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Evaluation of the use of fractional abundance of stable carbon and nitrogen isotopes and indigestible inert marker in diets and faeces to calculate apparent digestibility of carbon and nitrogen

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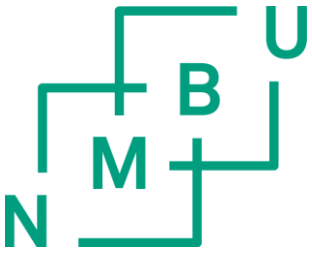

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Ås, 8.3.2018

Yue Song

Abstract

The study carried out to test the feasibility of calculating apparent digestibilities (AD) of carbon and nitrogen with diets in fishmeal (FM), soybean meal (SBM), and rapeseed meal (RSM) as the main sources of protein by calculating the fractional abundance of stable carbon and nitrogen isotopes (^{13}C and^{15}N) in diets to relative faeces with yttrium oxides ratios. In order to evaluate the potential of applying a fractional abundance of stable isotopes to test AD, the results of this study were taken to compare with AD estimated by quantifying total carbon and nitrogen in the previous study of (Shomorin 2017). Three different extruded diets, FM, SBM, and RSM used in this study, were fed to the triplicate groups of 120-g rainbow trout (*Oncorhynchus mykiss)* for once a day. The rearing temperature of freshwater was kept around 14 degrees constantly during the 22 days of this experiment. Two different faeces collection methods, stripping and sieving from retch wire screen (at 15 min and 240min, respectively) were utilized in this experiment. Dried freeze experimental diets and relative faeces were sampled around 0.15 mg in small plastic containers and then sent to the Institute for Energy Technology in Kjeller, Norway for stable isotope analysis.

Similar carbon isotope signatures were obtained by stable isotope analysis on FM, SBM and RSM, which were -22.57, -23.87 and -24.22, respectively. A significant differences were observed for the nitrogen isotope signatures among FM and rest of diets (SBM and RSM), which were 7.59, 3.56 and 4.22, respectively. This may be due to the more balanced amino acid profile in FM, compared with C_3 based plant protein (e.g., SBM and RSM). The stable isotope analysis on faeces collected by stripping illustrates fecal carbon isotopes was enriched relative to FM and SBM while depleted relative to RSM. On the other hand, nitrogen isotope signatures in faeces collected by stripping were depleted towards FM, SBM, and RSM. The ANOVA analysis showed the significant (<0.05) difference for the fecal carbon and nitrogen isotopes signatures with the increasing exposure time of faeces in water. The stable carbon isotope signatures in faeces originated from FM, SBM and RSM were all decreasing as the prolongation of exposure of faeces in water. This is an indication of fecal ^{13}C loss by nutrient leaching. However, a significant increase was found for the fecal stable nitrogen isotopes derived RSM during the first 15 min faeces collection intervals, and then decrease was observed from 15min to 240 min faeces collection intervals. Furthermore, the same pattern being directly from the stable isotope signatures was observed in the fractional abundance of stable isotopes in diets and faeces.

Apparent digestibilities estimated based on the fractional abundance of stable carbon and nitrogen isotopes in diets to faeces were not significantly different from that estimated by using total carbon and nitrogen for both faeces collection methods (stripping and retch wire screening). But the AD estimated by the fractional abundance of stable isotopes was generally higher than total elements among diets. This indicates that applying fractional abundance of stable isotopes to calculate AD is more quantitative, compared to using total elements. The AD estimated based on the fractional abundance of stable carbon isotopes ranked the digestibility of 3 experimental diets in this order (FM >SBM> RSM), and (SBM >FM> RSM) was observed when AD estimated based on the fractional abundance of stable nitrogen isotopes. The ranking trend observed in this study was in a line with the AD estimated based on corresponding total elements in the previous experiment of (Shomorin 2017).

Moreover, AD estimated by the fractional abundance of carbon and nitrogen isotopes obtained by collecting faeces on retch wire screen is higher than obtained by stripping methods, except for AD of ^{13}C observed for FM. This corresponds to the observation done by (Shomorin 2017) when the estimates were made based on total C in feeds and excreta.

Keywords: Apparent digestibility; fractional abundance of carbon and nitrogen isotopes, total carbon and nitrogen element, previous experiment, Stripping; Retch wire screen; Leaching; Fish meal; Soybean meal; Rapeseed meal; Rainbow trout (*Oncorhynchus mykiss)*

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1. Introduction

Apparent digestibility (AD) test is the useful measure to determine the amount of the nutrient utilized from diets by animals. However, overestimation or underestimation of the AD could happen. Underestimated AD value results from one or more factors that involve fecal loss of endogenous enzymes, cellular materials eroded from the intestinal lining and other materials secreted from the epithelial cells of the gastrointestinal system (McDonald et al. 2011). On the other hand, overestimated AD is mainly related to nutrient leaching after the faeces have been excreted into the water (Mu et al. 2000). Therefore, in order to minimize overestimation and underestimation of the AD, an effort has been directed toward on updating methodology of faeces sampling and methods used to estimate AD.

Quantitative collection of faeces for assessment of AD of nutrient is not feasible, since it is labor-consuming and not accurate. The use of indigestible makers, such as chromic oxides (Austreng 1978; Vandenberg & De La Noüe 2001; Windell et al. 1978) and different lanthanide oxides such as yttrium oxides (Austreng et al. 2000; Refstie et al. 1997; Storebakken et al. 1998; Sugiura et al. 1998) allow accurate AD estimates without quantitative collection. This method has been recognized as a reliable, in contrast to the quantitative collection of faeces sample. However, (Kavanagh et al. 2001) claimed that indigestible markers, such as chromic oxides, are not reliable to estimate AD because of poor recovery from dietary samples and chromic oxides was discovered to be pass through fish digestion system faster than feed nutrient when the concentration rate account for 2% in feed (Tacon & Rodrigues 1984). On the other hand, stable isotopes are found that they are able to trace feed nutrient digestion and assimilation in the animal body because stable isotopes ratio varies in a predictable way in accordance with the cycling elements in nature (Oliveira et al. 2008). In addition, the method that analyzing stable isotopes on naturally defecated faeces is noninvasive to experimental animals (Salvarina et al. 2013).

Therefore, the current experiment is carried out to estimate apparent digestibility (AD) of three experimental diets (fishmeal, soybean meal and rapeseed meal) by calculating the fractional abundance of stable isotopes in diets and respective faeces with yttrium oxides ratios. Furthermore, in order to evaluate the potential of applying fractional abundance of stable

isotopes in fish nutrient field, the apparent digestibility in this study is taken to compare with the AD estimated based on total elements with yttrium oxides ratios in previous experiment of (Shomorin 2017). We shared the same experimental fish, diets, faeces and faeces collection methods.

1.1 Natural stable isotopes: Definition, Isotopic abundance and Variation, and Measuring technique

Some chemical elements have plural forms with different numbers of the neutrons in the nucleus of each atom of that element. They are not radioactive and this plural form of the same element is known as natural stable isotopes (Michener & Schell 1994; West et al. 2006). Most chemical elements have two or more stable isotopes, and the abundance of stable isotopes are always presented by the ratio of heavy and light isotopes (Rundel et al. 2012). Isotopic compounds or molecules have a tiny difference in chemical and physical behavior. This is known as isotopic fractionation and occurs through the transition of a compound from one state to another state in chemical or physical equilibrium process (Peterson & Fry 1987). The variation on isotopic abundance through physical reaction, such as kinetic processes or thermodynamic equilibrium is very small in nature (Michener & Lajtha 2008). This tiny variation in isotopic composition in different substance with precise magnitude is obtained by the stable isotopic analysis based on measurement of the difference between defined standard isotope ratio and that of given sample (McKinney et al. 1950). This method can provide reliable result with high precision during long or short-time periods, which is usually expressed as delta value (e.g., recorded as $\delta^{13}C$ and $\delta^{15}N$) (Michener & Schell 1994; Rundel et al. 2012). Most importantly, the isotope analysis is processed by mass spectrometry. It is a precise analytical method introduced by (Nier 1947) and then improved by (McKinney et al. 1950) to measure the stable isotope ratio in the sample. Mass spectrometry measures stable isotope ratio of samples by three steps (Rundel et al. 2012). First-step is, combustion and ionization of the content of the samples. Secondly, charged particles are deflected and separated. Lastly, the ions are trapped in a series of ion collectors at the end of the flight tube. This technique has the power to sort the sample's ions based on their mass to charge ratio and depart heavier and lighter isotopes into separated groups for weighing. This measuring process is also knowns as stable isotope analysis (SIA) (Krueger 1998).

