyzed using SigmaPlot 11.0 statistical software (Systat software Inc., 2008). Protein DH percentage data from enzyme extract standardization were arcsine- and log10+1-transformed to statistically compare the effect of fish weight, feeding status and farming system on the hydrolytic performance of enzyme extracts (GOTELLI; ELLISON, 2004; ZAR, 1999). Plotting DH against the enzyme extract volumes after transformation resulted in a linear adjustment (y = a +bx). Slope (b) and intercept (a) values from the linear regressions of fish weight groups in the same feeding status were submitted to ANOVA and between feeding status of fish of the same weight group were submitted to t-test and significant differences between values were detected with the post-hoc Tukey test. Since the same data were compared twice (fed vs. unfed; fish weight groups) *P*-value was divided by two and difference considered significant at $P<0.025$ (ZAR, 1999). Ingredients DH data from hydrolysis with stomach, pyloric ceca/intestine extract and two-stage hydrolysis was submitted to one-way ANOVA. Ingredients DH data of the two Nile tilapia farming systems

were compared using t-test. Difference between means was detected with Tukey test at $P<0.05$.

1.3. Results

Mean (s.d.) values of fish body weight and total length, digestive organs weight, length and pH, are presented according to fish species, farming system and feeding status (Table 1.1). The relation between intestinal length and body length for rainbow trout and cobia was of 0.4 (0.06), whereas for Nile tilapia it was of 6.6 (1.4). Stomach pH ranged from 2.23 (0.54) to 6.66 (0.41) with lower values in unfed rainbow trout and higher values in unfed cobia. Rainbow trout and cobia pyloric ceca pH varied from 6.75 (0.26) to 7.75 (0.18). Nile tilapia intestine pH ranged from 6.27 (0.18) to 7.46 (0.36).

The recovered enzyme extracts showed consistent hydrolytic responses on analytical grade hemoglobin (stomach enzymes) and casein (pyloric ceca or intestine enzymes). The degree of protein hydrolysis (DH) of the substrate increased significantly ($P<0.05$) with the stomach and pyloric ceca/intestine enzyme extract volumes employed (50, 200, 600, 1000 μL), following a well-adjusted logarithmic function ($R^2 = 0.88 - 0.99$, n = 12, Fig. 1.2). As a log shaped model, higher DH increments were verified between 50 and 600 μL.

Table 1.1 – Mean values (s.d.) of individual body weight (g) and total length (cm), digestive tract organs - weight (g), length (cm) and pH, determined in juvenile rainbow trout (*Oncorhynchus mykiss*), cobia (*Rachycentron canadum*), and Nile tilapia (*Oreochromis niloticus*) farmed in raceways, concrete tanks and cage or pond, respectively, and at two feeding status (fed or unfed). n = 10.

Fish species	Farming system	Feeding status	Body		Stomach			Pyloric ceca [(1)]			Intestine		
			Weight (g)	Length (cm)	Weight (g)	Length (cm)	pH	Weight (g)	Length (cm)	pH	Weight (g)	Length (cm)	pH
Rainbow Trout (*Oncorhynchus mykiss*)	Raceway	Fed	165.2 (38.9)	22.2 (1.6)	2.7 (0.8)	5.0 (0.6)	3.40 (0.92)	2.9 (0.7)	3.1 (0.5)	7.40 (0.18)	1.8 (0.6)	9.2 (1.9)	7.80 (0.10)
			262.1 (22.5)	26.9 (0.9)	4.7 (1.3)	6.5 (1.2)	4.74 (0.39)	5.2 (1.5)	4.2 (0.4)	7.68 (0.27)	2.5 (0.6)	10.5 (1.7)	7.76 (0.14)
			393.1 (35.8)	30.7 (0.9)	8.2 (2.5)	7.3 (1.1)	3.86 (1.36)	8.1 (1.7)	4.2 (1.4)	7.75 (0.18)	5.2 (1.0)	14.1 (1.6)	8.01 (0.18)
		Unfed	165.2 (38.9)	22.2 (1.6)	2.8 (1.0)	5.8 (1.0)	2.29 (0.69)	2.6 (1.2)	3.5 (0.6)	7.58 (0.23)	1.8 (0.6)	9.5 (2.2)	7.68 (0.22)
			262.1 (22.5)	26.9 (0.9)	4.2 (1.9)	6.2 (1.3)	3.44 (0.64)	3.8 (1.2)	3.7 (1.1)	7.73 (0.14)	2.2 (0.7)	9.5 (1.4)	7.98 (0.27)
			393.1 (35.8)	30.7 (0.9)	8.7 (2.3)	7.0 (0.6)	2.23 (0.54)	5.8 (0.9)	4.4 (0.6)	7.64 (0.29)	3.9 (0.6)	13.1 (1.6)	7.91 (0.22)
Cobia (*Rachycentron canadum*)	Concrete Tank	Fed	550.5 (135.9)	39.9 (2.5)	10.92 (3.6)	6.01 (0.9)	4.68 (0.80)	15.39 (6.0)	3.04 (0.7)	6.95 (0.22)	3.61 (1.2)	14.5 (2.8)	6.94 (0.28)
			1052.6 (273.3)	48.1 (4.5)	19.01 (4.2)	7.41 (1.5)	4.39 (0.23)	29.73 (10.6)	4.24 (1.4)	6.75 (0.26)	6.15 (1.9)	18.7 (3.1)	6.80 (0.20)
		Unfed	550.5 (135.8)	39.9 (2.5)	11.00 (1.1)	5.99 (0.8)	6.66 (0.41)	13.04 (1.8)	2.48 (0.5)	7.02 (0.25)	2.82 (0.2)	14.8 (2.0)	7.08 (0.19)
			1052.6 (273.3)	48.1 (4.5)	21.07 (5.6)	7.44 (1.4)	6.42 (1.38)	26.77 (7.0)	3.25 (0.5)	7.03 (0.19)	5.33 (1.5)	18.3 (3.8)	7.13 (0.25)
Nile Tilapia (*Oreochromis niloticus*)	Cage	Fed	124.5 (23.2)	18.2 (1.1)	0.5 (0.2)	3.2 (0.7)	3.46 (0.75)				2.8 (0.4)	114.6 (16.8)	6.85 (0.18)
			372.3 (68.1)	24.8 (1.7)	1.5 (0.3)	6.1 (0.6)	3.34 (0.50)				8.5 (2.1)	191.1 (33.6)	6.44 (0.12)
			598.3 (110.1)	29.3 (1.7)	n.d.	7.1 (0.6)	3.97 (0.74)				n.d.	203.3 (26.0)	6.27 (0.18)
		Unfed	124.5 (23.2)	18.2 (1.1)	0.5 (0.2)	2.3 (0.4)	4.12 (1.69)				3.5 (1.0)	127.4 (19.9)	7.13 (0.32)
			372.3 (68.1)	24.8 (1.7)	n.d.	3.2 (0.5)	2.61 (0.57)				n.d.	149.5 (27.4)	6.81 (0.26)
			598.3 (110.1)	29.3 (1.7)	n.d.	3.9 (0.9)	5.08 (1.82)				n.d.	180.5 (30.0)	7.12 (0.12)
	Pond	Fed	116.1 (18.5)	18.1 (0.9)	0.71 (0.1)	2.5 (0.5)	2.55 (0.75)				5.89 (1.2)	123.0 (17.2)	6.64 (0.28)
			332.3 (38.2)	26.1 (1.0)	1.53 (0.2)	4.5 (0.8)	3.98 (0.95)				8.33 (1.0)	200.5 (36.0)	6.60 (0.34)
			669.7 (82.6)	32.3 (1.4)	2.80 (0.5)	4.74 (0.5)	3.89 (0.74)				16.16 (2.5)	242.6 (35.1)	6.92 (0.38)
		Unfed	116.1 (18.5)	18.1 (0.9)	0.54 (0.1)	2.04 (0.3)	3.01 (1.61)				3.55 (0.6)	104.2 (13.5)	7.46 (0.36)
			332.3 (38.2)	26.1 (1.0)	1.67 (0.2)	3.35 (0.5)	3.44 (0.73)				8.49 (1.5)	193.8 (30.2)	6.76 (0.37)
			669.7 (82.6)	32.3 (1.4)	2.97 (0.5)	3.93 (0.7)	2.91 (0.65)				13.62 (1.6)	202.8 (25.3)	6.86 (0.28)

[(1)] not present in *O. niloticus.*

n.d. = not determined.

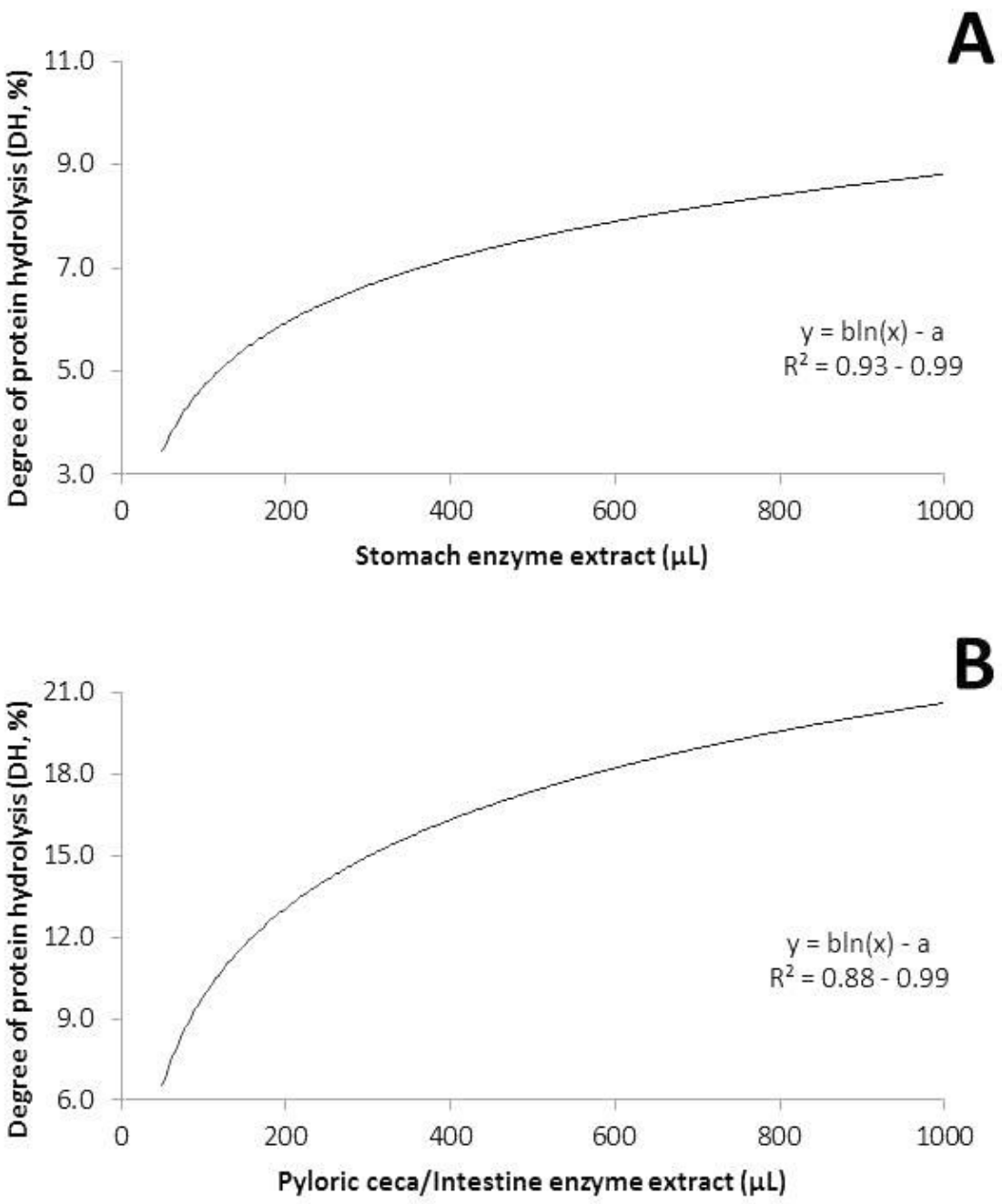

Figure 1.2 – Hydrolytic performance of the enzyme extracts recovered from (A) stomach and (B) pyloric ceca/intestine of juvenile rainbow trout, *Oncorhynchus mykiss*, cobia, *Rachycentron canadum* and Nile tilapia, *Oreochromis niloticus*. Analytical grade protein products (hemoglobin and casein for stomach and pyloric ceca/intestine enzymes, respectively) were used as substrates. Plotting *in vitro* pH-stat protein hydrolysis carried out for 60 min at 25 ± 0.2 ºC, with 80 mg protein substrate (y) and 50, 200, 600 and 1000 µL of enzyme extracts (n = 12) (x) resulted in well-adjusted logarithmic function [y = bLn(x) – a] with coefficient of determination R^2 = 0.88 – 0.99. Further details in Materials and Methods.

The coefficient of variation (n = 3) of DH under different enzyme extract volumes were of 4.24 ± 2.82% for stomach and of 4.48 ± 2.12% for pyloric ceca/intestine enzyme hydrolysis. The recovered species-specific crude enzyme extract batches could be standardized according to the hydrolytic capacity upon purified substrates. The intercept (a) and slope (b) values from the linearized regressions (y = a + bx) between DH and enzyme extract volumes for stomach and pyloric ceca/intestine, according to fish species, were obtained as additional comparison criteria, with the respective regression coefficients of determination (R^2) (Table 1.2). Accordingly, slope value indicate the rate at which DH increases with the enzyme extract volumes assayed, and intercept the overall DH level. With rainbow trout stomach enzyme extract, the rate of DH increase (b) was not statistically different ($P>0.025$) among fish weight or between feeding status, whereas with pyloric ceca extract it was significantly higher ($P<0.025$) in fed than unfed fish. With cobia stomach enzyme extract, fed fish showed significantly higher regression slope than unfed fish, regardless of fish weight, and with pyloric ceca extract, DH slope did not differ between fed or unfed fish. With stomach enzyme extracts of cage farmed Nile tilapia, the rate of DH increment was significantly higher in fed fish but with intestine extract, it was not affected by feeding status. In pond farmed Nile tilapia, no difference in DH increasing rate with stomach extract was observed between fed and unfed fish and with intestine enzyme extract, no clear trend of feeding status effect could be observed. Cage farmed Nile tilapia presented higher DH increasing rate (b) for stomach enzyme extract than pond farmed Nile tilapia, whereas the opposite was observed for intestine enzyme extract. On average, no clear trend of effect of fish weight, feeding status or farming system on DH increment rate could be determined for the three fish species.

Table 1.2 – Standardization of crude digestive enzyme extracts using the *in vitro* pH-stat degree of protein hydrolysis (DH) in rainbow trout (*Oncorhynchus mykiss*), cobia (*Rhachycentron canadum*), and cage and pond farmed Nile tilapia (*Oreochromis niloticus*). The hydrolytic capacity was standardized according to enzyme extracts from fish at different weight and feeding status. Standardization used hemoglobin and casein (80 mg protein per sample) as substrates for stomach and pyloric ceca/intestine assays, respectively. Enzyme extract volumes tested were 50, 200, 600 and 1000 µL, n = 12. Percentage DH data were arcsine- and log10+1-transformed for a linear adjustment (y = a + bx) with enzyme extract volume (x), and equation values are given as mean (s.d.). a = intercept or constant; b = slope; R^2= coefficient of determination for the regression. Different superscript upper-case letters indicate significant difference between fish of different feeding status but same weight and different superscript lower-case letters indicate significant difference between the different fish sizes at the same feeding status (P<0.025). Comparisons were made within the same fish species.

Fish species	Feeding status	Enzyme Extract (organ)	Fish size (g)	a	b	R^2
Rainbow trout (*Oncorhynchus mykiss*)	Fed	Stomach	165.2 (38.9)	0.335 (0.00789)$^{a, B}$	0.174 (0.00927)$^{a, A}$	0.97
			262.1 (22.5)	0.304 (0.00783)$^{c, B}$	0.179 (0.00920)$^{a, A}$	0.97
			393.1 (35.8)	0.316 (0.00739)$^{b, A}$	0.183 (0.00869)$^{a, A}$	0.98
	Unfed		165.2 (38.9)	0.360 (0.00877)$^{a, A}$	0.153 (0.01030)$^{b, B}$	0.96
			262.1 (22.5)	0.315 (0.01310)$^{b, A}$	0.171 (0.01540)$^{a, A}$	0.92
			393.1 (35.8)	0.299 (0.00761)$^{c, B}$	0.182 (0.00894)$^{a, A}$	0.98
	Fed	Pyloric ceca	165.2 (38.9)	0.359 (0.01700)$^{a, B}$	0.288 (0.02000)$^{b, A}$	0.95
			262.1 (22.5)	0.317 (0.01950)$^{b, B}$	0.337 (0.02290)$^{a, A}$	0.96
			393.1 (35.8)	0.366 (0.02080)$^{a, B}$	0.286 (0.02440)$^{b, A}$	0.93
	Unfed		165.2 (38.9)	0.449 (0.01140)$^{a, A}$	0.244 (0.01330)$^{a, B}$	0.97
			262.1 (22.5)	0.453 (0.02110)$^{a, A}$	0.237 (0.02480)$^{a, B}$	0.90
			393.1 (35.8)	0.422 (0.02030)$^{b, A}$	0.245 (0.02380)$^{a, B}$	0.91
Cobia (*Rachycentron canadum)*	Fed	Stomach	550.5 (135.9)	0.299 (0.00956)$^{a, B}$	0.193 (0.01120)$^{a, A}$	0.97
			1052.6 (273.3)	0.282 (0.00991)$^{b, B}$	0.198 (0.01160)$^{a, A}$	0.97
	Unfed		550.5 (135.9)	0.326 (0.01250)$^{a, A}$	0.177 (0.01470)$^{a, B}$	0.93
			1052.6 (273.3)	0.335 (0.01050)$^{a, A}$	0.164 (0.01230)$^{b, B}$	0.95
	Fed	Pyloric ceca	550.5 (135.9)	0.278 (0.01990)$^{b, A}$	0.322 (0.02330)$^{a, A}$	0.95
			1052.6 (273.3)	0.311 (0.01490)$^{a, A}$	0.321 (0.01750)$^{a, A}$	0.97
	Unfed		550.5 (135.9)	0.260 (0.02180)$^{a, B}$	0.222 (0.02560)$^{b, B}$	0.88
			1052.6 (273.3)	0.258 (0.03070)$^{a, B}$	0.326 (0.03610)$^{a, A}$	0.89
Nile tilapia (*Oreochromis niloticus*) cage farmed	Fed	Stomach	124.5 (23.2)	-0.0385 (0.0327)$^{a, A}$	0.429 (0.03840)$^{b, A}$	0.93
			372.3 (68.1)	-0.0450 (0.0254)$^{a, B}$	0.428 (0.02990)$^{b, A}$	0.95
			598.3 (110.1)	-0.0979 (0.0255)$^{b, B}$	0.446 (0.02990)$^{a, A}$	0.96
	Unfed		124.5 (23.2)	-0.0300 (0.0405)$^{b, A}$	0.434 (0.04760)$^{a, A}$	0.89
			372.3 (68.1)	0.0196 (0.0278)$^{a, A}$	0.390 (0.03260)$^{b, B}$	0.93
			598.3 (110.1)	-0.0181 (0.0295)$^{b, A}$	0.411 (0.03470)$^{b, B}$	0.93
	Fed	Intestine	124.5 (23.2)	0.280 (0.02170)$^{c, A}$	0.368 (0.02550)$^{a, A}$	0.95
			372.3 (68.1)	0.325 (0.02940)$^{b, A}$	0.339 (0.03450)$^{b, A}$	0.91
			598.3 (110.1)	0.418 (0.01650)$^{a, A}$	0.248 (0.01930)$^{c, B}$	0.94
	Unfed		124.5 (23.2)	0.261 (0.01870)$^{c, B}$	0.379 (0.02200)$^{a, A}$	0.97
			372.3 (68.1)	0.306 (0.02730)$^{b, A}$	0.358 (0.03210)$^{a, A}$	0.93
			598.3 (110.1)	0.352 (0.02700)$^{a, B}$	0.292 (0.03180)$^{b, A}$	0.89
Nile tilapia (*Oreochromis niloticus*) pond farmed	Fed	Stomach	116.1 (18.5)	0.110 (0.02720)$^{a, A}$	0.324 (0.03200)$^{b, A}$	0.91
			332.3 (38.2)	0.0429 (0.0193)$^{c, A}$	0.365 (0.02270)$^{a, A}$	0.96
			669.7 (82.6)	0.0871 (0.0176)$^{b, A}$	0.293 (0.02070)$^{c, B}$	0.95
	Unfed		116.1 (18.5)	0.123 (0.02670)$^{a, A}$	0.299 (0.03130)$^{c, A}$	0.90
			332.3 (38.2)	0.0408 (0.0263)$^{b, A}$	0.360 (0.03090)$^{b, A}$	0.93
			669.7 (82.6)	-0.150 (0.03150)$^{c, B}$	0.484 (0.03710)$^{a, A}$	0.94
	Fed	Intestine	116.1 (18.5)	0.431 (0.02380)$^{a, B}$	0.216 (0.02790)$^{c, A}$	0.86
			332.3 (38.2)	0.231 (0.02170)$^{c, B}$	0.412 (0.02550)$^{a, A}$	0.96
			669.7 (82.6)	0.259 (0.01010)$^{b, A}$	0.344 (0.01190)$^{b, B}$	0.99
	Unfed		116.1 (18.5)	0.463 (0.01680)$^{a, A}$	0.201 (0.01970)$^{c, A}$	0.91
			332.3 (38.2)	0.272 (0.02050)$^{b, A}$	0.378 (0.02410)$^{b, B}$	0.96
			669.7 (82.6)	0.220 (0.03650)$^{c, B}$	0.413 (0.04280)$^{a, A}$	0.90

The proximate composition of the set of 24 test ingredients of different sources and origins (Table 1.3) were within the ranges reported for standard raw materials (HERTRAMPF; PIEDAD-PASCUAL, 2000; NRC, 2011; ROSTAGNO, 2011; TACON et al., 2009). For the ingredients DH assays, the criteria adopted to choose the volume of stomach and pyloric ceca/intestine enzyme extract were the minimum volume resulting in maximum DH and also volumes that could fit in the test tube conditions. Additionally, selection of the enzyme extract batches was based on the extract standardization results, i.e., fed fish of harvest weight. For rainbow trout (393.1 ± 35.8 g) DH values with stomach and pyloric ceca extracts on hemoglobin and casein were 8.22 and 14.03%, respectively. For cobia (550.5 ± 135.9 g), DH values were 7.87 and 10.93%, respectively. For cage farmed Nile tilapia (598.3 ± 110.1 g), DH values with stomach and intestine extracts were 3.63 and 15.35% and for pond farmed fish (669.7 ± 82.6 g), DH values were 4.51 and 10.82%, respectively.

Table 1.3 – Proximate composition (g 100 g^{-1}) of the 24 tested ingredients (as-fed basis). Ingredient information in brackets refers to ingredient type and/or origin.

Test ingredient	IFN[a]	Moisture	Crude Protein[b]	Crude Fiber	Crude Fat	NFE[c]	Ash
Blood meal (Brazil)	5-00-380	5.8	90.6	0.0	0.3	1.0	2.2
Blood meal (USA)	5-00-380	7.2	89.3	0.0	0.2	0.6	2.7
Corn gluten meal (USA)	5-28-242	6.3	68.1	0.2	1.6	21.0	2.7
Corn grain (Brazil)	4-26-023	12.5	9.8	1.7	3.9	70.3	1.9
Cotton seed meal (Brazil)	5-01-621	8.0	41.1	14.8	3.1	27.2	5.8
Feather meal (Brazil)	5-03-795	6.5	82.9	0.0	7.1	0.3	3.2
Feather meal (European Union)	5-03-795	4.6	86.0	0.0	7.3	0.2	1.9
Fish meal (anchovy, Peru)	5-01-985	6.6	71.7	0.0	7.8	0.1	13.8
Fish meal (herring)	5-02-000	4.3	74.5	0.0	8.6	0.0	12.6
Fish meal (mackerel, Chile)	5-01-985	6.3	70.7	0.0	5.0	0.0	17.9
Fish meal (menhaden, USA)	5-02-009	5.7	66.3	0.0	7.7	0.8	19.5
Fish meal (by-product, Brazil)		6.8	61.1	0.1	5.2	4.0	22.8
Fish meal (salmon, Chile)	5-02-012	6.6	75.4	0.0	6.2	0.6	11.2
Poultry by-product meal (feed grade, Brazil)	5-03-798	12.3	61.0	0.2	12.5	0.8	13.1
Poultry by-product meal (feed grade, USA)	5-03-798	4.4	61.2	0.1	17.7	2.6	14.0
Poultry by-product meal (pet-food grade, USA)		3.7	67.4	0.3	13.2	1.5	13.8
Rapeseed meal (European Union)	5-06-145	19.3	28.5	9.8	3.5	33.0	5.9
Soybean meal (solvent extracted, Brazil)	5-20-637	11.3	47.4	6.0	0.5	28.5	6.3
Soybean meal (full fat, USA)	5-04-597	5.9	40.2	4.3	18.4	25.5	5.6
Soybean meal (solvent extracted, USA)	5-04-612	7.0	47.7	2.7	1.0	34.1	7.4
Soy protein concentrate (Brazil)	5-32-183	9.0	59.9	4.3	0.5	20.2	6.1
Wheat bran (Brazil)	4-05-190	9.0	16.7	7.2	3.3	58.9	4.8
Wheat flour (Brazil)	4-05-199	9.2	16.7	0.2	2.1	71.2	1.6
Wheat gluten (USA)	5-05-220	6.4	79.4	0.0	1.3	12.2	0.7

[a] International Feed Number
[b] N x 6.25
[c] Nitrogen-free extract = 100 – (crude protein + crude fiber + crude fat + ash + moisture)

In general, ingredient sample hydrolysis with stomach followed by pyloric ceca/intestine (two-stage DH) tended to be higher compared to results from hydrolysis with only stomach or pyloric ceca/intestine for rainbow trout and Nile tilapia (cage and pond farmed) (Figs. 1.3, 1.5). For rainbow trout, wheat gluten, soybean meal (USA), soy protein concentrate, cotton seed meal, blood meals, poultry by-product meals and feather meals presented the highest two-stage DH values (4.63 – 10.37%) and no significant difference (P>0.05) was observed for DH among fish meals (average 6.90 ± 0.43%). With cage farmed Nile tilapia, soybean meal (USA), soy protein concentrate, cotton seed meal and blood meal (Brazil) presented the highest two-stage DH values (5.92 – 8.86%). For pond farmed Nile tilapia, highest two-stage DH were determined with soy protein concentrate, cotton seed meal, blood meal (Brazil), poultry by-product meal (feed grade) and poultry by-product meal (pet food grade) (4.67 – 6.67%). For cobia, such trend could be observed in half of the tested ingredients, especially with fish meals (Fig. 1.4). High ingredient DH with stomach extract did not result in increased two-stage DH for soybean meal full-fat (rainbow trout), poultry by-product meals (cage farmed Nile tilapia), poultry by-product meal (pet food grade) (pond farmed Nile tilapia) and soybean meals, wheat gluten, blood meal (Brazil), poultry by-product meal (feed grade), poultry by-product meal (pet food grade), fish meal (menhaden) (cobia). Additionally, two-stage DH did not differ from hydrolysis with pyloric ceca extract for corn gluten meal, rapeseed meal, poultry by-product meal (feed grade) (rainbow trout), soybean meals (cobia), soybean meal full-fat, corn gluten meal, wheat flour (cage farmed Nile tilapia), soybean meal full-fat, soybean meal (Brazil), feather meals, poultry by-product meal (feed grade) and fish meals (pond farmed Nile tilapia). In general, ingredient two-stage DH from cage and pond farmed Nile tilapia followed a similar hydrolysis pattern (Fig. 1.5A, B).

