Fakulta rybářství
a ochrany vod
Faculty of Fisheries
and Protection
of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

# Digestibility of protein feeds in Tilapia

Koushik Roy, Jan Mráz

Edition of Methodics 186

Fakulta rybářství
a ochrany vod
Faculty of Fisheries
and Protection
of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

# Digestibility of protein feeds in Tilapia

Koushik Roy, Jan Mráz

**Czech Republic, Vodňany**

***The content of the methodology is the result of solving research projects:***
*Ministry of Agriculture of the Czech Republic*
*Project NAZV no. QK1810296 – 90%*
*a GAJU (GAJU 020/2020/Z Roy) – 10%*


No. 186

ISBN 978-80-7514-125-5

## CONTENT

## 1. INTRODUCTION

Globally, tilapias (*Orechromis* sp.) belong to the most important domesticated species of freshwater aquaculture, alongside cyprinids, salmonids and catfishes. Farmed tilapia currently comprises ~13% of the total global freshwater aquaculture production, both in terms of volume and value (FAO, 2020). The global production of farmed tilapia doubled (~213%) in the recent decade (2008–2018). Present level of production is about 6 million tons per annum, which is worth 11 billion USD (FAO, 2020). Furthermore, it is the most widely used species in aquaponics and in small-scale brackish water aquaculture in some parts of Asia and Africa. Tilapias are almost exclusively raised on compound aquafeeds. Globally, tilapias are the second largest consumer of compound aquafeed (following Chinese carp), consuming ~17.9% of the total global fish feed production (Boyd et al., 2020). As of 2017, the estimated total production of 'Tilapia feeds' globally is about 9.2 million tons, worth a market price of ~12.73 billion USD (Boyd et al., 2020). Usually, the commercial tilapia feeds have a price range of ~1.28–1.57 USD $kg^{-1}$, with company claimed FCRs between 1.25–1.6 (Štěpán Lang, Skretting/Trouw Nutrition Biofaktory Praha – Nutreco, personal communication).

Proteins and amino acids are critical molecules because of the role they play in the composition and metabolism of all living organisms. Fish cannot synthesize all amino acids and must acquire several in their diet, through consumption of protein or mixtures of amino acids. The terms "digestibility" and "availability" refer to the amount or proportion of nutrients, such as crude protein, that disappears from a meal as it passes through the digestive system and is egested in faeces. As for proteins, they are hydrolyzed to amino acids prior to absorption. Digested nutrients are presumably available to the organism for growth and metabolism (NRC, 2011).

In the context of fish feed formulation, 'protein sources' or 'protein feedstuffs' or 'protein feeds' refer to those feed ingredients that have crude protein content above 35%. These ingredients are selected to serve as a major source of protein and/or amino acids in a blended feed mixture. Other ingredients usually act as fillers (or, filler proteins) and/or energy sources in the formulation. By weight, protein sources are often the most expensive ingredient used in a feed formulation. Thus their rationalized use is important not only for animal growth, but also for economic reasons (least cost formulation). In recent decade, another aspect has been added – establishing sustainable and efficient (equally to fish meal) alternatives or complementary protein sources that can fully replace or to a large extent substitute fish meal in aquafeed formulations (NRC, 2011).

For the abovementioned reasons, the commercial aquafeed formulators use either digestibility trials developed in their own research or third-party digestibility results (e.g. cloud database services in premium feed formulation software) to formulate high-performance commercial feeds based on digestible protein (and digestible essential amino acids) content. Therefore, the digestibility trials on protein feeds are very important for the industry. Estimation of bioavailable or digestible proteins from the total crude protein fraction of protein feeds plays a key role in a formulation of 'realistic' feed for the industry. It benefits both the animals (e.g. guaranteed source of protein required for optimum growth) and the market (e.g. lower cost for manufacturers, better FCR, lower expenses for farmers; Roy and Mraz, 2020).

## 2. THE AIM OF THE TECHNOLOGY

The aim of the technology is to set 'practical guidelines' and 'quality control technicalities' for conducting easy, rapid and efficient digestibility trials on fish/protein feeds. The primary focus is to make the digestibility trial technology easy to understand, implement and interpret even for inexperienced personnel – either in small-scale aquafeed manufacturing companies or third-party laboratories assisting independent feed formulators.

## 3. NOVELTY OF THE TECHNOLOGY

- Use of established (already balanced) commercial tilapia feeds (e.g. Skretting TI-3 Tilapia 3.2 mm™) as a 'basal' or 'reference' diet to test the ingredients. This avoids the complications connected to the use of a home-made balanced feed serving as a basal diet, and then starting the trial.
- Lower experimental feed requirement (~4 kg of each experimental feed to successfully conduct the experiment), feeding at 2% of the body weight, instead of *ad libitum* feeding.
- Shortened trial period (12–14 days) achieved by collecting adequate high-quality faeces within a short time. In a 12 tank Guelph system, 3 test ingredients can be screened at once (providing 1 control every time). Within a month, at least 6 ingredients or protein feeds can be tested in one system.
- Biologically averaged faeces collection (and digestibility results) by using mixed size assortment of fishes (coefficient of size variation up to 40%) at relatively high densities (33 kg $m^{-3}$). High densities ensure voracious feeding and enough faeces production.

- Trial breakdown: gut emptying time (2 days); gut acclimatization period of 4 days (marker particles and experimental feeds); dedicated sampling window with round the clock sample collection (6–8 days), and; frequent faeces collection (4–6 hours interval) during 'peak poop pulse' of fish against a fixed feeding schedule.
- Use of yttrium oxide as a marker instead of conventionally used chromium oxide (which is more prone to leaching). Incubator based drying and refrigerator based chilling cycles to achieve well textured, water stable pellets ('water hardened').
- Special handling and processing of collected faeces: gravitational settling of collected faeces-water mix followed by decanting of excess water (wet mass immediately frozen). Later thawing and centrifuging to settle 'suspended faeces particles' altogether (removing water again) and finally subjecting to lyophilization for obtaining high-quality faeces dry matter powder.

## 4. PLACE WHERE TECHNOLOGY WAS VERIFIED

The technology was verified at the Laboratory of Nutrition, Faculty of Fisheries and Protection of Waters, University of South Bohemia in České Budějovice; address: Na Sádkách 1780, České Budějovice, 370 05 Czech Republic. The demonstration and validation of this technology was conducted on protein feeds and tilapia.

## 5. DESCRIPTION OF THE TECHNOLOGY AND RESULTS

The main stages and work process of the technology are illustrated in a flowchart (Fig. 1).

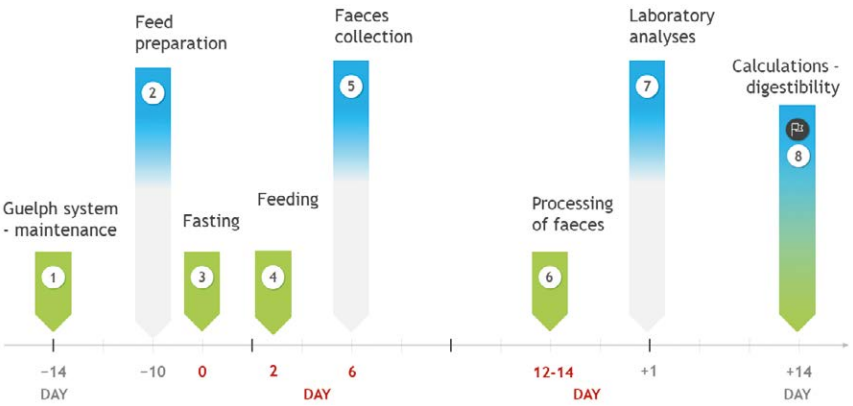


***Fig. 1.*** *Work process and timeline of the critical control points (CCPs) of the technology – digestibility estimation of feedstuffs. Days marked in red indicate the fixed and core experimental period. The flexible dependencies surrounding the core experiment are marked in grey. The CCPs are arranged in sequence (numbered from 1 to 8).*

### 5.1. Rationale of the technology

Determining digestibility of food and feeds in animals requires collection of faecal material. It involves feeding test feed ingredients singly or, more commonly, as a component of a diet. For example, to make a treatment diet, 30% of a test ingredient can be included in a basal (= control) diet containing some inert marker. This method relies on the collection of a representative sample of faeces free of uneaten feed particles and the use of a nontoxic, inert and indigestible digestion indicator, such as chromic oxide or yttrium oxide, added to the feed. The indicator passes through the digestive tract at the same rate as food and is unaffected by the digestive process, meaning it is not absorbed. Digestibility of nutrients is estimated on the basis of relative enrichment of the faeces with the digestion indicator compared to the level present in the feed (further demonstrated below). The fish can be held in normal fish rearing tanks or specialized tanks purpose-built for passive faeces collection. Passive faeces collection methods rely on the collection of faecal material naturally egested by the animal. Faecal material is collected from

tank effluent water by screening or filtering tank effluent water, or by settling suspended faecal material in low-flow apparatus. Faeces can also be collected by siphoning or netting (NRC, 2011).

Special tanks, referred to as the Guelph or modified Guelph system, are designed to facilitate feces collection by settling (Cho and Slinger, 1979; Hajen et al., 1993). In these systems, water flows are so adjusted that voided faeces are quickly swept out of the fish tank into a vertical settling column where slow water flow is allows faecal particles to settle. Effluent water exits gently at the top of the column. Typically, fish are fed at established time, such as late in the day, and tanks and collection columns are cleaned beforehand to ensure that no uneaten feed is present. Faeces collect overnight and are removed in the morning (NRC, 2011).

Sample collection is followed by the analyses of test ingredient, feeds (control and treatment) and faeces (control-derived and treatment-derived) using standard analytical procedures. Analyses conducted on dry samples usually include proximate composition, energy content, or contents of specific nutrients, e.g., amino acids or minerals. The concentration of the digestion indicator is also measured. All these data are required to calculate the ADC (apparent digestibility coefficient) values of the nutrients, first for whole diets and then for ingredient(s). The amount of faecal material needed for analysis depends upon the number of chemical analyses being conducted. Fresh faeces have approximately 90% moisture. So collection of 50 g of wet faeces will yield 5 g of dry material, enough for complete proximate, energy, and nutrient analysis. Smaller amounts are suitable when using analytical methods requiring small samples (NRC, 2011).