1.2 Use of stable isotopes analysis in fish nutrient research

The application of stable isotopes in the fish nutrition research is primarily facilitated by different feed sources having distinctively different isotopes signatures, such as C_3 and C_4 plant protein sources (Gannes et al. 1997). Stable isotope signatures ($\delta^{13}C$) of the C_4 plants (e.g. corn and wheat) are ranging from -16 ‰ to -10‰ while that of the C_3 plants (e.g. soybean and rapeseed) range from -33 ‰ to -24‰ (O'Leary 1988). This different stable isotope ratio among different plant-based feed sources is created by diffusion, dissolution, carboxylation of atmospheric CO_2 during the photosynthetic process (Gannes et al. 1998; Lajtha & Marshall 1994). On the other hand, $\delta^{15}N$ ratios vary among different plants with the variation of N-fixing rate from soil nitrogen source; which is also related to the depth of soil and seasonal changes (Delwiche et al. 1979; Peterson & Fry 1987). Thereafter, the distinct stable isotope signature in diets consumed by animals (including fish) incorporated into the tissue by nutrient absorption and incorporation (Gannes et al. 1998). The stable isotope variation from diets to tissues is related to fractionation occurring in the physical and biochemical reaction in the digestive tract of animals (Gamboa Delgado 2009). This fractionation results in the enrichment or depletion of one isotope relative to another (Gannes et al. 1998; Michener & Schell 1994). The rates of chemical reaction and isotopes transport between reactants and products depend on isotope nuclide mass and the partitioning of energy within molecules (Peterson & Fry 1987). Generally, the chemical bonds between light isotopes are weaker than that of heavy isotopes, hence lighter isotopes react faster than heavy isotopes (DeNiro & Epstein 1978; DeNiro & Epstein 1981). Ratios between stable isotope ratios in diets to their respective values in faeces has already shown to provide useful information on efficiency of nutrient digestion (Oliveira et al. 2008).

1.3 Dietary nutrient utilization of fish tested by stable isotopes analysis in diets to fish

Stable isotopes analysis conducted on samples of diets and fish have been used as a method to test the absorption rate of feed by fish in a series of closed laboratory studies (Beltran et al. 2009; Enyidi et al. 2013; Schlechtriem et al. 2004). An experiment with African Catfish, Clarias gariepinus, larva, showed higher ^{13}C and ^{15}N composition in their tissue when fed to FM, compared with feeding by corn meal (C_4 plant) and bambaranut meal (C_3 plant) (Enyidi et al. 2013). Increased fractionation of plant protein, compared with FM, has also been reported in

rainbow trout and gilthead sea bream (Beltran et al. 2009). However, the usefulness of stable isotope techniques to quantify feed intake and assimilation in aquatic larva is limited because of the small size of fish larva, complicated and faster growth (Le Vay & Gamboa-Delgado 2011). Furthermore, these limitations effect the accuracy of assesing nutrient incorporation by fish larave from their diets (Gamboa-Delgado & Le Vay 2009).

The most common used stable isotopes are carbon and nitrogen stable isotopes duo to wide natural abundance (Michener & Lajtha 2008), (DeNiro & Epstein 1978; Fry & Sherr 1989) observed less variation of carbon stable isotopes (1‰) between diets and organisms. However, differences at 3-4 ‰ have been observed for the $\delta^{15}N$ (Minagawa & Wada 1984). In addition, stable isotope analysis in diets to fish allow to route feed nutrient into some specific tissues, such as liver, blood, cartilage and muscles (MacAvoy et al. 2001; MacNeil et al. 2006). Different tissues reflect dieatry isotopes with different speed, more metabolically active tissses, such as liver and blood, were obsorbed to take months to reach equilibrium with new dietary $\delta^{15}N$ (MacNeil et al. 2006). Stable isotopes turnover time as long as years may require for muscle to obtain stable isotopes signatures that reflect the feed (Hesslein et al. 1993; MacAvoy et al. 2001; MacNeil et al. 2006).

1.4 The potential of dietary nutrient digestibility of fish tested by stable isotopes analysis on diets and relative faeces

Determination of feed digestibility based on estimating fractional abundance of stable isotopes in the feed to relative faeces have never done in the fish nutrient field, but this method is applicable according to the logistics that the stable isotopic signatures in faeces can reflect that of relative diets (Salvarina et al. 2013). Faeces can reflect the stable isotope of diets in land animals had been widely demonstrated by (Sponheimer, M et al. 2003; Steele & Daniel 1978; Sutoh et al. 1987; Sutoh et al. 1993). As for fish, the stable isotopes signatures of their diets and faeces had been separately analyzed in the study of detecting stable carbon and nitrogen isotope signatures in organic matter (Yokoyama et al. 2006), waste (McGhie et al. 2000) and particulate matter (Franco-Nava et al. 2004) in the sediments of fish cage. The result by (Yokoyama et al. 2006) revealed ^{15}N reduction in faeces relative to moist pellet (fish-derived meal) and extruded pellet (mainly C_3 derived meal). Similarly, the reduction of fecal ^{15}N relative to artificial diets and organic matter (diets) fed to seabass and salmonid also found in the study of (Franco-Nava et al.

2004; McGhie et al. 2000). On the other hand, the composition of carbon isotopes in faeces relative to diets are either enriched (McGhie et al. 2000; Ye et al. 1991) or depleted (Franco-Nava et al. 2004; Yokoyama et al. 2006). In addition, continually collected faeces for an extended period is more suitable to characterize long-term diets of an animal (Sponheimer, Matt et al. 2003). It is advantageous to sample faeces because sampling faeces from water can reduce the stress for fish by non-disturbance and do not need to kill fish. Most importantly, faeces have been reported to turnover feed nutrient with few hours or days depending on animal species (Jones et al. 1979; Salvarina et al. 2013; Udén et al. 1982). So that, there is no need for mathematical correction errors for feces because of faeces not like teeth or hair that have different growth rate and attenuation time (Ayliffe et al. 2004). The faster indigested dietary signatures reflected in the faeces of bats within 2-3 hours (Salvarina et al. 2013). Longer periods, such as 6 days and 14 days, was observed in feces of steers and rabbits (*Oryctolagus cuniculus*) to reflect their dietary $\delta^{13}C$ signatures when their diets switched from C_4 to C_3 and C_3 to C_4, respectively (Jones et al. 1979; Udén et al. 1982). In other words, faeces are short-term responsiveness of the dietary change in contrast to the sample from the whole body or some tissues. However, the potential utilization of fecal-based isotopic studies is limited by the over-representation of coarse refractory and an undigested component of diets in faeces (Codron et al. 2005). The other limitation in the study of fish nutrition is that nutrient leaching happened before collection of faeces in water.

2. Fecal collection methods

Digestibility is the main parameter to test the utilization of feed nutrient by animals (including fish). But, the result of most digestibility measurement is known as apparent digestibility (AD) because this value either higher or lower than true digestibility (TD). One of the main factors responsible for the difference between AD and TD is fecal collection methods. Fecal collection method can be divided into direct and indirect methods (Choubert Jr et al. 1979). The indirect fecal collection methods estimate the apparent digestibility coefficient by calculating the indigestible markers in diets to faeces (Austreng 1978; Choubert Jr et al. 1979). However, the direct fecal collection method requires calculating the difference between feed consumed and all excreted faeces (Choubert Jr et al. 1979; Smith 1971). Direct (Post et al. 1965;

Smith 1971) and indirect fecal collection methods (Austreng 1978; Windell et al. 1978) were both reported useful but still have insufficiency to estimate apparent digestibility coefficient.