Ingredient particle size distribution (Fig. 1.6) indicated wheat flour and wheat gluten were the ingredients with finest particle size (<250 μm), followed by blood meals, whereas soy protein concentrate and poultry by-product meal (pet food grade) were the coarser with more than 20% of particles >1000 μm. Fish meals particle size was mostly <500 μm. In general, ingredients particle size was <1000 μm but no clear relation between ingredient particle size distribution and DH could be detected in this study.

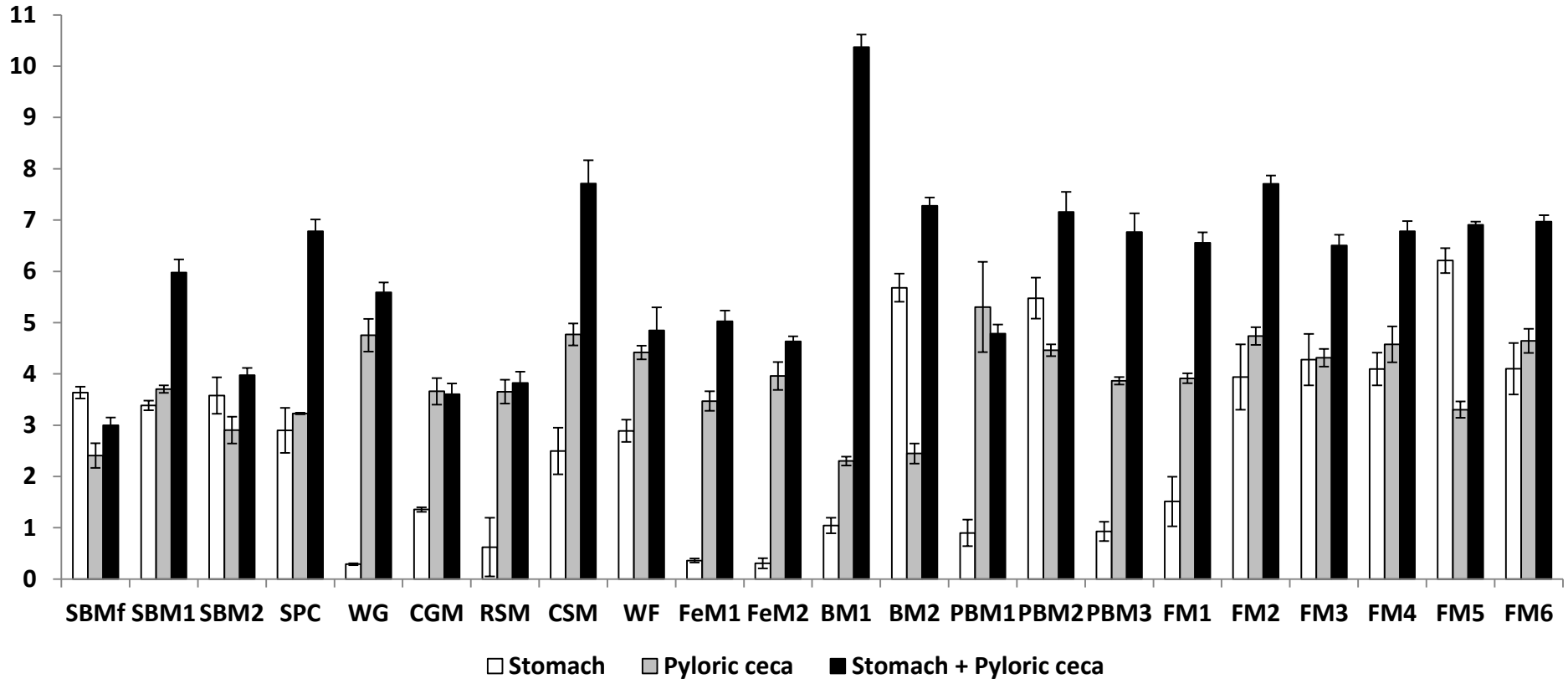

Figure 1.3 – *In vitro* pH-stat degree of protein hydrolysis (DH) of feed ingredients using digestive enzyme extracts from fed 400.5 ± 37.9 g rainbow trout, *Oncorhynchus mykiss*. Ingredients (80 mg crude protein per assay) were hydrolyzed for 60 min with (i) stomach, or (ii) pyloric ceca or 120 min with (iii) stomach (60 min) plus pyloric ceca (60 min) extracts at 25 ± 0.2 °C. SBMf: soybean meal full-fat; SBM1: soybean meal, USA; SBM2: soybean meal, Brazil; SPC: soy protein concentrate, Brazil; WG: wheat gluten, USA; CGM: corn gluten meal, USA; RSM: rapeseed meal, European Union; CSM: cotton seed meal, Brazil; WF: wheat flour, Brazil; FeM1: feather meal, Brazil; FeM2: feather meal, European Union; BM1: blood meal, Brazil; BM2: Blood meal, USA; PBM1: poultry by-product meal (feed grade), Brazil; PBM2: poultry by-product meal (pet food grade), USA; PBM3: poultry by-product meal (feed grade), USA; FM1: fish meal (by-product), Brazil; FM2: fish meal (anchovy), Peru; FM3: fish meal (herring); FM4: fish meal (mackerel), Chile; FM5: fish meal (menhaden), USA; FM6: fish meal (salmon), Chile. Results shown as mean (vertical bars) and s.d. (error bars), n = 4.

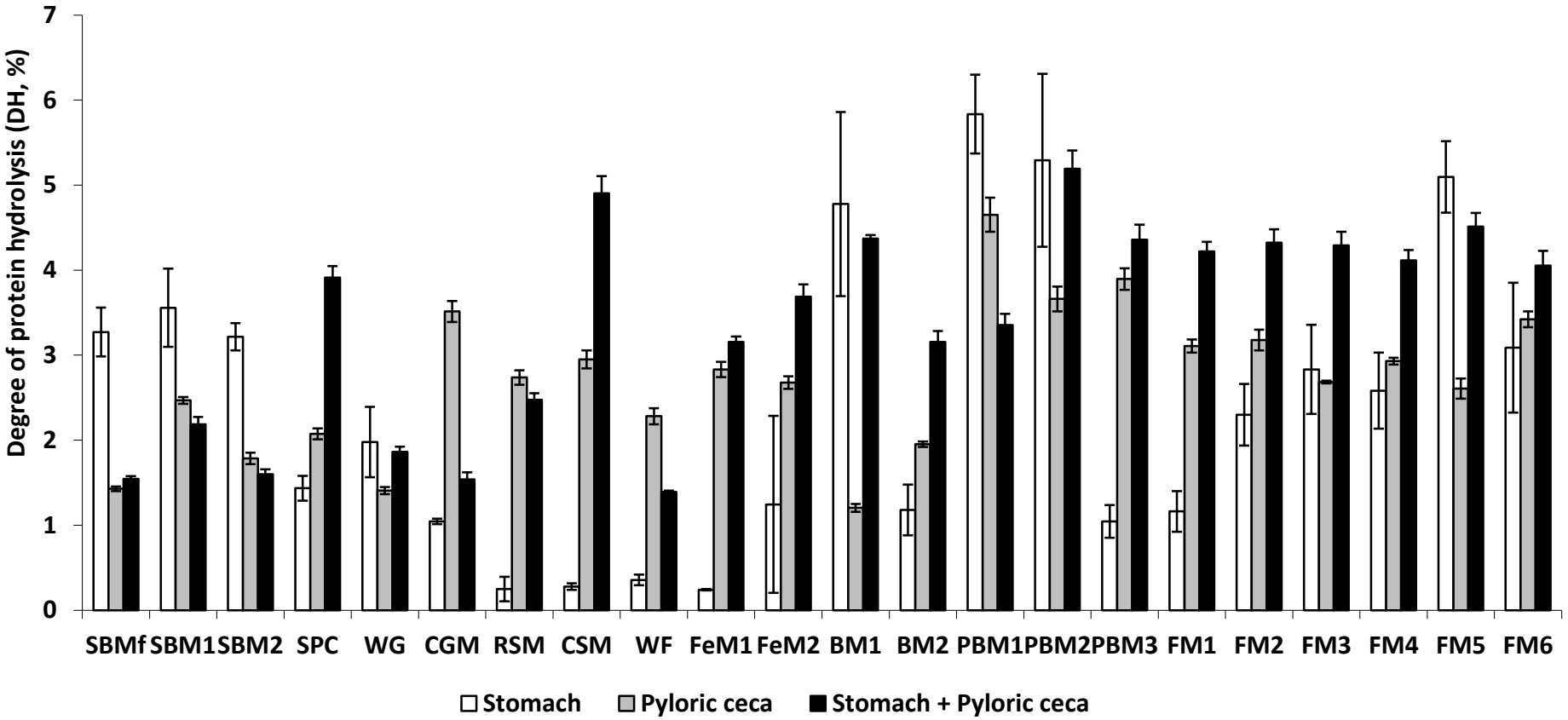

Figure 1.4 – *In vitro* pH-stat degree of protein hydrolysis (DH) of feed ingredients using digestive enzyme extracts from fed 542.80 ± 182.1 g cobia, *Rachycentron canadum*. Ingredients (80 mg crude protein per assay) were hydrolyzed for 60 min with (i) stomach, or (ii) pyloric ceca or 120 min with (iii) stomach (60 min) and pyloric ceca (60 min) extracts at 25 ± 0.2 °C. SBMf: soybean meal full-fat; SBM1: soybean meal, USA; SBM2: soybean meal, Brazil; SPC: soy protein concentrate, Brazil; WG: wheat gluten, USA; CGM: corn gluten meal, USA; RSM: rapeseed meal, European Union; CSM: cotton seed meal, Brazil; WF: wheat flour, Brazil; FeM1: feather meal, Brazil; FeM2: feather meal, European Union; BM1: blood meal, Brazil; BM2: Blood meal, USA; PBM1: poultry by-product meal (feed grade), Brazil; PBM2: poultry by-product meal (pet food grade), USA; PBM3: poultry by-product meal (feed grade), USA; FM1: fish meal (by-product), Brazil; FM2: fish meal (anchovy), Peru; FM3: fish meal (herring); FM4: fish meal (mackerel), Chile; FM5: fish meal (menhaden), USA; FM6: fish meal (salmon), Chile. Results shown as mean (vertical bars) and s.d. (error bars), n = 4.

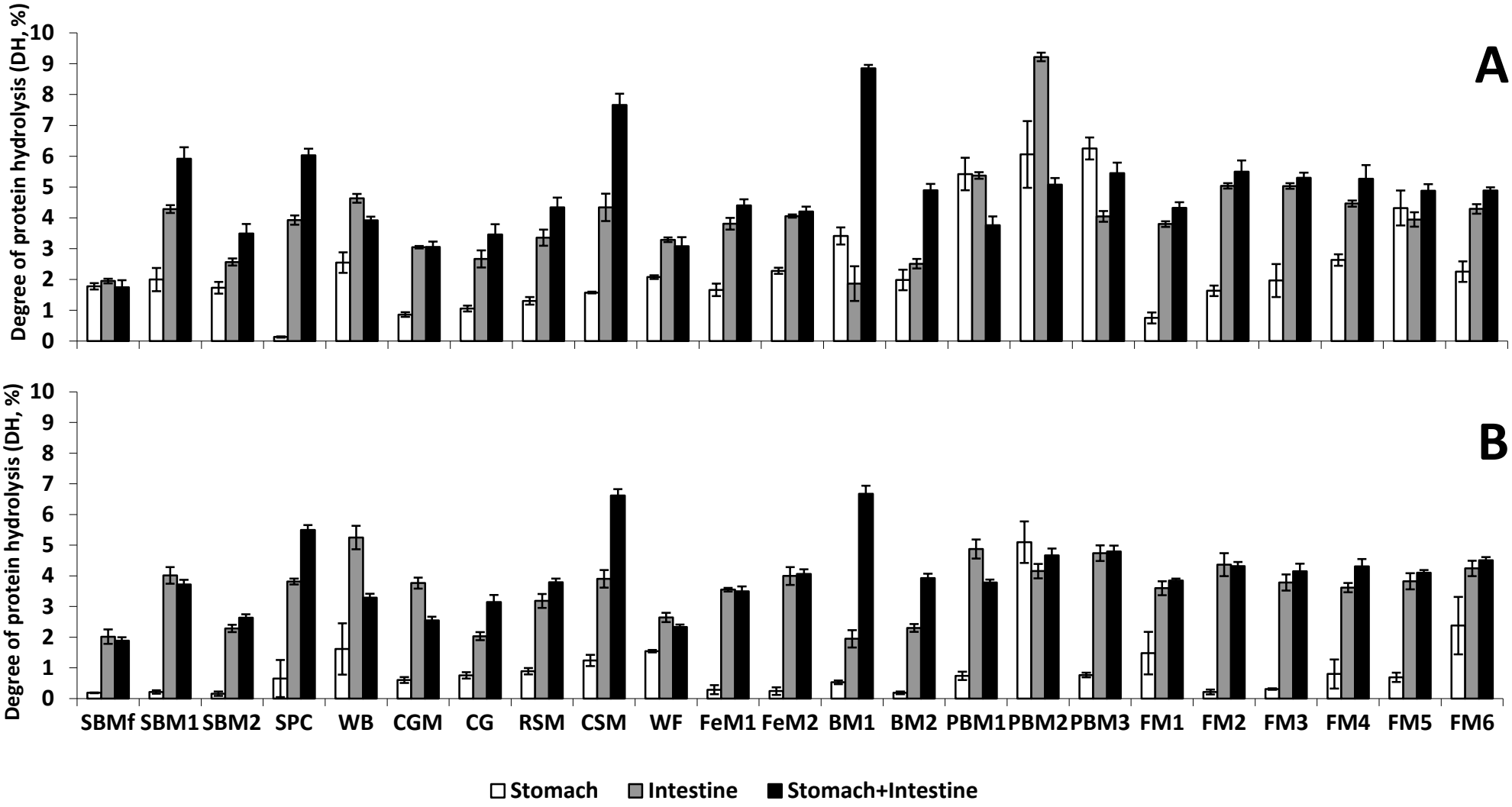

Figure 1.5 – *In vitro* pH-stat degree of protein hydrolysis (DH) of feed ingredients using digestive enzyme extracts from fed (A) cage farmed 573.0 ± 89.5 g and (B) pond farmed 696.7 ± 85.1 g Nile tilapia, *Oreochromis niloticus*. Ingredients (80 mg crude protein per assay) were hydrolysed for 60 min with (i) stomach, or (ii) intestine or 120 min with (iii) stomach (60 min) plus intestine (60 min) extracts at 25 ± 0.2 °C. SBMf: soybean meal full-fat, USA; SBM1: soybean meal, USA; SBM2: soybean meal, Brazil; SPC: soy protein concentrate, Brazil; CG: corn grain, Brazil; CGM: corn gluten meal, USA; RSM: rapeseed meal, European Union; CSM: cotton seed meal, Brazil; WB: wheat bran, Brazil; WF: wheat flour, Brazil; FeM1: feather meal, Brazil; FeM2: feather meal, European Union; BM1: blood meal, Brazil; BM2: Blood meal, USA; PBM1: poultry by-product meal (feed grade), Brazil; PBM2: poultry by-product meal (pet food grade), USA; PBM3: poultry by-product meal (feed grade), USA; FM1: fish meal (by-product), Brazil; FM2: fish meal (anchovy), Peru; FM3: fish meal (herring); FM4: fish meal (mackerel), Chile; FM5: fish meal (menhaden), USA; FM6: fish meal (salmon), Chile. Results shown as mean (vertical bars) and s.d. (error bars), n = 4.

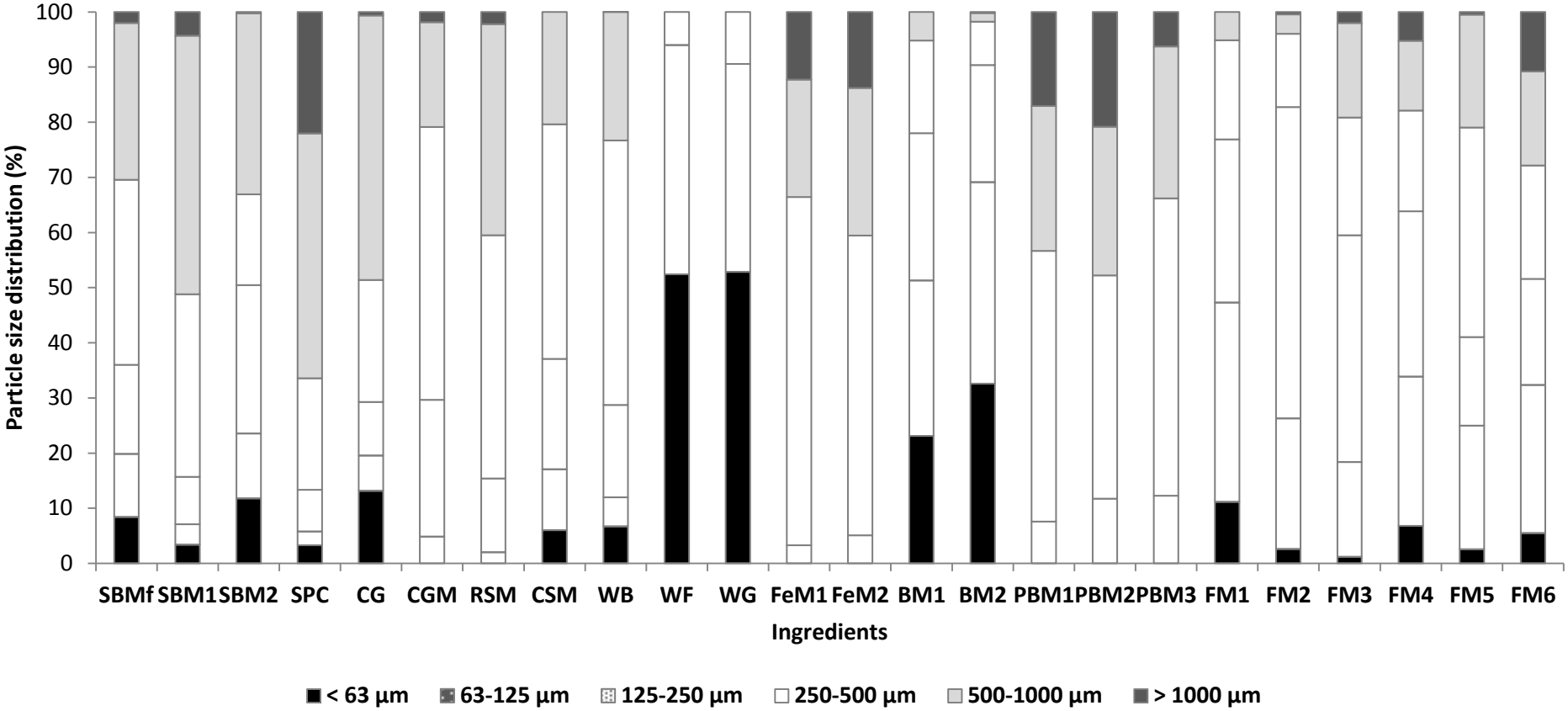

Figure 1.6 – Particle size distribution (%) for each of the 24 ingredients tested. Ingredients were processed 'as is'. Particle size ranges were <63 µm, 63 – 125 µm, 125 – 250 µm, 250 – 500 µm, 500 – 1000 µm, and > 1000 µm. SBMf: soybean meal full-fat, USA; SBM1: soybean meal, USA; SBM2: soybean meal, Brazil; SPC: soy protein concentrate, Brazil; CG: corn grain, Brazil; CGM: corn gluten meal, USA; RSM: rapeseed meal, European Union; CSM: cotton seed meal, Brazil; WB: wheat bran, Brazil; WF: wheat flour, Brazil; WG: wheat gluten, USA; FeM1: feather meal, Brazil; FeM2: feather meal, European Union; BM1: blood meal, Brazil; BM2: Blood meal, USA; PBM1: poultry by-product meal (feed grade), Brazil; PBM2: poultry by-product meal (pet food grade), USA; PBM3: poultry by-product meal (feed grade), USA; FM1: fish meal (by-product), Brazil; FM2: fish meal (anchovy), Peru; FM3: fish meal (herring); FM4: fish meal (mackerel), Chile; FM5: fish meal (menhaden), USA; FM6: fish meal (salmon), Chile.

1.4. Discussion

The use of species-specific crude enzyme extracts for *in vitro* digestion methods may be important since catalytic output may differ significantly among species (DIMES et al., 1994b; HAMDAN et al., 2009; LEMOS et al., 2004; MÁRQUEZ et al., 2013) and the donor organism (weight, age, feeding status, farming system) and habitat (water salinity, natural productivity) (BASSOMPIERRE, et al., 1998; DIMES; HAARD, 1994; LEMOS; NUNES, 2008; RUNGRUANGSAK-TORRISSEN et al., 2002). The consistency of *in vitro* techniques employing species-specific digestive enzymes depends on the standardization of the hydrolytic capacity of the recovered extract. Most commonly, extracts have been standardized to the activities of trypsin and or chymotrypsin (BASSOMPIERRRE, et al., 1997b; CHONG et al., 2002; DIMES; HAARD, 1994; GRABNER, 1985; RUNGRUANGSAK-TORRISSEN, et al., 2002; TIBBETTS et al., 2011a), or total alkaline proteinase (ALARCÓN, et al, 2002; EL-SAYED, et al., 2000; EZQUERRA et al., 1997; HAMDAN, et al., 2009; LEMOS et al., 2009; MÁRQUEZ et al., 2013). The present study introduces the degree of protein hydrolysis (DH) as a standardization method using analytical grade proteinaceous substrates under the same conditions (pH-stat) as of the *in vitro* digestion assays. Advantages of this approach would be the non-inclusion of buffers or other extra chemicals, stable pH during hydrolysis, and precision (average c.v. 4%). The adjusted function of DH output at fixed substrate protein quantity *versus* fish enzyme extract volume followed a similar trend (log function), either for stomach or pyloric ceca/intestine enzymes, regardless of the species or condition of the donor individuals (weight, feeding status, or farming system) (Fig. 1.2; Table 1.2). From such models, the volume of enzyme extract from different batches or species that may result in the same hydrolytic capacity (DH) may be obtained and then used for comparable determination of *in vitro* digestion of a given feedstuff. These methods of standardization according to digestive capacity may be more adequate than considering e.g. a fixed volume of enzyme extract to be used in the assays (DIVAKARAN et al., 2004; LAN; PAN, 1993).

For most of the ingredients tested incubation with stomach enzyme extracts prior to hydrolysis with pyloric ceca/intestine extracts (two-stage) resulted in higher *in vitro* DH values when compared to single stage hydrolysis (only stomach or pyloric ceca/intestine extract) (Figs. 1.3, 1.4 and 1.5). *In vivo*, the role of the stomach in protein digestion is to initiate breaking down the food, partially hydrolyzing proteins into peptides and/or mechanical disruption *via* muscular contraction in preparation for further hydrolysis in the

intestine (ALARCÓN et al., 2002; BASSOMPIERRE et al., 1997b; CLEMENTS; RAUBENHEIMER, 2006; HAMDAN et al., 2009). Gastric digestion could also increase the speed of intestinal hydrolysis, leading to a significant shift from soluble polypeptides to oligo- and dipeptides (GRABNER; HOFER, 1985, 1989) and potentially increasing availability of soluble protein by inactivation of protease inhibitors by low pH and/or pepsin activity (HAMDAN et al., 2009; PEDERSEN; EGGUM, 1983). This may be the case of some soybean meals tested that showed higher two-stage DH values (Figs. 1.3, 1.4 and 1.5). However, the meaning of measuring quantitatively the *in vitro* DH value of practical ingredients with fish stomach enzymes seems not well understood. For some of the ingredients, e.g., rapeseed meal and feather meal (European Union) for cobia and rainbow trout; soy protein concentrate, wheat bran, feather meals, fish meal (mackerel) for pond farmed Nile tilapia, the coefficient of variation in DH with stomach extracts was very high (> 50%), which may be due to the fact that they were comparatively coarser and less soluble than the substrate used for standardization (hemoglobin), a purified, highly soluble and digestible product (DÍAZ-LÓPEZ et al., 1998). Thus, present fish stomach enzymes seem to be sensible to variation in the purity of protein substrate but further studies are necessary to understand the significance of measuring the DH value with stomach enzyme extract on the protein hydrolysis of a feedstuff. The DH of tested ingredients with digestive enzymes from cage and pond farmed Nile tilapia followed the same trend, describing a significant correlation and a high determination coefficient regression, as verified in shrimp (*Litopenaeus vannamei*) under clear water *versus* pond conditions (LEMOS; NUNES, 2008).