### 5.2. Experimental system description

A 12-tank RAS (recirculating system) placed on galvanized iron frame with a bottom reservoir tank and an upper biofilter tank (with movable biofilter beds/ biofilter elements), each ~2 times more volume than the combined volume of all fish tanks combined. For example, our system comprised of 12 fish tanks (120 L each) with 2,000 L biofilter tank + 2,000 L reservoir tank. The circular tanks with centrally sloping bottom are placed on wooden frames between the reservoir tanks. The circular tanks (hereinafter referred to as 'fish tanks') are covered with elastic strapped meshed net with a hole in the center. Through the central hole in the cover net, a water supply inlet enters the tank as a T-shaped showerhead (with a water speed control valve fitted to its neck before bifurcation) hovering over the clockwise aerators (with duck-mouths) vertically attached to the tank wall. The water comes from the biofilter tank

above. Fish tanks are retro-fitted with duck-mouth aerator pipes (vertically attached to the inner tank wall), placed in clockwise direction to induce a gentle circular flow in the tanks. The air pipes are supplied with well networked air tubes connected centrally to two multi-pore extension pipes, which draw air from two air pumps with output capacity of 200 L $min^{-1}$ each. One set of air pipe(s) is for biofilter tank alone. The other set distributes air to the fish tanks.

The central outlet at the bottom of fish tanks (similar to a wash basin) opens to a carefully slanted (slope ratio 1:2.5; vertical drop: horizontal distance) transport tube leading into standing Guelph-tubes by the sides of each tank (double the diameter of the transport tube). The bottom of the Guelph tubes can be fitted with a cone and a drainable-tap or a silo and a tap. The water carrying the feces (and residual feed) from fish tanks suddenly stagnates into these standing (Guelph) tubes and as a result of the length/depth of the tubes the solids (feces, residual feeds) sink down by gravity over time, getting increasingly compacted into a cone, from which it becomes impossible for these solids to be re-suspended and travel all the way back to the top of the Guelph tubes and gush out. The feces are drained from the system using the taps at the end of these silos/cones. The upper layer of these broad Guelph tubes has a carefully made diameter hole (diameter slightly larger than the diameter of transport tubes) for 'gentle' bypassing of overflow water. Parallelly, the excess 'top-layer clear water' (with solids deposited in the bottom cone) in the Guelph tube gently drains out through this hole to a broader pipe collecting water from all individual Guelph tubes (per tank) and delivering it to the common bottom sump (reservoir tank). The bottom sump is fitted with two high power water pumps with output of 15,000 L $hour^{-1}$ each (with adjustable power regulator) that lift water from sump to the upper biofilter tank and it flows down to the fish tanks again through a central inlet water channel (which is connected to individual fish tanks via a T-shaped showerhead). One pump is operating while the other is kept as 'backup' and rotates fortnightly. The water hydraulics of the entire system (flow direction: bottom sump → biofilter → fish tanks → Guelph tubes → bottom sump) is adjusted by the power regulator of water pumps so that 1 complete system water turnover (recycling) takes 1 hour. The pace of the water turnover is subject to several rounds of internal calibrations. Overly fast water turnover is detrimental to feeding (feed pellets might drop before being eaten), effective functioning of feces sedimentation tubes and/or can cause fecal matter to disintegrate and dissolve. Photos and diagram of the system is given in Fig. 2 and 3, respectively.

### 5.3. Good management practices for reliable system operation

- Daily draining/flushing of the Guelph tubes by opening the taps for at least 12 seconds.
- Daily checking whether all the aerator pipes are working/bubbling out in the water of fish tanks; re-adjusting duck-mouth mouth direction if needed (in case a fish has collided with it and changed its position or disconnected the air pipe).
- Periodic cleaning of inner walls of the Guelph tubes and fish tanks, checking inlet shower heads for clogged solids (NOT during experiment) with a large/small custom made 'bottle-cleaning' brush.
- Never letting the biofilter compartment (upper tank) dry out. Installing sensors (alert by SMS) and maintaining water level in reservoir sump. Periodic changing/alternative use of pump-1 and pump-2 for easing pressure on each pump.
- Periodic cleaning (scrubbing) of bottom sump, removing dirty water and refilling with clean water. Using tempered water for refilling to prevent temperature shock to biofilter and fish.
- Adding a small amount of baking soda ($NaHCO_3$) every day, depending on pH drop from previous day. The aim is to maintain pH above 6.5 (ideally 7.3 units). For every 0.1-unit deviation of pH from the reference pH mark 7.0, use approximately 20 g of soda (dynamically change this dose depending upon system volume and internal calibration). If the pH is above 7.6 units, skip adding soda for a day. It usually depends on system volume and water buffer capacity (total alkalinity). Thus doses must be optimized based on internal calibrations.
- Using slaked lime in case of serious pH drop. In case of extreme pH drop, larger than 1 unit fall from previous day, soda will not solve the issue immediately. In this case, use slaked lime $Ca(OH)_2$, but WITH CAUTION. Add slowly with small split doses several times a day, followed by bihourly monitoring of pH. For every 0.1-unit deviation of pH from 7.0 units, add approximately 5–10 g of slaked lime; also subject to dynamic dose changing depending on system response and internal calibration.
- Regular flushing of Guelph tubes. The Guelph tubes should be flushed to keep them clean of uneaten feed particles about 1 hour after feeding, but not before. Flushing Guelph tubes immediately after feeding should be STRICTLY forbidden. This can be quite problematic due to sudden heavy clogging of hard pellets at the base of the tap/conus which are unable to be flushed out just by the force of water. More so, the remaining hard pellets from the fish tanks are also sucked into the tubes due to immediate flushing.

- Maintaining proper temperature. For tilapias, secure water temperature above 20 °C (ideally 23–25 °C) in fish tanks, which can be implemented by mixing sufficient amount of hot water with the existing cold water in system daily. Even better option is to have the system housed in a climate-controlled room, with room temperature set at around 27 °C. If the water temperature in the fish tanks drops below 17–19 °C, tilapias do not actively feed (meaning feeds remain in water longer; nutrient-marker leaches out) and even partially or completely reject the supplied pellets (loss of appetite). This can be a DETRIMENTAL situation during the core experiment.
- The level of dissolved oxygen in fish tanks should be above 3 mg.$L^{-1}$. If the dissolved oxygen drops below 2 mg.$L^{-1,}$ it has the same effect on feeding as low water temperature (as mentioned above). A basic aquaculture multi-meter to monitor basic water quality parameters suffices for daily checking and recording of these fundamental parameters.


***Fig. 2.*** *Snapshot of our in-house Guelph system used for digestibility trials. (Photo: laboratory archive).*

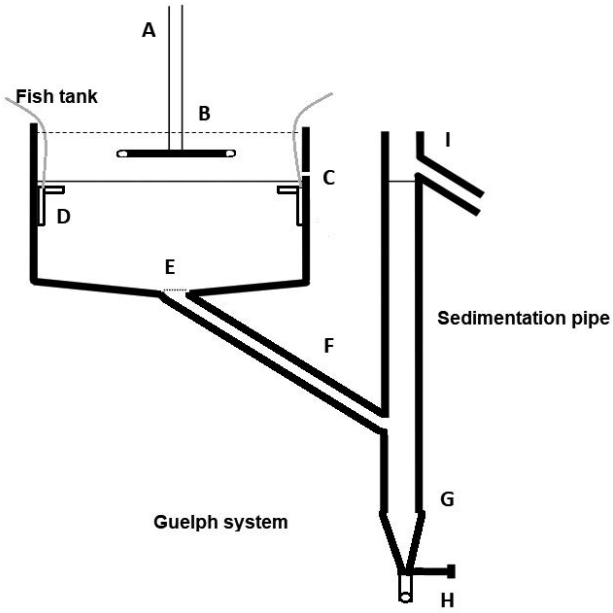


***Fig. 3.*** *Scheme of the Guelph system consisting of a connection of a fish tank and sedimentation tube.* **A =** *inflow water from biofilter tank (fitted with a T-shaped showerhead; clockwise);* **B =** *net covering of fish tank;* **C =** *overflow of any excess water from fish tank (directly into reservoir tank underneath);* **D =** *air pipes fitted with fish tank wall (in clockwise direction) and supplied with air tubes from air pump;* **E =** *sieve at the central outlet drain of fish tank (slightly sloped bottom);* **F =** *transport tube for water and solids from fish tank to sedimentation pipe;* **G =** *conical silo at the bottom of sedimentation tube for trapping sedimented solids;* **H =** *tap for draining trapped solids or feces;* **I =** *outflow of clear water (supernatant) post sedimentation of solids (into bottom reservoir tank).*

## 5.4. Experimental feed preparation guidelines

Experimental diets can be prepared using a common feed pelletizer, cold extruder or noodle maker (fitted with a motorized regulatable chopper/slicer; Fig. 4). The final pellets are usually of a sinking type. First, the ingredients and commercial pellets (control/basal diet) are ground to powder using a portable hammer mill/grinder. At least two diets are prepared. Control diet is prepared by weighing 1 kg of commercial pellet powder and adding 10 g of yttrium oxide to it (the indigestible marker for assessing digestibility). Treatment diet/s is/are prepared by mixing 700 g of commercial pellet powder and 300 g of test ingredient powder. Again, 10 g of yttrium oxide is added to the mixture. Please be careful to mix the powder with marker first (in dry form; without adding water). Make sure that the marker (yttrium oxide – looks whitish) particles are thoroughly mixed with the feed or feed + ingredient powder mix and that there are no clusters of 'white powder' visible in the mixture (revolving inside the mixer).

