

RESEARCH ARTICLE

Towards sustainable and ocean-friendly aquafeeds: Evaluating a fish-free feed for rainbow trout (*Oncorhynchus mykiss*) using three marine microalgae species

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Aquaculture, the fastest growing food sector, is expected to expand to produce an additional 30 million metric tons of fish by 2030, thus filling the gap in supplies of seafood for humans. Salmonids aquaculture exploits the vast majority of fishmeal and fish oil rendered from ocean-dwelling forage fish. Most forage fish diverted to these commodities are human-food grade, and all are primary prey for marine predators. Rising costs, price volatility, and environmental sustainability concerns of using these commodities for aquaculture feed are driving the global search for alternatives, including marine microalgae originating from the base of marine food webs but produced in culture. We report the first evaluation of two marine microalgae, *Nannochloropsis* sp. and *Isochrysis* sp., for their potential to fully replace fishmeal and fish oil in diets of rainbow trout (*Oncorhynchus mykiss*), an important model for all salmonid aquaculture. We conducted a digestibility experiment with dried whole cells of *Nannochloropsis* sp. and *Isochrysis* sp., followed by a growth experiment using feeds with different combinations of *Nannochloropsis* sp., *Isochrysis* sp., and *Schizochytrium* sp. We found that digestibilities of crude protein, crude lipid, amino acids, fatty acids, omega 3 polyunsaturated fatty acids (n3 PUFA), docosahexaenoic acid (DHA), n6 (omega 6) PUFA in *Isochrysis* sp. were significantly higher than those in *Nannochloropsis* sp. Digestibility results suggest that for rainbow trout diets *Isochrysis* sp. is a better substitute for fishmeal and fish oil than *Nannochloropsis* sp. The lower feed intake by fish fed diets combining multiple microalgae, compared to fish fed the reference diet, was a primary cause of the growth retardation. In trout fillets, we detected an equal amount of DHA in fish fed fish-free diet and reference diet. This study suggests that *Isochrysis* sp. and *Schizochytrium* sp. are good candidates for DHA supplementation in trout diet formulations.

Keywords: Marine microalgae; Salmonids; Ocean-derived fishmeal and fish oil; Substitution; Nutrient; Digestibility; Growth

1. Introduction

Aquaculture is the world's fastest growing food sector (FAO, 2018), and its global activities signal a major change in humanity's relationship with the ocean (Duarte et al., 2009). For the first time in human history, aquaculture now accounts for more human food than wild-captured seafood or beef (FAO, 2016), for example, producing 53% of total food fish in 2016 (FAO, 2018). Landings from

capture fisheries have leveled off in recent decades, an outcome of stocks being overexploited or fully exploited (FAO, 2018), yet the world will need to produce an additional 30 million metric tons of fish by 2030 to meet projected rising demand (OECD-FAO, 2015; FAO, 2016). Aquaculture is expected to expand to fill this gap and supply two-thirds of global fish consumption by 2030 (World Bank, 2013; FAO, 2018; Plagányi, 2019). Mariculture production (in marine and coastal waters or saltwater tanks on land) is led by aquatic plants (macroalgae) and unfed filter-feeding molluscs, followed by finfish (mostly salmonids), and then shrimp and prawns (FAO, 2016; Clavelle et al., 2019). Excluding aquatic plants, mariculture comprised 36% of aquaculture (26.7 million t) and 18.25% of all edible seafood in 2014 (FAO, 2016) and is projected to increase (FAO, 2018).

Underpinning these trends is a historic shift in what farmed fish are fed, away from extensive aquaculture

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relying on natural foods to intensive aquaculture using formulated feeds (aquafeeds). Intensive aquaculture grew by approximately 15 million t (97%) to 30 million t between 2000 and 2010 (Shepherd and Jackson, 2013). Aquafeeds include ocean-derived ingredients rendered from 'forage' fish, small and intermediate-sized pelagic fish (e.g., sardines, anchovy, herring, mackerel) that are primary food sources for marine predators including fish, mammals and seabirds (Pikitch et al., 2014). Currently, one-sixth of global capture fisheries are rendered into fishmeal and fish oil commodities (Cashion et al., 2017), despite 90% of these harvested fish being food-grade for human consumption (Cashion et al., 2016). The fraction of global supplies of fishmeal and fish oil going to aquafeeds has risen rapidly, primarily due to growth of salmonid aquaculture: from 10% of fishmeal in 1980 and 20% of fish oil in 1990 to 73% of fishmeal and 71% of fish oil in 2010 (Shepherd and Jackson, 2013; Chauton et al., 2015; Cashion et al., 2016; FAO, 2016). The aquaculture industry has preferred these feedstuffs because fishmeal contains high-quality proteins rich in essential amino acids and fish oil is rich in essential omega-3 long chain polyunsaturated fatty acids (n3 LC-PUFA), particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Salmonids require high levels of nutrient-dense protein and essential n3 LC-PUFA in their diets. Not surprisingly, aquafeeds for farmed salmonids—primarily Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) grown in marine and freshwaters—used 24% of fishmeal and 50% of fish oil of global aquaculture in 2010 (Shepherd and Jackson, 2013).

