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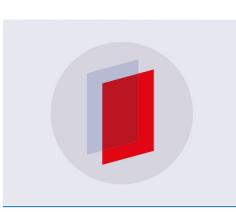
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Comparison of three inert markers in measuring apparent nutrient digestibility of juvenile abalone under different culture condition and temperature regimes

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Abstract. A comparative research using three inert markers, chromic oxide, yttrium and ytterbium to measure the apparent nutrient digestibility of experimental feed in juvenile Hybrid abalone (*Haliotis rubra* X *H. laevigata*) and Greenlip abalone (*H.laevigata*) revealed that apparent digestibility of crude protein (AD_{CP}) measured using yttrium and ytterbium in hybrid abalone were significantly different across the treatments. Protein digestibility measured in experimental tanks was higher than those measured in indoor and outdoor commercial tanks, regardless of inert marker used. Chromic oxide led to overestimated AD_{CP} compared to when measured using yttrium and ytterbium. There were no significant interactions between temperature and inert markers when measuring AD_{CP} and apparent digestibility of gross energy (AD_{GE}). However, there was a significant difference of AD_{CP} amongst inert markers when measured in greenlip abalone cultured at two temperatures. While measurements of AD_{GE} calculated using three inert markers shared the same value.

1. Introduction

Determining the nutrient digestibilities of certain feed ingredients is pivotal to provide credible information to formulate a cost effective formulated feed. Measuring nutrient digestibility directly in Haliotids is difficult due to the problems related to the accurate measurements of feed consumed and faeces voided [1]. Digestibility determination can be affected by time between the production of faeces and their collection [2]. If faeces are exposed to water over time, nutrients may leach from the feed and faeces, which may lead to overestimation of AD. Additionally, experimental feeds tend to disintegrate in water, causing collection of whole uneaten feed to be difficult with potential contamination problem between feed and faeces [3]. Therefore, it is requisite to use indirect method by using inert markers that cannot be absorbed by the animal, should pass through the digestive system at a similar rate as the other ingredients, it must be inert and not impede the digestive processes, consistently included into the feed and can be easily and accurately analysed even at very low concentrations, and be nontoxic to people and environment [4, 5].

There are various possible markers for use to measure digestibility of particular nutrients in Haliotids, In a study conducted by [1] using blacklip abalone and greenlip abalone, it was concluded that markers including insoluble mineral components determined as acid insoluble ash (AIA), hydrolysis resistant organic matter (HROM) and crude fiber content of the feed were inappropriate due to the abalone capability to digest complex carbohydrates, which create a component of these markers. However, amongst all of the observed markers in this study, AIA and chromic oxide (Cr_2O_3) were found to be the promising markers.

Chromic oxide found to be reliable in determining protein digestibility of greenlip abalone [3, 6, 7]. However, it was not accurate to determine carbohydrate or cholesterol digestibility due to a low correlation between the movement of the marker to carbohydrate and cholesterol [8]. Trivalent oxides of yttrium and lanthanides (rare earth metals) have been investigated as inert markers for protein digestibility experiments since the beginning of 1990's [4]. Those inert markers appear to satisfy the desirable attributes of inert markers. They are moderately soluble in weak acid, can be detected by plasma emission spectroscopy and atomic absorption, therefore they can be analyzed in feed and feces even at low concentrations (in the $mg \cdot kg^{-1}$ range) [4, 9].

Another rare earth metal that can be used as inert marker is ytterbium. Ytterbium has not been used as an inert marker in mollusc, particularly in haliotids. However, this inert marker has been successfully used to determine nutrient digestibility in humans, salmonid fish and crustaceans [4, 10, 11]. Ytterbium is useful since it is unabsorbed across the digestive system and does not interfere with the metabolic processes or react with digestive enzymes [12]. These markers have replaced the use of chromic oxide in most aquaculture nutrition research in recent years due to more accurate recovery of marker, better homogeneity in feces and more accurate measurement techniques at low concentration. Thus, evaluation of ytterbium as inert markers in determining digestibility of abalone feed is of significance.