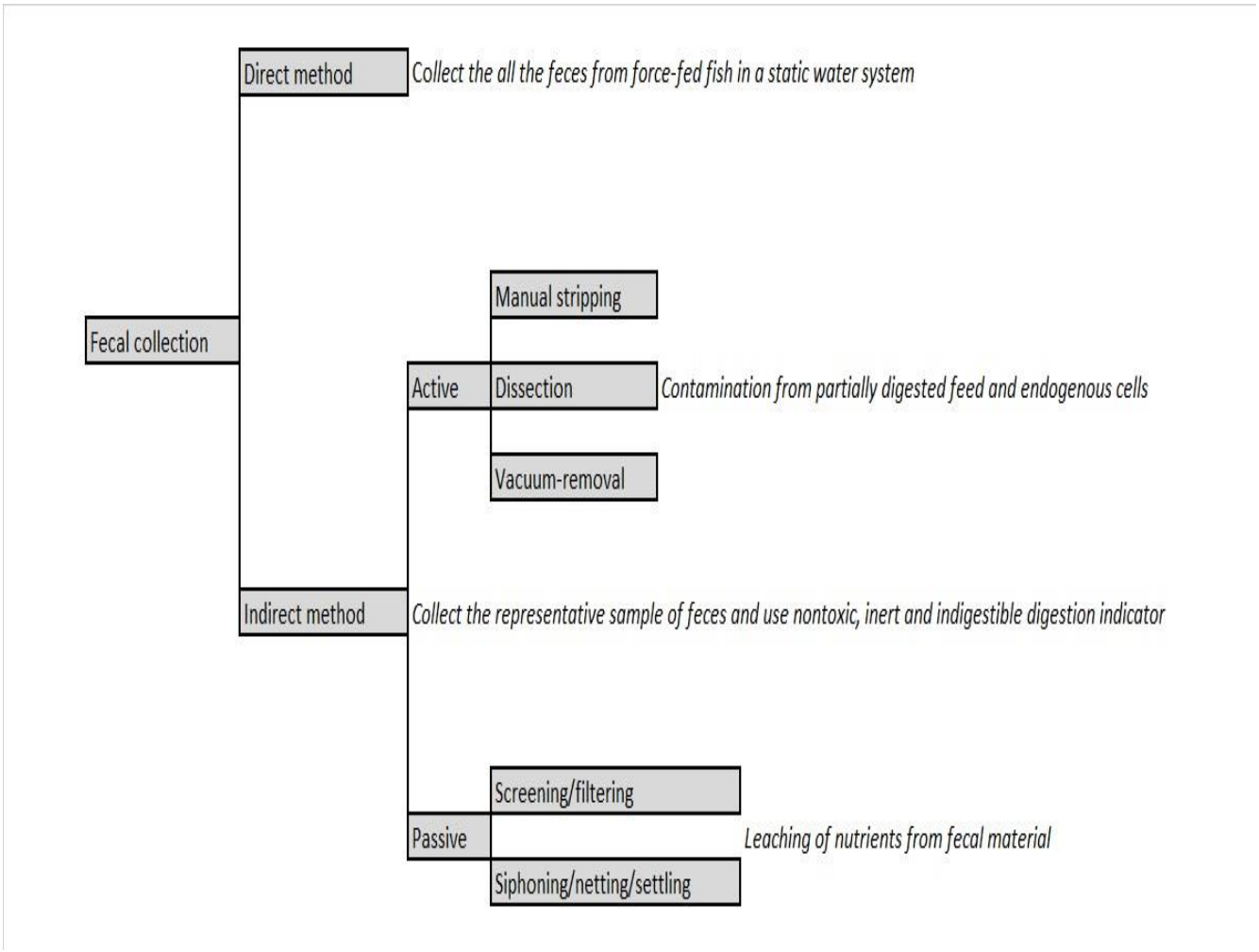

Figure 1. Classification of fecal collection methods

2.1 Direct fecal collection

The metabolic chamber used to rear fish for direct fecal collection methods was introduced by (Smith 1971). This metabolic chamber allows collecting faeces quantitatively in the water medium and allows separate collection of urine by inserting a catheter in the urinary tract and ammonia from the gills (Smith et al. 1980). The use of this equipment, however, requires force feeding, and the use of anesthetized fish force feeding limits nutrient leaching from the feed into the water (Post et al. 1965). However, the report by (Tunison et al. 1942) has shown that apparent digestibility estimates are not accurate when using direct fecal collection methods. The

fish also gets stressed and swimming ability is limited (Post et al. 1965; Smith 1971). In conclusion, digestibility assessment based on complete collection of digesta is not a recommended procedure.

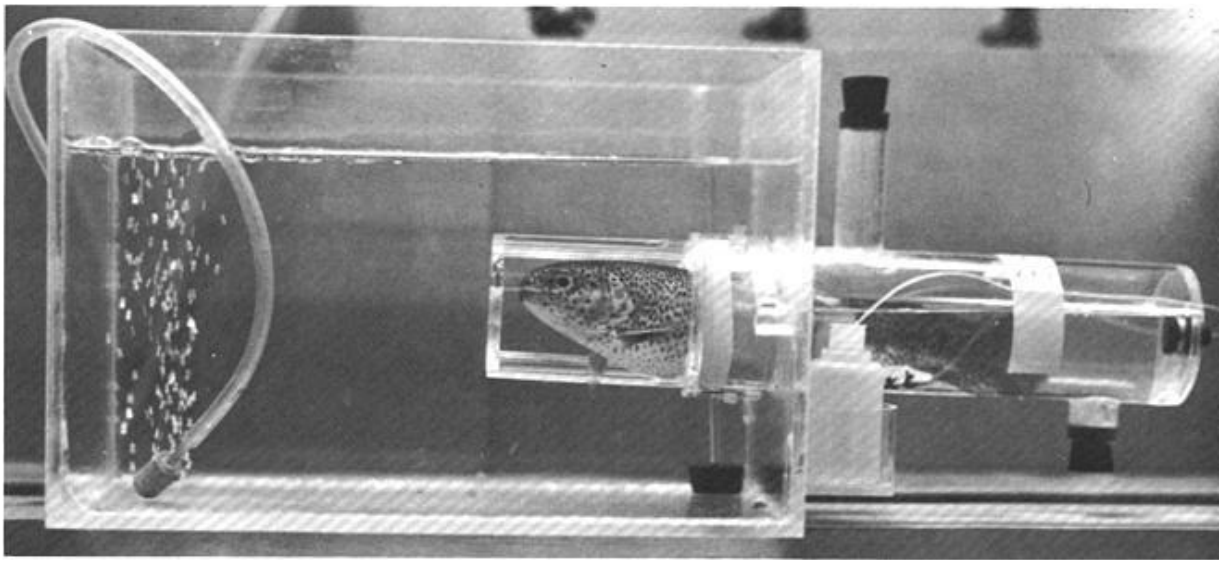

Figure 2. Metabolic chamber for direct fecal collection by *(Smith 1971)*

2.2 Indirect fecal collection

Indirect methods may classified as active or passive (Halver & Hardy 2002). The active method includes stripping (Austreng 1978; Shomorin 2017; Vens-Cappell 1985; Windell et al. 1978), dissection (Austreng 1978; Windell et al. 1978) and anal suction (vacuum removal) (Spyridakis et al. 1989). AD may be slightly underestimated by all these 3 procedures, due to the fecal sample being contaminated with undigested feed, endogenous fluids or epithelial cells (Cho et al. 1982; Hajen et al. 1993a; Storebakken et al. 1998). Faeces collection by these three methods requires external force. Stripping is conducted by applying abdominal pressure to force faeces from the posterior intestine of anesthetized fish (Austreng 1978). This squeezing may cause the sample to be contaminated by body mucus, sexual products, and urine along with feces (Windell et al. 1978). Faeces in the distal intestine is sucked out by the use of a glass cannula inserted in the anus of anesthetizing fish (Spyridakis et al. 1989). The main limitation of anal suction is the induction of sudden defecation and acceleration of the intestinal transit (Spyridakis et al. 1989). The difference of dissection methods, compared with the other two active fecal

collection methods, is required to kill fish, thus faeces are dissected out from the alimentary canal (Austreng 1978). (Hajen et al. 1993a) have shown that apparent digestibility estimated by dissection and stripping make some errors, with the reasons of attributed to incomplete digested nutrient and endogenous contaminant in collected fecal samples. However, the result of (Shomorin 2017) shown that applying gentle abdominal pressure to strip out faeces can provide reliable AD estimates in rainbow trout.

In order to prevent the underestimation of apparent digestibility by active faeces collection methods, the methods of passive collection of faeces with different fecal collecting devices, such as netting (Windell et al. 1978), siphoning (immediate pipetting) (Spyridakis et al. 1989), settling (Cho 1979; Cho et al. 1982; Hajen et al. 1993a) and screening/filtering (Choubert et al. 1982; Choubert Jr et al. 1979; Shomorin 2017; Vens-Cappell 1985) have been used in a series of studies. High apparent digestibility estimates obtained by faeces settling (Guelph system), compared with stripping and dissection, was observed by (Hajen et al. 1993a). The similar finding also observed in (Vens-Cappell 1985), showing AD of protein and energy digestibility being 1-2.3 % units higher when using continuous filtering fecal collection method than stripping. It strongly highlighted the efficiency of continuous filtration method, in contrast to the other passive fecal collection methods. This may due to automatic rapid fecal removing speed of this device with 6-15 seconds, which results in lower apparent digestibility by reducing the contact between water and faeces to the minimum (Choubert et al. 1982; Choubert Jr et al. 1979).