Then, 300–400 ml of water is added per 1 kg of each mix (control/diet), and it is well blended in a high-power food processor/stand mixer. The mixer must have a chimney-like opening to pour/add water while the contents inside are churning at full speed. Remember to add water slowly, not at once, to avoid unwanted clumps. Stop mixing once the dough inside is stuck and unable to rotate further/shaking inside (Fig. 4).

The moist mix is put into the extruder/pelletizer, at first to see whether the noodles/pellet threads are coming out as required. At this stage we have to make sure that the selected dicer (containing holes) has the optimum diameter (which results into a fixed diameter of pellets). The holes of the dicer also need to be open/clean and not clogged with old remnants. The dicer is then tightly secured/installed on the extruding mouth of the extruder. Inside the extruder, there should be a horizontal rotating screw, driving the feed through the tight cavity of the machine and through the holes of the dicer. The screw also needs to be clean without any clogging. At the mouth of the dicer, the motorized rotor with cutter knife is tightly secured. The speed is adjusted, and the sliced pellet threads fall in a bucket underneath as discrete pellets. Higher rotor speed usually gives shorter pellets (because of faster slicing) and slower speed yield longer pellets. It is important to remember that pellet diameter is not determined by the motorized chopper but only by the dicer. A combination of both factors (dicer selection and motor speed optimization) is necessary to produce pellets of optimum diameter and length, that is eatable for the experimental fish. In experiments with small tilapia, determining optimum pellet size might be crucial for its success (Fig. 4).

Finally, the pellets are spread on trays and left to sun-dry or room-dry (using a fan). Leaving the pellets room-dry overnight (with a rotating table fan) proves a good strategy. Next day those semi-dried pellets should be put in driers/incubators for 24–48 hours at 45 °C until they are dry to touch and feel lightweight (Fig. 5). Depending on the size of the dryer, it is recommended not to place more than 2 kg of pellets to dry at once (making more than 2 kg of experimental feed in one batch should be well considered). When taken out of the dryer, hot pellets should be kept at room temperature for some time to cool down. If immediately packed and stored, the pellets may develop invisible mold growth at the core or excessive formation of dust. It is essential to let the pellets 'breathe' for a while and only then pack the cooled pellets into sealable (zip lock) plastic pouches, with no more than 1 kg of dried pellets per pouch. The pouches are transferred to a classic refrigerator (preferably in dry mode setting) and kept there overnight, keeping the pouch open/unsealed. The next day, the plastic pouches can be sealed and stored in refrigerator till further use. This step apparently hardens the pellets (external surface) and prevents further excessive losses through disintegration of the pellets. This careful post-manufacturing protocols ensure longer or better water stability of the manufactured experimental pellets. It has many benefits: (a) securing that the feed is available to the fish in intact form for a longer time to enable guaranteed feeding; (b) even if some feed passes from fish tanks into the Guelph tube bottoms, it will remain intact until the regular flushing (1 hour after feeding) and will not dissolve/suspend inside the tube (c) greater water stability means less chances of marker and nutrients leaching out into water and thereby affecting the data; (d) longer shelf life before the beginning of the experiment (given the feeds are left in fridge in labelled airtight zip bags). Putting the sliced pellets immediately into closed dryers/incubators might cause fungus/mold growth (due to closed, dark, hot and moist conditions). Keeping the sliced pellets too long at the room temperature can also cause fungus/mold growth on the pellets. Such pellets are not fit for feeding animals or for experiment and should be safely disposed of (with all units cleaned and sterilized to destroy remaining inoculants/spores) (Fig. 5).

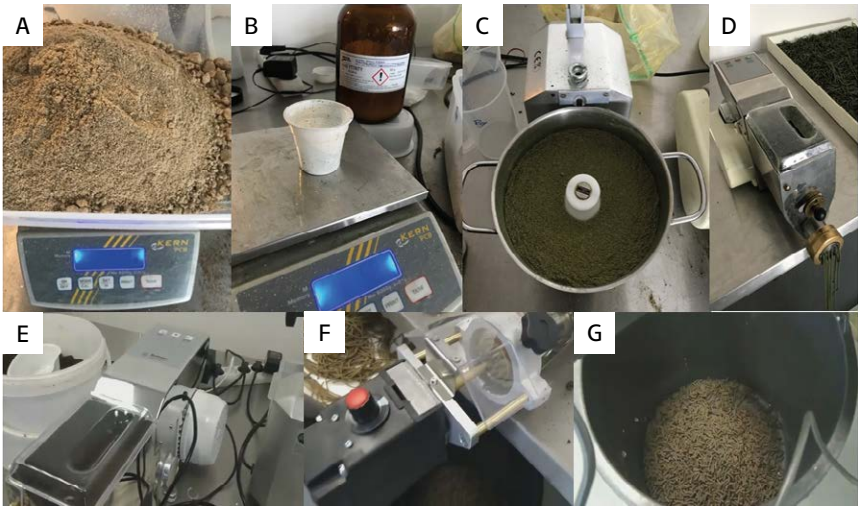


***Fig. 4.*** *Feed preparation – **A:** weighing of ingredients/feed; **B:** addition of marker; **C:** mixing with water to make dough; **D:** noodle maker machine (pelletizer); **E:** loading compartment of feed mix to pelletizer; **F:** motorized noodle cutter making pellets; **G:** final pellets. (Photos: laboratory archive).*

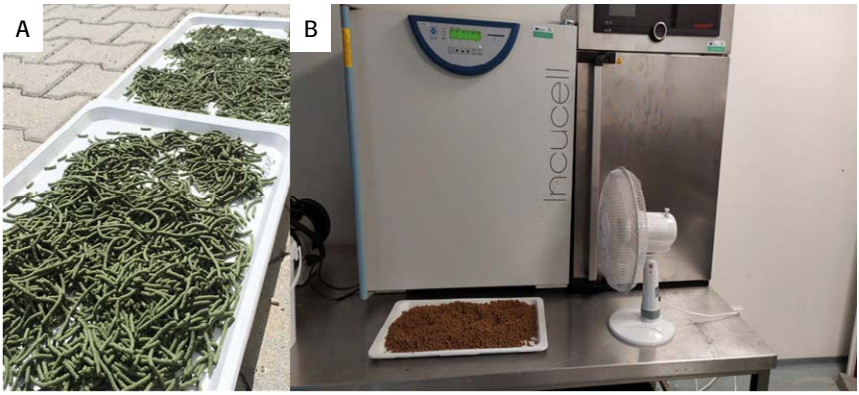


***Fig. 5.*** *Drying of prepared feed – **A:** open air sun drying followed by placing the pellets in preheated oven/incubator, **B:** indoor drying initially under a fan followed by placing the pellets in preheated oven/incubator. (Photos: laboratory archive).*

### 5.5. Feeding with experimental feed

At the beginning, the stocking density of fish per tank is limited to 33 $kg.m^{-3}$. For this, operational water volume of the fish tank is first measured and then fish are carefully weighed and stocked (e.g. 4 kg per 120 L tank). It is important to re-weigh the fish and re-adjust stocking density before another round of digestibility trial is initiated, if the conclusion of the first round takes longer (~3 weeks). After stocking, the fish are starved for 48 hours (2 days). This is called 'gut emptying period' and it helps to get rid of the remnants of previous feed. Additionally, it makes the fish hungry, which in return ensures experimental feed to be readily accepted by the fish stock upon feeding for the first time. Feeding itself is started on day 3. Maintaining a fixed feeding schedule, or more precisely, following a fixed time of feeding is important for obtaining freshly defaecated faecal matter in sufficient quantity. In other words, it ensures good quality samples obtained in the shortest period of sample collection possible. The strategy is to collect the majority of the freshly defaecated faeces within the peak excretion pulse (or peak circadian gut evacuation) and the faeces derived from latest feeding (fed in one whole day). The recommended feeding ration is 2% of the body weight and it is split into two equal doses (i.e. feeding two times a day). Feeding is best done by hand at water temperature around 23 °C and dissolved oxygen above 3 $mg.L^{-1}$. First feeding can be done at 08:00 HRS and second feeding at 12:00 HRS, i.e. with an average interval of 4 hours between the two feedings. The second feeding is the last feeding of the day.

Three hours after the last feeding, i.e. at 15:00, the Guelph tubes are flushed to get rid of uneaten feed or sedimented feed fragments. 12-second flushing should be sufficient to get rid of any solids in the Guelph tube that accumulated through the first and second feeding. If it is not flushed at this point, the feed will accumulate and compromise the faeces sample to be collected (in the next step). The contamination of faeces with feed is highly undesirable in digestibility studies. It renders the faeces with higher nutrient content (due to addition of nutrient rich feed pellets). As a result, the formula-based calculations demonstrate low digestibility coefficients (i.e. underestimation of actual digestibility). The next step is to refill the water to the system, preferably using water of similar temperature to the culturing conditions to avoid significant temperature drop of the system water that might shock the tilapia and cause loss of appetite.

### 5.6. Faecal sample collection guidelines

Collection of faeces should start after 4 days (96 hours) of feeding with experimental feed(s). These initial 4 days of feeding with experimental feed when faeces collection is not performed is called 'gut acclimatization period'. It helps to homogenize the fish gut with marker particles, uniformly distribute experimental feed along the gut (undergoing different stages of digestion) and helps to adapt the digestive process to the newly introduced feed. Faeces collection starts on day 7 (if counted from the beginning of experiment) or on day 5 of feeding and continues onwards. Given that last flushing took place in the afternoon (15:00; see previous section), faeces collection should start in the evening (19:00), then continue at midnight (23:00–00:00) and last one should take place in the early morning (04:00–05:00, +1 day). The experiment can be conducted under general photoperiod regime (12–14 hours light: 10–12 hours dark). A low illumination of normal lights in the room may enable working from evening to early morning, but at the farthest corner of the room (from the system; not to stress the fish). In fact, the room housing the system (with fish) must have large glass windows or enough white/golden lighting to maintain a normal photoperiod regime for experimental stock (light – dark circadian cycles).