The *in vitro* pH-stat protein digestion assays of the present study were standardized adopting conditions that may not exactly simulate digestion in some fish species. Ingredient incubation time, temperature, and enzyme:substrate ratio were chosen considering previous studies (LEMOS et al., 2009; MORALES; MOYANO, 2010), laboratory convenience and output rates. Since assay temperature and salinity may not be natural for rainbow trout and cobia, respectively, ingredient DHs should not be compared between these species. On the other hand, the quantitative meaning of DH enables the differentiation of ingredients according to their potential to be digested and also the comparison between feedstuffs digestion by different species. Further aspects regarding assay conditions should be considered for the consistency of the *in vitro* determination. Sample preparation step to stabilize pH ensures that variation in pH during hydrolysis is due only to the enzyme activity and not to any possible sample instability (DIMES; HAARD, 1994; LEMOS et al., 2009;

PEDERSEN; EGGUM, 1983). Accordingly, ingredient particle size plays a significant role in aqua feed manufacturing (CHAMBERLAIN; BORTONE, 2006; TACON, 1988) and may affect feed digestion. Unfortunately, the influence of the ingredient particle size distribution on DH could not be detected in the present study, as individual particle size assays were not performed.

The pH-stat application to determine *in vitro* digestion with species-specific enzymes has shown to be a fast and precise method with reported use for different aquatic species such as fish (DIMES et al., 1994a,b; TIBBETTS et al., 2011a), shrimp (EZQUERRA et al., 1997; LEMOS et al., 2009), and mollusks (AGUILLAR, et al., 2012). Significant relationship between *in vivo* apparent protein digestibility and *in vitro* DH have been reported for some feedstuffs using enzyme extracts from digestive organs of the target species (Table 1.4), indicating DH as capable of predicting digestibility in the live animal. Nevertheless, the correlation between *in vivo* apparent protein digestibility and *in vitro* DH digestion may be essentially dependent on the consistency and experimental details of feeding trials involved in the determination of apparent digestibility (FULLER, 1991; TACON, 1996). This includes mainly (a) the formulation of experimental diets, types and inclusion levels of test ingredients, (b) the feeding regime, including feeding method and strategies for feces collection, to minimize nutrient leaching, and (c) applied research and development focused on specific practical raw materials, manufacturing and farming conditions. Apart from correlation and method validation, the pH-stat *in vitro* DH technique may be important to qualify or distinguish raw materials, indicating how much it could be potentially hydrolyzed in the fish digestive tract and, to a certain extent, the availability for absorption. The complex nature of food digestion in the live fish may not be easily replicated and it may also not be strictly required for consistent screening of feed protein quality, as demonstrated for farmed land and aquatic species (BASSOMPIERRE et al., 1997b; BOISEN; EGGUM, 1991; BOISEN; FERNÁNDEZ, 1995; DIMES et al., 1994a,b; DONG; HARDY, 2000; EZQUERRA et al., 1997; HARDY; BARROWS, 2002; PEDERSEN; EGGUM, 1983; SHIPTON; BRITZ, 2002; TIBBETTS et al., 2011b). The DH has emerged with potential as accessory method in quality control of raw materials in the aqua feed manufacturing industry (DE MUYLDER et al., 2008; LEMOS; TACON, 2011). For feed manufacturers, the DH could assist ranking protein from different ingredients or a certain ingredient from different suppliers, as an additional criterion for the quality control of aqua feeds. It could be helpful also in the preliminary

assessment of the effectiveness of feed additives (e.g. exogenous proteases) on the nutritional value of raw materials (GONZÁLEZ-ZAMORANO et al., 2013).

Table 1.4 – Prediction of *in vivo* apparent protein digestibility (APD) of feed ingredients by *in vitro* pH-stat degree of protein hydrolysis (DH) using digestive enzyme extract from fish (rainbow trout, *Oncorhynchus mykiss*, haddock, *Melanogrammus aeglefinus*, Atlantic cod, *Gadus morhua*) and whiteleg shrimp (*Litopenaeus vannamei*). R^2= coefficient of determination between *in vivo* APD and *in vitro* DH. n = number of feed ingredients tested.

Species	R^2	n	Ingredient [a]	Reference
Rainbow trout	0.87	5	FM	Dimes et al. (1994a)
	0.94	8	FM, MBM, FeM, PBM, BM	El-Mowafi et al. (2000)
	0.81	8	FM	Lemos and Yasumaru (2010)
Haddock	0.80	7	FM, CrM, ShM, SBM, CM, CGM, CAS	Tibbets et al. (2002)
Atlantic cod	0.61-0.99	21	FM, PBM, FeM, SBM, SPC, SPI, KM, CrM, SM, CM, CPC, FSM, CGM, WG, PPC, WLM	Tibbets et al. (2011b)
Whiteleg shrimp	0.81	7	FM, SBM, LM	Ezquerra et al. (1998)
	0.69	6	KH, FH, SM	Córdova-Murueta and Garcia Carreño (2002)
	0.86	26	BM, CAS, CGM, CrM, DDGS, FeM, FM, GEL, KM, KF, PBM, SBM, SPI, SM, WG	Lemos et al. (2009)

[a] BM: blood meal, CAS: casein, CGM: corn gluten meal, CM: canola meal, CPC: canola protein concentrate, CrM: crab meal, DDGS: distiller's dried grains with solubles, FeM: feather meal, FH: fish hydrolysate, FM: fish meal, FSM: flaxseed meal, GEL: gelatin, KF: krill flour, KH: krill hydrolysate, KM: krill meal, LM: langostilla meal, MBM: meat and bone meal, PBM: poultry by-product meal, PPC: pea protein concentrate, SBM: soybean meal, ShM: shrimp meal, SM: squid meal, SPC: soy protein concentrate, SPI: soy protein isolate, WG: wheat gluten, WLM: white lupin meal.

The regular use of DH by the industry as a standard method may be still restrained by the source of the enzyme extract, which is obtained from individuals of the target species. More practical sources and analytical routines should also be developed, e.g., easy-to-handle freeze-dried crude extracts and products to be supplied for prompt use in the feed industry. Additional studies should include the determination of a possible relationship between *in vitro* pH-stat DH and individual amino acid availability. Protein digestibility may not be the only factor that affects feed quality, but objective assessment of feedstuffs are valuable inputs for aquaculture nutritionists in using different raw materials, achieving adequate, cost-effective, and sustainable species-specific feed solutions (GLENCROSS, et al, 2007).

1.5. Conclusions

The *in vitro* pH-stat species-specific method to determine the degree of protein hydrolysis (DH) has shown to be a useful tool to distinguish the raw materials available for feed production. The hydrolytic capacity of fish enzyme extracts could be standardized by the DH in the same conditions (pH-stat) as of the *in vitro* digestion assays. The significance of quantitatively measuring the DH with stomach enzyme extract is still not well understood but, overall, the pre-hydrolysis of feedstuffs with stomach enzymes contributed to elevate pyloric ceca/intestine DH. Ingredient DH presented distinct patterns and may be compared among different species when enzyme extracts are originated from individuals reared under practical conditions. Assay conditions e.g. temperature, medium salinity, incubation time, and enzyme:substrate ratios according to the fishes physiological status should be taken into account for the consistency of the *in vitro* determination. Routine use of the method may yet be dependent on the prompt availability of more practical sources of enzyme extracts.

Chapter 2

Soluble phosphorus released by *in vitro* (pH-stat) digestion of commercial feeds for Nile tilapia, *Oreochromis niloticus* (L.)

Abstract

The objective of this study was to determine the amount of soluble phosphorus (P) released from feeds submitted to *in vitro* digestion with stomach, intestine, and stomach followed by intestine (two-stage) enzyme extracts. Nine commercial feeds from different manufacturers with similar crude protein content (32%, label value) and pellet size (4-6 mm) were tested. Digestive enzyme extracts were recovered from growing Nile tilapia, *Oreochromis niloticus*, commercially farmed in cages in a freshwater reservoir (fed individuals, 598.3 ± 110.1 g, n=10). Release of soluble P (%) was determined after feed samples were incubated in distilled water (pH 6.4 ± 0.4), in distilled water at pH 2.0, in distilled water at pH 8.0, and after feed digestion with stomach and intestine extracts separately and sequentially (two-stage digestion). The amounts of enzyme extracts tested were 50, 100 and 200 μL. Values were compared between commercial feeds and between digestion procedures. Correlations between feed total P content and *in vitro* release of soluble P were also tested. In general, released soluble P with stomach digestion was higher (9.85 – 15.22%) when compared to intestine digestion (0.55 – 6.78%) or two-stage digestion (3.49 – 10.58%). Solubility of P appeared to be more related to medium pH rather than enzymatic hydrolysis. Significant correlations ($P<0.05$) were observed between feed total P and soluble P released in distilled water and between soluble P released in distilled water and soluble P released in intestinal digestion. This *in vitro* method was found reproducible as variation was low (< 5% c.v.). Further studies would be warranted to determine any correlation with *in vivo* P digestibility values.

2.1. Introduction

Although fish have the ability to absorb soluble phosphorus (P) through the skin, fins and gills, the concentration of phosphates in fresh and sea water is low, thus, phosphorus requirements for growth and metabolism are usually satisfied from dietary sources (TACON, 1988; LALL, 2002). In compound feeds, phosphorus may derive from animal and plant ingredients and also from inorganic supplements, with varying levels of P digestibility. In ingredients of animal origin, usually by-products, P is present primarily as calcium phosphate (hydroxyapatite), which is partially digestible to fish, depending on digestive physiology, i.e., with or without acid and gastric secretions (SATOH et al., 1992; SUGIURA et al., 2006). Animal organic P bound to protein, lipids and sugar might be highly digestible as they may be hydrolyzed by alkaline phosphatases (HUA; BUREAU, 2006; MORALES; MOYANO, 2010). In ingredients of plant origin, most (>60%) of the total P is present as phytate, predominantly as magnesium and potassium salt of phytic acid (myo-inositol hexaphosphate) (RAVINDRAN et al., 1994; RAVINDRAN et al., 1995, SELLE et al., 2012), which is poorly digested by fish by the lack of phytase, the enzyme that is able to hydrolyze phytic acid (LALL, 2002). Digestibility of inorganic P is usually affected by their solubility (SATOH et al., 1992; SATOH et al., 1997; HUA; BUREAU, 2006).

In vitro methods can be useful tools in predicting nutrient digestibility and also variation in digestibility of feed ingredients and may vary from simple solubility tests to simulation of the digestive process (VAN DER KLIS; PEDERSEN, 2010). There are some studies on *in vitro* phosphorus (P) solubility or availability from ingredients for monogastric animals such as pigs and poultry (LIU et al., 1997, 1998; BOLLINGER et al., 2004; ZYLA et al., 1995). For fish, *in vitro* P solubility has been tested by fractionated extraction with deionized water, acetic acid and hydrochloric acid and compared to *in vivo* P availability for rainbow trout, *Oncorhynchus mykiss*, and common carp, *Cyprinus carpio* (SATOH et al., 1992, 1997). Also, *in vitro* P digestibility was assessed by mimicking digestion of feedstuffs with stomach and/or pyloric ceca enzyme extracts from rainbow trout (WEERASINGHE et al. 2001; MORALES; MOYANO, 2010). However, those studies with fish digestive enzymes were performed at a temperature (37 °C) above physiological in an attempt to reduce reaction time, and tested only as individual feedstuffs not as compound feeds. Nevertheless, the major challenge in developing an *in vitro* method for P digestion is the different properties of plant and animal sources of ingredients (WEERASINGHE et al., 2001).

Nile tilapia, *Oreochromis niloticus*, was chosen for this study due to its relevance in the aquaculture industry. Nile tilapia is the most aquacultured species in Brazil (287,000 tonnes) and third globally (3.2 million tonnes), being geographically the most wide spread species for aquaculture production (approximately 140 countries and territories) (FAO, 2014). The estimated requirement of total P for Nile tilapia may vary from 0.3 to 1.10% (FURUYA et al., 2008; NRC, 2011; QUINTERO-PINTO et al., 2011; YAO et al., 2014) and from 0.46 to 0.75% available P (QUINTERO-PINTO et al., 2011) – requirement being higher at initial stages and gradually reducing with fish growth and available P for maximum bone mineralization are higher than that for growth (QUINTERO-PINTO et al., 2011). P in aquaculture effluents is considered a significant source of eutrophication of freshwater ecosystems (SUGIURA et al., 2004). Considering that compound feeds for Nile tilapia contain animal, plant and inorganic supplements of P, usually in excess to meet requirement for growth and metabolism, and that levels above requirement are excreted either as solid waste in feces or soluble P through urine and gills, aquaculture is required to reduce the amount of P in its effluents (SUGIURA et al., 2004). Although it is known that bioavailability of minerals, including P, is affected by many factors, such as intake level, chemical form, digestibility, particle size, interactions with other nutrients, chelators, inhibitors, physiological and pathological states of the animal, water chemistry, feed processing, and fish species (LALL, 2002).

The objective of this study was to assess the amount of soluble P released after *in vitro* (pH-stat) digestion procedures by incubating commercial feeds with digestive enzyme extracts recovered from Nile tilapia, i.e., stomach and intestine enzyme extracts, separately and sequentially (two-stage) at 25 °C. Soluble P was measured as reactive P or orthophosphate, i.e., the form in which P is absorbed by the fish. For such, samples were submitted only to the hydrolysis by digestive enzymes and not to further dry or wet ashing with strong concentrated acids (nitric, perchloric or hydrochloric acid). Amount of soluble P released were compared between compound feeds according to digestion procedure and amount of enzyme extract used. Also, relationships between feed total P and soluble P released after *in vitro* digestions; and between soluble P released after *in vitro* digestion with stomach or intestine extracts and soluble P released after a two-stage digestion (stomach followed by intestine) were determined.

2.2. Materials and Methods

2.2.1. Crude enzyme extract

Stomach and intestine crude enzyme extracts were recovered from Nile tilapia, *Oreochromis niloticus* (fed individuals, 598.3 ± 110.1 g, n=10) commercially farmed in cages in a freshwater reservoir in Paranapanema river, Ipaussu, São Paulo. Fish were killed by pithing (driving a sharp spike into the brain) and digestive organs removed. Stomach and intestine were emptied, cleansed with distilled water, pooled, immediately frozen and transported to the laboratory. Enzyme extracts were obtained by adapting method developed for marine shrimp (GARCÍA-CARREÑO et al., 1997). Pooled stomach samples were allowed to thaw partially and then homogenized (T25 digital Ultra-Turrax®, 18G dispersing element, IKA WORKS, Inc., Wilmington, NC, USA) with cold distilled water (1:3, w/v) in glass beakers on ice. Homogenate was centrifuged in 1.5 mL microcentrifuge tubes at 16,800 × g for 30 min at 4 °C (Centrifuge 5417R, Eppendorf, Hamburg, Germany). The supernatant, i.e., stomach crude enzyme extract was recovered and pooled in glass beakers on ice and pH adjusted to 2.0 by adding HCl 0.1 N. For such, pipette tip was carefully introduced in the centrifuge tubes through the thin lipid layer and liquid aspired with a 1-mL pipette. Pooled intestine sample was homogenized with cold distilled water (1:1, w/v), centrifuged and supernatant recovered equally as done with stomach sample. The pH of the recovered intestine crude enzyme extract was adjusted to 8.0 by adding NaOH 0.1 N. Stomach and intestine enzyme extracts were stored in 2-mL polypropylene cryogenic vials at – 20 °C until used.

2.2.2. Commercial feed samples

Nine commercial extruded feeds from different manufacturers with pellet size 4 – 6 mm for Nile tilapia in grow-out phase were purchased in the local market. The nine brands were the most commonly used by commercial Nile tilapia farmers in the São Paulo state region (in alphabetical order: Acqua Fish – Supra Alisul; Acqua Pesca 32 – Matsuda; API Peixe – Malta Cleyton; Fri-Aqua – Fri-Ribe Nutreco; Laguna – Socil; Nutripiscis – Presence; Pirá Ideal – Guabi; Poli-Peixe – Polinutri; Tilápia Crescimento G320AM – Amicil). The values informed in the feed labels were 32% crude protein (min), 8 - 13% moisture (max), 4 - 7% ether extract (min), 6 - 10% crude fiber (max), 10.0 - 12.5% ash (max), and 0.4 - 1.2%

total phosphorus (min). Proximate analysis of the nine commercial feeds is presented in Table 1. Analysis was done based on the AOAC methods described in Silva and Queiroz (2009). Dry matter was determined by oven drying at 105 °C for 15 hours (overnight), ash by incineration in muffle furnace at 600 °C for 6 h, and crude fiber by acid and alkaline hydrolysis with 1.25% sulfuric acid and 1.25% potassium hydroxide, respectively. For crude fat, extruded feed samples were submitted to acid hydrolysis with ethanol and 8 N HCl for 40 min at 80 °C with shaking prior to ether extraction and gravimetric determination (SINDIRAÇÕES, 2009). For the calculation of crude protein (N x 6.25), nitrogen content was determined in an elemental analyzer (Elemental Analyzer 2400 CHN, Perkin Elmer, Waltham, MA, USA). Briefly, 2 mg of sample was completely oxidized at 950 °C in excess of pure oxygen; the gases produced were carried by helium gas through a metallic copper reduction column, and the elements separated by gas chromatography using a thermal conductivity detector, and result calculated as percentage. Feed total phosphorus (P) was determined by submitting sample to acid digestion with concentrated HNO_3 in microwave oven at 160 °C for 20 min and analyzed by inductively coupled plasma optic emission spectrophotometer (ICP-OES, Arco, Spectro, Kleve, Germany). Nitrogen-free extract was calculated by difference. Analysis was performed in duplicate, except for crude protein (single analysis) and results expressed as percentages.

Table 2.1 – Proximate composition (%, 'as fed' basis) of the nine commercial extruded feeds for Nile tilapia, *Oreochromis niloticus*, tested for the *in vitro* release of soluble phosphorus using enzyme extracts from stomach and intestine of Nile tilapia (fed individuals, 598.3 ± 110.1 g, n=10).

Feed	Moisture	CP	CF	EE	NFE	Ash	Total P
R1	9.0 (0.00)	28.5	4.9 (0.03)	5.9 (0.14)	41.6	10.1 (0.04)	1.5 (0.03)
R2	9.8 (0.06)	26.3	4.9 (0.14)	6.3 (0.13)	44.6	8.1 (0.08)	0.9 (0.00)
R3	10.3 (0.01)	28.9	2.7 (0.05)	7.7 (0.05)	40.9	9.5 (0.07)	1.3 (0.05)
R4	6.7 (0.01)	33.8	3.8 (0.03)	7.9 (0.09)	37.2	10.6 (0.09)	1.6 (0.00)
R5	7.5 (0.01)	31.8	5.5 (0.14)	6.0 (0.10)	39.1	10.1 (0.12)	1.2 (0.00)
R6	6.7 (0.03)	34.0	4.6 (0.20)	7.3 (0.07)	38.5	8.9 (0.07)	1.2 (0.05)
R7	8.8 (0.01)	27.6	3.8 (0.05)	6.9 (0.02)	45.4	7.5 (0.17)	0.9 (0.00)
R8	4.8 (0.11)	30.3	5.9 (0.03)	8.1 (0.17)	39.1	11.8 (0.16)	1.4 (0.01)
R9	8.0 (0.01)	30.7	3.7 (0.01)	6.7 (0.06)	40.2	10.7 (0.16)	1.5 (0.01)

CP, crude protein (N x 6.25); CF, crude fiber; EE, ether extract; NFE, nitrogen free extract – calculated by difference (NFE = moisture + CP + CF + EE + ash); P, phosphorus. Value represents mean (s.d.) of duplicate analysis, except for crude protein (single analysis) and nitrogen free extract (calculated from means).

2.2.3. In vitro *(pH-stat) digestion and soluble phosphorus determination*

The *in vitro* pH-stat procedure was designed to simulate digestion in fish by incubating feed with enzyme extracts recovered from fish stomach and intestine. Assays consisted of feed digestion with stomach and intestine enzyme extracts separately and a combined two-stage digestion with stomach digestion followed by intestine digestion. The assays were carried out under stable pH using two automated titrators (Titrando 836, Titrando 907, Metrohm AG, Switzerland), connected to a single controlling and data logging software (Tiamo™ v. 2.3, Metrohm AG, Switzerland). A diagram of the *in vitro* pH-stat digestion and soluble phosphorus (P) determination is presented in Figure 1. The amount of total P (3.33 mg) was calculated based on the average crude protein and total P content in the feeds in relation to the amount of CP (80 mg) in the protein hydrolysis assay. For the digestion with stomach enzyme extract, ground feed samples containing 3.33 mg total phosphorus (P) was stirred in distilled water and the pH adjusted to 2.0 by the addition of HCl 0.1 N. After 30 min of pH stabilization of the solution, stomach enzyme extract was added and the mixture was incubated at 25 ± 0.5 °C for 60 min. During this digestion stage HCl 0.1 N was automatically added by the titrators to maintain pH stable at 2.0. For digestion with intestine extract, the same protocol was followed except for the addition of NaOH 0.1 N to maintain pH set at 8.0.

For the two-stage digestion, the procedure for digestion with stomach extract was immediately followed by the procedure for digestion with intestine extract, i.e., reaction pH was kept stable at 2.0 for 30 min, stomach enzyme extract added and sample digested at pH 2.0 for 60 min, then pH was adjusted to 8.0, kept stable for 30 min, intestine enzyme extract added and sample digested for 60 min. Three volumes of stomach and intestine enzyme extracts were tested (50, 100 and 200 µL). In the two-stage digestion, the same amounts of stomach and intestine enzyme extracts were added (50-50, 100-100 and 200-200 µL). Feed samples incubated with distilled water without adding enzyme extract were also run in pH 2.0, pH 8.0, and at pH 2.0 followed by pH 8.0. Feed samples with distilled water without enzyme extract and without pH adjustment were also run (pH 6.4 ± 0.4). All assays were run in duplicate at 25 ± 0.5 °C. After each reaction, 3-mL aliquots were taken from the digestion mixture for the determination of soluble phosphorus (P) content. For higher precision of analysis, centrifugation (16800 × g at 4 °C for 15 min) and filtration of the total mixture volume were tested. Compared to subsampling 3-mL aliquots, centrifugation and filtration reduced variation between replicates to less than 5% (c.v.). Filtration was preferred over

centrifugation because it was faster and less laborious. Thus, total volume of mixtures after stomach digestion, intestine digestion and two-stage digestion was filtered through qualitative filter paper (4-12 µm particle retention range, 80g m^2, 20-25 s filtration speed according to DIN 53137) with the aid of a vacuum pump and the filtrate analyzed for soluble P content (reactive phosphorus or orthophosphate) by the vanadomolybdophosphoric acid colorimetric method (EATON et al., 1995) with slight adaptations. Briefly, to 1 mL of the filtrate 4 mL of vanadate-molybdate reagent was added and let stand for 10 min at room temperature. Reaction solution was centrifuged at 16800 × g at 4 °C for 15 min and absorbance of the supernatant was measured at 470 nm (Genova, Jenway, UK) using a 1-mL quartz cuvette. Soluble P content was also determined in the enzyme extracts from stomach and intestine and the value accounted for in the soluble P value after the *in vitro* digestion. The vanadate-molybdate reagent was prepared as follows: Solution A: 25 g ammonium molybdate, $(NH_4)_6Mo_7O_{24}.H_2O$, was dissolved in 300 mL distilled water; Solution B: 1.25 g ammonium metavanadate, NH_4VO_3, was dissolved in 300 mL boiling distilled water, cooled, 330 mL concentrated HCl was added and cooled. Solution A was poured into Solution B, mixed, and dilute to 1L. Calibration curve of phosphorus content (0.005 – 0.05 mg P mL^{-1}) at 470 nm was prepared from a standard phosphorus solution (0.4394 g anhydrous monopotassium phosphate, KH_2PO_4, dilute to 50 mL distilled water; 1.00 mL = 2.0 mg PO_4^{3-}-P). Soluble P content was calculated based on the concentration obtained with the equation from the calibration curve ($y = 14.575x + 0.041$; $R^2 = 0.99$) multiplied by the total volume of the digestion mixture. The amount of released soluble P was then calculated as percentage of the initial total P (3.33 mg). Released soluble P content was analyzed in duplicate and expressed as percentage.