Inert markers provide the opportunity to measure apparent digestibility in both experimental systems but also in commercial tank setting. The majority of the data used in feed formulation is derived from experimental measurements conducted in laboratory systems [13–21], whereas in commercial scale abalone also have access to biofilms on tank surfaces and are exposed to different light levels, temperature and water flows according to the farm design. Justifying the results gained from experimental systems and digestibility measurements made on farm is of interest to know if in-tank commercial measurements might be valid during times of rapid commercial growth or during adverse culture conditions.

Providing fundamental data about nutrient digestibility of greenlip and hybrid abalone at different temperature regimes in different culture systems is of important in order to formulate cost-effective formulated feed that can be adjusted to the temperature seasonal changes. Moreover, a reliable inert marker to measure digestibility of feed ingredients should be investigated to provide accurate estimation of digestibility of the feed. This will assure optimal growth rates and reducing grow out period through the formulation of feeds specific to species, size and culture conditions.

The aims of this study were to compare yttrium, ytterbium and chromic oxide as inert digestibility markers to measure apparent protein digestibility in subadult hybrid abalone (*H. rubra x H. laevigata*) and greenlip abalone (*H.laevigata*), in experimental and commercial tanks.The second one was to compare yttrium, ytterbium and chromic oxide as inert digestibility markers to measure apparent protein and energy digestibility at two temperatures in subadult greenlip abalone *(H. laevigata)*.

2. Materials and method

2.1. Experiment 1 (protein digestibility and inert markers comparison in subadult hybrid abalone)

2.1.1. Experimental animals
Subadult hybrid abalone with initial body weight 33.72 ± 5.7 g and initial shell length 60.18 ± 3.2 mm (*n* = 600 animals) were used in this experiment. The abalone were provided by AbTas, Clarence Point, Tasmania.

2.1.2. Experimental system
The experimental systems consisted of 4 square outdoor tanks, 4 square indoor tanks (780 L Solid Nally Megabins, MS7800; Viscount Plastics Pty Ltd, Hawthorn East, Victoria, Australia), and 20 rectangular white plastic tanks (41 cm L × 27 cm W × 16 cm H). Outdoor and indoor tanks were cleaned but biofilm was not removed, while the rectangular white plastic tanks were installed in a controlled room to resemble the experimental tanks used in laboratory based nutrition research. Concrete hides were placed into each indoor and outdoor tank to provide more space, while there were no hides placed in experimental tanks. To avoid abalone escaping, 40 mm wide black stick on hook tape (Velcro Australia Pty Ltd., Hallam, Victoria, Australia) was installed around the top inside perimeter of experimental tanks. The experiment was conducted in a flow-through sea water system with ambient temperature conditions (13.6 °C ± 0.046). The tanks were cleaned every day at 8 AM to remove overnight feces and uneaten feed.

2.1.3. Experimental protocol
The abalone were gently removed from a commercial tank surface using a scraper. The animals were weighed and measured using a digital balance and a caliper. Each indoor and outdoor commercial tank was randomly stocked with 60 animals, while six animals were randomly stocked into each experimental tank. Animals were acclimated to the systems and diet for one week. During the acclimation period, one mortality was replaced by a new animal from the same population with similar weight and size.

2.2. Experiment 2 (Protein, energy digestibility and inert markers comparison in subadult greenlip abalone)

2.2.1. Experimental animals. Subadult greenlip abalone (initial shell length 51.3 mm ± 0.075, initial body weight 20.450 g ± 0.032, Mean ± SE) were used in this experiment. The juvenile were obtained from commercial abalone farms and pre-conditioned in SARDI Aquatic Science Centre, Adelaide and fed commercial diet prior to stocking in the experimental system.