For collection of samples, one large glass beaker (1,000–2,000 ml) per fish tank or Guelph tube is needed. If 3 ingredients (treatment diets) and 1 control diet is being tested, 12 such beakers are needed. Faecal sample replicates are kept separately at par with fish tank replicates (i.e. 3 tanks or 3 samples per group). We recommend this approach for more accurate estimate of digestibility. Additional 7 to 10 days might be needed to collect enough sample(s) for laboratory analysis using this approach.

However, if there is a time constraint (i.e. the trial need to be finished within 2 weeks), a four-beaker sampling may be applied (1 beaker or sample per group). In this case, faeces from 3 tanks or replicates of a group is pooled/combined (i.e. physically averaged). Replicates of collected faeces sample (pooled) are made only in the laboratory before actual analysis. We have quantified the expected differences in digestibility results for individual samples (tank replicates + laboratory replicates) versus pooled samples (only laboratory replicates). The observed coefficient of variance (CV) of nitrogen (Kjeldahl-N) or yttrium content in pooled faeces versus replicate faeces is 7.2%. This means calculated ADC values from pooled faeces might vary ±7% on an average from ADC values of well replicated faeces sample. Such deviation should be anticipated. Even an adjusted (corrected) ADC range may be calculated considering ±7% as a standard deviation from the central value (=value obtained from pooled faeces).

Coming back to faeces collection, the beakers are held at the bottom of Guelph tubes. The tap is quickly opened, draining the 'visibly black/brown sludge water' into the beakers and swiftly closed with the first sight of clear/ glassy white water. The tap should be opened 100% at once, quite swiftly, and NOT slowly in sequential turns. This allows the sedimented faeces to suddenly gush out 'in cluster' by action of gravity and maximum force of suddenly released water. This assures safe delivery of faecal sediments into beaker without breaking down and mixing furhter with water. The same swiftness should be applied when closing the taps; the taps need to be closed AT ONCE. This prevents excess of clear, faeces-free water or minimizes entry of colloidal water which was above the sedimented faeces layer (in Guelph tubes) from draining into the beaker containing 'tufts/ dense clouds' of faecal matter-water mix. The skillful operation of taps while draining faecal matter needs prior training/exposure of personnel and taps strong and sleek enough to be operated in this manner. It should be borne in mind that bad samples should be discarded and only good quality sample (Fig. 6) used for analysis.

The beakers are then left on a tabletop undisturbed to let the suspended faecal particles settle down by gravity for at least 7 minutes. The beakers should be not be touched at this time. After a couple of minutes, the contents inside the beaker start to visibly separate into two segments – a sedimented 'faecal mud' and overlying water. The sedimented faecal mud occupy only up to 10% of the beaker height, while the rest 90% of the beaker height is filled with water. This overlying water is then carefully decanted by cautious tilting of the beakers over a drain. The priority is to get rid of the excess water but retain the faecal matter in beaker as concentrated as possible. Repeat the process of gravitational settling and decanting if required, but not more than twice (further repetition will most likely result in draining suspended faecal matter). The concentrated/compacted faecal mass, still in the watery medium (but without a visible prominent separate layer of water), is drained to sample collection tubes. The sample collection tubes are mega-centrifuge compatible Nalgene™ Wide-Mouth HDPE Bottles with capacity 200–250 ml, with airtight cap. They can withstand centrifugation up to 8,000 g. The faeces sample tubes are capped and placed in a refrigerator (Fig. 6).

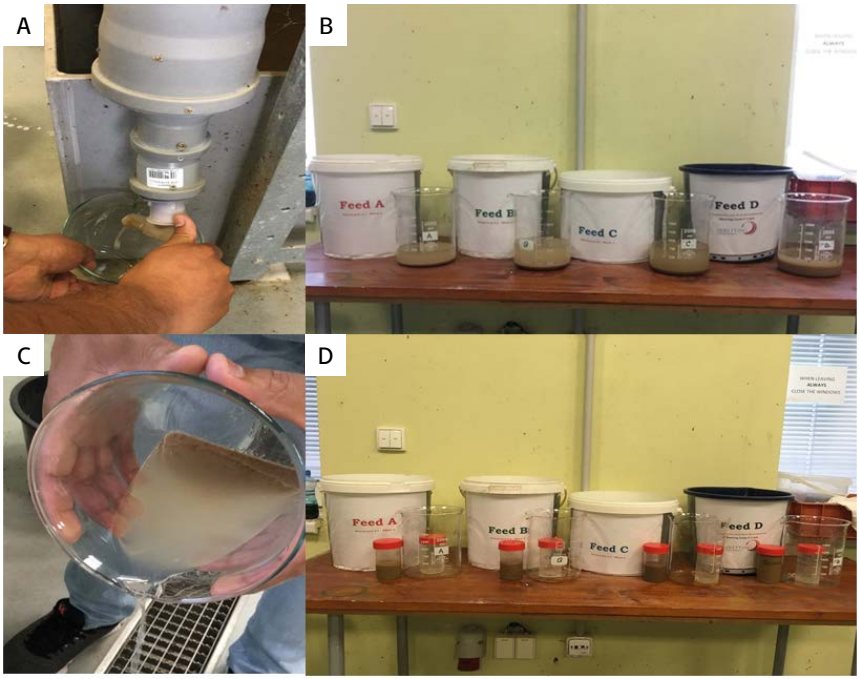


***Fig. 6.*** *Faeces collection –* **A:** *draining faeces sample from Guelph tube;* **B:** *gravitational sedimentation;* **C:** *decanting excess water;* **D:** *storing concentrated samples in sample tubes and preparation for next sampling. (Photo: laboratory archive).*

Each time faeces are sampled (over the course of night till early morning), the concentrated faeces from the beakers are filled into the tubes kept in a refrigerator since the previous sample collection, until the tubes are completely full or having remaining space filled with water (visible excess water). At any sampling point, if the tubes from previous sampling) show excess water layer above faecal sediment, they should be decanted/drained as well. This makes the faecal samples inside the tubes more concentrated. If the tubes are already filled from the previous sampling (without excess water), ignore this step and take new tubes to fill. This is an important technicality because all the tubes should be filled completely to allow thawing and centrifugation at later stage. Tubes with uneven contents/volume create disbalance in a centrifuge machine and might cause an accident by making the machine unstable or even prevent centrifugation. The key is to keep the sample tubes full of concentrated faeces. The 'completely packed' tubes (with faeces) should be immediately transferred

from refrigerator to freezer and frozen. Per treatment, 8 completely full tubes should be enough to finish the experiment. A photo gallery of faeces collection steps and collected faeces in tubes is provided as Fig. 6 and 7.

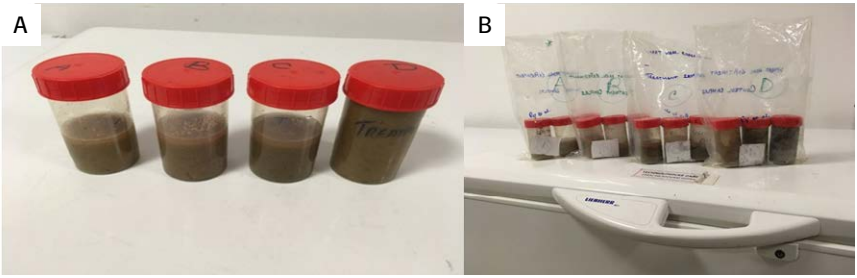


***Fig. 7.*** *Storage of collected faeces sample.* **A:** *Storage of collected and concentrated faeces sample from repeated samplings in one day (attempting to collect a tube full of the sample material), and,* **B:** *immediate freezing of collected faeces sample from one day of full sampling (irrespective of whether the tube is full). (Photo: laboratory archive).*

### 5.7. Processing of collected faecal sample

The frozen faecal sample (approximately 6–8 tubes per group; each 200 ml) are thawed in a regular deep wash basin/bucket filled with hot tap water. The tubes are left afloat to completely thaw the iced contents. About 30 minutes is needed for completion of thawing process. In between, complete exchange of 'cooled water' with hot water is done to keep the tubes in a constant hot water bath. Once the contents are thawed, individual tubes are opened fully and then semi-closed again (i.e. the cap is closed half, letting it cling-on to the tube but not tightly secured). The semi-closed tubes are put in the mega-centrifuge machine (4 tubes at a time) and centrifuged at 3,800 g for 8 minutes at 21–22 °C. The acceleration rate of machine should be set to 90%, but deceleration rate to 70%. It should be noted that if the machine shows instability (or jumps) longer than 7–8 seconds post-start, or gives an error for 'disbalance', the cycle should be aborted urgently. After aborting, the tubes should be checked for unequal volume of water and solids. On spotting the tube(s) with relatively lower content, the 'deficient' tube is filled with water in order to match the mass in other 3 tubes. In other words, make sure that the 'filled volume' of all 4 tubes is equal (i.e. 200 ml × 4); if not, substitute the missing mass by filling the tubes with water.

After the centrifugation cycle is finished, the tubes are carefully taken out of the buckets in the centrifugal rotor or holder. The centrifuged tube

has two distinct layers – (a) a consolidated faeces sediment with a visibly smooth top layer, and, (b) a clear, golden or straw yellow supernatant water. The supernatant water is completely discarded. If the faeces sediment is breaking up and getting re-suspended during discarding of supernatant, another centrifugation cycle might be necessary. It is crucial to make sure that the post-centrifugation supernatant liquid is clear and not muddy. A clear supernatant liquid means that 99% of the nutrient in faecal solids or faecal matter *per se* are deposited from water-colloidal phase to solid sediment (at the bottom of tubes) by the centrifugal force, minimizing the chances of nutrients being leached from faeces to water. Through centrifugation step it was estimated that leaching nutrient loss from faeces were made negligible (i.e. <1% of original nutrient locked in faeces escaped to water; Roy and Mraz, unpublished). The tubes with faeces sediment are then capped and transferred to a lyophilizer or freeze-drying machine.