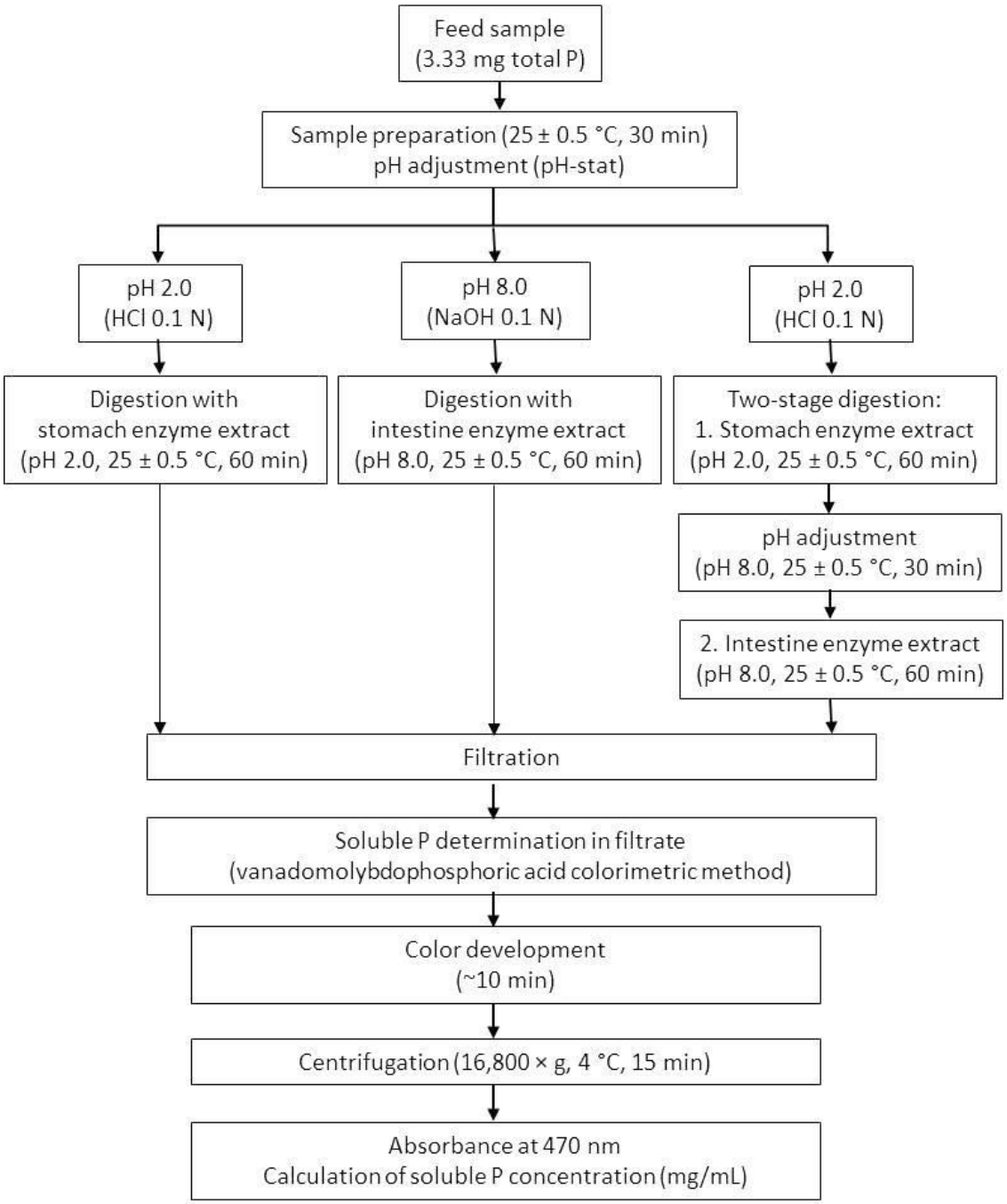

Figure 2.1 – Diagram of released soluble phosphorus (P) determination after *in vitro* pH-stat digestion of feeds for Nile tilapia, *Oreochromis niloticus*, with stomach, intestine and sequential stomach and intestine (two-stage) enzyme extracts recovered from commercially cage farmed Nile tilapia (fed individuals, 598.3 ± 110.1 g, n=10).

2.2.4. Statistical analysis

Normality and equal variance of raw data for feed total P and soluble P released after *in vitro* pH-stat digestion procedures were determined by the Shapiro-Wilk test (SigmaPlot® v.11.0). Values were entered in the statistical software as mean value, standard deviation and sample size per enzyme extract volume, and subjected to one-way analysis of variance (ANOVA). Difference between treatment means was detected by Tukey test. Data was also subjected to Pearson product moment correlation test to determine correlation coefficients (r) and also regressed linearly to determine the coefficient of determination (R^2) between total P and soluble P release after *in vitro* digestion and between digestion with stomach or intestine enzymes and the two-stage digestion procedure. Values were considered significant at $P<0.05$.

2.3. Results

Total phosphorus (P) content of the nine commercial feeds tested ranged from 0.9% (R2 and R7) to 1.6% (R4) (Table 2.1). The release of soluble P after *in vitro* incubation of feeds in distilled water without enzyme extracts at pH 2.0, pH 8.0, pH 2.0 followed by pH 8.0, and with enzyme extracts (50, 100 and 200 μL) are presented in Figure 2. After 1 h of *in vitro* pH-stat incubation of feeds in distilled water at pH 2.0 without enzyme extracts, soluble P ranged from 10.98% (R7) to 15.22% (R3). In distilled water at pH 8.0 values were lower than in pH 2.0 and ranged from 0.97% (R5) to 3.53% (R1). In the two-stage incubation without enzymes, where sample were incubated in distilled water at pH 2.0 and subsequently at pH 8.0, soluble P values ranged from 5.30% (R8) to 10.58% (R4). The addition of stomach digestive enzyme extracts (50, 100 or 200 μL) resulted in lower release of soluble P in most feeds (R1, R2, R3, R5, R6, R9), whereas no significant difference ($P>0.05$) was observed between release of soluble P with or without stomach enzyme extract in the others (R4, R7, R8). The addition of intestine enzyme extract did not increase significantly ($P>0.05$) the release of soluble P in feeds R3, R8 and R9. With feeds R1, R2, R6, and R7 the addition of intestine enzyme extract reduced release of soluble P, whereas with feeds R4 and R5, the release was increased. In the two-stage digestion, feeds R2 and R8 did not present significantly different ($P>0.05$) release of soluble P with or without enzyme extracts from stomach and intestine. In most of the commercial feeds tested, the addition of enzyme extracts reduced the release of soluble P in the two-stage digestion. Overall, release of soluble P after *in vitro* pH-stat digestion procedures with stomach enzyme was higher than two-stage digestion, and digestion with intestine enzyme resulted in low values.

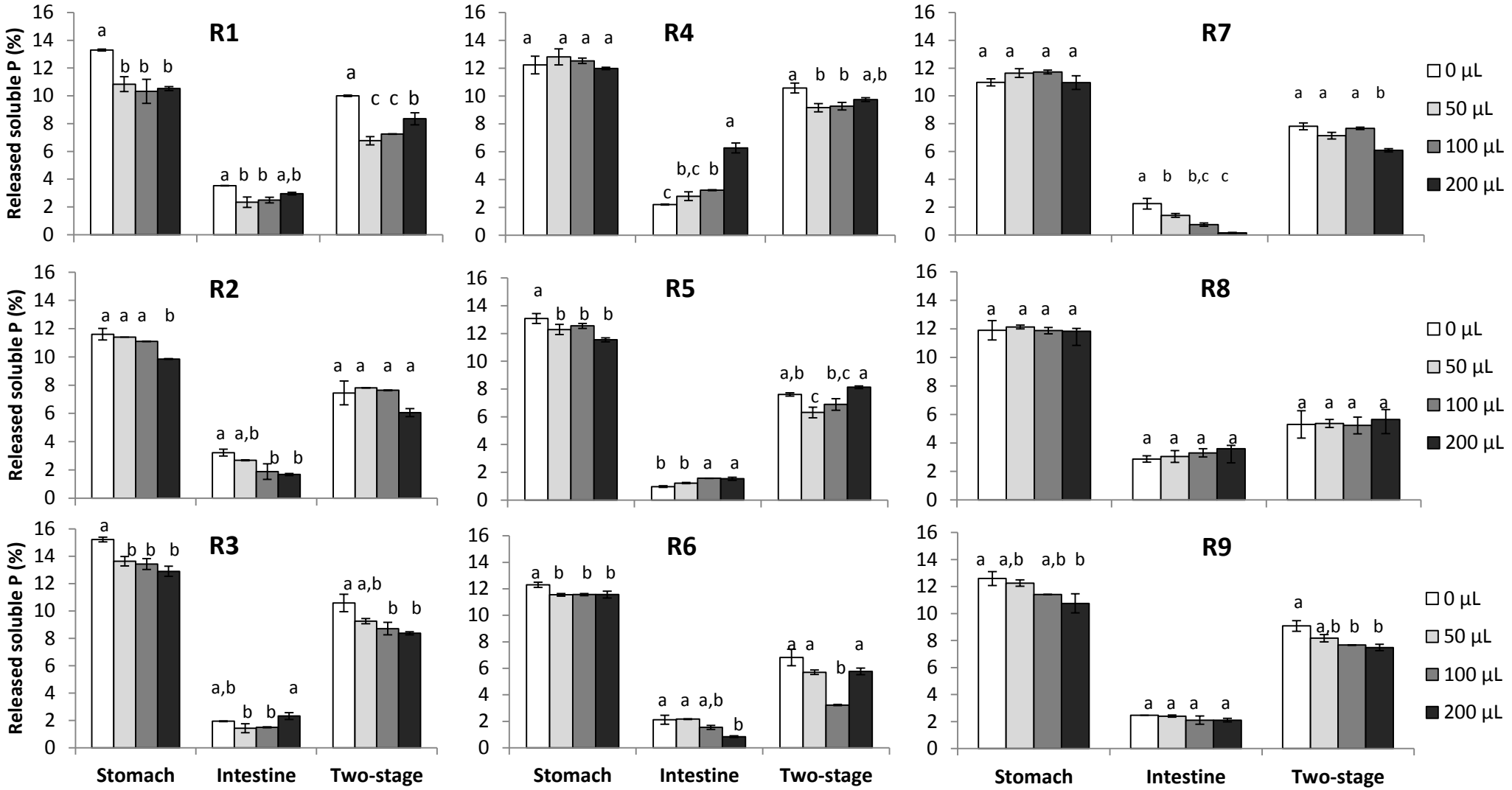

Figure. 2.2 – Mean percentage of released soluble phosphorus (P) from nine commercial feeds (R1 – R9) for Nile tilapia, *Oreochromis niloticus*, submitted to *in vitro* (pH-stat) digestion with -specific enzyme extracts. Feed samples were hydrolyzed with 50, 100 or 200 µL enzyme extracts from stomach (pH 2.0), intestine (pH 8.0) and with stomach extract followed by digestion with intestine extract at pH 2.0 and 8.0 (two-stage). 0 µL means incubation of feed with distilled water at pH 2.0 (stomach), pH 8.0 (intestine) and pH 2.0 and pH 8.0 (two-stage) without addition of enzyme extract. In the two-stage digestion, volumes of stomach and intestine enzyme extracts added were the same. R1 – R9, commercial extruded feeds for growing Nile tilapia (32% crude protein, 4-6 mm pellet size, label values). Each column represents the mean (s.d. as error bar) of duplicate analysis. Different superscript letters indicate significant difference (P<0.05) within the same digestion procedure.

Between the feeds (Table 2.2), R4 with higher total P content (1.6%) resulted in higher release of soluble P in distilled water (6.51%), in the two-stage without enzyme extract (10.58%), in the digestion with intestine enzyme extract (2.80 – 6.27%), and in the two-stage with enzyme extracts (9.16 – 9.75%). R2 and R7, with lower total P content (0.9%), presented low values of soluble P release in distilled water (R7, 2.09%), in distilled water at pH 2.0 (R2, 11.61%; R7, 10.98%), with stomach enzymes (R2, 9.85 – 11.40%), and with intestine enzymes (R7, 0.14 – 1.41%). In distilled water without pH-stat (mean pH 6.4 ± 0.4), R4 released the highest amount of soluble P (6.51%) and R5, the lowest (2.02%). In pH 2.0 without addition of enzyme extract, highest release of soluble P was in R3 (15.22%) and lowest in R7 (10.98%). In pH 8.0, highest and lowest release of soluble P were with R1 (3.53%) and R5 (0.97%), respectively. In the two-stage without enzymes, highest values were with R3 and R4 (10.58%) and lowest with R8 (5.30%). With stomach enzyme extract, intestine enzyme extract, and two-stage digestion the highest release of soluble P were with R3 (12.90 – 13.63%), R4 (2.80 – 6.27%), and R4 (9.16- 9.75%), respectively. Overall, the feed with highest total P content (R4) resulted in high release of soluble P in all the *in vitro* procedures tested, whereas R5 with intermediate total P content presented poor results of release of soluble P.

Table 2.2 – Mean (s.d.) percentage values of soluble phosphorus (P) released from nine commercial extruded feeds (R1 – R9, 32% crude protein, 4-6 mm pellet size, label values) for Nile tilapia, *Oreochromis niloticus*, after incubation in distilled water without pH-stat (6.4 ± 0.4, water), with pH-stat at 2.0, 8.0, 2.0 followed by 8.0 (two-stage); after *in vitro* pH-stat digestion procedures with stomach enzyme extract, intestine enzyme extract and stomach followed by intestine (two-stage). Digestive enzyme extract volumes tested were 50, 100, and 200 µL. Value is mean of duplicate analysis. Different superscript letters within the same column indicate significant difference between means (P<0.05).

		In vitro release of soluble P (%) in												
		Distilled water				Stomach			Intestine			Two-stage		
Feed	Total P %	water	pH 2.0	pH 8.0	pH 2.0-8.0	50 µL	100 µL	200 µL	50 µL	100 µL	200 µL	50-50 µL	100-100 µL	200-200 µL
R1	1.5	5.12	13.30	3.53	10.00	10.84	10.33	10.52	2.35	2.49	2.96	6.77	7.25	8.35
	(0.03)	(0.34)^{b,c}	(0.07)^{b}	(0.01)^{a}	(0.05)^{a,b,c}	(0.54)^{d}	(0.86)^{d}	(0.14)^{c,d}	(0.37)^{a,b}	(0.21)^{a,b,c}	(0.09)^{b,c}	(0.30)^{e}	(0.02)^{c}	(0.44)^{b}
R2	0.9	3.75	11.61	3.22	7.45	11.40	11.10	9.85	2.69	1.88	1.68	7.81	7.64	6.06
	(0.00)	(0.03)^{c}	(0.41)^{c}	(0.25)^{a,b}	(0.85)^{c,d,e}	(0.01)^{c,d}	(0.01)^{c,d}	(0.03)^{d}	(0.03)^{a}	(0.55)^{b,c}	(0.07)^{d,e}	(0.02)^{c,d}	(0.02)^{b,c}	(0.28)^{c}
R3	1.3	3.18	15.22	1.94	10.58	13.63	13.42	12.90	1.42	1.49	2.31	9.25	8.71	8.37
	(0.05)	(0.07)^{c,d}	(0.17)^{a}	(0.03)^{d}	(0.64)^{a}	(0.34)^{a}	(0.39)^{a}	(0.37)^{a}	(0.32)^{b,c}	(0.03)^{c,d}	(0.24)^{c,d,e}	(0.19)^{a}	(0.46)^{a,b}	(0.11)^{b}
R4	1.6	6.51	12.23	2.20	10.58	12.81	12.53	11.99	2.80	3.23	6.27	9.16	9.28	9.75
	(0.00)	(0.70)^{a}	(0.63)^{b,c}	(0.03)^{c,d}	(0.35)^{a}	(0.58)^{a,b}	(0.20)^{a,b}	(0.08)^{a,b}	(0.31)^{a}	(0.03)^{a}	(0.36)^{a}	(0.30)^{a,b}	(0.27)^{a}	(0.14)^{a}
R5	1.2	2.02	13.09	0.97	7.61	12.28	12.55	11.55	1.22	1.57	1.53	6.31	6.89	8.13
	(0.00)	(0.20)^{e}	(0.36)^{b,c}	(0.06)^{e}	(0.12)^{c,d}	(0.36)^{a,b,c}	(0.18)^{a,b}	(0.14)^{b,c}	(0.04)^{c}	(0.01)^{b,c,d}	(0.12)^{e,f}	(0.38)^{e,f}	(0.42)^{c}	(0.09)^{b}
R6	1.2	3.79	12.30	2.11	6.82	11.55	11.57	11.56	2.17	1.54	0.84	5.70	3.23	5.77
	(0.05)	(0.29)^{c}	(0.19)^{b,c}	(0.34)^{c,d}	(0.63)^{d,e}	(0.10)^{b,c,d}	(0.08)^{b,c,d}	(0.25)^{a,b,c}	(0.03)$_{a,b,c}$	(0.15)^{b,c,d}	(0.06)^{f,g}	(0.17)^{f}	(0.05)^{e}	(0.25)^{c}
R7	0.9	2.09	10.98	2.25	7.81	11.64	11.73	10.97	1.41	0.74	0.14	7.14	7.66	6.08
	(0.00)	(0.10)^{d,e}	(0.25)^{d}	(0.38)^{c,d}	(0.25)^{b,c,d}	(0.32)^{b,c,d}	(0.14)^{b,c}	(0.49)^{b,c,d}	(0.13)$_{b,c}$	(0.12)^{d}	(0.03)^{g}	(0.24)^{d,e}	(0.08)^{b,c}	(0.11)^{c}
R8	1.4	5.30	11.90	2.87	5.30	12.12	11.87	11.83	3.04	3.30	3.60	5.37	5.23	5.65
	(0.01)	(0.03)^{b}	(0.68)^{b,c}	(0.22)^{a,b,c}	(0.96)^{e}	(0.13)^{b,c,d}	(0.22)^{b,c}	(0.20)^{a,b,c}	(0.41)$_{a}$	(0.29)^{a}	(0.22)^{b}	(0.28)^{f}	(0.59)^{d}	(0.69)^{c}
R9	1.5	4.06	12.59	2.47	9.08	12.26	11.41	10.76	2.41	2.10	2.11	8.17	7.67	7.49
	(0.01)	(0.12)^{c}	(0.51)^{b,c}	(0.00)^{b,c}	(0.40)^{a,b,c}	(0.23)^{b,c}	(0.01)^{b,c,d}	(0.70)^{b,c,d}	(0.07)$_{a}$	(0.31)^{b,c}	(0.13)^{d,e}	(0.27)^{b,c}	(0.01)^{b,c}	(0.23)^{b}

Relationships between feed total P content and *in vitro* release of soluble P in the different procedures and also the relationships between the soluble P in the *in vitro* procedures are presented in Table 2.3. Positive and significant ($P<0.05$) correlations were observed between feed total P content and soluble P in distilled water without pH-stat, and after digestion with intestine extract (100 and 200 µL). Feed total P content did not correlate with stomach digestion or two-stage digestion ($P>0.05$). Soluble P in distilled water without pH-stat (pH 6.4 ± 0.4) correlated significantly ($P<0.003$) with soluble P after digestion with intestine extract (50, 100 and 200 µL). Release of soluble P in pH 2.0 or pH 8.0 did not correlate with released soluble P in the two-stage (pH2.0 – 8.0) incubation ($P>0.05$). Released soluble P in distilled water at pH 8.0 correlated negatively ($P = 0.02$) with released soluble P after stomach digestion (100 µL) and positively ($P = 0.04$) with released soluble P after intestine digestion (50 µL). Released soluble P after two-stage incubation (pH2.0 – 8.0) correlated significantly ($P<0.02$) with released soluble P after two-stage digestions (50-50, 100-100 and 200-200 µL). Positive but not significant correlations were observed between released soluble P after stomach digestion and 50-50 µL two-stage digestion ($P = 0.09$) and between released soluble P after intestine digestion and 200-200 µL two-stage digestion ($P = 0.07$). For the significant correlations, predictive linear regression equations and the coefficients of determination (R^2) are also presented (Table 2.3).

Table 2.3 – Correlation coefficients (r) and *P*-values of the relationships between feed total phosphorus (P) and *in vitro* soluble P released from feeds incubated in distilled water at different pH and after digestion with stomach, intestine and sequentially with stomach and intestine (two-stage) extracts. Extract volumes tested were 50, 100 and 200 µL. In distilled water pH was 6.4 ± 0.4. Predictive equation is given for significant correlation ($P<0.05$). N = 9. Further details in Materials and Methods.

Variables		*In vitro* release of soluble P in												
						Stomach digestion			Intestine digestion			Two-stage digestion		
		Distilled water	pH 2.0	pH 8.0	pH 2.0-8.0	50 µL	100 µL	200 µL	50 µL	100 µL	200 µL	50-50 µL	100-100 µL	200-200 µL
Feed total P	r	0.75	0.41	0.09	0.43	0.31	0.09	0.40	0.42	0.76	0.40	0.19	0.14	0.62
	P-value	0.02*	0.27	0.82	0.25	0.41	0.81	0.26	0.26	0.02*	0.01*	0.62	0.72	0.07
Soluble P in														
Distilled water			0.05	0.53	0.22	0.00	0.20	0.06	0.85	0.91	0.87	0.15	0.09	0.33
			0.89	0.14	0.57	0.99	0.60	0.87	0.003*	<0.001*	0.002*	0.70	0.82	0.39
pH 2.0					0.57	0.56	0.47	0.57	-0.37	-0.03	0.13	0.37	0.24	0.55
					0.11	0.12	0.20	0.11	0.32	0.93	0.74	0.32	0.52	0.13
pH 8.0					-0.01	-0.57	-0.75	-0.57	0.68	0.40	0.18	-0.05	-0.01	-0.23
					0.97	0.11	0.02*	0.11	0.04*	0.29	0.65	0.91	0.99	0.55
pH 2.0-8.0						0.38	0.21	0.20	-0.19	0.03	0.40	0.83	0.75	0.86
						0.32	0.59	0.60	0.63	0.95	0.29	0.006*	0.02*	0.003*
Stomach digestion														
50 µL												0.59	0.44	0.45
												0.09	0.24	0.22
100 µL												0.40	0.29	0.34
												0.28	0.44	0.37
200 µL												0.19	0.05	0.33
												0.62	0.91	0.38
Intestine digestion														
50 µL												-0.05	-0.17	-0.12
												0.91	0.75	0.76
100 µL												-0.01	0.05	0.29
												0.99	0.89	0.45
200 µL												0.37	0.40	0.63
												0.32	0.28	0.07

*Predictive regression equations.

Soluble P in water = 4.365 (feed total P) – 1.532 $R^2 = 0.56$

Soluble P with intestine digestion (100 µL) = 2.520 (feed total P) – 9.890 $R^2 = 0.57$

Soluble P with intestine digestion (200 µL) = 5.494 (feed total P) – 4.045 $R^2 = 0.61$

Soluble P with intestine digestion (50 µL) = 0.384 (soluble P in water) + 0.763 $R^2 = 0.73$

Soluble P with intestine digestion (100 µL) = 0.521 (soluble P in water) + 0.218	$R^2 = 0.83$
Soluble P with intestine digestion (200 µL) = 1.051 (soluble P in water) – 1.289	$R^2 = 0.76$
Soluble P with stomach digestion (100 µL) = - 0.898 (soluble P in water pH 8.0) + 13.987	$R^2 = 0.56$
Soluble P with intestine digestion (50 µL) = 0.599 (soluble P in water pH 8.0) + 0.856	$R^2 = 0.46$
Soluble P with two-stage digestion (50-50 µL) = 0.639 (soluble P in water pH2.0-8.0) + 2.083	$R^2 = 0.68$
Soluble P with two-stage digestion (100-100 µL) = 0.756 (soluble P in water pH2.0-8.0) + 0.999	$R^2 = 0.57$
Soluble P with two-stage digestion (200-200 µL) = 0.686 (soluble P in water pH2.0-8.0) + 2.092	$R^2 = 0.74$

2.4. Discussion

In compound feeds for fish, phosphorus (P) may be provided by animal and plant ingredients and also from inorganic supplements. Organic P from animal origin are presumably highly digestible, and digestibility of inorganic P might depend on acidic digestion (SATOH et al.; 1992, SUGIURA et al.; 2006), whereas most plant P (phytate) is poorly digested by fish (LALL, 2002). Nevertheless, in hybrid tilapia (*Oreochromis niloticus* × *O. aureus*) phytic acid hydrolyzing enzyme activity has been reported in the intestinal brush border, providing a probable mechanism by which these fish could utilize phytate P effectively (ELLESTAD et al., 2002; LA VORGNA et al., 2003). Digestibility of inorganic P is usually dependent on their solubility (SATOH et al., 1992; SATOH et al., 1997; HUA; BUREAU, 2006). In the present study, the *in vitro* release of soluble P appeared to be more dependent on medium pH than on enzymatic hydrolysis, as in most cases released soluble P was higher from feeds incubated in distilled water at pH 2.0 without stomach enzyme extract. Nevertheless, release of soluble P with stomach enzyme extracts at pH 2.0 was higher than the release of soluble P after digestion with intestine (pH 8.0) or with sequential stomach and intestine enzyme extracts (two-stage digestion). In general, one of the mechanisms underlying P digestibility would be the acidity of fish stomachs (SUGIURA et al., 2006). In fish feed, P of animal origin (hydroxyapatite) would require strong acidity (≤ pH 2.0) to be solubilized in the stomach of rainbow trout for subsequent absorption in the intestine (SUGIURA et al., 2006). It has been reported that *in vitro* solubility of P from fish meal based diets without inorganic P supplement was higher when incubated in acidic medium with 80% acetic acid and 0.25 M HCl than in distilled water (SATOH et al., 1992). At neutral or alkaline pH, P is mostly precipitated (SUGIURA et al., 1998, HUA; BUREAU, 2006, MORALES; MOYANO, 2010). Release of soluble P was low when feeds were digested with intestine enzyme extracts when compared to gastric or two-stage digestions possibly because P might have been precipitated by the change in the medium from acidic to alkaline pH. Another factor that can causes precipitation of P in the intestinal lumen is the antagonistic relation between calcium and P, forming precipitates as calcium phosphates (SUGIURA et al., 1998), most probably dibasic calcium phosphates as the divalent orthophosphate (HPO_4^{2-}) is predominant at or above neutral pH (HUA; BUREAU, 2006). Furthermore, excess of iron, aluminum and magnesium may precipitate phosphorus by forming insoluble salts (WEBSTER; LIM, 2002). Nevertheless, the release of soluble P after the two-stage digestion was higher than the digestion with intestine extract, indicating the importance of including the digestion with

stomach enzyme extracts to *in vitro* digestion analysis (WEERASINGHE et al., 2001; MORALES; MOYANO, 2010).

The decrease in the *in vitro* release of soluble P from the feeds with increasing digestive enzyme extract volume was generally observed but reasons for such response are still uncertain. In an *in vitro* study with pyloric ceca enzyme extracts from juvenile rainbow trout, *Oncorhynchus mykiss*, enzyme-mediated decrease in P solubility from plant origin feedstuffs was attributed to possible alteration in the ionic environment of the digest caused by deamination of asparagine and glutamine residues during digestion, which resulted in P precipitation (WEERASINGHE et al., 2001). On the other hand, release of *in vitro* soluble P was increased by the addition of juvenile rainbow trout stomach and pyloric ceca enzyme extracts in the digestion mixture with fish meal and soybean meal and such increase was attributed to possible hydrolysis of organic phosphates covalently linked to proteins by alkaline phosphatases present in pyloric ceca extracts (MORALES; MOYANO, 2010). It is difficult to compare those findings with feed ingredients with the results of this study with compound feeds, as compound feeds may include P of animal, plant and inorganic sources, all with different properties and antagonistic or synergistic interactions between nutrients. Nevertheless, it may be hypothesized that further to pH effect, some product resulting from the enzymatic hydrolysis might also interfere in the release of soluble P.