2.2.2. Experimental system. The experiment was carried out in a temperature controlled flow-through UV treated salt water system (Model 025120-2, 120w, Emperor Aquatics, Pottstown, PA, USA). The experimental system consisted of six circular shaped tanks, assigned in pairs to two temperature levels, 14 °C and 18 °C. The 14 °C temperature water was the ambient temperature of the flow through system, while the 18°C temperature water was controlled using immersion heater (3 kw, 240 V, 3 kw, JQ20; Austin & Cridland, Carlton, NSW, Australia). The water flow was maintained at a rate of 2 L ·min⁻¹ and the water level was maintained at 14.5 cm. Water temperature and dissolved oxygen (ppm and % saturation) were measured on a daily basis using an OxyGuard™ Handygamma dissolved oxygen meter (OxyGuard International A/S, Birkerod, Denmark). pH was measured using a pH meter (Oakton pHtester 20; Oakton Instruments, Vernon Hills, IL, USA). Salinity (ppt) was measured daily using a portable refractometer, model RF20 (Extech Instruments, Nashua, NH, USA).

2.2.3. Experimental feeds. Feeds for experiment 1 and 2 were manufactured using the SARDI formulation, contained 33% crude protein. Dry ingredients were initially mixed (Brice, Model: VFM-2 °C, Brice Australia Pty. Ltd). The dough mixture was then pelleted using a commercial pasta machine (ITALPAST, Food Machinery International Pty.Ltd, Australia**),** and pellet**s** steamed for 15 mins using a domestic food steamer (TEFAL Steam Cuisine, Series S04, China**)**. Feed was then dried to 10% moisture using fan forced fumehood for 4 days and stored at -20^{0}C prior to the experiment.

2.2.4. Fecal collection. Collection of feces from experiment 1 was conducted 2 times a day at 2 h intervals (1100 hours to 1500 hours) for 2 weeks. Feces were collected using plastic pipette onto 63 μm screen before transferring into plastic tubes. The samples were then stored frozen at -20°C prior to analysis.

Feces from experiment 2 were collected using plastic pipette into labeled 1.5 ml eppendorf tubes. All eppendorf tubes were kept on ice during collection. Collection was conducted 3 times a day in 2 h intervals (1000 hours to 1900 hours) for 4 weeks. The feces samples then centrifuged for 30 s at 1000 rpm and excess water was removed by plastic pipette. Samples were stored frozen at -20°C prior to analysis.

2.2.5. Feeding and cleaning. Animals were fed to excess in all experiments. Feeding was conducted at 4:00 PM daily. Diets were randomly allocated to each tank. Cleaning and collection of uneaten feed was accomplished by screening the tank contents using either a fine meshed net or a feeding tray to retain pellets.

2.2.6. Chemical analysis. Prior to sample analysis, all samples were freeze dried until no loss of moisture was detected. All of the samples were then pooled and ground to fine homogenous powder. Gross energy (GE) was determined by complete combustion using a bomb calorimeter (Gallenkamp Autobomb). Due to the silica contamination, gross energy of samples from experiment 2 could not be analysed. Crude protein was determined as $N \times 6.25$ where nitrogen (N) was measured after sulphuric acid digestion, auto Kjeldahl (Kjeltec^{TM} 8100, Foss, Sweden) distillation, and titration against hydrochloric acid as described in the Association of Official Analytical Chemists (AOAC) guidelines for chemical testing [22]. Lipid was determined by chloroform methanol extraction as described by [23]. The inert markers (yttrium, ytterbium and chromic oxide) concentration in the diet and feces were digested in 3:1 nitric:hydrochloric acid solution for 4 days then centrifuged for 10 mins at 3000 rpm. Centrifuged samples were diluted volumetrically in distilled water, then transferred into acid-washed test tubes before analysis using inductively coupled plasma atomic emission spectrophotometry (ICP-AES) based on method described by [24]. Total nitrogen, carbon, hydrogen and sulfur was determined using elemental analysis (Thermo Finnigan EA 1112 Series Flash Elemental Analyser). Ash was determined gravimetrically by incineration in a muffle furnace at 600^0 C for 2 h NFE was calculated by difference.