To extract dry matter, the faeces (maximum 14 centrifuged tubes per run) are lyophilized for 24 hours. For an effective lyophilization of wet faeces mass, the machine needs to be manually set to 14 hours main drying followed by 10 hours final drying (total = 24 hours). Additional 10 hours (5 hours main drying + 5 hours final drying) might be required if the faeces are not 'completely' dried (symptom = wet core of the dried mass). It should be noted that before actual lyophilization, the machine needs to get prepared through a freezing (for preparing freezing tubes; 5 minutes) and warm up (for preparing vacuum pump; 15 minutes) cycle taking about 20 minutes in total. Without this essential step, the faeces might not have completely dried even after 24 hours. The completely dried faeces come out of the tubes as 'round, soft cakes' sticking to the bottom of the tube while holding it inverted (upside-down). Make sure to immediately store them in air-tight plastic zip lock pouches. The texture of a lyophilized faeces resembles that of ultra-light cotton fabric or flower spores (snowball spores), which can fly with the slightest blow of the air. This dry matter is ideal for the laboratory analysis. A photo gallery of processing of faeces sample is given in Fig. 8.

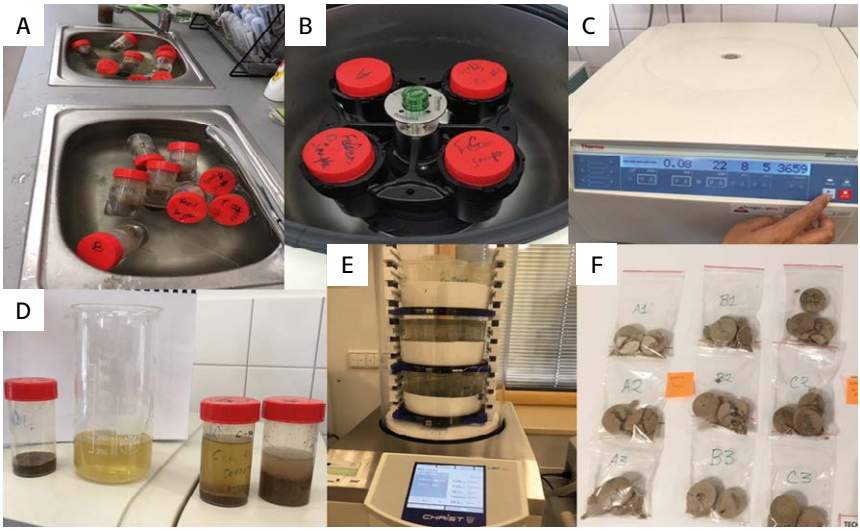


***Fig. 8.*** *Processing of collected faeces sample –* **A:** *thawing of frozen samples;* **B** *and* **C:** *centrifugation of thawed faeces;* **D:** *separation of water supernatant from faeces by decanting (after centrifugation);* **E:** *lyophilization of sedimented faeces;* **F:** *final faecal dry matterfor analysis. (Photo: laboratory archive).*

## 5.8. Samples for laboratory analyses

For ultimate calculation of digestibility estimates through formula (next sub-chapter), following samples should be sent out for analysis. The list applies for each test ingredient.

- ✓ Control feed (= 1 sample).
- ✓ Treatment feed (= 1 sample).
- ✓ Control feed derived 'control faeces' (= 1 pooled or 3 replicate samples).
- ✓ Treatment feed/s derived 'treatment faeces' (= 1 pooled or 3 replicate samples).
- ✓ Test ingredient (= 1 sample).

For calculating protein digestibility, following parameters need to be analyzed from each of the these samples.

- ✓ Dry matter (in %).
- ✓ Kjeldahl-N (which is further multiplied by 6.25 to get protein values; in %).
- ✓ Yttrium (for all samples, except the ingredient; in $mg.kg^{-1}$).

If digestibility estimation of **complete macronutrient fraction** of an ingredient is targeted, crude lipid, total ash and total fiber should be included in the analysis. In this case, NFE (nitrogen free extract or carbohydrate minus fibers) is calculated by subtracting the total values (on dry matter basis) of protein + lipid + ash + fiber from 100. If digestibility estimation of **energy** in an ingredient is targeted, then gross energy of samples needs to be calculated by multiplying calorific value of each proximate fraction (protein 4 $cal.g^{-1}$; NFE 4 $cal.g^{-1}$; lipid 9 $cal.g^{-1}$) by their content in sample. Additionally, **phosphorus** can also be analyzed.

We recommend sending the samples to an accredited third-party laboratory specialized in biochemical analysis. Detailed explanation of these standard protocols is beyond the purview of the present methodology. Refer to Tab. 1 for being redirected to the analytical SOPs (standard operating procedures) relevant to the parameters mentioned above.

***Tab. 1.*** *Standard operating procedures (SOPs) of dry matter, protein and yttrium.*

| Parameter | Accredited method | Link |
|---|---|---|
| Dry matter | ISO 11465:1993 | https://www.iso.org/standard/20886.html |
| Protein or N | ČSN EN ISO 16634-1 | https://www.iso.org/standard/46328.html |
| Yttrium | ISO 17294-2:2016 | https://www.iso.org/standard/62962.html |
| | **Additional** | |
| Lipid | ISO 1443:1973 | https://www.iso.org/standard/6038.html |
| Ash | ISO 1575:1987 | https://www.iso.org/standard/6170.html |
| Fiber | ISO 5498:1981 | https://www.iso.org/standard/11544.html |
| Phosphorus | ISO 6491:1998 | https://www.iso.org/standard/12864.html |

### 5.9. Calculation of apparent digestibility coefficients (ADCs)

It should be noted that although ADC is called 'coefficient', it is usually expressed in percentage (%) multiplied by 100. On the contrary, when the ADC values are used to estimate digestible content from crude content of an ingredient, the 'real coefficient' is used for multiplication with crude contents. The coefficient is usually in decimals and must be ≤1 (=100%); i.e. without multiplication by 100 or dividing the % value by 100.

Apparent digestibility coefficient is calculated on two levels: first, apparent digestibility coefficient of diet ($ADC_{Diet}$), and second, apparent digestibility coefficient of ingredient ($ADC_{Ingredient}$). It should be noted that $ADC_{Ingredient}$ CANNOT be calculated without calculating $ADC_{Diet}$ first.

✓ First, the protein digestibility of diet is calculated (Protein $ADC_{Diet}$) following the formula (NRC, 2011):

$$\textbf{Protein ADC}_{\textbf{Diet}}\ \textbf{(\%)} = \left[1 - \frac{(Yttrium\ in\ Feed)x(Protein\ in\ Faeces)}{(Yttrium\ in\ Faeces)x(Protein\ in\ Feed)}\right] x\ 100$$

It should be noted that **two** $ADC_{Diet}$ values are calculated: one for control feed **(Protein $ADC_{Basal\ Diet}$)** and one for treatment feed **(Protein $ADC_{Treatment\ Diet}$)** (of a test ingredient).

✓ Second, the protein digestibility of ingredient (Protein $ADC_{Ingredient}$) is calculated following the formula (NRC, 2011):

$$\textbf{Protein ADC}_{\textbf{Ingredient}}\ \textbf{(\%)} = ADC\ of\ Treatment\ diet + \left\{(ADC\ of\ Treatment\ diet - ADC\ of\ Basal\ diet)\ x\ \left(\frac{0.7\ x\ Protein\ in\ basal\ diet}{0.3\ x\ Protein\ in\ ingredient}\right)\right\}$$

In the formula above, values of 0.7 and 0.3 indicate 70% basal diet and 30% test ingredient in the treatment diet. The formula is valid if ingredients are tested at 30% inclusion ratio (or, 300 g test ingredient per 1 kg of feed).

**Caution:** These proportions or coefficients help to minimize the error in calculated values caused by differences in the protein content between the treatment feed and basal feed caused by protein-rich test ingredients. In other words, it avoids the complications of isonitrogenous diet formulations (i.e. control feed, treatment feed with equal protein content) when conducting experiments. Therefore, these proportions (70 diet: 30 ingredient) MUST BE 'precisely' followed during feed preparation (see NRC, 2011). Otherwise, the ADC values will be calculated incorrectly (=values considerably over 100, or, considerably below 0).

The abovementioned formulas can also be used for estimating digestibility of **other proximate fractions** like lipid, ash, fiber, NFE, energy and phosphorus. It can be done by simply exchanging the 'protein values' in these formulas with the 'proximate fraction values' (which is being targeted for ADC estimation). For environmental concerns associated with aquafeed or ingredients, ADC of phosphorus often serves as a marker, besides being an essential mineral for the fish.

## 6. VALIDATION OF THE TECHNOLOGY

Using the abovementioned approaches, we have tested 6 contemporary protein feedstuffs for tilapia – **feather meal, insect meal, corn gluten meal, fish meal, blood meal** and **soybean meal**. The results from our experiment are summarized in Tab. 2 (samples and composition), 3 (Protein $ADC_{Diet}$) and 4 (Protein $ADC_{Ingredient}$).

The ADCs of protein feedstuffs from our experiment were matched with the published estimates on those ingredients in available literature. For this purpose, an exhaustive search of peer-reviewed published articles on Google scholar was conducted using search keywords like digestibility trial, apparent digestibility coefficient, digestibility, tilapia, *Oreochromis* and ingredient (in different combinations). About **105 articles** were collected in total. From this collection, reported **Protein $ADC_{Ingredient}$ (%)** of our six test ingredients (feather meal, insect meal, corn gluten meal, fish meal, blood meal and soybean meal) were acquired.