Since the ingredient composition of the compound feeds tested in the present study is not known the meaning of the values of released soluble P with stomach (9.85 – 15.22%), intestine (0.55 – 6.78%) or two-stage digestion (3.49 – 10.58%) may not be attributed to or correlated with the ingredient composition or the source of P, i.e., if animal, plant or inorganic supplement. Because Nile tilapia is an omnivorous fish species, it is likely that large amount of plant ingredients have been included in the feeds to reduce costs. Up to 70% of the P in plant feedstuff may be in the form of phytic acid or phytate, which is poorly digestible, i.e., it is not available to most monogastric animals as they lack the enzyme phytase to release P from the phytate molecule (RAVINDRAN et al, 1995; LALL, 2002; HUA; BUREAU, 2006), although phytase activity has been reported for hybrid tilapia and these fish might be able to hydrolyze phytate P (ELLESTAD et al., 2002; LA VORGNA et al., 2003). Inorganic P sources would be mostly soluble in water (SATOH et al., 1992) and bone P is expected to be highly solubilized in acidic medium (SUGIURA et al., 2006). Nonetheless, a significant correlation ($P<0.05$) was observed in this study between soluble P released in deionized water

(pH 6.4 ± 0.4) and soluble P released after digestion with intestine enzymes (pH 8.0). It could be speculated that such result with only intestinal digestion is comparable to what may be found in carps that could only utilize water soluble P by the lack of acidic digestion (SATOH et al., 1992). Additionally, rainbow trout fed diets with inorganic P supplementation, the fraction of P soluble in deionized water corresponded to digestible P but when fed unsupplemented fish meal-based diets, digestible P corresponded to the sum of soluble P in deionized water and acidic medium (SATOH et al., 1992). In this study, all commercial feed tested included supplementation of inorganic dicalcium phosphate, except in R5 (label information). Possibly, the release of soluble P observed in the present study, as seen in R4, is mainly of inorganic P, and release of soluble P in R5 was low maybe because it was not supplemented with inorganic P.

The *in vitro* method developed in this study to measure amount of soluble P release after digestion with stomach, intestine, and stomach and intestine enzyme extracts can be considered to be reproducible as variation between replicates was low (<5% c.v.). Total feed P correlated significantly ($P<0.05$) with released soluble P in distilled water (pH 6.4) and with released soluble P after intestine digestion (pH 8.0; 100 and 200 µL). Also, significant correlation ($P<0.05$) was observed between soluble P released in distilled water (pH 6.4) and soluble P released after intestine digestion (pH 8.0; 50, 100 and 200 µL). Digestion in acidic pH seems to affect more the release of soluble P than digestion in alkaline pH, as digestion with stomach enzyme extracts resulted in higher values. Furthermore, gastric digestion showed to be important in the two-stage digestion, yielding results higher than intestine digestion only. *In vitro* studies on release of soluble P for fish are scarce for salmonid (MORALES; MOYANO, 2010; WEERASINGHE et al., 2001) and omnivorous fish (ELLESTAD et al., 2002; LA VORGNA et al., 2003). To our knowledge the present study was the first to assess *in vitro* pH-stat release of soluble P using species-specific enzyme extracts for Nile tilapia and so the meaning of the values as measured in the present study remains unclear. Therefore, further studies including *in vivo* P digestibility should be carried out to provide information to elucidate the meaning and potential utility of this *in vitro* method.

Chapter 3

In vivo apparent protein and phosphorus digestibility and *in vitro* (pH-stat) degree of protein hydrolysis and release of soluble phosphorus of commercial feeds for Nile tilapia, *Oreochromis niloticus* (L.)

Abstract

The present study aimed at assessing crude protein and phosphorus digestibility coefficients of commercial feeds simulating commercial stocking density, using AIA as internal marker, further to evaluate fish growth and the potential correlation of *in vivo* apparent digestibility data with *in vitro* protein digestion and released soluble phosphorus from commercial feeds for Nile tilapia. For the digestibility and growth trial, Nile tilapia (57.4 g average individual weight) were stocked in thirty-six 300-L circular fiberglass tanks connected to a recirculation system, at a density of 24 fish per tank for 66 days at 26.0 ± 0.4 °C. Acid-insoluble ash was used as the internal marker. Nine commercial feeds for Nile tilapia (32% crude protein, 4-6 mm pellet size, declared values) were fed to fish in four replicate tanks, four times a day to apparent satiation. Feces were collected daily between meals throughout the trial. *In vitro* pH-stat degree of protein hydrolysis (DH) and the release of soluble phosphorus (P) of the nine commercial feeds were analyzed by incubating feed samples with Nile tilapia stomach or intestine enzyme extract, separately or sequentially (two-stage digestion). Assays were run at 25 ± 0.5 °C for 60 min. Growth performance between feeds were not significantly different ($P>0.05$) with average weight gain, daily weight gain and feed conversion ratio of 189.5 g, 3.0 g, and 1.2, respectively. The variation of AIA content between feed replicates was high, between feces replicates per tank variation was low, but between tank replicates it was high. The *in vitro* DH did not correlate significantly ($P>0.05$) with ADC of CP, but it was possible to discriminate between the highest and lowest ADC of CP. *In vitro* release of soluble P in distilled water and after digestion with intestine extract correlated significantly ($P<0.05$) with total feed P and apparently digestible P. Further studies are necessary to increase analytical precision of the AIA determination and also to improve methods to possibly predict *in vivo* digestibility values with *in vitro* methods.

3.1. Introduction

In Brazil, Nile tilapia, *Oreochromis niloticus*, has been commercially farmed in cages in freshwater reservoirs at high densities varying from 80 to 120 kg m^{-3}, at high production costs (AYROZA et al., 2011; MARENGONI, 2006). Thus, fish stocking density below 200 fish m^{-3} are recommended for efficient production (AYROZA et al., 2011). Nile tilapia is the most farmed species in the country but studies under practical farming conditions are very scarce, including tests with commercial feeds, and most studies are performed at laboratory scale and for short periods. Practical studies should be carried out to provide advisory service to the farmer and also to formulate cost-effective diets (VILLAMIDE et al., 2009).

For aquacultured species, determination of digestibility is most widely done by the indirect method, based on the collection of representative sample of feces and the addition of an inert external indicator to the feed, such as chromic oxide or yttrium, which is not absorbed and should pass through the digestive tract at the same rate as food (NRC, 2011). However, when testing commercial compound feeds addition of such digestion indicator and reprocessing would affect the original characteristics, thus, an internal marker, such as acid insoluble ash (AIA), which is primarily silica, or insoluble siliceous compounds, and naturally occurring in the feed ingredients would be of particular interest. AIA has been validated as internal marker for digestibility trials in swine (McCARTHY et al., 1974), poultry (VOGTMANN et al., 1975), ruminants (VAN KEULEN; YOUNG, 1977), salmonid fish (ATKINSON et al., 1984), and Nile tilapia (GODDARD; McLEAN, 2001; ADEPARUSI; JIMOH, 2002).

To predict the nutritional value of compound feeds and raw materials is one of the main goals of fish nutritionists and feed formulators and, to be adequate at industrial level, assessment methods should be fast, precise and reproducible, which has motivated the development of *in vitro* methods (PEDERSEN; EGGUM, 1983; GRABNER, 1985). Previous studies using the *in vitro* pH-stat determination of degree of protein hydrolysis have reported its potential to distinguish feedstuff quality and also to predict ingredient protein digestibility (DIMES et al., 1994a,b; LEMOS et al., 2009; TIBBETTS et al., 2011b; YASUMARU; LEMOS, 2014). However, commercial or compound feeds have not been tested. In this way, the aim of the present study was to assess protein and phosphorus digestibility coefficients of

commercial feeds simulating commercial stocking density, using AIA as internal marker, further to evaluate fish growth and the potential correlation of *in vivo* apparent digestibility data with *in vitro* protein digestion and soluble phosphorus release from commercial feeds for Nile tilapia.

3.2. Materials and Methods

3.2.1. In vivo apparent digestibility and growth trial

3.2.1.1. Fish and feeds

Three-thousand temperature-masculinized GIFT (F3) Nile tilapia, *Oreochromis niloticus*, fingerlings (0.5-1.0 g) were purchased from a commercial hatchery (Piscicultura S3, Registro, São Paulo, Brazil) and stocked in a 18-m^3 circular tank. Four times per day fingerlings were hand-fed commercial extruded feeds [1 mm diam., 45% crude protein (CP) and 1.7 mm, 40% CP, values informed by manufacturer on label] at a rate of 10% biomass. When fish reached an average weight of 20 g, commercial extruded feed (2-4 mm, declared minimum 32% CP) was fed four times per day at 7% biomass until average 50 g of individual weight was reached for the digestibility and growth trial.

Nine commercial extruded feeds (R1 – R9) from different manufacturers with pellet size 4 – 6 mm for Nile tilapia in grow-out phase were purchased in the local market in 25 kg bags with production dates between 19/12/2012 and 16/02/2013, and minimum validity of 4 months and maximum of 12 months. The nine brands (in alphabetical order, Acqua Fish – Supra Alisul, Acqua Pesca 32 – Matsuda, API Peixe – Malta Cleyton, Fri-Aqua – Fri-Ribe Nutreco, Laguna – Socil, Nutripiscis – Presence, Pirá Ideal – Guabi, Poli-Peixe – Polinutri, Tilápia crescimento G320AM – Amicil) were the most commonly used by commercial Nile tilapia farmers in the São Paulo state region. The declared values on the feed labels were 32% crude protein (min), 8 - 13% moisture (max), 4 - 7% ether extract (min), 6 - 10% crude fiber (max), 10.0 - 12.5% ash (max), and 0.4 - 1.2% total phosphorus (min). Feeds were not reprocessed to add any external marker, e.g. chromic oxide (Cr_2O_3). An internal marker, i.e., the acid insoluble ash (AIA) fraction, was used to determine apparent digestibility coefficients.

3.2.1.2. Digestibility system and growth trial

The digestibility system consisted of thirty-six 500-L capacity circular fiberglass tanks (300 L water volume) connected to a recirculation system with two 1000-L header tanks. The system was originally designed for digestibility trials with marine shrimp,

Litopenaeus vannamei with tank bottom slightly sloped toward the central drain and feces settlement column and conical plastic collection tube attached to it (CARVALHO et al., 2013). Tanks were individually aerated and water flow was adjusted to 4 L min^{-1}. Water temperature was thermostatically kept at 26.0 ± 1.4 °C with heaters in the header tanks. Dissolved oxygen was measured daily with a portable oxygen meter (YSI Model 55, OH, USA). pH, ammonia N, nitrite, alkalinity and hardness were monitored weekly with colorimetric water analysis kit (kit técnico água doce, Alfakit, Florianópolis, SC, Brazil). Twenty-four juvenile Nile tilapia (57.40 ± 4.81 g individual mean wet weight ± standard error of the mean) were stocked in each of the 36 tanks with 300 L of water volume. Each of the nine commercial feeds was fed to four replicate tanks. Fish were acclimated to the system and the commercial feeds for 12 days prior to the beginning of the trial. During the 66-day experimental period, fish were hand-fed four times per day (0900, 1130, 1430 and 1730h) to apparent satiety and feed intake measured daily. Every morning, before the first feeding, tank wall and bottom were cleaned with a mop, settling column and feces collection tubes cleaned using a water spray gun. For the growth trial, fish were group weighed to determine initial body weight and individually weighed at days 20, 41, 55 and 66 of the experiment to monitor weight gain. Fish were fasted overnight before weighing and fed only on the following day. Feces were collected five times per day (1100, 1300, 1400, 1600 and 1700h) into 50 mL conical centrifuge tubes, filtered each time through qualitative filter paper with the aid of a vacuum pump, pooled per tank and stored at -20 °C. Feces were collected five days per week (25 days in total), except on the day of weighing, one day before and one day after. At the end of the experimental period, fecal samples were lyophilized, ground in a mortar with a pestle and stored in amber glass flasks at 4 °C until further analyses.

3.2.1.3. Chemical analysis and calculation of *in vivo* apparent digestibility coefficients

Dry matter, crude fiber, and ash content of the commercial feeds were analyzed following the AOAC methods as described by Silva and Queiroz (2009) by the Bromatology and Mineral Analysis Laboratory of the Animal Science Institute (APTA, SAA). Briefly, dry matter was determined by drying sample at 103-105 °C overnight; ash by burning in a muffle furnace at 600 °C for 6 h, and crude fiber by acid and alkaline hydrolysis. Crude fat was determined by the petroleum ether extraction with acid pre-hydrolysis (SINDIRAÇÕES, 2009). Nitrogen-free extract was calculated by difference [100 – (crude protein + crude fat + ash + moisture)]. In fish feeds and feces protein content was calculated as N × 6.25 and N

determined in an elemental analyzer (Elemental Analyzer 2400 CHN, Perkin Elmer, Waltham, MA, USA). For total phosphorus (P), feed and feces samples were submitted to acid digestion with concentrated HNO_3 in microwave and analyzed by an inductively coupled plasma atomic emission spectrophotometer (ICP-OES, Arco, Spectro, Kleve, Germany). Nitrogen and total P was analyzed by the Chemistry Institute analytical laboratory, University of Sao Paulo. The internal marker (AIA) in fish feeds and feces was analyzed following the method proposed by Atkinson et al. (1984) with slight modification. Briefly, 2-g sample was weighed into porcelain crucible and placed in a muffle furnace. Temperature was gradually increased until 600 °C and sample ashed for 6 h. Sample was transferred to desiccator, cooled and weighed. The resulting ash was boiled in 25 mL HCl 2N for 5 min, filtered with the aid of a vacuum pump through ashless filter paper (Whatman 41) and residue washed with hot water. Filter paper and residue were placed in porcelain crucible and ashed for 6 h at 600 °C. Resulting AIA was transferred to desiccator, cooled and weighed. Acid-insoluble ash fraction was analyzed in five replicates per feed. For feces, AIA content was determined in five replicates per tank.

Apparent digestibility coefficient of protein and phosphorus were calculated as $ADC_n = 100 - (100 \times Feces_n/Feed_n \times Feed_{AIA}/Feces_{AIA})$, where subscript n means nutrient and AIA the internal marker concentrations (%). Fish specific growth rate (SGR) was calculated as $SGR\ (\%) = (\ln W_f - \ln W_i \times 100)/t$, where $\ln W_f$ and $\ln W_i$ are the natural logarithms of the final and initial weight, respectively, t is the time in days between $\ln W_f$ and $\ln W_i$. Average total feed conversion ratio (FCR) was calculated as feed intake/weight gain in the four replicates per test feed.

3.2.2. In vitro *degree of protein hydrolysis (DH) and release of soluble P*

The recovery of crude enzyme extract and *in vitro* pH-stat assays to determine degree of protein hydrolysis (DH) and the amount of soluble phosphorus (P) released from the nine commercial feeds were conducted as described previously (YASUMARU; LEMOS, 2014). Stomach and intestine enzyme extract donor organisms were cage farmed Nile tilapia (fed individuals, 598.3 ± 110.1 g). For the *in vitro* protein digestion, the amount of ground feed sample containing 80 mg of CP was placed in a 20-mL tube with distilled water and the suspension (6 and 8 mL for digestion with stomach and intestine extract, respectively) stirred for 30 min to stabilize pH at 2.0 (stomach) with 0.1 N HCl or 8.0 (intestine) with 0.1 N

NaOH. Feed samples were submitted to hydrolysis with stomach and intestine extracts separately and also subsequently (two-stage digestion). The pH-stat protein hydrolysis assays were tested with three enzyme extract volumes (50, 200, 800 μL) from Nile tilapia stomach and intestine, and a blank (without enzyme extract). All assays were run in duplicate at 25 ± 0.5 °C for 60 min. The assays were carried out simultaneously in two automated titrators (Titrando 836, Titrando 907, Metrohm AG, Switzerland), connected to a single controlling and data logging software (Tiamo™ v. 2.2, Metrohm AG, Switzerland). The degree of protein hydrolysis (DH) at pH 2.0 was calculated according to Diermayr and Dehne (1990):

$$\%DH = [(V \times N)/E] \times (1/P) \times F_{pH} \times 100$$

Where:

V = volume of HCl consumed in the hydrolysis reaction (mL)

N = normality of HCl

E = mass of substrate protein (g)

P = number of peptide bonds cleaved (mol g protein^{-1}). For proteins which amino acid composition is not determined, P is generally suggested as 8.0

F_{pH} = 1.08 (constant for pH 2.0 at 25 °C)

And at pH 8.0, DH was calculated according to Adler-Nissen (1986):

$$\%DH = B \times Nb \times (1/\alpha) \times (1/MP) \times (1/Htot) \times 100$$

Where:

B = volume of NaOH consumed (mL)

Nb = normality of the base

α = average degree of dissociation of the α-NH groups ($1/\alpha$ = 1.50 for pH 8.0 at 25 °C)

MP = mass of protein (g)

Htot = total number of peptide bonds in the protein substrate [average value of 8.0 meqv g protein^{-1} was assumed (ADLER-NISSEN, 1986)].

In the pH-stat assay for the determination of soluble P released from the nine commercial feed samples, procedure was as in the protein hydrolysis assay differing only in

the amount of ground feed samples, which contained 3.33 mg total P and the volume of Nile tilapia stomach and intestine enzyme extracts tested were 50, 100 and 200 μL. The amount of total P (3.33 mg) was calculated based on the average crude protein and total P content in the feeds in relation to the amount of CP (80 mg) in the protein hydrolysis assay. The enzyme extract volumes were different from the DH protein hydrolysis assays due to the limited availability of extracts. Further to the blank without enzyme extracts assays with distilled water without pH adjustment were also run. Following hydrolysis suspensions were filtered and the filtrate analyzed for soluble P (reactive phosphorus or orthophosphate) content by the vanadomolybdophosphoric acid colorimetric method read at 470 nm (Eaton et al., 1995). A calibration curve of phosphorus content (0.005 – 0.05 mg P mL^{-1}) was plotted using a standard phosphorus solution (0.4394 g anhydrous monopotassium phosphate, KH_2PO_4, dilute to 50 mL distilled water; 1.00 mL = 2.0 mg PO_4^{3-}-P) and the equation used to calculate the soluble P content in the commercial feeds submitted to the different *in vitro* assays (Fig. 3.1). Soluble P content was calculated based on the concentration obtained with the equation from the calibration curve ($y = 14.575x + 0.041$; $R^2 = 0.99$) multiplied by the total volume of the digestion mixture. The amount of released soluble P was then calculated as percentage of the initial total P (3.33 mg). Released soluble P content was analyzed in duplicate and expressed as percentage.

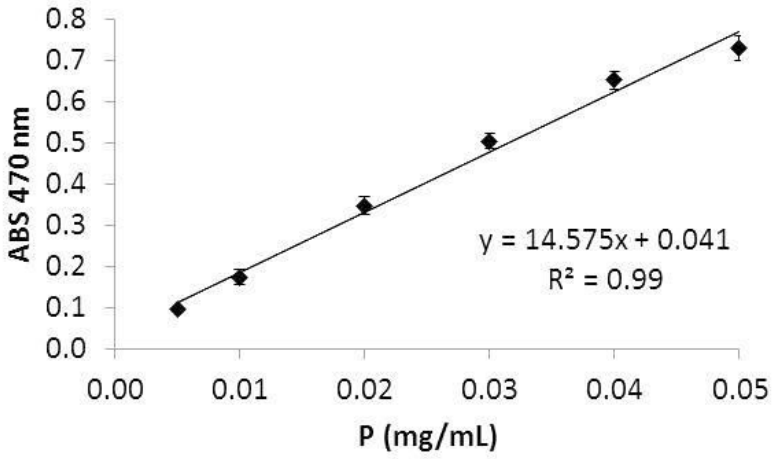

Figure 3.1 – Calibration curve for the determination of soluble phosphorus (P) content in nine commercial feeds for Nile tilapia, *Oreochromis niloticus*, submitted to *in vitro* pH-stat hydrolysis with stomach or intestine enzyme extract, separately or sequentially. Standard phosphorus solution prepared with anhydrous monopotassium phosphate, KH_2PO_4. P = PO_4^{3-}-P (phosphate).

3.2.3. Statistical analysis

In vivo crude protein apparent digestibility coefficients values normal distribution and constant variance were confirmed by the Shapiro-Wilk test (SigmaPlot® v.11.0) and were subjected to one-way analysis of variance (ANOVA) and the Tukey multiple comparison test. *In vivo* phosphorus apparent digestibility coefficient values did not pass the normality test and were subjected to the non-parametric Kruskal-Wallis test and to Student-Newman-Keuls (SNK) multiple comparison procedure. Normality and equal variance of raw data for feed sample DH and released soluble P were determined by the Shapiro-Wilk test (SigmaPlot® v.11.0). Values were entered in the statistical software as mean value, standard error and sample size per enzyme extract volume, and subjected to one-way ANOVA. Difference between treatment means was detected by Tukey test. *In vivo* and *in vitro* data was also subjected to Pearson product moment correlation test to determine correlation coefficients (r) and also regressed linearly to determine the coefficient of determination (R^2) between *in vivo* apparent protein digestibility and *in vitro* degree of protein hydrolysis, and between *in vivo* phosphorus digestibility and *in vitro* release of soluble phosphorus. Results were considered significant at $P<0.05$.

3.3. Results

The mean value for dissolved oxygen throughout the trial was of 4.26 ± 0.87 mg/L (54.8 ± 11.0% saturation) and mean temperature was 26.0 ± 1.4 °C. Results for pH, ammonia N, and nitrite were 6.0 ± 0.7, 1.7 ± 1.4 mg/L N-NH_3, 0.60 ± 0.56 mg/L N-NO_2, respectively. Fish mortality was not caused by water quality parameters but rather by fish scape over the tanks.

The proximate composition of the nine commercial feeds tested is presented in Table 3.1. Crude protein content varied from 26.3 (R2) to 34.0% (R6) and only two feeds (R4 and R6) met the minimum declared value of 32%. Total phosphorus (P) content varied from 0.9 (R2 and R7) to 1.6 (R4). Moisture, ether extract, crude fiber, ash and total phosphorus contents were within the declared ranges. The acid-insoluble ash content in the feeds varied from 0.23 ± 0.03% in R3 to 1.05 ± 0.05% in R5, with coefficient of variation between replicates of 5% (R5) to 21% (R8).

Table 3.1 – Proximate composition of the nine (R1 – R9) commercial extruded feed fed to juvenile Nile tilapia, *Oreochromis niloticus* (% 'as is' basis).

Feed	Moisture	CP	CF	EE	NFE	Ash	Total P	AIA
R1	8.9 (0.0)	28.5	4.9 (0.0)	5.9 (0.2)	41.7	10.1 (0.0)	1.5 (0.0)	0.34 (0.04)
R2	9.8 (0.1)	26.3	5.0 (0.2)	6.3 (0.1)	44.5	8.1 (0.1)	0.9 (0.0)	0.82 (0.11)
R3	10.5 (0.1)	28.9	2.8 (0.1)	7.7 (0.2)	40.6	9.5 (0.1)	1.3 (0.0)	0.23 (0.03)
R4	7.1 (0.1)	33.8	3.8 (0.0)	7.9 (0.1)	36.8	10.6 (0.1)	1.6 (0.0)	0.55 (0.04)
R5	8.0 (0.1)	31.8	5.4 (0.2)	6.0 (0.1)	38.7	10.1 (0.1)	1.2 (0.0)	1.05 (0.05)
R6	7.2 (0.1)	34.0	4.5 (0.2)	7.3 (0.1)	38.1	8.9 (0.1)	1.2 (0.1)	0.29 (0.05)
R7	9.6 (0.1)	27.6	3.8 (0.1)	6.9 (0.0)	44.6	7.5 (0.2)	0.9 (0.0)	0.24 (0.03)
R8	5.1 (0.1)	30.3	5.9 (0.0)	8.5 (0.2)	38.8	11.8 (0.2)	1.4 (0.0)	0.24 (0.05)
R9	8.1 (0.1)	30.7	3.7 (0.0)	6.7 (0.1)	40.1	10.7 (0.2)	1.5 (0.0)	0.47 (0.04)

CP, crude protein (N x 6.25); CF, crude fiber; EE, ether extract; NFE, nitrogen free extract – calculated by difference (NFE = moisture + CP + CF + EE + ash); P, phosphorus; AIA, acid-insoluble ash. Value represents mean (s.d.) of duplicate analysis, except for crude protein (single analysis), nitrogen free extract (calculated from means), and AIA (five replicates).

In the growth trial, no significant differences (P>0.05) were observed for final body weight, feed intake, weight gain, specific growth rate, feed conversion ratio and survival rate between the fish groups fed the nine commercial feeds (Table 3.2). Fish daily weight gain varied from 2.6 ± 0.4 g (R8) to 3.1 ± 0.3 g (R4), which corresponded to a specific growth rate (SGR) of 2.2% per day. Average fish weight gain, feed conversion ratio and survival rate

were 246.5 ± 24.4 g, 1.2 ± 0.2, and 86.8 ± 8.6%, respectively. Although not significant (P>0.05), R4 resulted in lowest feed intake (207.8 ± 19.7 g) and highest weight gain (202.2 ± 26.2 g). Fish initial, intermediate and final weight values are presented in Figure 3.2. From day 0 to day 41, fish daily individual weight gain varied from 2.20 (R1) to 2.97 g (R2). In the following interval, fish daily individual weight gain varied from2.64 (R8) to 3.50 g (R2). In the last interval, fish daily individual weight gain was increased, varying from 3.04 (R6) to 5.32 g (R4). The average SGR was higher in the first 20 days (3.2%) and reduced in the following intervals to 1.9 – 1.7%.