2.2.7. Digestibility determination. Animals were fed to excess in all experiments. Feeding was conducted at 4:00 PM daily. Diets were randomly allocated to each tank. Cleaning and collection of uneaten feed was accomplished by screening the tank contents using either a fine meshed net or a feeding tray to retain pellets. The apparent digestibility of protein and energy of diets was calculated using the following equation [25]:

$$Digestibility = 100 - \left(100\, \frac{I\,(feed)}{I\,(feces)} \times \frac{N\,(feces)}{N\,(feed)} \right)$$

Where N = nutrient (protein or energy), I = indicator (yttrium, ytterbium and chromic oxide)

2.2.8. Statistical Analysis.. Residual plots were used to examine the homogeneity and normality of the data. A two way ANOVA was performed to determine the interaction between three different inert markers and applied treatments. Tukey's Post-hoc test was used to distinguish significant differences between means. All statistical analysis were done using IBM SPSS version 20 for Macintosh (IBM SPSS Inc., Chicago, IL). Data are presented as mean ± SE.

3. Results and Discussion

3.1. Results

3.1.1. Experiment 1 (Protein digestibility and inert markers comparison in subadult hybrid abalone). There were significant interactions between treatments and marker (P < 0.05). Chromic oxide in outdoor and indoor tanks displayed higher AD_{CP} than calculated according to digestibility of yttrium and ytterbium (Figure 1).

Results calculated using ytterbium and yttrium were the same across all treatments (P > 0.05). Chromic oxide results were higher than yttrium and ytterbium in all experimental tanks. There were significant differences between AD_{CP} of chromic oxide in outdoor and indoor tanks with AD_{CP} of yttrium and ytterbium under the same treatments, however there were no significant differences between AD_{CP} of all inert markers in experimental tanks.

There was no significant difference for AD_{CP} of chromium oxide in all treatments (P > 0.05). However, AD_{CP} measured using yttrium and ytterbium were significantly different across the treatments. AD_{CP} of yttrium and ytterbium was higher in experimental tanks compared to AD_{CP} by these two inert markers in outdoor and indoor tanks. Outdoor tanks exhibited the lowest apparent crude protein digestibility.
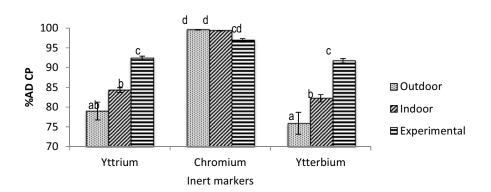

Figure 1. Apparent crude protein digestibility of subadult hybrid abalone (*H. laevigata* x *H. rubra*) fed 33% dietary protein levels with 3 inert markers at three treatments (outdoor, indoor and experimental tanks). Values that share the same superscript are not significantly different (P > 0.05).

3.1.2. Experiment 2 (Protein, energy digestibility and inert markers comparison in subadult greenlip abalone). There were no significant interactions by two way ANOVA between temperature and inert markers (P > 0.05). There were also no significant differences between the temperature (P > 0.05) but there were significant differences amongst inert markers (P < 0.05) (Figure 2)

AD_{CP} when measured using chromic oxide was higher (81.9 ± 1.6%) than yttrium (76.9 ± 0.9%) but not significantly different to AD_{CP} of ytterbium (77.2 ± 0.8%).
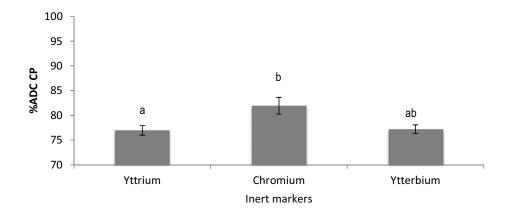
Figure 2. Apparent crude protein digestibility of subadult greenlip abalone (*H. laevigata*) fed 33% dietary protein levels at two different temperatures (14°C and 18°C). Values that share the same superscript are not significantly different (P > 0.05).

There were no significant interactions by two way ANOVA between temperature and markers for apparent gross energy digestibility (P> 0.05). Moreover, AD_{GE} calculated using the three inert markers was the same (P > 0.05).

3.2. *Discussion*

3.2.1. *Inert markers digestibility comparison*
The recent study verified the feasibility of yttrium and ytterbium as inert markers for use in protein digestibility experiments with juvenile greenlip and hybrid abalone. However, some caution should be given to results of AD_{CP} obtained using Cr_2O_3 as a marker.