A comparative account is given in Fig. 9 and 10, comparing our results (indicated by black horizontal dash) with the global metadata collection of the tested protein feedstuffs (indicated by boxplot). The closest published estimates to our obtained results are highlighted in Tab. 4. Our results on soybean meal might have been caused by poor quality of soybean meal tested (crude protein only 36.2%). A good soybean meal (to be considered as a protein source in aquafeed) should have a crude protein level around 50% (IAFFD, 2020). As only one previous estimate on blood meal exists (Davies et al., 2011), it is difficult to validate our blood meal ADC estimate. However, seeing the variability in other ingredients (e.g. feather meal, insect meal; see Fig. 9) our present result on blood meal seems plausible. Overall, our results closely correspond with the published estimates (Hanley, 1987; Watanabe et al., 1996; Degani et al., 1997; Fontainhas-Fernandes et al., 1999; Maina et al., 2002; El-Sayed, 2004; Sklan et al., 2004; Köprücü and Özdemir, 2005; Borgeson et al., 2006; Gaber, 2006; Drew et al., 2007; Borghesi et al., 2008; Goddard et al., 2008; Guimarães et al., 2008a, 2008b, 2012; Agbo et al., 2009; Dong et al., 2010; Davies et al., 2011; Ribeiro et al., 2011; Tram et al., 2011; Pereira et al., 2012; Ramos et al., 2012; Rodrigues et al., 2012; Zhou and Yue, 2012; Geremew et al., 2015; Serrano et al., 2015; Vidal et al., 2015, 2017; Godoy et al., 2016; Novelli et al., 2017; Putri et al., 2017; Barone et al., 2018; Farahiyah et al., 2018; Fontes et al., 2019; Tran-Ngoc et al., 2019). It fell within the expected range of global metadata (Fig. 9). Thus, our proposed technology is successfully validated.

***Tab. 2.*** ***Protein and yttrium content (%) of basal feed, test ingredients, treatment feed and faeces.*** *All results are based on 100% dry matter.*

| S. No. | Item | Protein (%) | Yttrium (%) |
|---|---|---|---|
| | **Experiment – I** | | |
| 1. | Feather meal | 93.75 | * |
| 2. | Feather meal feed | 55 | 0.98 |
| 3. | Feather meal faeces | 31.25 | 2.76 |
| 4. | Insect meal | 62.5 | * |
| 5. | Insect meal feed | 45.87 | 0.81 |
| 6. | Insect meal faeces | 30.12 | 2.53 |
| 7. | Corn gluten meal | 68.75 | * |
| 8. | Corn gluten meal feed | 46.93 | 0.87 |
| 9. | Corn gluten meal faeces | 26.56 | 2.82 |
| 10. | Commercial feed | 37.62 | 0.81 |
| 11. | Commercial feed faeces | 24.75 | 3.11 |
| | **Experiment – II** | | |
| 12. | Fish meal | 75 | * |
| 13. | Fish meal feed | 49 | 0.76 |
| 14. | Fish meal faeces | 21.12 | 4.03 |
| 15. | Blood meal | 96.25 | * |
| 16. | Blood meal feed | 56 | 0.82 |
| 17. | Blood meal faeces | 43.31 | 2.61 |
| 18. | Soybean meal | 36.18 | * |
| 19. | Soybean meal feed | 36.93 | 0.786 |
| 20. | Soybean meal faeces | 34.18 | 2.95 |
| 21. | Commercial feed | 37.75 | 0.8 |
| 22. | Commercial feed faeces | 20.12 | 2.82 |

***Tab. 3.*** *Apparent digestibility coefficients of protein in experimental feed (control and treatment). All results are based on 100% dry matter.*

| S. No. | Experiment feed name – (% of test ingredient) | Protein $ADC_{Diet}$ (%) |
|---|---|---|
| | **Experiment – 1** | |
| 1. | Feather meal feed – (30% feather meal) | 79.87 |
| 2. | Insect meal feed – (30% insect meal) | 78.98 |
| 3. | Corn gluten meal feed – (30% corn gluten meal) | 82.56 |
| 4. | Skretting TI-3 Tilapia 3.2 mm™ – (control; 0% ingredient) | 82.89 |
| | **Experiment – 2** | |
| 5. | Fish meal feed – (30% fish meal) | 91.91 |
| 6. | Blood meal feed – (30% blood meal) | 75.76 |
| 7. | Soybean meal feed – (30% soybean meal) | 75.34 |
| 8. | Skretting TI-3 Tilapia 3.2 mm™ – (control; 0% ingredient) | 84.84 |

***Tab. 4.*** *Apparent digestibility coefficient of the protein feedstuffs for Tilapia (tested ingredients) and its cross-validation with published estimates. All results are based on 100% dry matter.*

| S. No. | Protein feedstuff | Protein $ADC_{Ingredient}$ (%) | Top matching evidences* (result in parentheses) |
|---|---|---|---|
| 1. | Feather meal | 77.03 | Hanley, 1987 (73.9); Guimarães et al., 2008a (79.7) |
| 2. | Insect meal | 73.48 | Köprücü and Özdemir, 2005 (75.8); Fontes et al., 2019 (69.7, 70) |
| 3. | Corn gluten meal | 82.14 | Watanabe et al., 1996 (89.3); Davies et al., 2011 (83.03); Ribeiro et al., 2011 (90.07) |
| 4. | Fish meal | 99.22 | Fontainhas-Fernandes et al., 1999 (96.9); Borgeson et al., 2006 (93); Dong et al., 2010 (99.4) |
| 5. | Blood meal | 67.45 | Davies et al., 2011 (85.8)[1] |
| 6. | Soybean meal | 52.22** | Gaber, 2006 (77.4)[2] |

*Nearest published estimate(s) to our results; from available literature on tilapia. For full reference see the bibliography section.
[1]The only existing estimate on blood meal. Difficult to compare.
**Might be underestimated. Probably due to poor quality of soybean meal tested (see item 18 in Tab. 1).
[2]Lowest estimate on soybean meal encountered in the available literature (majority of ADC >88%; see Fig. 10).

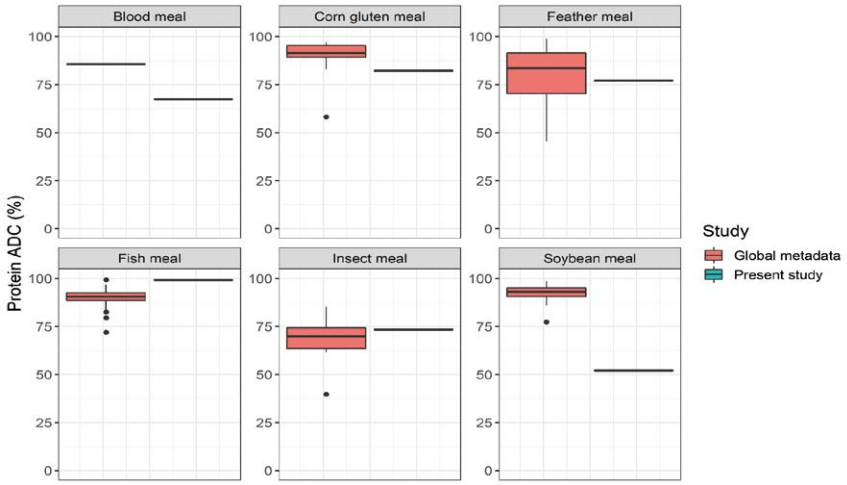


***Fig. 9.*** *Comparison of results obtained using the technology (digestibility of protein) with global metadata on the same protein feedstuffs for tilapia. Reliable results (black horizontal line) were obtained for all protein feedstuffs (i.e. near/within global metadata range; pink boxplot), except soybean meal; see the text for detailed explanation and references.*

## 7. ECONOMIC BENEFITS

High performance diets are often formulated based on digestible nutrient-energy values of ingredients, fulfilling the specific 'nutrient-energy-nutrient: energy balances' of the target fish species. Digestibility trials help to determine such bioavailable nutrient and energy values for each ingredient. Formulations based on these values ensure better growth with lower feed conversion ratio (FCR), meaning smaller amount of feed is required for target production. This would not be achievable if the feeds were formulated by crude nutrient-energy content basis or crude values of the ingredients. Therefore, for an effective feed formulation or to help in the decision-making process of aquaculture nutritionists (feed formulators), digestibility trials should be an indispensable part of a manufacturer's research and development. The total operational cost of this technology and its breakup is given in Tab. 5. The fixed asset investments like setting up of 12 tank RAS-Guelph system, weighing balance, noodle maker (pelletizer) machine, motorized noodle cutter, hot air dryer (incubator), refrigerator, freezer, centrifugation machine and freeze-drying machine are not included (Tab. 5).

Usually, the commercial tilapia feeds have a retail price range of ~30–37 CZK $kg^{-1}$, with company claimed FCRs between 1.25–1.6 (Štěpán Lang, Skretting/ Trouw Nutrition Biofaktory Praha – Nutreco, personal communication). If this final price (retail price) is assumed 2 times over the actual formulation cost to cover the manufacturing-packaging-logistics costs and sales profit margin, the current formulation cost of most commercial tilapia feed might be ~15–18.5 CZK $kg^{-1}$. Estimating the retail price of presently marketed commercial feeds from probable formulation costs may be misleading. Although formulations based on digestible values with cheaper and alternative feed ingredients may be cost-effective, it is not the complete picture. An ingredient or formulation cost is just a small part of the bigger picture. The present retail price of most commercial fish feed is highly competitive and based on R&D, manufacturing, packaging and supply chain and marketing costs. We have previously demonstrated the economic benefits of feed formulation using digestible values of ingredients – through another published methodology (*suggested reading*: Roy and Mraz, 2020). We found out that fishmeal (FM) free feed formulations can be ~10–27% cheaper than conventional FM-based formulation. The FM free feed with amino acid supplementation have lower or comparable formula cost to that of a conventional FM-based feed (Roy and Mraz, 2020).