Table 3.2 – Mean (s.e.m) growth performance values of juvenile Nile tilapia, *Oreochromis niloticus*, fed nine (R1 – R9) commercial feeds for 66 days. Values are means of four replicate tanks.

Commercial Feed	BWi (g fish^{-1})	BWf (g fish^{-1})	Total feed intake (g fish^{-1})	Total weight gain (g fish^{-1})	Average daily weight gain (g fish^{-1})	SGR (% day^{-1})	FCR	Survival (%)
R1	56.9 (3.9)	245.4 (38.4)	217.0 (9.6)	188.5 (38.7)	2.9 (0.5)	2.2 (0.2)	1.2 (0.2)	89.6 (8.7)
R2	57.7 (3.0)	259.2 (15.9)	246.1 (24.0)	201.5 (15.5)	3.1 (0.2)	2.3 (0.1)	1.2 (0.0)	86.5 (6.3)
R3	56.3 (9.9)	231.7 (34.5)	203.7 (32.0)	175.4 (25.2)	2.7 (0.3)	2.1 (0.1)	1.2 (0.1)	90.6 (7.1)
R4	57.2 (3.7)	259.5 (26.8)	207.8 (19.7)	202.2 (26.2)	3.1 (0.3)	2.3 (0.2)	1.1 (0.2)	86.5 (11.5)
R5	57.2 (3.4)	240.2 (11.3)	227.1 (18.7)	186.4 (8.6)	2.8 (0.1)	2.2 (0.1)	1.3 (0.1)	93.8 (2.4)
R6	57.1 (7.1)	252.6 (20.0)	234.4 (24.5)	195.5 (14.3)	3.0 (0.2)	2.3 (0.1)	1.2 (0.0)	87.5 (10.8)
R7	57.3 (4.2)	247.0 (16.6)	216.3 (18.9)	189.6 (15.9)	2.9 (0.2)	2.2 (0.1)	1.2 (0.1)	83.3 (12.3)
R8	58.3 (3.9)	229.9 (32.1)	222.8 (22.8)	171.7 (28.5)	2.6 (0.4)	2.1 (0.1)	1.3 (0.1)	83.3 (9.0)
R9	58.6 (5.8)	253.3 (13.5)	240.5 (20.0)	194.6 (12.1)	2.9 (0.2)	2.2 (0.1)	1.3 (0.1)	80.2 (5.2)

BWi, initial body weight; BWf, final body weight; SGR, specific growth rate SGR (%) = $(\ln BW_f - \ln BW_i \times 100)/t$; FCR, feed conversion ratio (feed intake/weight gain).

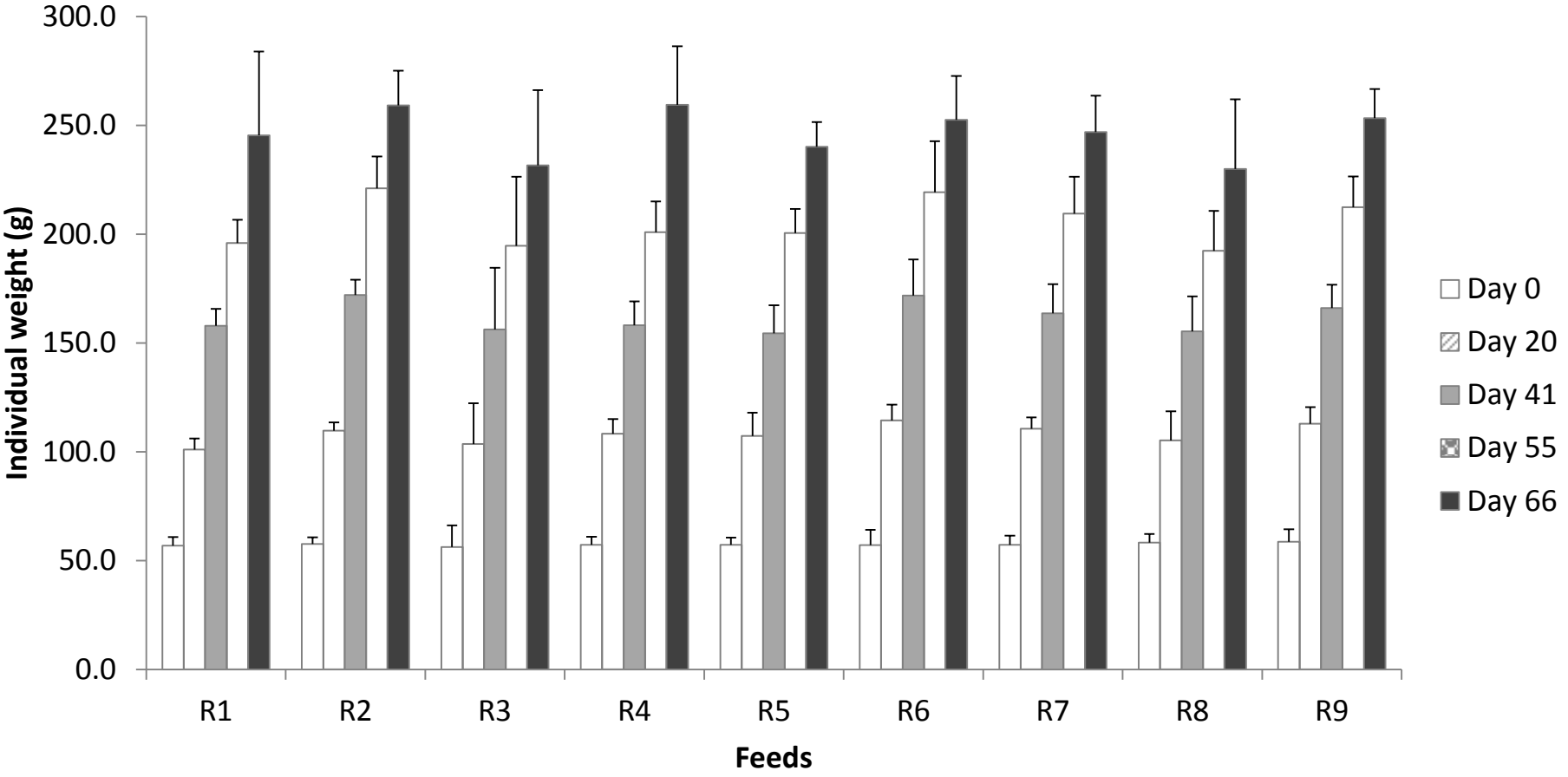

Figure 3.2 – Mean individual body weight (g) of Nile tilapia, *Oreochromis niloticus* fed nine different commercial feeds (R1 – R9) for 66 days at 26.0 ± 1.4 °C. During the trial fish were individually weighed five times (Day 0: initial weight; Day 20, 41 and 55: intermediate weighing; Day 66: final weight). Each bar is the mean of four replicate tanks and error bar is the standard error of the mean.

For the digestibility trial, concentration of the internal marker (AIA) was determined in the feeds and feces, which presented coefficients of variation between replicates of 5.2 and 21.0% in feeds and of 3.0 to 53.2% in feces (Table 3.3). In feces, such variation was higher between the four replicate tanks, but less than 8% between the five replicates of feces sample from the same tank. However, the coefficients of variation of the resulting ADC values of CP and P were reduced to less than 3.8% for CP and between 3.7 and 16.4% for P. ADC of CP varied significantly between the commercial feeds – the highest value was observed in R7 (93.8 ± 2.3%) and the lowest with R5 (83.7 ± 1.0%). Apparent digestibility coefficient of total P did not differ significantly between most of the feeds tested, except R5 that presented the lowest value (51.0 ± 8.3%). Applying ADC values, available protein varied from 25.8 ± 0.3 to 30.0 ± 1.1% and available phosphorus from 0.6 ± 0.1 to 1.1 ± 0.0%.

Table 3.3 – Mean (s.e.m) value and coefficient of variation (cv) of internal marker (acid insoluble ash – AIA) in feed and feces (% dry matter), apparent digestibility coefficients (ADC) of dry matter (DM), crude protein (CP) and total phosphorus (P) and available crude protein and phosphorus of the nine commercial feeds (R1 – R9) fed to juvenile Nile tilapia, *Oreochromis niloticus* for 66 days at 26.0 ± 1.4 °C. Feed AIA content value is the mean of five replicates per feed sample and per tank. Other values are means of four replicate tanks.

Feed	AIA % feed	cv	AIA% feces	cv	ADC % DM	cv	ADC% CP	cv	ADC% P	cv	Available % CP	cv	Available % P	cv
R1	0.38 (0.05)	12.2	1.62 (0.30)	18.6	75.9 (4.3)^{b,c}	5.7	90.3 (2.0)^{a,b}	2.2	61.2 (7.1)^{a,b}	11.6	28.3 (0.6)	2.2	1.0 (0.1)	11.6
R2	0.91 (0.12)	13.0	3.93 (0.40)	10.3	76.7 (2.3)^{b,c}	3.0	88.5 (1.5)^{b,c}	1.7	60.0 (4.3)^{a,b}	7.1	27.6 (0.4)	1.6	0.6 (0.0)	7.1
R3	0.25 (0.03)	12.9	2.18 (0.56)	25.7	88.0 (2.7)^{a}	3.0	93.0 (1.4)^{a,b}	1.6	74.1 (5.9)^{a}	8.0	27.1 (0.5)	1.7	1.1 (0.1)	8.0
R4	0.59 (0.03)	7.5	3.28 (0.71)	21.5	81.4 (4.1)^{a,b}	5.0	90.4 (2.6)^{a,b}	2.8	66.8 (7.0)^{a,b}	10.5	26.5 (0.9)	3.5	1.1 (0.1)	10.5
R5	1.14 (0.06)	5.2	3.90 (0.12)	3.0	70.7 (0.9)^{c}	1.3	83.7 (1.0)^{c}	1.1	51.0 (8.3)^{b}	16.4	25.8 (0.3)	1.3	0.7 (0.1)	16.4
R6	0.31 (0.05)	16.4	2.01 (0.75)	37.5	83.3 (4.7)^{a,b}	5.6	92.9 (2.0)^{a,b}	2.2	69.8 (8.8)^{a,b}	12.6	26.9 (0.7)	2.7	0.9 (0.1)	12.6
R7	0.27 (0.03)	10.9	2.64 (1.40)	53.2	88.1 (4.2)^{a}	4.8	93.8 (2.3)^{a}	2.4	75.6 (9.1)^{a}	12.1	27.9 (0.7)	2.5	0.7 (0.1)	12.1
R8	0.26 (0.05)	21.0	1.84 (0.92)	50.1	83.5 (6.6)^{a,b}	7.9	92.3 (3.1)^{a,b}	3.3	72.2 (9.7)^{a,b}	13.4	28.9 (1.0)	3.4	1.1 (0.1)	13.4
R9	0.51 (0.04)	8.3	2.67 (0.31)	11.5	80.7 (2.2)^{a,b}	2.7	87.9 (3.4)^{b,c}	3.8	66.9 (2.5)^{a,b}	3.7	30.0 (1.1)	3.7	1.1 (0.0)	3.7

Different superscript letters within the same column indicate significant difference ($P < 0.05$) for ADC values.

No significant correlation was observed between CP or total P dietary content and respective ADC values. Figure 3.3 shows the linear relationship between feed ash and total P contents (R^2 = 0.73; *P* = 0.004). The total P content in feeds also correlated positively with total P in feces (R^2 = 0.70; *P* = 0.005). The same trend was observed for the inert marker, acid insoluble ash (R^2 = 0.78; *P* = 0.002; Fig. 3.3). However, no significant correlation was observed between CP in feed and CP in feces (*P* >0.05). Apparent digestibility coefficient of P and of dry matter (DM) correlated significantly with dietary crude fat content (Fig. 3.4A, B). Although not significant, ADC of DM tended to decrease with increased dietary crude fiber content (R^2 = 0.36, *P* = 0.08) (Fig. 3.4C). Apparent digestibility coefficient of total P was highly correlated with ADC of DM (R^2 = 0.96; *P*<0.001) and with ADC of CP (R^2 = 0.85; *P*<0.001). The ADC of CP and DM were also highly correlated (y = 0.501x + 49.742, R^2 = 0.79; *P* =0.001) (Fig. 3.5).

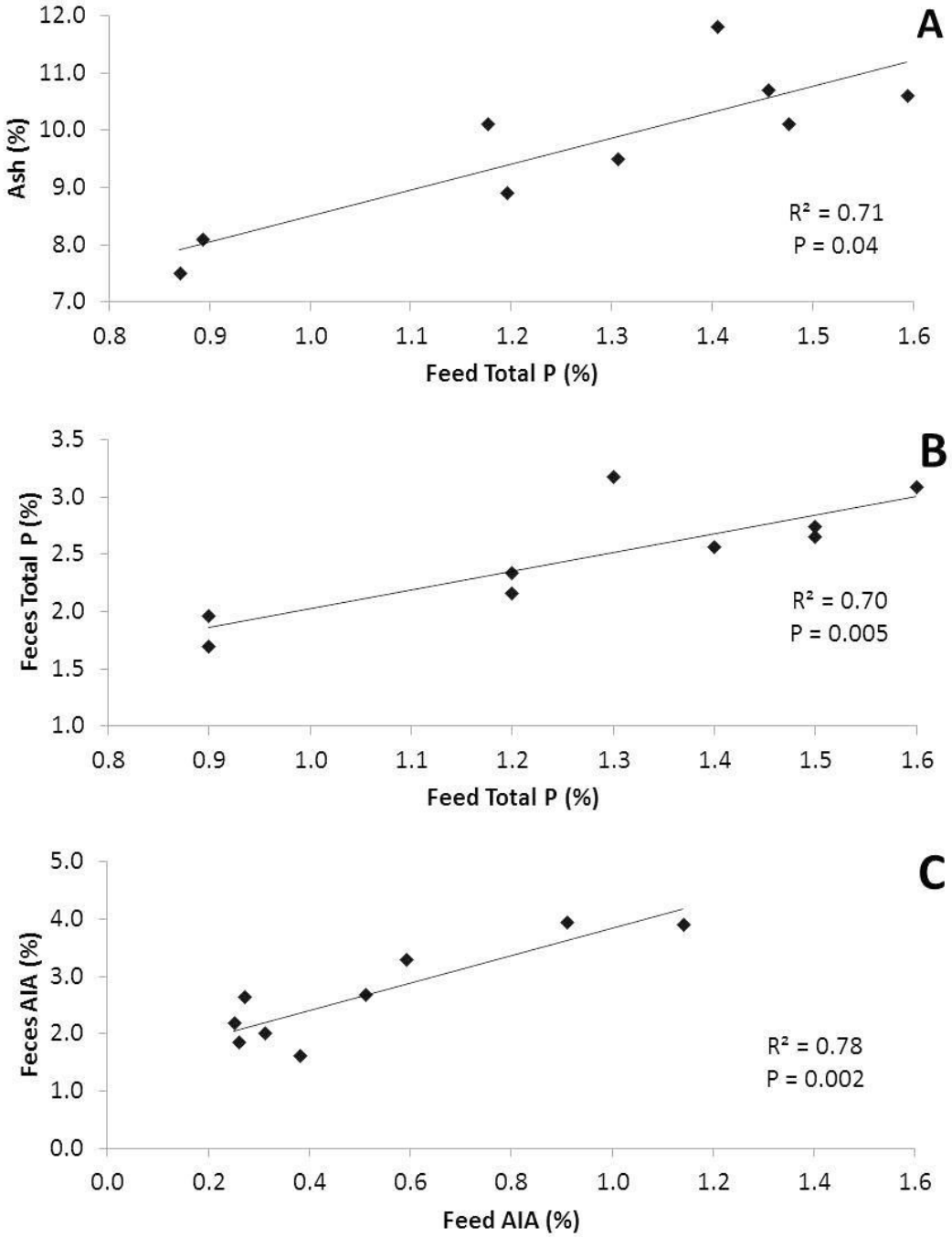

Figure 3.3 – Relationships between (A) dietary total phosphorus (P) and ash contents, (B) dietary total P and feces total P, and (C) internal marker acid insoluble ash (AIA) in feed and feces.

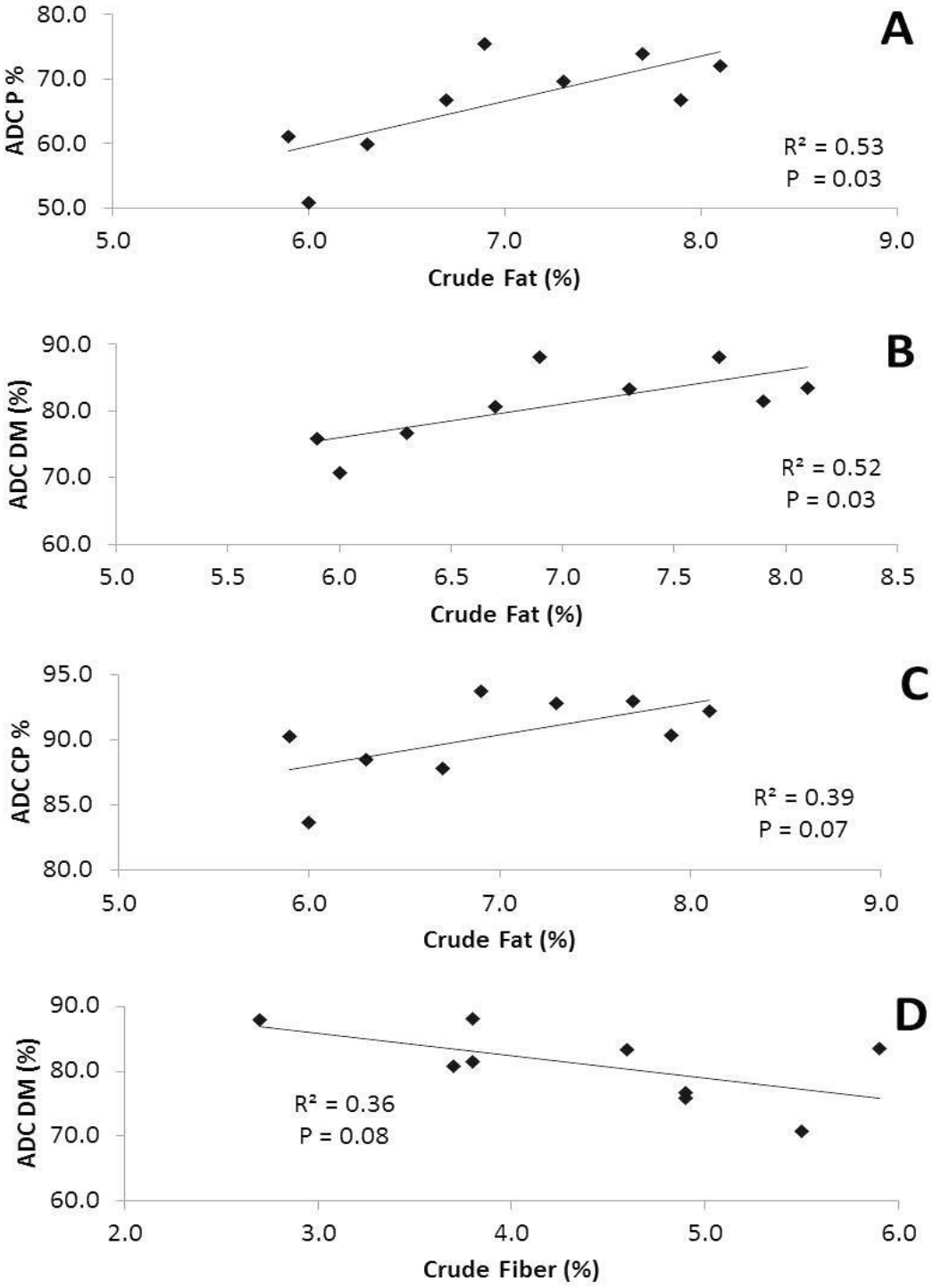

Figure 3.4 – Relationship between dietary crude fat content and apparent digestibility coefficient (ADC) of (A) total phosphorus (P), (B) dry matter (DM) and (C) crude protein (CP), and (D) crude fiber and ADC of DM of nine commercial feeds for Nile tilapia, *Oreochromis niloticus* cultured at 26.0 ± 1.4 °C.

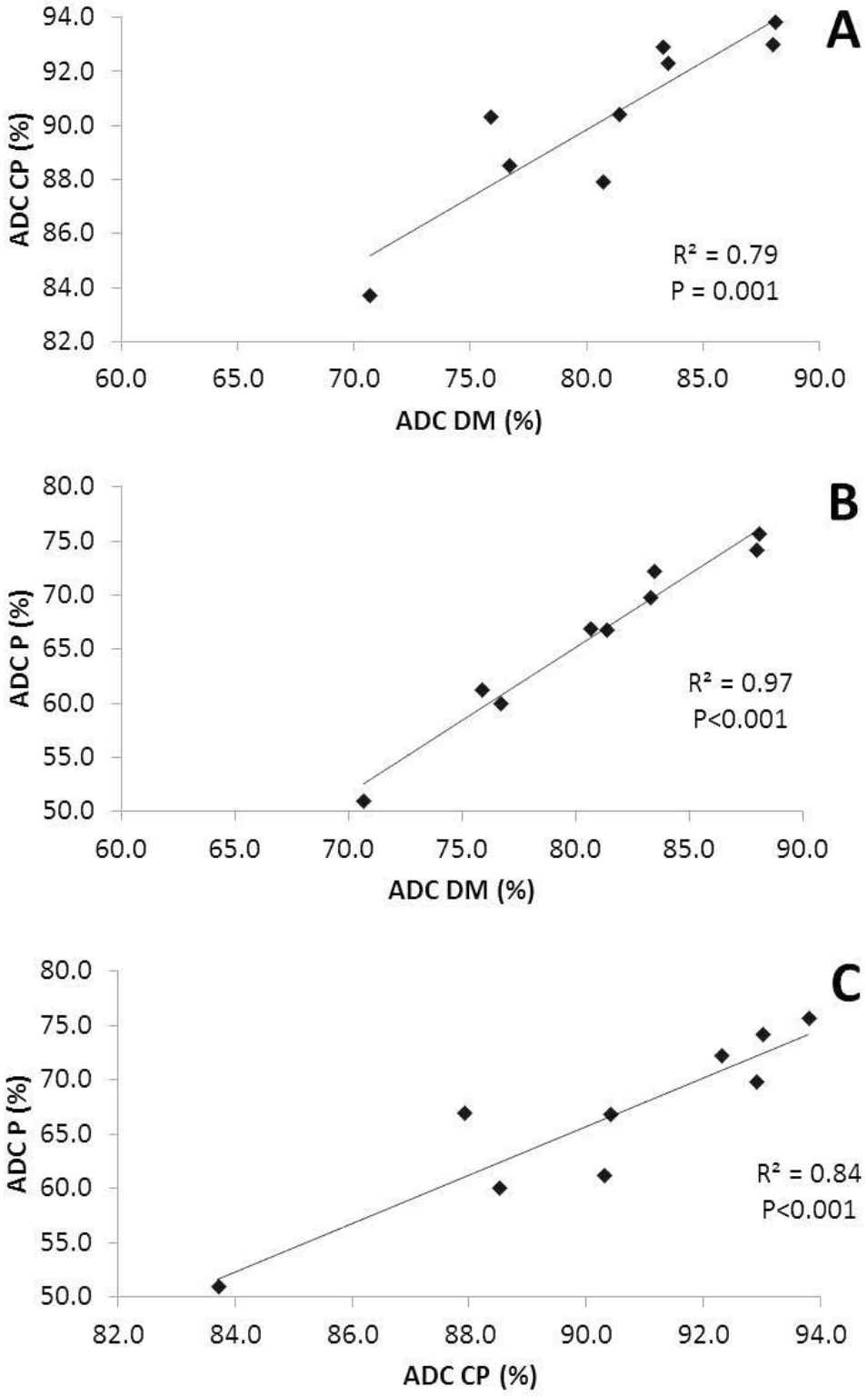

Figure 3.5 – Relationship between (A) apparent digestibility coefficient (ADC) of crude protein (CP) and dry matter (DM), (B) ADC of phosphorus (P) and DM, and (C) ADC of CP and P of nine commercial feeds for Nile tilapia, *Oreochromis niloticus* at cultured at 26.0 ± 1.4 °C.

For all commercial feeds tested, with increased addition of enzyme extract, maintaining the substrate constant, the DH resulted in a log-shaped function (Fig. 3.6). *In vitro* pH-stat degree of protein hydrolysis (DH) with stomach extract presented very high variation between replicates (Fig. 3.7A). Blank assays, i.e., feed sample in distilled water without addition of stomach enzyme extract resulted in DH values sometimes higher than with extract. The DH of feed samples with intestine extract or two-stage digestion (digestion with stomach enzyme extract followed by digestion with intestine enzyme extract) with 50 or 200 µL enzyme extract did not differ significantly ($P>0.05$) between the commercial feeds. With 800 µL enzyme extract significant difference ($P<0.05$) was found between feeds in the digestion with intestine extract and the in the two-stage digestion (Fig. 3.7B,C). With intestine extract and two-stage hydrolysis highest DH was for R7 (12.81% and 13.46%, respectively). With intestine extract, lowest DH was for R5 (8.46%) and with two-stage DH, for R4 (9.11%). No significant difference ($P>0.05$) between DH values with intestine extract and two-stage digestion was found for any of the volumes tested (50, 200, 800 µL extract). The DH with 800 µL intestine enzyme and the two-stage digestion with 800-800 µL were able to discriminate the feeds with highest (R7, 93.8 ±2.3%) and lowest (R5, 83.7 ± 1.0%) ADC of CP (Fig. 3.7B,C).