This study was the first study that investigated the application of ytterbium as inert marker on abalone nutrient digestibility determination, and the observed significant AD_{CP} between yttrium and ytterbium across all treatments suggest a possibility to use ytterbium as inert marker in digestibility study on abalone.

The AD_{CP} measured using chromic oxide overestimated AD_{CP} and AD_{GE} when compared to yttrium and ytterbium when using standard digestion and analytical technique for inductively coupled plasma atomic emission spectrophotometry (ICP-AES). These three inert markers were digested using the hydrochloric:nitric acid method while [4] suggested the digestion of chromic oxide should use phosphoric acid since solubilisation of chromic oxide could be incomplete if using hydrochloric:nitric acid method [4]. Using standard faecal digestion protocols provide unsatisfactory chromic oxide recovery and results.

Chromic oxide has been widely used as inert digestibility markers of aquatic animals [26], Nevertheless, its reliability as an inert marker cannot be validated due to the analytical variability and lower recovery rate [27]. [28] measured feed digestibility in green abalone (*H. fulgens*), found that chromic oxide resulted in lower digestibility coefficient values compared to acid insoluble ash. However, it was digested using perchloric acid and not phosphoric acid as [4] have suggested. Moreover, negative apparent digestibility coefficients were observed when using chromic oxide as inert marker in digestibility study of *H. midae* [29]. The recovery rate value of chromic oxide was over 100% and apparent digestibility coefficient were higher than the other two inert markers demonstrating the limitation of using chromic oxide using standard digestion techniques. Moreover, to

avoid analytical variation, the inclusion of chromic oxide into the feed should be in high concentration (5–10 g kg^{-1} feed) [4], while the amount of the chromic oxide that included into the formulated feed in this study was 0.02 g 100g^{-1}. The inclusion of chromic oxide in high concentrations is to be avoided as it interferes with the absorption and metabolism nutrients and may affect the feed consumption[4, 30].

Compared to chromic oxide, yttrium and ytterbium provided consistent and accurate estimates of apparent digestibility at lower concentrations (100 mg.kg^{-1}) than chromic oxide [4].

3.2.2. Experimental approach
Results from this study assessed the possibility to obtain an accurate estimation of nutrient digestibility of feed in a commercial system. Conducting digestibility research in commercial systems seemed to be inappropriate due to several limiting factors. Contamination of silica in the feces samples occurred when conducting this study. This contamination was likely caused by unfiltered sea water used on farm that brings suspended solids and sediments from the sea, particularly during storm events. This contamination led to an inability to determine AD$_{GE}$ of all samples collected from the experiment conducted on abalone farm due to imperfect combustion of the samples when determining gross energy using the bomb calorimeter. A different result was obtained when determining gross energy of samples from experimental tanks that had no contamination where the samples ignited perfectly.

Culture conditions including water temperature, water flow, tank design and the presence of microflora in tanks might have influenced the nutrient digestibility. Microflora that grows in the tanks is a food source for abalone containing nitrogen and energy. Therefore, instead of consuming the feed provided, the animals might preferentially to graze on microflora bed. Visual observation during the sample collection showed the growth of microflora bed in outdoor tanks, however further investigation is required to confirm this event was occurred.

On farm management was also a limiting factor in doing on farm digestibility research. The workers might accidentally feed the experimental animals with their commercial feed. During this study, despite clear instructions and training, on one occasion farm workers fed experimental tanks with incorrect feeds. Therefore, the samples from the contaminated tank were not used, reducing the samples available to be analysed. This highlights potential limitation of commercial-based trials where feeding regime is critical to results.

4. Conclusion
Ytterbium and yttrium are reliable inert markers in determining digestibility of feed ingredients. An appropriate method in chromic oxide digestion should be applied to obtain a more accurate estimation of nutrient digestibility. It is recommended to conduct nutrient digestibility research under experimental conditions to avoid sample contamination. Doing on-farm digestibility research should be well considered and all limiting factors should be mitigated.

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