However, the main drawback of using passive fecal collection methods remains nutrient leaching from faeces into water (Hajen et al. 1993a; Shomorin 2017; Vens-Cappell 1985; Windell et al. 1978). For instance, 15% decrease gained for the protein content of faeces after remained in the water for 5 min, while lower leaching rate (around 6%) was observed within 5-60 min exposure time intervals (Possompes 1973 as cited by Vens-Cappell 1985). Similarly, significant leaching and elevation of AD were evident already 15 min after feces had been trapped on a retch wire screen (Shomorin 2017). Nutrient leaching from faeces had been tested after 1, 4, 8 and 16 h) in water (Windell et al. 1978) and a significant e nutrient losses were observed already during the first hour, while no significant leaching occurred from 4 to 16 hours after defecation.

3. The purpose of current experiment

In current experiment, the apparent digestibility of three experimental diets (FM, SBM, and RSM) for rainbow trout (*Oncorhynchus mykiss*) is estimated based on the fractional abundance of natural stable carbon and nitrogen isotopes with yttrium oxides ratios in diets to faeces.

1) To assess variation of stable carbon and nitrogen isotopes ratios in diets and relative faeces.

2) To compare the apparent digestibility estimated based on fractional abundance of carbon and nitrogen stable isotopes in diets to faeces with that of estimation based on total carbon and nitrogen in a previous experiment of (Shomorin 2017), respectively with the different time intervals (0, 15 and 240 min, respectively).

3) To investigate the difference of nutrient leaching rate with different time intervals by comparing AD estimated based on two different fecal collection methods used in the current study and previous study by (Shomorin 2017).

4. Materials and Methods

4.1 Experimental design

This experiment aimed to compare AD estimated by different two methods with different time intervals (0, 15 and 30 min). The estimating methods can be termed as new and classic, in order to differentiate clearly. The new estimating method used in current study applied the fractional abundance of stable carbon and nitrogen isotopes with yttrium oxides ratio in diets to faeces. But the classic estimating method used in the previous study of (Shomorin 2017) only calculate the yttrium oxides in diets to faeces. The AD estimated by classic method is showed in **Table1.**

Table 1. Apparent digestibility coefficients obtained from fecal collection at different time intervals (0, 15 and 240 min) with classic estimation method in the previous experiment of (Shomorin 2017)

Diet	Fishmeal	Soybean meal	Rapeseed meal	P-value
AD Carbon, %				
Stripping	89.3 ± 0.2^{a}	82.8 ± 0.2^{b}	73.9 ± 0.1^{c}	0.0001
15 min	$89.3 \pm 0.1^{a\,Y}$	86.9 ± 0.7^{b}	79.2 ± 0.6^{c}	<0.0001
240 min	$91.2 \pm 0.4^{a\,X}$	87.9 ± 0.5^{b}	79.6 ± 0.4^{c}	<0.0001
AD Nitrogen, %				
Stripping	90.7 ± 0.2^{b}	91.8 ± 0.1^{a}	85.1 ± 0.3^{c}	<0.0001
15 min	$93.4 \pm 0.1^{b\,Y}$	95.9 ± 0.1^{a}	91.9 ± 0.03^{c}	<0.0001
240 min	$93.8 \pm 0.3^{b\,XY}$	96.1 ± 0.1^{a}	92.4 ± 0.4^{b}	0.0026

Mean ± Standard Error.
^{abc} Indicate significant ($P \leq 0.05$) differences among diets within a row.
^{XYZ} Indicate significant differences among time of collection for individual diets within a column.
AD: Apparent digestibility

4.2 Experimental diets and fish

The Norwegian University of Life Science Center for Feed Technology, Ås, Norway provided three different diets pre-incorporated with yttrium oxides for this study. Three different diets are recorded as Diet 1 (fishmeal), Diet 2 (soybean meal) and Diet 3 (rapeseed meal), respectively, with supplementation of the other ingredients (corn gluten meal, vital wheat gluten, wheat, fish oil and rapeseed oil). The sample of each experimental diets was stored in the freezer

with -20°C, preparing for further stable isotope analysis. The experimental fish- Rainbow trout (*Oncorhynchus mykiss*) were provided by the fish lab of NMBU, and reared in a total of 9 different tanks with 14 degree, fed with three replicates for each experimental diet in the fish lab. The rearing condition in tanks are standardized and mortality did not happen during the whole 22 days of experimental periods.

4.3 Faeces collection methods

After fish adaptation of experimental diets, faeces were collected by stripping and retch wire screening, respectively in this study. Stripping of faeces conducted on fish with applying pressure from the posterior intestine (pectoral fin), after they were anesthetized (***Figure 3***). On the other hand, faeces were collected from retch wire screen with at 15 and 240 min different time intervals, respectively (***Figure 4***). After that, collected faeces were immediately stored in a freezer with -20°C, prepared for further stable isotopes analysis.

Figure 3. Image of fecal stripping , pictured by *(Shomorin 2017)*

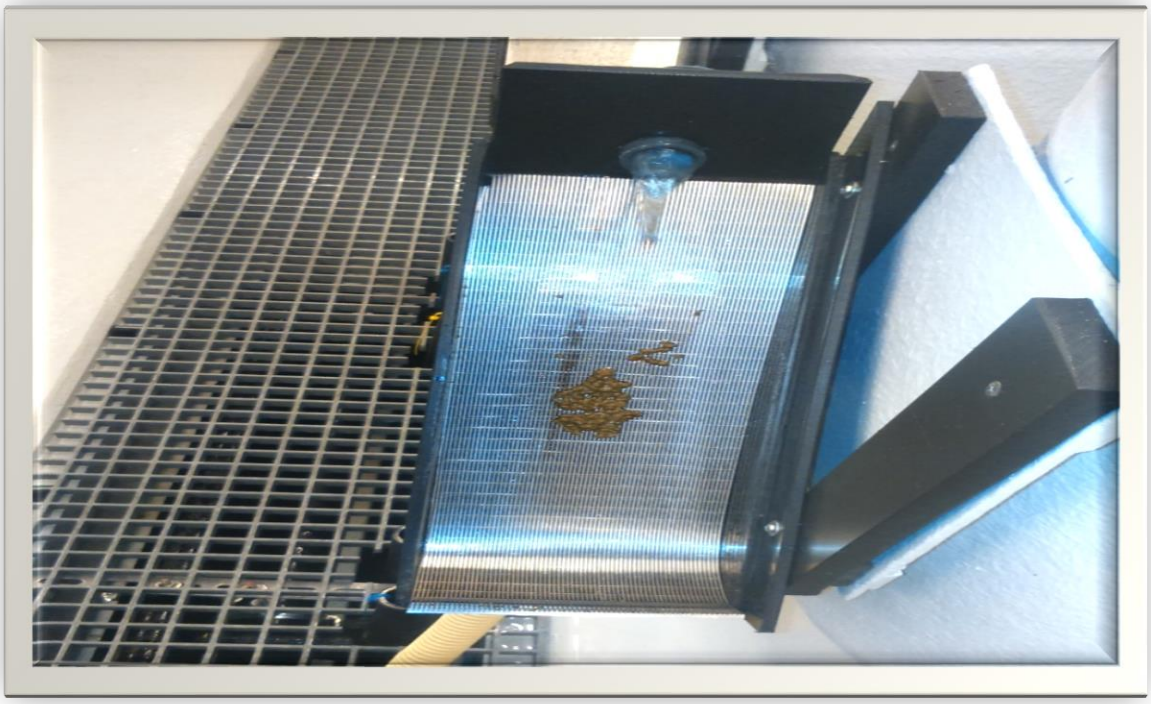

Figure 4. Image of fecal collection from retch wire screen, pictured by (Shomorin 2017)

4.4 Stable isotope analysis

Sampled around 0.15 mg freeze and dried dietary ingredients, diets (FM, SBM and RSM), and relative faeces, respectively in small plastic containers, and then the further processing of stable isotope analysis of carbon and nitrogen in diets and faeces were carried out in Institute for Energy Technology in Kjeller which located just outside Oslo. The stable isotope composition of diets and supplementary ingredients were shown in **Table 2,** respectively. The isotope ratios are expressed as parts per thousand, or "per mill", differences (‰) from a standard reference material. The standard reference materials for carbon and nitrogen are Pee Dee Belemnite and nitrogen gas in the atmosphere, respectively.

$$\delta X = \frac{R_{Sample} - R_{standard}}{R_{standard}} \times 1000$$

Where X represents ^{13}C and ^{15}N, R is a corresponding ratio, $^{13}C/^{12}C$ or $^{15}N/^{14}N$, δ is the measure of the ratio of heavy to light isotopes in samples. Positive or less negative δ value

indicate an increase in a sample isotope interest, relative to a standard. On the opposite, lower or more negative values denote decrease.