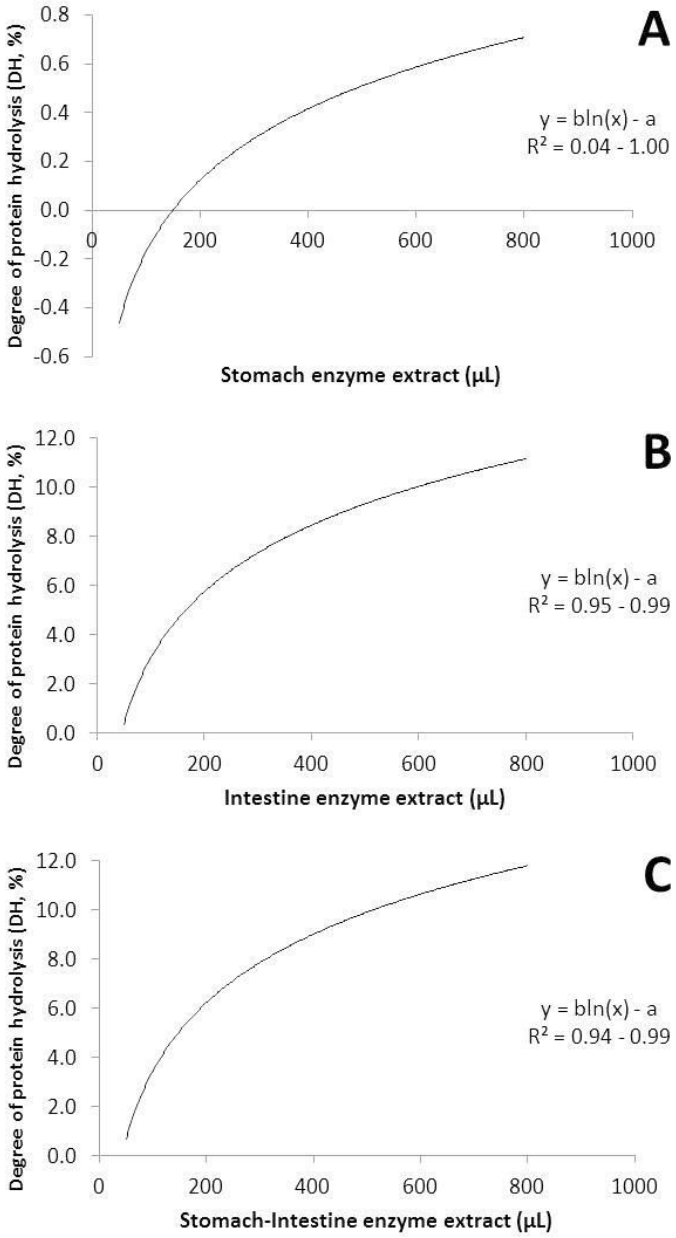

Figure 3.6 – Hydrolytic performance of enzyme extracts recovered from cage farmed Nile tilapia, *Oreochromis niloticus* (fed individuals, 598.3 ± 110.1 g). Substrates were nine commercial feeds for Nile tilapia (4 -6 mm pellet size, 32% crude protein, declared values) submitted to digestion with (A) stomach, (B) intestine, and (C) stomach followed by intestine enzyme extract (two-stage). Plotting *in vitro* pH-stat protein hydrolysis carried out for 60 min at 25 ± 0.5 ºC, with 80 mg protein substrate and 50, 200, and 800 µL of enzyme extracts (n = 6) resulted in well-adjusted logarithmic function [y = bLn(x) – a]. Further details in Materials and Methods.

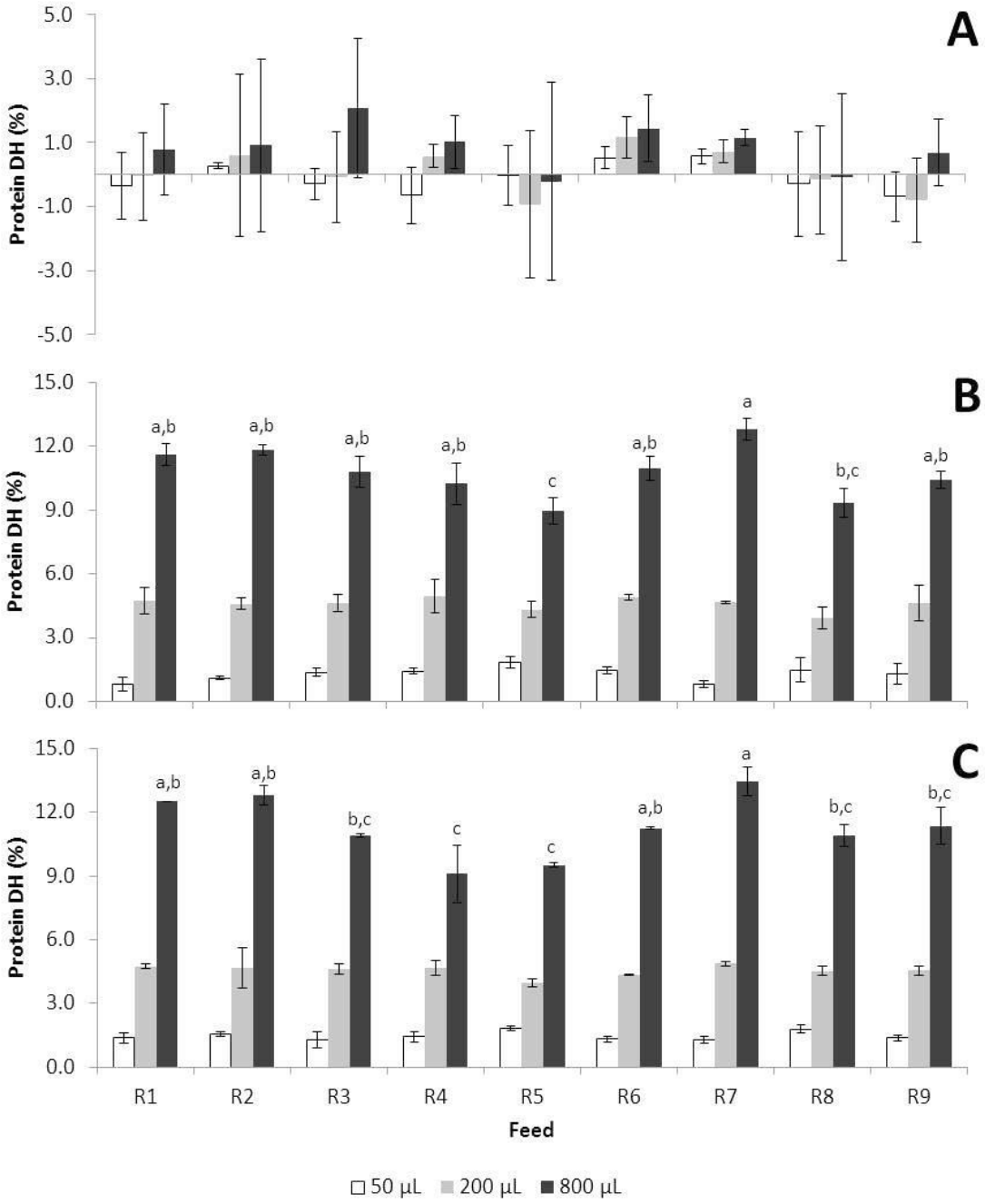

Figure 3.7 – Degree of protein hydrolysis (DH, %) of the nine commercial feeds (R1 – R9) for Nile tilapia, *Oreochromis niloticus.* DH values of feeds incubated with (A) stomach enzyme extracts, (B) intestine enzyme extracts, and (C) sequential (two-stage) digestion with stomach followed by intestine extract. Each bar is the mean of two replicate enzyme volumes (error bar is the standard deviation). Each feed was tested with three extract volumes for stomach, intestine and two-stage digestion (50, 200 and 800 µL). For the two-stage digestion, stomach and intestine extract volumes were the same. Different superscript letters indicate significant difference (P<0.05) between feeds using the same enzyme extract volume. Further details in Materials and Methods.

Significant correlation was found between feed DH with intestine (800 µL) and two-stage DH with 200-200 µL (r = 0.76; *P* = 0.02) and with 800-800 µL enzyme extract (r = 0.83; *P*=0.005) (Table 3.4). No significant correlation was detected between *in vitro* DH with stomach, intestine, and two-stage digestion, and feed crude protein content, weight gain, feed intake or *in vivo* protein ADC.

No correlation was observed between ADC of total P and *in vitro* release of soluble P in distilled water, at pH 2.0, pH 8.0 or after *in vitro* digestion with fish stomach or intestine enzyme extracts. However, correlation between total P and digestible P (r = 0.77; *P* = 0.002) and soluble P (r = 0.75; *P* = 0.02) was observed (Table 3.5). Furthermore, correlation between digestible P and *in vitro* release of soluble P in water (r = 0.70; *P* = 0.03) and between digestible P with *in vitro* release of soluble P with 200 µL intestine extract (r = 0.67; *P* = 0.05) were also observed.

Table 3.4 – Correlation coefficient (r) and *P*-value of the relationship between *in vitro* degree of protein hydrolysis (DH), feed crude protein (CP) content, apparent digestibility coefficient (ADC) and apparently digestible (AD) CP, and daily weight gain with nine commercial feeds for Nile tilapia, *Oreochromis niloticus*. Two-stage digestion consisted of incubating feed with stomach enzyme extract and sequentially incubating with intestine extract. (*) Significant correlation at $P<0.05$.

Variables				*In vitro* (pH-stat) degree of protein hydrolysis with								
				Stomach digestion			Intestine digestion			Two-stage digestion		
		ADC CP	AD CP	50 µL	200 µL	800 µL	50 µL	200 µL	800 µL	50-50 µL	200-200 µL	800-800 µL
Feed CP	r	-0.10	-0.34	-0.23	0.04	-0.10	0.66	0.24	-0.56	0.10	-0.51	-0.78
	P-value	0.80	0.37	0.55	0.92	0.79	0.05	0.54	0.11	0.79	0.16	0.01
ADC CP			0.20	0.26	0.65	0.61	-0.46	0.12	0.42	-0.69	0.63	0.41
			0.61	0.50	0.05	0.08	0.21	0.76	0.26	0.04	0.07	0.28
AD CP				-0.31	-0.24	-0.12	-0.46	-0.09	-.37	0.36	0.53	0.43
				0.42	0.53	0.76	0.21	0.82	0.33	0.34	0.14	0.24
Daily weight gain		-0.13	-0.23									
		0.74	0.55									
In vitro DH with Stomach digestion												
50 µL										-0.41	-0.10	0.54
										0.28	0.80	0.13
200 µL										-0.74	0.39	0.36
										0.02	0.30	0.34
800 µL										-0.79	0.47	0.25
										0.01	0.20	0.35
Intestine digestion												
50 µL										0.48	-0.86	-0.85
										-0.18	0.003	0.003
200 µL										-0.50	0.34	0.06
										0.17	0.36	0.88
800 µL										-0.51	0.76	0.83
										0.16	0.02*	0.005*

Table 3.5 – Correlation coefficient (r) and *P*-value of the relationships between *in vitro* release of soluble phosphorus (P) and feed total P, apparent digestibility coefficient (ADC) of P, apparently digestible (AD) P, and between daily weight gain and ADC and AD P of nine commercial feeds for Nile tilapia, *Oreochromis niloticus*. (*) Significant correlation at $P<0.05$.

Variables				*In vitro* release of soluble P in												
								Stomach digestion			Intestine digestion			Two-stage digestion		
		ADC P	AD P	Distilled water	pH 2.0	pH 8.0	pH 2.0-8.0	50 µL	100 µL	200 µL	50 µL	100 µL	200 µL	50-50 µL	100-100 µL	200-200 µL
Feed total P	r	0.00	0.77	0.75	0.41	0.09	0.43	0.31	0.09	0.40	0.42	0.76	0.40	0.19	0.14	0.62
	P-value	0.98	0.002*	0.02*	0.27	0.82	0.25	0.41	0.81	0.26	0.26	0.02*	0.01*	0.62	0.72	0.07
ADC P			0.48	0.11	-0.06	0.16	-0.03	0.28	0.21	0.40	0.07	-0.08	-0.03	0.14	-0.05	-0.29
			0.20	0.87	0.87	0.68	0.94	0.47	0.58	0.29	0.85	0.84	0.98	0.71	0.89	0.44
AD P				0.70	0.40	0.17	0.42	0.44	0.20	0.54	0.39	0.61	0.67	0.29	0.14	0.43
				0.03*	0.29	0.67	0.25	0.24	0.60	0.13	0.29	0.08	0.05*	0.44	0.72	0.24
Daily weight gain		-0.24	-0.26													
		0.53	0.50													

3.4. Discussion

Nile tilapia cage farming in freshwater reservoirs in Brazil are recommended to use fish stocking densities below 200 fish m^{-3} (*ca.* 40 g individual weight) aiming at efficient production, which means highest weight gain (accepted by the consumer) with low feed conversion rate, within a short period of time (SCHIMITTOU, 2006; MARENGONI, 2006) in addition to reduced stress with manipulation (GARCIA et al., 2013). Stocking density of 100 fish m^{-3} may result in a specific growth rate (SGR) of 3.9% (6.45 g day^{-1}) and a feed conversion ratio (FCR) of 0.94 in 58 days with fish initial and final weight of 43.16 ± 5.34 g and 415.52 ± 34.85 g, respectively (AYROZA et al., 2011). In a similar study with cages in a freshwater reservoir, 78 g Nile tilapia were stocked at a density of 133 fish m^{-3} for 41 days and resulted in a final weight of 311.2 g, SGR of 3.4% (5.73 g day^{-1}) and a FCR of 0.95 at an average temperature of 29 °C (GARCIA et al., 2013). Compared to such studies in commercial farming conditions in freshwater reservoirs, the present study yielded lower growth values possibly due to lower water temperature (26 °C) and high total ammonia nitrogen and nitrite, and low pH values (6.0), as these parameters may have stressed fish and affected the results. In the present study feed was provided to apparent satiation to avoid feed waste, as may occur in commercial farms where feed is given as percentage of biomass. Additionally, tanks were cleaned every day to avoid periphyton formation, which can be an important food source in commercial cages and, consequently, result in better FCR values.

For the digestibility trial, dietary acid insoluble ash fraction (AIA) proved to be an efficient indicator of digestibility in testing commercial feeds, as reported previously for rainbow trout (ATKINSON et al., 1984), Australian freshwater crayfish (JONES; DA SILVA, 1998), Nile tilapia (GODDARD; McLEAN, 2001; ADEPARUSI; JIMOH, 2002), and bullfrog (CASTRO et al., 2012). In the present study, the AIA content in the nine commercial feeds ranged from 0.25 to 1.14% (dry matter basis) and was positively correlated with the AIA content in feces. The high coefficient of variation of up to 21% in feed AIA content may be a result of analytical error, possibly because the AIA fraction in the feeds was low and the amount of feed sample analyzed was insufficient. Increasing feed sample to 5 g or more might result in higher AIA content and consequently reduce the coefficient of variation. The addition of 1% celite (a form of diatomaceous silica) has shown to increase the precision of the AIA measurement with c.v. of 1.6% (ATKINSON et al., 1984). Analysis of AIA content in feeds still needs improving. The coefficient of variation of AIA content in feces between

the five replicate samples from pooled feces from one tank was less than 8%. Possibly the amount of feces sample analyzed was enough to reduce variation, as the AIA content in feces was at least three times and up to ten times (R3, R7) the AIA content in feeds. The coefficient of variation of AIA content in feces between replicate tanks was high in two cases (about 50% in R7 and R8) but it did not affect coefficient of variation in apparent digestibility coefficient (ADC) of dry matter (<8%), crude protein (<4%) or phosphorus (<13%). The reason for high variation between replicate tanks but not between replicate within a tank is not clear. Considering the results, the relative simple chemical analysis, and increase in the measurement precision, the AIA fraction has the potential to be used routinely as indigenous marker in digestibility studies of commercial feeds or formulated diets with practical ingredients.

Feeds with high fat content resulted in higher ADC of dry matter, whereas feeds with high fiber content tended to decrease ADC of dry matter, which may demonstrate a more efficient digestion of lipids than fiber. Dietary fiber of plant origin could be partly digested by cellulase and other non-starch polysaccharides degrading enzymes produced by Nile tilapia gut bacterial flora (SAHA et al., 2006; BAIRAGI et al., 2002, HLOPHE et al., 2014), but other sources of dietary fiber such as chitin, a non-starch polysaccharide from crustacean exoskeleton, is not digested and additionally, dietary fiber might influence nutrient movement along the gastrointestinal tract reducing nutrient absorption (SHIAU; YU, 1999). Crude protein ADC was positively correlated with dry matter ADC, showing that crude protein was efficiently digested. Crude protein ADC was above 83% but only two (R8 and R9) of the nine commercial feeds met the minimum recommendation 29% digestible protein (NRC, 2011). Crude protein in feces did not correlate with crude protein levels in the feed.

Ash content in the feeds correlated with total phosphorus, indicating that increased phosphorus in the feed increased dietary ash. In fish, absorption of inorganic phosphorus (Pi) may be downregulated by its concentration in the intestinal lumen, i.e., when luminal P concentration is high, above requirement, uptake is passive but when it is low, uptake may be mediated by sodium-phosphate (NaPi) cotransporter (AVILA et al, 2000; SUGIURA; FERRARIS, 2004). In Nile tilapia mRNA of such co-transporter was detected in the proximal intestine (SUGIURA, 2009). Total phosphorus ADC varied between 51 and 75.6 %, which resulted in available P varying from 0.6 to 1.1%, which meets the dietary requirement reported for Nile tilapia of 30 - 146 g and of 150 – 350 g (0.52 and 0.45% available P,

respectively) (FURUYA et al., 2008; QUINTERO-PINTO et al, 2011). Total phosphorus in the feed correlated positively with total phosphorus in the feces, which may be an indication that phosphorus was in excess in the feeds or that it is not available to the fish. The reason for the positive correlation between ADC of total P and ADC of crude protein observed in the present study is not clear. For pigs and poultry, the interaction between phytate and protein is reported as forming insoluble binary and ternary protein-phytate complexes, depending on the surrounding pH, in the gastrointestinal tract and such interactions may be fundamental to the negative impact of phytate on protein or amino acid availability (SELLE et al., 2012). In an *in vitro* study with rainbow trout stomach and pyloric ceca proteases reported reduction in protein solubility from casein and also the phytate-protein complex formation may have reduced the activity of the gastric and intestinal proteases, except chymotrypsin (MORALES et al., 2011). In the present study, according to label information in the commercial feed, inorganic P (Pi) was not included in the composition of R5. Therefore, the lowest total P ADC (51.0%) could be possibly explained by the absence of inorganic supplementation, as P from plant ingredients (phytate) or from bones (calcium phosphates) would be less soluble and, thus, less digestible (HUA et al., 2005). Based on low ADC of total P, possibly caused by high phytate content, the formation of such phytate-protein complex that reduce protein solubility and digestive enzyme activity in the fish gut may be hypothesized, which consequently reduced ADC of crude protein in R5.

In nature, organic P such as phytate excreted in the feces may be mineralized by bacteria and made available in the water, whereas calcium-bound P is usually inert in normal environmental conditions and have little potential to stimulate eutrophication (CHO; BUREAU, 2001). In addition to the particulate form of P loss through feces, all the excess P after absorption would be excreted through urine and gills as inorganic soluble P (orthophosphate), contributing to eutrophication as it is the form most available for algal growth (CHO; BUREAU, 2001). Therefore, quantification of different forms of P in feeds would be interesting to better understand apparent digestibility of P (HUA et al., 2005). Unfortunately, the total P content in fish body was not measured in the present study so as to determine P retention and to estimate excretion of soluble P.

The nine commercial feeds tested in the *in vivo* trial were also tested *in vitro* to determine the degree of peptide bond hydrolysis (DH). The DH values from the digestion with Nile tilapia stomach extract were highly variable between replicates. This may indicate

that the technique (pH-stat) may not work in pH 2.0 (ADLER-NISSEN, 1986) or the method still requires refining, as it worked well with purified substrate such as hemoglobin (YASUMARU; LEMOS 2014). In a previous study with feed ingredients, the meaning of measuring DH in digestion with stomach extract was not clear but the incubation with stomach extract prior to digestion with intestine extract (two-stage) resulted in higher DH when compared to digestion with intestine extract only (YASUMARU; LEMOS, 2014). In the present study, compound feeds of unknown composition were tested and DH with two-stage digestion did not differ from DH with intestine extract digestion. For some reason the digestion with stomach extract did not increase DH value in the two-stage digestion of the compound feeds. Nevertheless, significant difference could be detected between feed DH (with 800 µL of enzyme extract) in the digestion with intestine extract and in the two-stage digestion. In this way, the compound feeds might be ranked according to intestine or two-stage DH.

In the present study, correlation between *in vivo* ADC of crude protein and *in vitro* DH could not be observed with the nine commercial feeds tested. However, with the *in vitro* DH it was possible to detect the feeds with highest and lowest ADC of CP. The absence of significant correlation in this study could be partially explained by the differences inherent to each method, e.g., *in vivo* digestibility trials may be affected by endogenous losses, which does not occur in the *in vitro* digestion assays (VILLAMIDE et al. 2009), and *in vivo* digestibility is a measure of the disappearance of a nutrient (Bureau, 2008; NRC, 2011) and *in vitro* digestion calculates only the amount of peptide bonds cleaved, free amino acids, if present, are not included in the calculation. However, with feed ingredients and also diets, correlation between *in vivo* and *in vitro* data has been reported for some fish and crustacean (DIMES et al., 1994b; EZQUERRA et al., 1998; EL-MOWAFI et al., 2000; CÓRDOBA-MURUETA; GARCIA-CARREÑO, 2002; TIBBETTS et al., 2002; LEMOS et al., 2009; LEMOS; YASUMARU, 2010; TIBBETTS et al., 2011b). Correlation between *in vivo* apparent phosphorus digestibility of animal origin ingredients and *in vitro* digestibility with digestive enzymes has been reported for rainbow trout (WEERASINGHE, 2001). In the present study, correlation of feed total phosphorus with apparently digestible P and *in vitro* released soluble P were observed. Also, correlations between *in vivo* apparently digestible P and *in vitro* release of soluble P either in distilled water or after digestion with intestine extract (200 µL) were observed. A study carried out with common carp and rainbow trout, a stomachless fish and a fish with a true stomach, respectively, demonstrated that digestible P to

carp corresponded mostly to the soluble P fraction in water (SATOH et al., 1992), as P contained in fish meal is mainly in the form of tricalcium phosphate, very poorly absorbed by common carp which have no stomach (OGINO et al., 1979). When inorganic P was supplemented to a fish meal based diet, the digestible P for rainbow trout was the equivalent to the water soluble P but in a diet without supplementation rainbow trout utilized P from the fish meal (SATOH et al., 1997). According to the correlations found between apparently digestible P and released soluble P, there may be an indication that Nile tilapia absorbs the inorganic P supplement until the requirement is reached and above such level, it may be excreted in the feces. Therefore, feeds should be formulated to contain the minimum P requirement to avoid waste and minimize eutrophication (SUGIURA, 2000; SUGIURA et al., 2006; SATOH et al., 2003).

In conclusion, it was not possible to predict apparent digestibility of crude protein or phosphorus under the methods tested in the present study, as *in vivo* apparent digestibility and *in vitro* digestion methods are affected by different factors. However, the value of degree of protein hydrolysis (DH) from the *in vitro* pH-stat digestion method could discriminate between the feeds with highest and lowest ADC of CP. And some inferences could be made on the relationships between released soluble P in distilled water and *in vitro* pH-stat digestion with intestine extract and total feed P and apparently digestible P. The AIA fraction may be an alternative as internal marker in digestibility studies if analytical precision is improved. Further studies are necessary to overcome the differences inherent to each method and, eventually, develop a method capable of predicting nutrient digestibility in fish.

GENERAL CONSIDERATIONS

The primary goal of this thesis was to assess species-specific crude digestive enzymes from fish to develop an *in vitro* method for the digestion of protein and phosphorus. The pH-stat technique was utilized for both protein and phosphorus digestion. For protein, the result is expressed as degree of protein hydrolysis (DH, %), which is a measure of the amount of peptide bonds cleaved from a raw material or finished feed. For a consistent analysis, enzyme extracts should be standardized so that influence of factors as fish size, age, farming condition, and strain is minimized and different batches of the same species or of different species may result in the same hydrolytic capacity (DH). Results in **Chapter 1** indicate that enzyme extracts could be standardized by the DH method and values could be compared between the feed ingredients. The study also included a gastric digestion or acid pre-digestion, which values were not conclusive but, overall, the inclusion of the procedure increased DH results. The sample preparation step, when sample pH is stabilized appears to be very important for the precision and reproducibility of the digestion assay, as instability may lead to high variations between replicates and poor results. The *in vitro* pH-stat method of protein digestion developed using species-specific enzyme extracts proved to be precise and with potential application in the industry to rank raw materials or finished feeds but some improvements is still necessary. These would include testing more practical sources and analytical routines, and determination of possible relationship with apparent protein digestibility or amino acids availability.

Chapter 2 presented the development of an *in vitro* pH-stat of phosphorus digestion. The method showed to be precise, based on the low coefficient of variation (< 5% cv). Ingredient composition of the nine commercial feeds tested was not available but some inferences could be made. The acidic medium (pH 2.0) greatly affected phosphorus solubility, compared to alkaline medium (pH 8.0), in which it may form insoluble compounds with calcium for example. The release of soluble phosphorus after the two-stage digestion indicated the importance of including the digestion with stomach enzyme extracts to *in vitro* digestion analysis. The decreased release of soluble phosphorus with the addition of digestion enzyme extract could be explained by the formation of insoluble complexes between phytate and protein (from the enzyme extract), which reduces activity. The released soluble phosphorus measured could be basically inorganic supplements, as feed without supplementation (R5, label information) resulted in low solubility phosphorus. Some

relationships could be verified, e.g., total feed phosphorus with released soluble phosphorus in distilled water (pH 6.4) ($P<0.05$) and with released soluble P after intestine digestion (pH 8.0; 100 and 200 µL). Also, significant correlation ($P<0.05$) was observed between soluble P released in distilled water (pH 6.4) and soluble P released after intestine digestion (pH 8.0; 50, 100 and 200 µL). Digestion in acidic pH seems to affect more the release of soluble P than digestion in alkaline pH, as digestion with stomach enzyme extracts resulted in higher values. Furthermore, gastric digestion showed to be important in the two-stage digestion, yielding results higher than intestine digestion only. *In vitro* studies on release of soluble P for fish are scarce, thus, the meaning of the values of released soluble P as measured in the present study remains unclear. Therefore, further studies including *in vivo* P digestibility should be carried out to try to elucidate the meaning and potential utility of this analysis.