For example – using our internally developed tilapia feedstuff inventory database (Roy and Mraz, unpublished), we could develop a formulation for tilapia, which is FM-free, includes alternative protein sources + non-conventional energy sources + essential amino acids + mineral supplementation. The formulation cost came out to be 12 CZK $kg^{-1}$, which is 20–35% cheaper than the existing commercial formulation price. A snapshot of that formulation based on digestible values of the ingredients is given in Fig. 10 (intended as an example). Such theoretical suggestions may be practically beneficial for the feed companies only if they enable them to make extra sales margin, given the already high manufacturing, packaging and supply chain and marketing costs. Professionally formulated feed based on good digestibility trial results in most cases seems beneficial for the farmers, the reason being that such formulations assure easy fulfillment of species optimum requirements (nutrition, energy) and may be reflected in lower, better FCR. This means a lower amount of feed (≈capital) might be needed than in ordinary cases (i.e. crude content-based formulations), to obtain the same yield. **Such helpful formulations are made possible only by technologies like the present one.**

| Ingredient Name | Formula (% share) | Rules |
|---|---|---|
| Crab meal | 3.76 | - |
| Feather meal | 12.16 | - |
| Black soldier fly meal | 8.66 | - |
| Yellow mealworm meal | 14.24 | - |
| Banana meal | 19.99 | - |
| Cottonseed hulls | 6.6 | - |
| Palm kernel meal | 15.66 | - |
| Potato protein | 13.53 | - |
| Safflower oil | 2.86 | - |
| Glycerol | 0.31 | - |
| Lysine | 0.08 | Max 1% |
| DCP | 0.15 | Max 1% |
| Calcium carbonate | 2 | Max 2% |
| SUM | 100 | |

| | | |
|---|---|---|
| Formula Cost /unit (kg) | 0.52 USD/ kg | 0.44 EUR/ kg |
| Formula Cost /Bag | 5.25 USD/ bag | 4.43 EUR/ bag |
| (Bag Size = 10 kg) | | |

**Model feed formula for Tilapia grow-out (FCR ≤ 1)**

| Nutrient | Set - MIN | Set - MAX | Analysis/ Outcome | Crude level (calculated)* |
|---|---|---|---|---|
| Dry Matter %age | - | 100 | 93.9 | 93.9 |
| Digestible Fibre (%) | - | 15 | 7.3 | 11.0 |
| Digestible Ash (%) | - | 15 | 2.2 | 4.7 |
| Digestible Protein (%) | 29 | 32 | 32.0 | 39.1 |
| Digestible Lipid (%) | - | 8 | 8.0 | 9.1 |
| Digestible Energy (kcal/kg) | 3400 | - | 3400.0 | 4624.6 |
| DP-DE Ratio (mg/kcal) | 70 | 130 | 87.7 | - |
| Digestible Arginine (%) | 1.2 | - | 2.1 | - |
| Digestible Histidine (%) | 0.5 | - | 0.7 | - |
| Digestible Isoleucine (%) | 0.9 | - | 1.6 | - |
| Digestible Leucine (%) | 1.3 | - | 2.8 | - |
| Digestible Lysine (%) | 1.6 | - | 2.1 | - |
| Digestible Methionine (%) | 0.7 | - | 0.7 | - |
| Dig Phenylalanine (%) | 1 | - | 1.6 | - |
| Digestible Threonine (%) | 1.5 | - | 1.5 | - |
| Digestible Tryptophan (%) | 0.3 | - | 0.5 | - |
| Digestible Valine (%) | 1 | - | 2.1 | - |
| Digestible Calcium (%) | 0.7 | - | 0.7 | 2.1 |
| Digestible Phosphorus (%) | 0.4 | - | 0.4 | 0.7 |
| Digestible PUFA (%) | - | - | 3.2 | 3.8 |
| Digestible Omega3 (%) | 0.6 | - | 0.8 | - |
| Digestible Omega6 (%) | 0.5 | - | 2.7 | - |
| Dig Phospholipid (%) | 0.5 | - | 0.5 | - |

*Crude level was calculated from the calculator/ converter (Scroll Left)

***Fig. 10.*** *An example of application of results derived from digestibility assessment technology. A model tilapia grow-out feed formulation, created by a feed formulator software according to the optimum species requirement and least-cost formulation environment – utilizing the digestible values of feedstuffs (Roy and Mraz, unpublished).*

***Tab. 5.*** *Breakdown of operational cost of the technology and digestibility assessment of 6 feedstuffs in one month (round 1 = 3 ingredients; round 2 = 3 ingredients; 14 days per round)***.** *The approximate breakdown of 'operational cost' is based on our internal budget and expenditure.*

| S. No. | Expenditure | Particulars | Maximum Budget (CZK) |
|---|---|---|---|
| 1. | Fish# | Monosex red tilapia (GIFT strain). Must include experimental stock (48 kg in 12 tanks) + 12 kg backup (for mortality compensation). 60 kg of fish in total. | 16,380 |
| 2. | Feed | Commercial tilapia feed. For maintenance and use as basal feed during experiment. 45 kg = 3 full bags. | 1,303 |
| 3. | Ingredients | Six protein feedstuffs (test ingredients). 5 kg per ingredient = 30 kg in total. | 2,500 |
| 4. | Consumables# | Gloves, sample tubes, glass beakers, zip lock bags, fish nets, buckets, trays, etc. | 2,500 |
| 5. | Laboratory analysis | Complete proximate fraction – dry matter, protein, lipid, fiber, ash, phosphorus and yttrium. Prices for analysis of 22 samples in an accredited third-party laboratory (tax included). Includes samples of ingredient, experimental feeds and faeces. | 73,205 (43,897*) |
| 6. | Energy and water | Energy and water charges – for all processes and daily system operation. | 5,000 |
| 7. | Manpower | Personnel wage – 1 full-time person or 2 partly employed | 25,000 |
| | | **Grand total** | 125,888 |
| | | **Cost per test ingredient** | 20,981 |
| | | **Cost per test ingredient (if only protein is tested)** | 16,097 |

*cost of analysis with only dry matter, protein and yttrium analyzed.
#The one-time investment is carried forward to the next month and for future trials.

**Note:** The prices are approximate, only for laboratory-scale digestibility trials and should not be compared with commercial feed costs or R&D costs of large-scale aquafeed manufacturers.

## 8. THE APPLICATION OF TECHNOLOGY IN PRODUCTION

The technology presented in this paper is intended for the application by aquafeed manufacturers/independent feed formulators, consultancy farms/ fish nutritionists, and researchers/third-party laboratories rendering such service to small scale fish feed producers or even ingredient suppliers. The technology will be useful for assessing and promoting novel or alternative feedstuffs in fish feed formulations, and ultimately to produce high-performance fish feed (Fig. 10).

## 9. REFERENCES

Agbo, N.W., Madalla, N., Jauncey, K., 2009. The biochemical composition of some Ghanaian oilseed by-products and their digestibility by Nile tilapia, *Oreochromis niloticus* L. Journal of the Ghana Science Association 11: 136–148.

Barone, R.S.C., Sonoda, D.Y., Lorenz, E.K., Cyrino, J.E.P., 2018. Digestibility and pricing of *Chlorella sorokiniana* meal for use in tilapia feeds. Scientia Agricola 75: 184–190.

Borgeson, T.L., Racz, V.J., Wilkie, D.C., White, L.J., Drew, M.D., 2006. Effect of replacing fishmeal and oil with simple or complex mixtures of vegetable ingredients in diets fed to Nile tilapia (*Oreochromis niloticus*). Aquaculture Nutrition 12: 141–149.

Borghesi, R., Portz, L., Oetterer, M., Cyrino, J.E.P., 2008. Apparent digestibility coefficient of protein and amino acids of acid, biological and enzymatic silage for Nile tilapia (*Oreochromis niloticus*). Aquaculture Nutrition 14: 242–248.

Boyd, C.E., D'Abramo, L.R., Glencross, B.D., Huyben, D.C., Juarez, L.M., Lockwood, G.S., McNevin, A.A., Tacon, A.G.J., Teletchea, F., Tomasso, J.R., Jr, Tucker, C.S., Valenti, W.C., 2020. Achieving sustainable aquaculture: Historical and current perspectives and future needs and challenges. Journal of the World Aquaculture Society 51: 578–633.

Cho, C.Y., Slinger, S.J., 1979. Apparent digestibility measurement in feedstuffs for rainbow trout. In: Proceedings of the World Symposium on Finfish Nutrition and Fish Feed Technology, vol. 2, June 20–23, 1978, Hamburg, Germany, pp. 239–247.

Davies, S.J., Abdel-Warith, A.A., Gouveia, A., 2011. Digestibility characteristics of selected feed ingredients for developing bespoke diets for Nile tilapia culture in Europe and North America. Journal of the World Aquaculture Society 42: 388–398.

Degani, I., Biola, S., Yehuda, Y., 1997. Apparent digestibility of protein and carbohydrate in feed ingredients for adult tilapia (*Oreochromis niloticus* x *O. aureus*) hybrid. Bamidgeh 49: 15–123.

Dong, X.H., Guo, Y.X., Ye, J.D., Song, W.D., Huang, X.H., Wang, H., 2010. Apparent digestibility of selected feed ingredients in diets for juvenile hybrid tilapia, *Oreochromis niloticus* × *Oreochromis aureus*. Aquaculture Research 41: 1356–1364.

Drew, M.D., Borgeson, T.L., Thiessen, D.L., 2007. A review of processing of feed ingredients to enhance diet digestibility in finfish. Animal Feed Science and Technology 138: 118–136.

El-Sayed, A.F.M., 2004. Protein nutrition of farmed tilapia: searching for unconventional sources. In: New dimensions in farmed tilapia: proceedings of the Sixth International Symposium on Tilapia Aquaculture, September 12–16, 2004, Manila, Philippines, pp. 364–378.

FAO (Food and Agriculture Organization), 2020. The State of World Fisheries and Aquaculture 2020. Sustainability in action. Rome. https://doi.org/10.4060/ca9229en

Farahiyah, I.J., Mardhati, M., Yong, S.T., 2018. Effects of using puffed feather meal on the growth performance and carcass characteristics of red hybrid tilapia. Malaysian Journal of Animal Science 21: 99–107.

Fontainhas-Fernandes, A., Gomes, E., Reis-Henriques, M.A., Coimbra, J., 1999. Replacement of fish meal by plant proteins in the diet of Nile tilapia: digestibility and growth performance. Aquaculture International 7: 57–67.