Table 2. Stable isotope composition of diet ingredients and diets

Diet	$\delta^{13}C$	$\delta^{15}N$
FM	-24.22	7.59
SBM	-23.87	3.56
RSM	-22.57	4.22
Ingredients		
FM	-20.67	11.92
SBM	-25.92	0.48
RSM	-26.35	3.60
Vital wheat gluten	-27.62	3.70
Corn gluten	-14.00	-0.28
Wheat	-27.59	3.66

4.5 Calculation formula and other statistical analysis

According to the known δX value from the result of stable isotope analysis, the fractional abundance of carbon and nitrogen stable isotopes in diets and faeces were both calculated by the formula based on the report of (Hayes): $^{13}F/^{15}F = \delta X / 1+ \delta X$, in which ^{13}F and ^{15}F represent fractional abundance of heavy carbon and nitrogen, respectively. Besides, the proposed mathematical formula (listed below) created based on the knowledge of fractional abundance of stable isotope and the calculation of apparent digestibility described by (Maynard & Loosli 1969). Comparison between dietary and fecal isotope signatures, the difference between the apparent digestibilities estimated based on total elements and fractional abundance of stable isotopes were performed using two-way ANOVAs in SAS software package (SAS/STAT Version 9.4. SAS Institute, Cary, NC, USA). Statistically significant ($P<0.05$) differences were ranked by the Pdiff procedure in SAS, and indicated by superscript letters. The results are presented as a mean ± standard error. The proposed formula used in this experiment to calculate the apparent digestibility (AD):

$$AD=100-100*(IMR_{diets} / IMR_{faeces} *(CT_{faeces} \times FA_{faeces} / CT_{diets} \times FA_{diets}).$$

IMR_{faeces} represent inert maker ratio in faeces

IMR_{diet} represent inert maker ratio in diets

CT_{diet} represent concentration of total element in diets

CT_{faeces} represent concentration of total element in faeces

FA_{diet} represent fractional abundance of stable isotopes in diets

FA_{faeces} represent fractional abundance of stable isotopes in faeces relative to diets

5. Results

5.1 Stable isotope signatures in samples

Table 3. Isotope signatures in diets and faeces

Diet	Element	Isotope signature in diet	Stable isotope signatures in faeces			P (time)
			Stripping	Retch wire screening		
Time (min）			0	15	240	
FM	C	-22.57	-20.39±0.08^{b}	-21.97± 0.07^{a}	-21.95±0.09^{a}	<0.0001
	N	7.59	5.13±1.10^{b}	5.57±0.36^{b}	4.92±0.39^{a}	0.0011
SBM	C	-23.87	-23.11±0.11^{b}	-24.33±0.20^{a}	-24.58±0.14^{a}	0.0012
	N	3.56	3.20±1.43^{c}	-1.16±0.88^{b}	-1.98±0.30^{a}	0.0011
RSM	C	-24.22	-24.34±0.11^{b}	-25.46±0.04^{a}	-25.55±0.08^{a}	<0.0001
	N	*4.22*	2.49±0.42^{b}	3.08±0.92^{c}	-0.70±0.37^{a}	0.0011

Mean ± standard error
a,b,c indicate significant difference for carbon and nitrogen stable isotope signatures in faeces with the different time intervals
Stable isotope signatures = $\delta^{13}C$ or $\delta^{15}N$

Based on the Table 3, the stable isotope signatures of carbon did not so much differ among the diets, and the values were -22.57 ‰, -23.87 ‰ and -24.22 ‰ for FM, SBM, and RSM, respectively. However, for nitrogen, the stable isotope signatures in FM was clearly different from plant protein sources (SBM and RSM). 7.59 ‰ was determined for the nitrogen stable isotope signature in FM while that of SBM and RSM ranged from 3.56 ‰ to 4.22 ‰, respectively. Faeces were enriched in ^{13}C relative to FM and SBM, but depletion was found for the RSM. On the other hand, faeces were depleted in ^{15}N relative to FM, SBM, and RSM. The analysis of variance showed a significant difference for stable carbon isotopic signatures in faeces collected by stripping and retch wire screening method.

To be more specific, the values of $\delta^{13}C$ in faeces collected by stripping was significantly ($P<0.05$) differ from that of faeces collected from retch wire screen at 15 or 240 min after defecation. However, the variation of $\delta^{13}C$ in faeces was not significant from 15 min to 240 min fecal exposure time intervals. For instance, the stable carbon isotope signatures in faeces collected by striping was -20.39, -23.11 and -24.34 for FM, SBM, and RSM, respectively. In comparison, the lower carbon stable isotope signatures of faeces collected from retch wire screen at 15 min and 240 min time intervals were ranging from -21.97 to -21.95, -24.33 to -24.58 and -25.46 to -25.55 for FM, SBM, and RSM, respectively.

The analysis of variance illustrated significant (<0.05) difference of fecal $\delta^{15}N$ with the different time intervals (0, 15 and 240 min, respectively). The similar fecal $\delta^{15}N$ relative to FM was absorbed between faeces collected by stripping and collected by retch wire screen at 15 min, which was significantly higher, compared with the value of faeces sample collected by retch wire screen at 240 min. On the other hand, $\delta^{15}N$ in faeces derived from SBM and RSM were significantly different at each different time points. The fecal $\delta^{15}N$ originated from SBM showed a decreasing trend as the extension of faeces in the water. However, the $\delta^{15}N$ in faeces derived from RSM increased during the first 15 min collection intervals and then decreased to – 0.70‰ at 240 min collection intervals.

5.2 Fractional abundance of stable isotopes in samples

Table 4. Fractional abundance (FA) of stable isotopes in diets and faeces

Diet	Element	FA of isotope in diets	FA of isotope in feces			P (time)
Time			0	15	240	
FM	C	0.0108642	$0.0108882 \pm 0.9*10^{-6\ b}$	$0.0108709 \pm 0.8*10^{-6\ a}$	$0.0108710 \pm 0.1*10^{-6\ a}$	<0.0001
	N	0.0036260	$0.0036177 \pm 3.8*10^{-6\ b}$	$0.0036190 \pm 1.5*10^{-6\ b}$	$0.0036167 \pm 1.2*10^{-6\ a}$	<0.0001
SBM	C	0.0108500	$0.0108583 \pm 1.2*10^{-6\ b}$	$0.0108449 \pm 2.2*10^{-6\ a}$	$0.0108421 \pm 1.5*10^{-6\ a}$	0.0011
	N	0.0036120	$0.0036103 \pm 5.3*10^{-6\ c}$	$0.0035947 \pm 4*10^{-6\ b}$	$0.0035920 \pm 1.1*10^{-6\ a}$	<0.0001
RSM	C	0.0108461	$0.0108448 \pm 1.2*10^{-6\ b}$	$0.0108325 \pm 0.4*10^{-6\ a}$	$0.0108315 \pm 0.9*10^{-6\ a}$	<0.0001
	N	0.0036140	$0.0036080 \pm 1.5*10^{-6\ b}$	$0.0036103 \pm 3.4*10^{-6\ c}$	$0.0035963 \pm 1.2*10^{-6\ a}$	<0.0001

Mean ± standard error

a,b,c indicate significant difference (<0.05) for the fractional abundance of carbon and nitrogen in feces with the different time intervals

FA= fractional abundance

The same pattern being directly from the stable isotope signatures was observed in the fractional abundance of stable isotopes in diets and faeces, which was illustrated in Table 4. However, the fractional abundance of stable isotopes in diets and relative faeces with different collection time intervals were very small. The fractional abundance of carbon isotopes in faeces derived from FM, SBM and RSM were significantly ($p<0.05$) different through the different time intervals, respectively. The fractional abundance of carbon isotopes in faeces were decreasing as the extension of faeces exposed in water and significant (<0.05) difference was observed between faeces collected by stripping and different time intervals (15 and 240 min, respectively). But the difference of carbon fractional abundance was not significant in the faeces collected by retch wire screen at 15 to 240 min. On the other hand, the result of ANOVA analysis showed significant different for fractional abundance of nitrogen isotopes in faeces during the different time intervals. The FA of nitrogen isotopes in faeces showed decreasing trend as the extension of faeces in water, except the FA of nitrogen in faeces derived from RSM at the collection of 15 min intervals that was higher than fecal FA of nitrogen collected by stripping.

5.3 Comparison of apparent digestibility estimated by the new method with classic method

Table 5. Mean values ± s.e.m. of main effects for diet fish meal (FM), soybean meal (SBM) and rapeseed meal (RSM) and faeces sampling procedure (stripping and 15 or 240 minutes post defecation), and two-way analysis of variance for apparent digestibility of ^{15}N and ^{13}C and the difference between apparent digestibility (AD) estimated from total N or C and AD of ^{15}N or ^{13}C.