In **Chapter 3**, an *in vivo* trial was carried out to determine apparent digestibility of protein and phosphorus. Nile tilapia were stocked in a recirculated water system designed originally for shrimp. Large amount of feces could be collected throughout the trial, which provided more than enough samples for the chemical analysis. Although fish did not appear to be under stress or with any pathology, the water quality and temperature might have influenced the growth results that were below those obtained in the field, in commercial cage farms in freshwater reservoirs. The acid-insoluble ash fraction seemed to be a potential inert marker for more routinely use. The analytical protocols should be revised and possibly by increasing the amount of sample, marker concentration may be increased and coefficient of variation reduced. This inert marker would have some advantages over the added external markers, e.g., acid-insoluble ash, which is primarily silica, is present in the feedstuffs and should pass through the fish digestive tract at the same pace as the rest of the digesta and does not affect pellet palatability or appearance. A correlation was observed between apparent digestibility coefficients of crude protein and phosphorus. Possibly phosphorus in the form of phytate interacted with protein, both in the stomach and the intestine, which resulted in precipitation or unavailability. Unfortunately, no body phosphorus content was measured, which would be an important data on phosphorus retention and estimate soluble waste of tilapia. For future studies, determination of body phosphorus content and the analysis of phytate and bone phosphorus should be included to maybe provide better understanding or correlation. The results from the *in vitro* method did not correlate with *in vivo* apparent digestibility but the *in vitro* method was able to distinguish between high and low DH feeds.

Intensive work, both on laboratory bench and at the experimental station was required for the accomplishment of the goals. Nevertheless, some improvements or future studies still could be made, e.g., testing higher amounts of feed sample in the attempt to reduce variation between replicates in the acid-insoluble ash fraction analysis; dimension the recirculated system at the experimental station, including efficient and adequate biological filtration, and cover or lid to the fish tanks; determine fish body phosphorus content when studying apparent phosphorus digestibility to estimate soluble losses through, e.g., urine and gills; formulate experimental diets with ingredients with known phosphorus content and test for apparent digestibility and also *in vitro* release of soluble phosphorus and analyze any possible relationship between phosphorus solubility and source, i.e., bone phosphorus, phytate phosphorus; test commercially available enzymes to compare with digestive enzymes from fish, such as Nile tilapia, cobia or any other species of interest to aquaculture, searching for a possible correlation so that the method can have commercial application; and also run more *in vitro* phosphorus digestion assays to try to understand what might happen in the fish gut, the interactions between nutrients.

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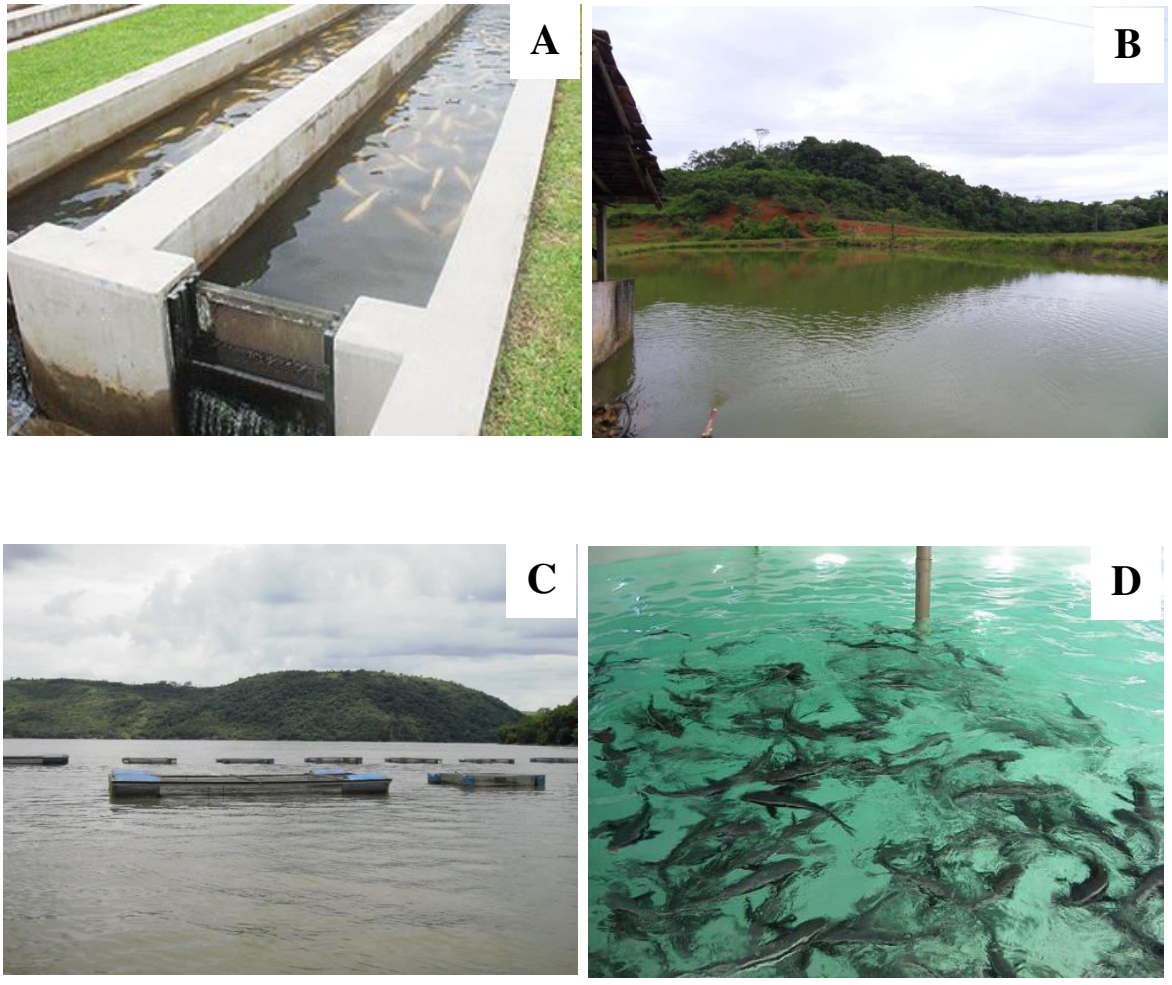
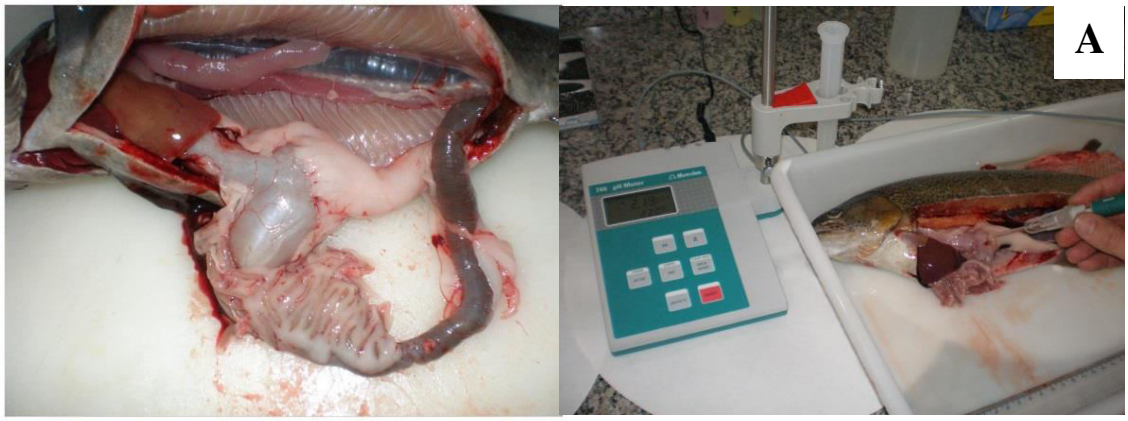
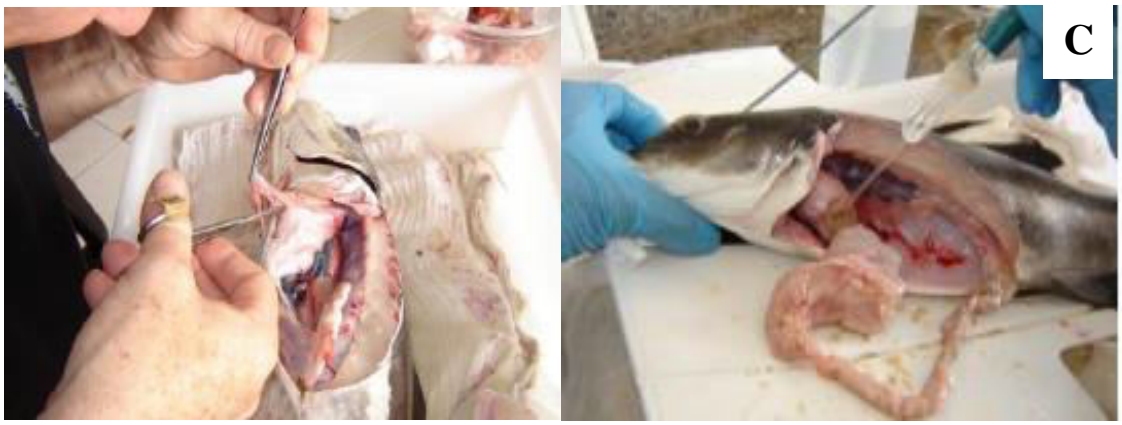
APPENDIXES

APPENDIX A – Fish sampling sites. (A) rainbow trout, *Oncorhynchus mykiss* in raceways (Campos do Jordão, SP); (B) Nile tilapia, *Oreochromis niloticus* in cages in a freshwater reservoir (Ipaussu, SP); (C) Nile tilapia, *Oreochromis niloticus* in fertilized earthen ponds (Joinville, SC); and (D) cobia, *Rachycentron canadum* in concrete tanks with flow-through seawater (Ipojuca, PE).

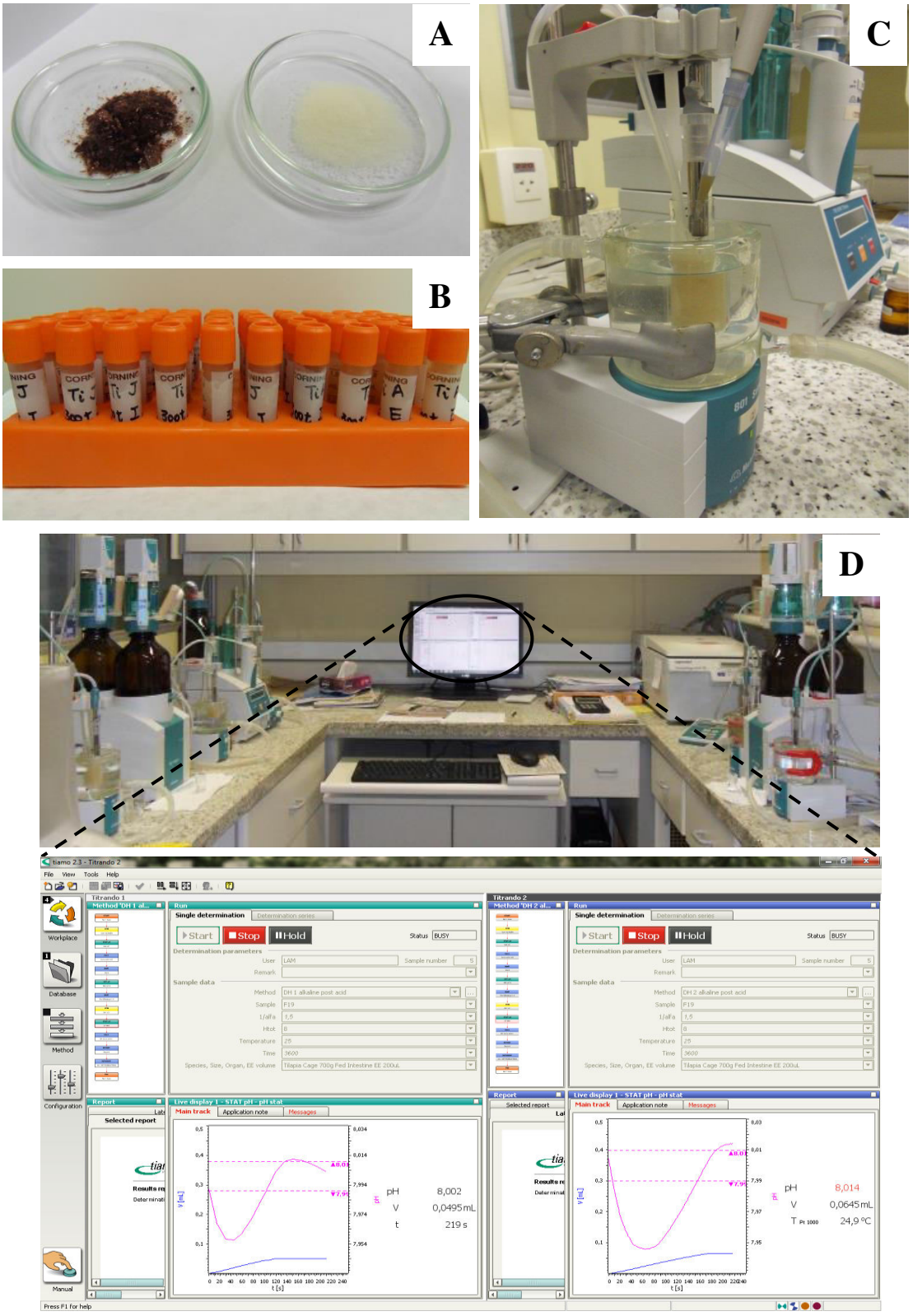


APPENDIX B – Digestive tract of sampled fish and pH measurement in each organ (stomach, pyloric ceca and/or intestine) with a combined glass electrode connected to a pHmeter (7 mm immersion depth and 3 mm electrode tip diameter – Biotrode 744 pHmeter, Metrohm AG, Switzerland). (A) rainbow trout, *Oncorhynchus mykiss*, (B) Nile tilapia, *Oreochromis niloticus*, and (C) cobia, *Rachycentron canadum*.

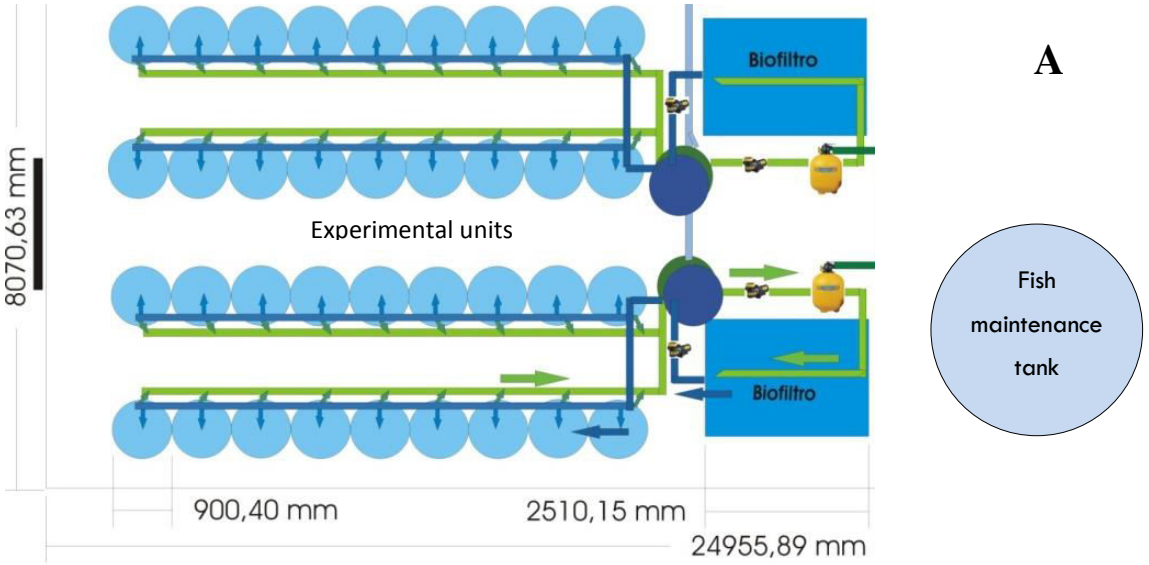




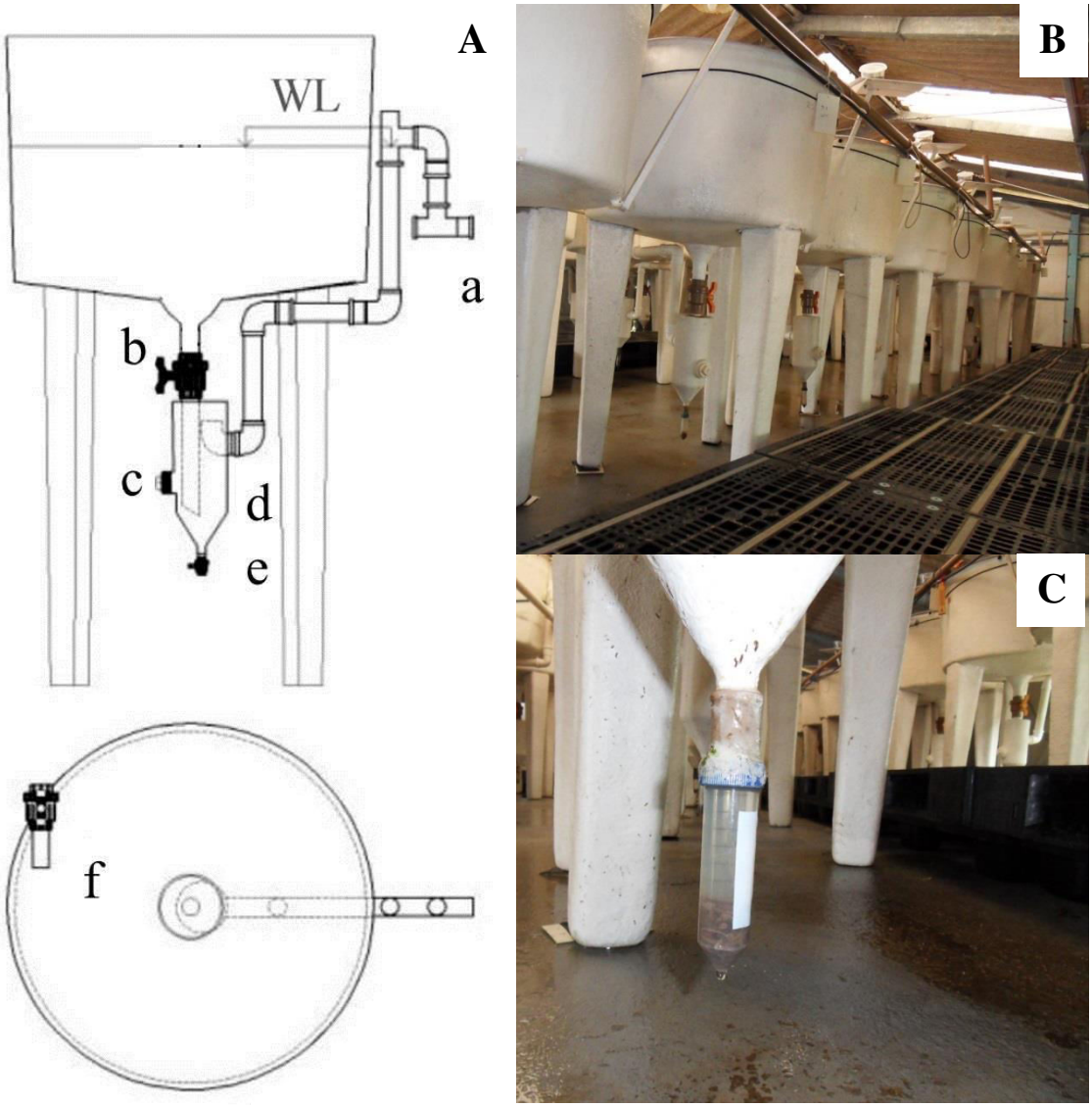
APPENDIX C – Digestive crude enzyme extract hydrolytic capacity standardization. (A) Analytical grade protein substrates (hemoglobin and casein for stomach and pyloric ceca/intestine digestion, respectively); (B) crude enzyme extracts from stomach and pyloric ceca/intestine of rainbow trout, *Oncorhynchus mykiss*, cobia, *Rachycentron canadum* , and Nile tilapia, *Oreochromis niloticus*; (C) pH-stat protein hydrolysis assay; (D) pH-stat titrators coupled (two double and one single titrator) to a data logging software and standardization curves in the detail.



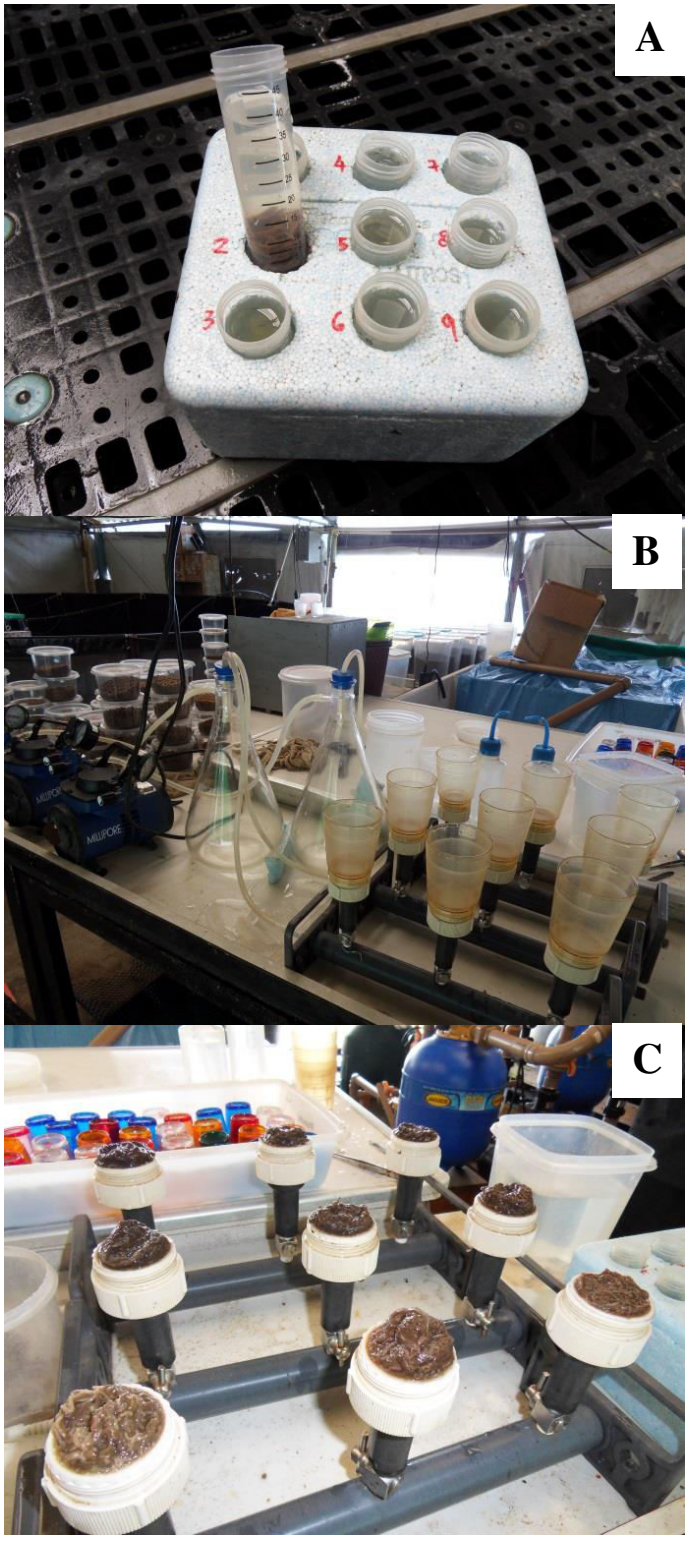
APPENDIX D – *In vivo*digestibility trial. (A) layout of experimental units and recirculating system; (B) commercial feeds tested; (C) experimental tanks; (D) Nile tilapia, *Oreochromis niloticus* in the experimental tank; (E) water header tanks (top), water collecting tanks (bottom), water pumps (black and yellow), sand filters (blue) and beads biofilter (green, on the left).



APPENDIX E – Experimental tanks. (A) and (B) schematic drawing of tank used for the growth and digestibility trials with Nile tilapia, *Oreochromis niloticus*. (a) Primary drain, (b) secondary drain, (c) adapter and plug for cleaning purpose (d) settling column, (e) outlet for sampling and solids removal, (f) water inlet, WL – water level. Tank measured 1000 x 960 x 670 mm (upper x lower diameter x height); bottom sloped 5 ° toward the drain; drain measured 170 x 50 x 65 mm (upper x lower diameter x height). Tank total volume 500 L, water volume 300 L (water depth 400 mm). From Carvalho et al. (2013); (C) feces collecting tube.



APPENDIX F – Feces collection. (A) Feces collection tubes; (B) vaccum filtration manifolds for feces filtration using qualitative filter paper (4-12 µm particle retention range, 80g m^2, 20-25 s filtration speed according to DIN 53137); (C) Nile tilapia, *Oreochromis niloticus*, feces after filtration. Feces pooled per tank were stored in freezer at – 20 °C in 250 mL plastic containers until processed.



APPENDIX G – Analysis of acid insoluble ash (AIA) content in feed and feces samples. (A) Feed or feces samples after ashing (6 h at 600 °C) cooling in dissecator prior to weighing; (B) Feed or feces ash samples boiled with HCl 2N (25 mL HCl 2N for 5 min); (C) samples filtered with the aid of a vacuum pump through ashless filter paper (Whatman 41) and residue washed with hot water. Filter paper and residue were placed in porcelain crucible and ashed for 6 h at 600 °C; (D) Resulting AIA was transferred to desiccator, cooled and weighed.