Fontes, T.V., de Oliveira, K.R.B., Gomes Almeida, I.L., Maria Orlando, T., Rodrigues, P.B., Costa, D.V.D., 2019. Digestibility of insect meals for Nile tilapia fingerlings. Animals 9: 181.

Gaber, M.M., 2006. The effects of plant-protein-based diets supplemented with yucca on growth, digestibility, and chemical composition of Nile tilapia (*Oreochromis niloticus*, L) fingerlings. Journal of the World Aquaculture Society 37: 74–81.

Geremew, A., Getahun, A., Rana, K., 2015. Digestibility of soybean cake, Niger seed cake and linseed cake in juvenile Nile tilapia, *Oreochromis niloticus* L. Journal of Aquaculture Research & Development 6: 333.

Goddard, S., Al-Shagaa, G., Ali, A., 2008. Fisheries by-catch and processing waste meals as ingredients in diets for Nile tilapia, *Oreochromis niloticus*. Aquaculture Research 39: 518–525.

Godoy, A.C., Fries, E., Corrêia, A.F., Melo, I.W.A., Rodrigues, R.B., Boscolo, W.R., 2016. Apparent digestibility of fish meat and bone meal in Nile tilapia. Archivos de Zootecnia 65: 341–348.

Guimarães, I.G., Pezzato, L.E., Barros, M.M., 2008a. Amino acid availability and protein digestibility of several protein sources for Nile tilapia, *Oreochromis niloticus*. Aquaculture Nutrition 14: 396–404.

Guimarães, I.G., Pezzato, L.E., Barros, M.M., Tachibana, L., 2008b. Nutrient digestibility of cereal grain products and by-products in extruded diets for Nile tilapia. Journal of the World Aquaculture Society 39: 781–789.

Guimarães, I.G., Pezzato, L.E., Barros, M.M., Fernandes, R.D.N., 2012. Apparent nutrient digestibility and mineral availability of protein-rich ingredients in extruded diets for Nile tilapia. Revista Brasileira de Zootecnia 41: 1801–1808.

Hajen, W.E., Beames, R.M., Higgs, D.A., Dosanjh, B.S., 1993. Digestibility of various feedstuffs by post-juvenile chinook salmon (*Oncorhynchus tshawytscha*) in sea water. 1. Validation of technique. Aquaculture 112: 321–332.

Hanley, F., 1987. The digestibility of foodstuffs and the effects of feeding selectivity on digestibility determinations in tilapia, *Oreochromis niloticus* (L). Aquaculture 66: 163–179.

IAFFD (International Aquaculture Feed Formulation Database), 2020. Feed Ingredients Composition Database (FICD) v5.1. Available online at https://www.iaffd.com/ (accessed on 29 September 2020)

Köprücü, K., Özdemir, Y., 2005. Apparent digestibility of selected feed ingredients for Nile tilapia (*Oreochromis niloticus*). Aquaculture 250: 308–316.

Maina, J.G., Beames, R.M., Higgs, D., Mbugua, P.N., Iwama, G., Kisia, S.M., 2002. Digestibility and feeding value of some feed ingredients fed to tilapia *Oreochromis niloticus* (L.). Aquaculture Research 33: 853–862.

Novelli, P.K., Barros, M.M., Pezzato, L.E., de Araujo, E.P., de Mattos Botelho, R., Fleuri, L.F., 2017. Enzymes produced by agro-industrial co-products enhance digestible values for Nile tilapia (*Oreochromis niloticus*): A significant animal feeding alternative. Aquaculture 481, 1–7.

NRC (National Research Council), 2011. Nutrient Requirements of Fish and Shrimp. National Academy Press, Washington DC, 376 pp.

Pereira, R., Valente, L.M., Sousa-Pinto, I., Rema, P., 2012. Apparent nutrient digestibility of seaweeds by rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*). Algal Research 1: 77–82.

Putri, N.T., Jusadi, D., Setiawati, M., Sunarno, M.T.D., 2017. Potential use of green algae *Caulerpa lentillifera* as feed ingredient in the diet of Nile tilapia *Oreochromis niloticus*. Jurnal Akuakultur Indonesia 16: 184–192.

Ramos, A.P.D.S., Braga, L.G.T., Carvalho, J.S.O., Oliveira, S.J.R.D., 2012. Digestibility of agro-industrial byproducts in 200 and 300-g Nile tilapia. Revista Brasileira de Zootecnia 41: 462–466.

Ribeiro, F.B., Lanna, E.A.T., Bomfim, M.A.D., Donzele, J.L., Quadros, M., Cunha, P.D.S.L., 2011. True and apparent digestibility of protein and amino acids of feed in Nile tilapia. Revista Brasileira de Zootecnia 40: 939–946.

Rodrigues, A.P.O., Gominho-Rosa, M.D.C., Cargnin-Ferreira, E., de Francisco, A., Fracalossi, D.M., 2012. Different utilization of plant sources by the omnivores jundiá catfish (*Rhamdia quelen*) and Nile tilapia (*Oreochromis niloticus*). Aquaculture Nutrition 18: 65–72.

Roy, K., Mraz, J., 2020. Alternative feed components to replace fishmeal and fish oil in carp feed. In: Edition of Methodics, University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Vodňany, no. 184, 30 pp.

Serrano Jr., A.E., Declarador, R.S., Tumbokon, B.L.M., 2015. Proximate composition and apparent digestibility coefficient of *Sargassum* spp. meal in the Nile tilapia, *Oreochromis niloticus*. Animal Biology & Animal Husbandry 7: 159–168.

Sklan, D., Prag, T., Lupatsch, I., 2004. Apparent digestibility coefficients of feed ingredients and their prediction in diets for tilapia *Oreochromis niloticus* × *Oreochromis aureus* (Teleostei, Cichlidae). Aquaculture Research 35: 358–364.

Tram, N.D.Q., Ngoan, L.D., Hung, L.T., Lindberg, J.E., 2011. A comparative study on the apparent digestibility of selected feedstuffs in hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) and Nile tilapia (*Oreochromis niloticus*). Aquaculture Nutrition 17: e636–e643.

Tran-Ngoc, K.T., Haidar, M.N., Roem, A.J., Sendão, J., Verreth, J.A., Schrama, J.W., 2019. Effects of feed ingredients on nutrient digestibility, nitrogen/energy balance and morphology changes in the intestine of Nile tilapia (*Oreochromis niloticus*). Aquaculture Research 50: 2577–2590.

Vidal, L.V.O., Xavier, T.O., de Moura, L.B., Graciano, T.S., Martins, E.N., Furuya, W.M., 2017. Apparent digestibility of soybean coproducts in extruded diets for Nile Tilapia, *Oreochromis niloticus*. Aquaculture Nutrition 23: 228–235.

Vidal, L.V.O., Xavier, T.O., Michelato, M., Martins, E.N., Pezzato, L.E., Furuya, W.M., 2015. Apparent protein and energy digestibility and amino acid availability of corn and coproducts in extruded diets for Nile Tilapia, *Oreochromis niloticus*. Journal of the World Aquaculture Society 46: 183–190.

Watanabe, T., Takeuchi, T., Satoh, S., Kiron, V., 1996. Digestible crude protein contents in various feedstuffs determined with four freshwater fish species. Fisheries Science 62: 278–282.

Zhou, Q.C., Yue, Y.R., 2012. Apparent digestibility coefficients of selected feed ingredients for juvenile hybrid tilapia, *Oreochromis niloticus* × *Oreochromis aureus*. Aquaculture Research 43: 806–814.

**Analytical standards**

ISO 11465: 1993. Soil quality — Determination of dry matter and water content on a mass basis — Gravimetric method. https://www.iso.org/standard/20886.html

ČSN EN ISO 16634-1. Food products - Determination of the total nitrogen content by combustion according to the Dumas principle and calculation of the crude protein content — Part 1: Oilseeds and animal feeding stuffs. https://www.iso.org/standard/46328.html

ISO 17294-2:2016. Water quality — Application of inductively coupled plasma mass spectrometry (ICP-MS) — Part 2: Determination of selected elements including uranium isotopes. https://www.iso.org/standard/62962.html

ISO 1443:1973. Meat and meat products — Determination of total fat content. https://www.iso.org/standard/6170.html

ISO 1575:1987. Tea — Determination of total ash. https://www.iso.org/standard/6170.html

ISO 5498:1981. Agricultural food products — Determination of crude fibre content — General method. https://www.iso.org/standard/11544.html

ISO 6491:1998. Animal feeding stuffs — Determination of phosphorus content — Spectrometric method. https://www.iso.org/standard/12864.html

***External reviewer***

*Štěpán Lang, Ph.D.*
*Trouw Nutrition Biofaktory, Na Chvalce 2049,*
*193 00 Praha 9 – Horní Počernice*

***Internal reviewer***

*Carlos Yanes-Roca, Ph.D.*
*University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Institute of Aquaculture and Protection of Waters, Zátiší 728/II, 38925 Vodňany, www.frov.jcu.cz*

***Verification and application of the technology in 2020***

*Trouw Nutrition Biofaktory, s.r.o., Na Chvalce 2049,*
*193 00 Praha9 – Horní Počernice*

***Affiliation***

*M.Sc. Koushik Roy – 50%*
*Assoc. Prof. Jan Mráz – 50%*

*University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Institute of Aquaculture and Protection of Waters, Na Sádkách 1780, 370 05 České Budějovice, www.frov.jcu.cz*

***Preferred citation***

*Roy, K., Mráz, J., 2021. Digestibility of protein feeds in Tilapia. Edition of Methodics, University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Vodňany, Czech Republic, No. 186, 38 pp.*

*In the edition of Methodics published by University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, editorial board: Antonín Kouba, Ph.D., Language correction: M.Sc. Kateřina Kovářová, published in 2021.*

Fakulta rybářství
a ochrany vod
Faculty of Fisheries
and Protection
of Waters

Jihočeská univerzita
v Českých Budějovicích
University of South Bohemia
in České Budějovice

Edition
of Methodics

ISBN 978-80-7514-125-5