	Diet				Sample				
	FM	SBM	RSM	P(diet)[1]	Stripping	Retch wire screening		P(sample)[1]	P(interaction)[1]
					0 min	15 min	240 min		
Apparent digestibilities of ^{15}N and ^{13}C									
AD $^{15}_{N}$, %	92.7±0.5^{b}	94.6±0.7^{c}	89.8±1.2^{a}	<0.0001	89.2±1.0^{x}	93.7±0.6^{y}	94.1±0.6^{y}	<0.0001	<0.0001
AD $^{13}_{C,}$ %	89.9±0.4^{c}	85.9±0.8^{b}	77.6±1.0^{a}	<0.0001	82.0±2.2^{x}	85.2±1.5^{y}	86.3±1.7^{z}	<0.0001	<0.0001
Difference between AD[2] N or C total and AD ^{15}N or ^{13}C									
AD ^{15}N - AD N total	0.017±0.003	0.014± 0.004	0.024±0.005	0.18	0.017±0.006	0.013±0.003	0.025±0.003	0.12	0.063
AD ^{13}C- AD C total	-0.012±0.003^{a}	0.001±0.003^{b}	0.019±0.004^{c}	<0.0001	-0.011±0.004^{x}	0.007±0.005^{y}	0.011±0.005^{y}	<0.0001	0.55

[1] Probability level in the ANOVA.
[2] The differences are estimated based on the fractional abundance of ^{15}N or ^{13}C in diets and faeces minus AD of total N or C.

^{abc} indicate significant (P<0.05) differences of apparent AD among different diets.
^{xyz} indicate significant (P<0.05) differences among feces-sampling procedures

The ANOVA revealed significant differences among all three diets in apparent digestibility of ^{15}N and ^{13}C. The highest AD estimated by fractional abundance (FA) of ^{15}N in diets to faeces was obtained for the SBM diet, followed by the FM and RSM diets. The apparent digestibility of ^{13}C was also significantly different among all three diets but was ranked different than the estimates for ^{15}N, with the fish meal diet giving the highest value, followed by the SBM and RSM diets.

Faeces collected procedure also resulted in significant differences in AD of ^{15}N. The lower apparent digestibility estimated by fractional abundance of ^{15}N in diets to faeces was gained when the faeces was collected by stripping, in contrast to collected by retch wire screening at different time intervals (15 and 240 min, respectively). But, the AD of ^{15}N was not significantly different for the faeces collected by retch wire screen at 15 and 240 min. On the other hand, the similar pattern was also observed for the stripping and retch wire screening methods when the apparent digestibility was estimated by the fractional abundance of ^{13}C in diet to faeces. However, the less significant difference was obtained for the AD of ^{13}C when the faeces collected by retch wire screen at 15 and 240 mins, which was the difference in compared with the pattern observed for AD estimated by ^{15}N ratio in diets to faeces during the same periods. Overall, diets and sample collected at different time intervals have significant interactional effect on apparent digestibility estimated by FA of ^{15}N and ^{13}C in diets to faeces.

The two-way analysis of variance showed the individual effect of diets and samples collected at different time intervals was not significant on the difference between apparent digestibility of total nitrogen (ADN) and nitrogen isotopes ($AD^{15}N$). Furthermore, there was no significant interactional effect of diets and samples on this difference as well. This may be due to the less concentration of ^{15}N in faeces sample. In this experiment, the ratio of stable nitrogen isotopes in fecal samples (0.15 mg) was run twice for the stable isotope analysis (SIA) because the result from first running was not explainable. And then the uniformly homogenized faeces samples (0.15 mg) was prepared for the second running of SIA. The result from second running of SIA is much better than the first one, but it is still far away from expected accuracy. So, it is recommended to try sampling larger amount of faeces (> 0.15 mg) for the next SIA. This may give more explainable result. On the other hand, diets had a significant effect on the difference between apparent digestibility estimated by FA of carbon isotopes and total carbon, similar

significant effect was also found among the different fecal collection intervals (0, 15 and 240 min). But the interactional effect of diets and different fecal collection time intervals was not significant for this differences

5.4 Apparent digestibility estimated based on fractional abundance of stable isotopes in diets to faeces

Table 6. Significant interactions in the 2-way ANOVA.

Diet	FM			SBM			RSM		
Time	0	15	240	0	15	240	0	15	240
AD^{15}_{N}, %	90.76 ± 0.23^{b}	93.37 ± 0.08^{e}	93.82 ± 0.26^{e}	91.77 ± 0.03^{c}	95.96 ± 0.10^{f}	96.14 ± 0.55^{f}	85.10 ± 0.25^{a}	91.89 ± 0.03^{c}	92.46 ± 0.42^{c}
AD^{13}_{C},%	89.23 ± 0.15^{e}	89.31 ± 0.12^{e}	91.23 ± 0.40^{f}	82.76 ± 0.25^{c}	86.95 ± 0.67^{d}	87.94 ± 0.45^{d}	73.92 ± 0.10^{a}	79.26 ± 0.62^{b}	79.64 ± 0.36^{b}

Mean ± Standard Error.

^{abcdef} Indicate significant ($P\leq0.05$) differences of apparent digestibility estimated based on stable isotopes in diets to faeces in different diets during different time intervals in a row.

AD^{15}_{N} = AD estimated based on fractional abundance of stable nitrogen in diets to faeces

AD^{13}_{C} = AD estimated based on fractional abundance of stable carbon in diets to faece

5.4.1 AD estimated by fractional abundance of stable nitrogen isotopes in diets to faeces

The ANOVA analysis illustrated AD estimated by fractional abundance of ^{15}N in diets to faeces showed significant differences among the diets during the different time intervals (Figure 5). The highest apparent digestibility of ^{15}N was observed for the SBM, followed by FM and RSM when the faeces was collected by stripping method. The AD of ^{15}N was significantly lower for stripping than retch wire screening methods among diets. But no significant difference was found for the AD of ^{15}N in each diet between 15 and 240 min fecal collection intervals by retch wire screening method. However, the significant difference of AD estimated by FA of ^{15}N in diets to faeces was observed among the diets from 15 to 240 min exposure time of faeces in water. The highest and lowest AD of ^{15}N were belong to the SBM and RSM, respectively during the periods from 15 to 240 min fecal collection intervals.

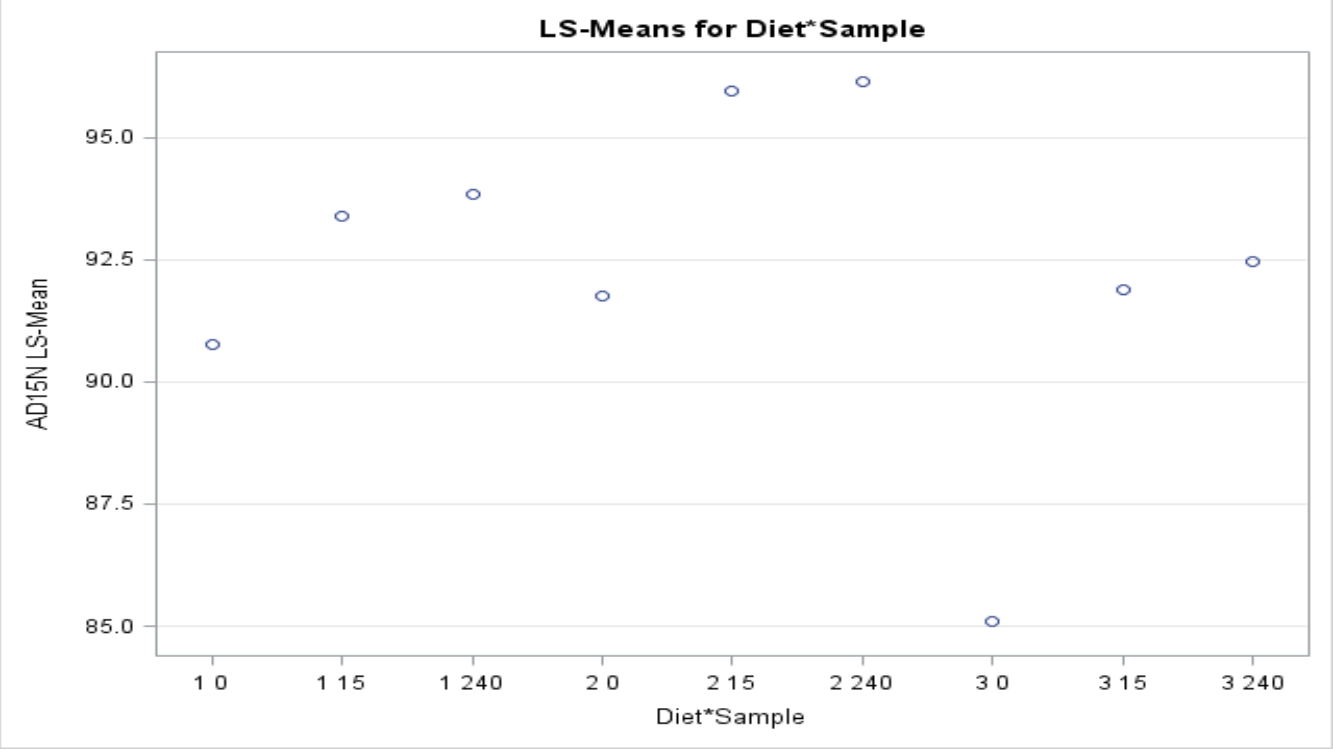

Figure 5. Apparent digestibility of ^{15}N among diets during different time intervals

5.4.2 AD estimated by fractional abundance of stable carbon isotopes in diets to faeces

The two-way analysis of variance showed a significant difference for an AD of ^{13}C among diets during different fecal collection intervals (Figure 6). Different from the ranking of AD estimated by FA of ^{15}N among diets at different time intervals, the estimation based on FA of ^{13}C in diets to faeces gave the highest AD for the FM, followed by SBM and RSM for the stripping method. The apparent digestibility of ^{13}C was lower for stripping than retch wire screening at different time intervals (15 and 240 min) among diets, except for the FM. There were no significant differences for the AD of ^{13}C in FM when faeces were sampled by stripping and at 15 min fecal collection time intervals after defecation. However, the significant difference was found for the AD of ^{13}C in FM during the 15 and 240 min fecal collection intervals, which was higher than the AD estimated by FA of ^{13}C in SBM and RSM to relative faeces during the same periods. On the opposite, the AD estimated by FA of ^{13}C in SBM and RSM was significantly different between 0 and 15 min faeces collection intervals, respectively. But it became not significant from 15 to 240 min fecal exposure time in water after excretion and the AD of ^{13}C were always higher for SBM than RSM in this periods.

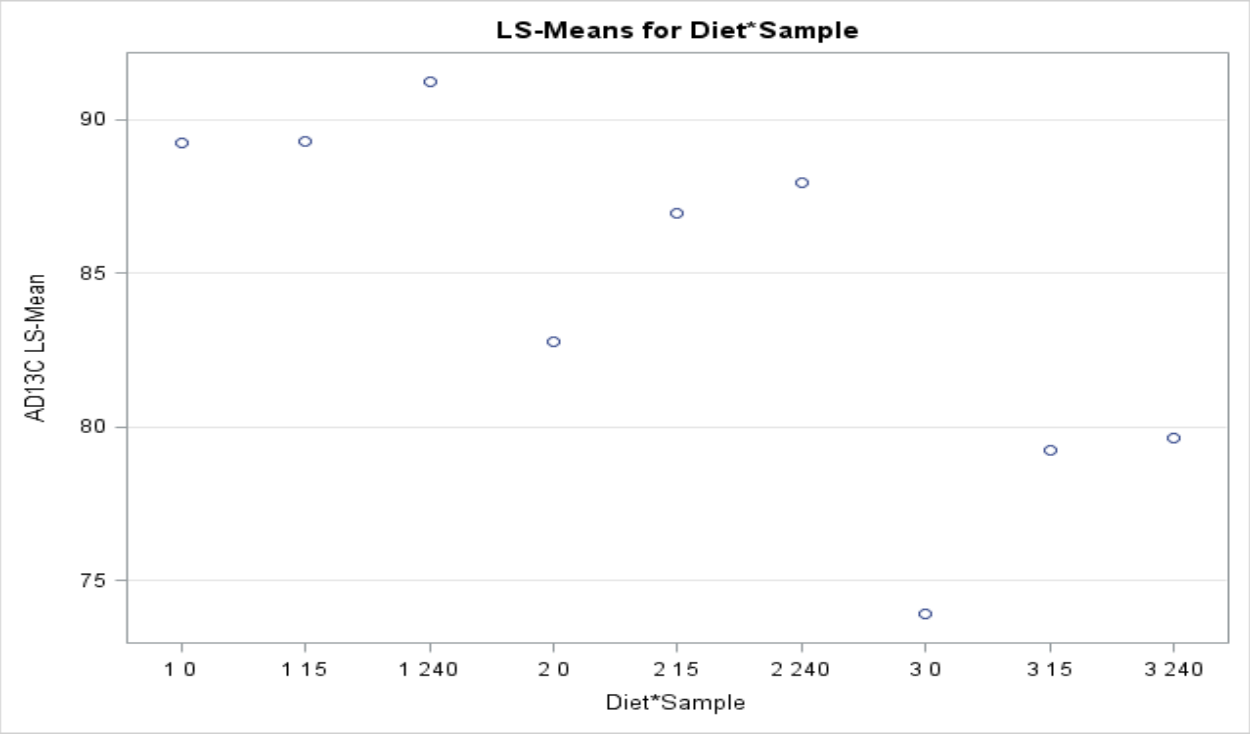

Figure 6. Apparent digestibility of ^{13}C among diets during different time intervals

6. Discussion

6.1 Variation of carbon stable isotope ratios in diets and faeces

The current study demonstrates the isotopic change of rainbow trout (Oncorhynchus mykiss) feed after digestion. $\delta^{13}C$ in faeces collected by stripping were -20.39 ‰ and -23.11 ‰, which were enriched relative to FM (-22.57 ‰) and SBM (-23.87 ‰), respectively. This is in a line with the previous findings by (Ye et al. 1991), it resulted fecal $\delta^{13}C$ was -20.48 ‰ relative to fishmeal (-21.53 ‰) when faeces collected from the hindgut of freshly killed salmonids. However, the depletion of ^{13}C in faeces (-24.34 ‰) relative to rapeseed meal (-24.22 ‰) was observed in this study, which fits with the result of most previous controlled feeding experiments on land animals (Sponheimer, Matt et al. 2003) and fish (Franco-Nava et al. 2004). For example, the study conducted on alpaca, cattle, goat, llama, rabbit, and horse illustrated that the value of $\delta^{13}C$ in faeces was lower than relative diets (Alfalfa and Coastal Bermuda grass) (Sponheimer, Matt et al. 2003). For the seabass, it was found that fecal $\delta^{13}C$ enriched with -22.1‰ relative to commercial fishmeal (-24 ‰) (Franco-Nava et al. 2004). For the reasons why faeces either enriched or depleted with $\delta^{13}C$ relative to different experimental diets in this study is not clear. This may attribute to mixed experimental diets containing a variety of ingredients with disparate carbon isotopic compositions. Diets of the current study can consider as mixed diets with a range of different ingredients with different isotopic signatures. For instance, FM, SBM and RSM diets in this study all contain high amount (150 g/kg) of C_4 plant ingredient (e.g., corn gluten meal) and generally the use of corn gluten meals especially in cold-water or marine species has limitation because of the non-soluble carbohydrate (Spannhof & Plantikow 1983). In addition, carbon isotopes composition in the C_4 plant is higher than a C_3 plant (O'Leary 1988). Therefore, it was possible that poorly digested ^{13}C derived from corn gluten source in FM and SBM diets were overrepresented in faeces, which led to the enrichment of ^{13}C in faeces. The other added ingredients in diets, fish oil and rapeseed oil, are the main energy source in rainbow trout feed (Caballero et al. 2002). The main component of fish/ rapeseed oil is lipids that are depleted in ^{13}C compared with protein and carbohydrate sources (DeNiro & Epstein 1977; Focken & Becker 1998). Rainbow trout may preferentially use lipid as an energy source and excrete excess amount of undigested carbohydrate or protein source as waste in faeces may responsible for the fecal enrichment of $\delta^{13}C$ relative to FM and SBM. On the opposite, fecal $\delta^{13}C$ depletion relative to RSM may result from highly use of carbohydrate and protein as an

energy source and excreting undigested lipid in faeces. Therefore, further research needs to be going to analyze the carbon stable isotopes ratios in protein, carbohydrate, and lipids in diets to relative faeces, which may confirm this assumption. The potential mechanism behind variation of carbon isotope composition in faeces relative to diets and tissue/body is still not well known and it was reported that consumer tissue normally enriched in ^{13}C relative to diets because lighter isotopes (e.g. ^{12}C) are more easily used in the metabolic process (DeNiro & Epstein 1978).

6.2 Variation of nitrogen stable isotope ratios in diets to faeces

In terms of the fecal $\delta^{15}N$ in this study, the depletion with 5.13 ‰, 3.20 ‰ and 2.49 ‰ in faeces relative to FM (7.59 ‰), SBM (3.56 ‰) and RSM (4.22 ‰), respectively is in a line with previous studies in fish nutrition (Franco-Nava et al. 2004; McGhie et al. 2000; Yokoyama et al. 2006). It may result from the enrichment of ^{15}N in body tissues relative to dietary ^{15}N (Minagawa & Wada 1984). Compared with other body tissue, faeces are normally depleted in ^{15}N (Franco-Nava et al. 2004) and the tissue of consumers will be enriched in heavier isotopes (e.g., ^{15}N and ^{13}C) over time (DeNiro & Epstein 1978; DeNiro & Epstein 1981). The lighter isotopes (e.g.^{14}N and ^{12}C) are relatively active during reaction compared to heavier isotopes because of the weaker bonds between ^{14}C lighter isotopes (Hoefs & Hoefs 1997). The biological reaction (e.g., transamination and deamination) will discriminate against lighter isotopes (Gaebler et al. 1966; Macko et al. 1986). In other words, the heavier isotopes tend to accumulate more in tissues of the consumers than in faeces. Furthermore, rainbow trout and gilthead sea bream showed decreasing $\delta^{15}N$ value in their tissue with the replacement of fishmeal diets by plant protein diets (Beltran et al. 2009). The enrichment of ^{15}N in tissue is gained by the process of deamination in situ or synthesis of a protein with ^{15}N enriched amino acids (Gannes et al. 1998). So, the depletion of ^{15}N in faeces relative to FM, SBM, and RSM in this study is determined by the enrichment of ^{15}N in tissues through intricacies of protein catabolism. If analyzing ^{15}N in isolated proteins of individual diets and relative faeces in further research will give more clear information about the digestive mechanism. However, the result in this study is not consistent with previous findings (Sponheimer, M et al. 2003; Steele & Daniel 1978; Sutoh et al. 1987; Sutoh et al. 1993), and their results showed that the faeces of cattle (Steele & Daniel 1978; Sutoh et al. 1987) , pig and goats (Sutoh et al. 1987), sheep (Sutoh et al. 1993), and llamas (Sponheimer, M et al. 2003) were enriched in ^{15}N compared to their diets. The enrichment of ^{15}N in faeces possibly

caused by endogenous nitrogen loss (Van Soest 1994). Because it is possible sloughed endogenous tissues and microbial cells are enriched in ^{15}N, compared to diets. The difference of ^{15}N in faeces relative to consumed feed by fish and land animals may attribute to their different digestion system. The mechanism responsible for in the variation of ^{15}N in diets to relative faeces during the digestion process of fish need to step forward research. Nonetheless, the report by (Boutton 1991; Griffiths 1991) indicated that uniformed sample preparation for stable isotope analysis is very important for the result with high precision.

6.3 AD estimates using fractional abundance of carbon stable isotopes

AD estimates using a fractional abundance of carbon stable isotopes was higher for FM, compared with SBM and RSM for both methods of faeces collection in this study. This may be related to higher intake of FM in rainbow trout, compared with SBM and RSM, because FM has high palatability and digestibility (Hardy 2010). Additionally, it cannot deny the possibility that tiny amount of crude starch was failed to gelatinized through technical feed treatment process in this study. Although their existence did not make visible or detective negative effect on the performance of rainbow trout, they may responsible for a lower AD of ^{13}C in SBM and RSM, in compared FM. Because the AD of ^{13}C is estimated based on very precise tiny fractional abundance variation of ^{13}C in diets to relative faeces. Furthermore, rainbow trout, cold-water carnivores fish, are not able to digest crude starch contained in plant protein originated diets (e.g., SBM and RSM) as efficiently as omnivore fish (Wilson 1994) because amylase secreted by rainbow trout easily absorbed by starch, which prohibits the hydrolysis of starch (Spannhof & Plantikow 1983). The other factor may ascribe to this lower digestibility may due to anti-nutritional factors present in SBM and RSM impact the digestion of main carbon skeletons (^{12}C and ^{13}C), such as protein, carbohydrate, and lipid. Besides, carbon fraction of indigestible anti-nutritional factors in SBM and RSM may contain a high amount of ^{13}C and it may decrease the AD of ^{13}C as well. But this prediction still needs to further study. The lower ^{13}C digestibility in rapeseed meal, compared to SBM, in this study may result from the high content of fiber in RSM (Higgs et al. 1995). It was reported that fiber reduces apparent digestibility of carbon by decreasing lipid digestion (Hajen et al. 1993b). However, lipid was reported to depleted in ^{13}C relative to protein and carbohydrate (DeNiro & Epstein 1977; Focken & Becker 1998). So, it is possible fiber contain a high amount of ^{13}C. Less change was observed for the AD of ^{13}C in fishmeal during first 15 min faces collection, but it changed rapidly during 15 to 240 min fecal collection

intervals, which indicates rapid ^{13}C containing nutrient leaching occurred through 15 to 240 min after defecation. On the opposite, the apparent digestibility of ^{13}C in SBM and RSM estimated on the faeces collected by stripping was significantly different from those faeces collected by screening at 15min, but there was no significant differences between AD of ^{13}C at 15 and 240 min time. This means nutrient loss with ^{13}C in faces derived from SBM and RSM mainly happened during the first 15 min fecal collection.

6.4 AD estimates using fractional abundance of nitrogen stable isotopes

The highest AD estimated based on the fractional abundance of nitrogen stable isotopes was obtained for the SMB, and the lowest value was observed for RSM, which corresponds to the AD estimated based on total nitrogen ratios in diets to faeces by (Shomorin 2017). This indicates optimal heat processing of SBM was applied in this study. Because the natural ^{15}N is mainly from protein content of FM or alternative plant protein sources had been reported by (Beltran et al. 2009), and the lower digestibility of protein in plant-based protein source, compared with FM may attribute to ANFs (Romarheim et al. 2006; Storebakken et al. 2000). ANFs, such as protease inhibitors in SBM, are irreversibly bind with the proteolytic enzymes (trypsin and chymotrypsin) in the intestinal tract and the excreted with faeces, which decrease the protein digestibility due to both enzyme and enzyme inhibitors are protein themselves (Barth et al. 1993; Dabrowski et al. 1989). However, protease inhibitors in SBM are heat liable and can be removed when there is optimal moist heat treatment around 15-30 min (Francis et al. 2001). The lowest AD estimated by FA of nitrogen isotopes in diets to faeces for the RSM than other experimental diets in this study may attribute to the high level of phytic acid and fiber content in RSM. It was reported that phytic acid in canola/rapeseed meal reduces protein digestibility by forming complexes with proteins at acidic pH intestinal environment (Higges et al., 1995 as cited in Mwachireya et al. 1999). The fiber was pointed out to decrease absorption of amino acids and peptides by shorting the gut transit time (Krogdahl 1989 as cited in Mwachireya et al. 1999). Higher AD of ^{15}N was observed in FM, SBM, and RSM with stripping methods, compared with retch wire screening, but there was no significant differences between AD of ^{15}N at 15 min and 240 min fecal collection intervals. This indicates the ^{15}N containing nutrient rapidly loss from faeces during the first 15 min fecal collection intervals while less ^{15}N containing nutrient lost from 15 to 240 min fecal collection intervals.

7. Conclusion

The two-way analysis of variance revealed no significant difference between AD estimated based on the fractional abundance of stable nitrogen, carbon isotopes, and total nitrogen, carbon elements with yttrium oxides ratios in experimental diets (FM, SBM and RSM) to faeces, respectively. Furthermore, it was found that AD estimated by the fractional abundance of stable isotopes in diets to faeces was higher than that estimated by total elements, which indicated that using new AD estimating method in this study gives more quantitative value than using classic methods. But, further research required to do more specific stable isotope analysis on protein, lipid, and other carbohydrate sources (e.g., starch, NSPs or carbon-containing ANFs) in experimental diets and relative faeces. This would enable to give more detailed information about dietary individual multinutrient to relative faeces during the digestion process.

The ranking of the apparent digestibility based on the fractional abundance of stable carbon isotopes among the 3 diets to relative faeces showed the same order as that estimated by total carbon in the previous study by (Shomorin 2017), which revealed this order (FM >SBM >RSM). On the other hand, the ranking of the apparent digestibility based on the fractional abundance of stable nitrogen isotopes among the 3 diets to relative faeces followed the order of SBM >FM >RSM, which is in line with the result of (Shomorin 2017) as well. In addition, it is highly recommended to sample more amount (>0.15 mg) of faeces for stable isotope analysis in next study, which may increase the accuracy of AD by increasing concentration of stable carbon and nitrogen isotopes in sample of faece.

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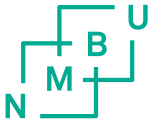

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