At current rates of commercial demand, the world could exhaust marine forage fisheries by 2040, with disastrous consequences for food security of billions of humans and for wild marine fish, mammals and seabirds that forage on them (Duarte et al., 2009; FAO, 2010, 2012; Tacon and Metian, 2013; Pikitch et al., 2014; Cashion et al., 2016). The aquafeed industry also feels the pressure of unfavorable market trends for fishmeal and fish oil: increased price volatility and rising prices, partly due to competition from higher value uses of fish oil for human nutrition and pharmaceuticals (Klinger and Naylor, 2012). These concerns have motivated aquafeed producers to reduce their use of forage fish via partial substitution of fishmeal and fish oil with ingredients from terrestrial farmed crops, e.g., soy, corn, canola (Shepherd and Jackson, 2013; Fry et al., 2016). If this approach causes a net increase in industrial agriculture of these crops (Froelich et al. 2018), aquaculture could exacerbate environmental damage from their industrial farming (Boissy et al., 2011), including land use change from deforestation (Galford et al., 2010), eutrophication of natural waters from fertilizer runoff (Klinger and Naylor, 2012; Troell et al., 2014), and increased indirect freshwater footprint (Gephart et al., 2017; Pahlow et al., 2015). Analysts also anticipate major shortages of soy-based ingredients over the next decade, which will drive soybean prices higher (Whittaker, 2015). Moreover, terrestrial plant ingredients commonly used in aquafeed have the following nutritional disadvantages: terrestrial crop proteins have low digestibility, high levels of anti-nutritional factors, and are deficient in certain essential amino

acids such as lysine, methionine, threonine and tryptophan (Li et al., 2009; He et al., 2013); and terrestrial crop oils lack n3 LC-PUFA (Turchini et al., 2009).

Recent industrial-scale production of microalgae for biofuels and human nutritional supplements has stimulated interest in their use in animal feeds (Gouveia et al., 2009; Hemaiswarya et al., 2011; Ryckebosch et al., 2012) and especially for aquafeeds (Beal et al., 2018; Shah et al., 2018). Marine microalgae show promise as potential replacements for fishmeal and fish oil in feeds for salmonids and other finfish because of their elevated fatty acid profiles and high protein content (Walker and Berlinsky, 2011; Tibaldi et al., 2015; Kiron et al., 2016; Sarker et al., 2016a, 2016b, 2018; Gong et al., 2018; Sorensen et al., 2017; Bélanger-Lamonde et al., 2018). Our recent studies showed that whole cell biomass of *Schizochytrium* sp. is a highly digestible source of nutrients for rainbow trout and tilapia and is a potential substitute for fish oil in aquafeed (Sarker et al., 2016b; Bélanger-Lamonde et al., 2018). Aquafeed companies have begun including DHA-rich oil from *Schizochytrium* sp. into salmon feeds (Tocher et al., 2019). *Nannochloropsis* sp. and *Isochrysis* sp. are two potential species for aquaculture feeds rich in EPA and DHA as well as protein, amino acids, lipid, and various minerals (Sukenik et al., 1993; Walker and Berlinsky, 2011; Kagan et al., 2013; Sarker et al., 2018). These microalgae could thus be good candidates for salmonid feed ingredients.

We focused the research presented here on *Oncorhynchus mykiss* (rainbow trout) because this fish species is an important model for all farmed salmonids, which dominate finfish aquaculture. Mariculture of *O. mykiss* raises fish derived from ocean-migrating populations whereas freshwater culture of *O. mykiss* uses freshwater-residing populations and comprises the most widely cultivated cold freshwater finfish in the world. Whether salmonids can thrive on a fish-free diet is an open question given that salmonids are naturally piscivorous. The literature lacks data on the digestibility of the protein, lipid, individual amino acids and fatty acids of algal biomass in rainbow trout. Also, the potential of marine microalgae to fully substitute fishmeal and fish oil and to maintain growth and fillet quality in rainbow trout has not been investigated previously. Thus, we first conducted an experiment to determine the apparent digestibility in rainbow trout of the macronutrients, amino acids and fatty acids in the commercially produced microalgae *Nannochloropsis* sp. and *Isochrysis* sp. We then conducted a nutritional feeding experiment using different combinations of dried whole cells of these microalgae and *Schizochytrium* sp. in diets designed to fully replace fishmeal and fish oil and to assess effects on fish growth and deposition levels of n3 LC PUFA in trout filets and liver.

2. Materials and Methods

2.1. Determination of *Nannochloropsis* sp. and *Isochrysis* sp. digestibility

2.1.1. Dietary design

In the digestibility experiment the nutritional values of *Nannochloropsis* sp. and *Isochrysis* sp. were evaluated by determining the apparent digestibility coefficients

(ADCs) of protein, lipid, and energy, amino acid, as well as fatty acid availability in the microalgae and test diets. We prepared a high-quality reference diet (Table S1) and combined it with each test microalga species (whole dried cells) at a 7:3 ratio to produce two test diets (one for each microalga species) following the standard apparent digestibility protocol (Cho et al., 1982; Bureau and Hua, 2006; Sarker et al., 2016a, 2018). We obtained dried *Nannochloropsis* sp. and *Isochrysis* sp. from Reed Mariculture, Inc., Pasadena, CA, USA. For the digestibility measurement of the diet, 1% Sipernat 50™ (Degussa AG, Frankfurt, Germany) was added to the diet as an indigestible marker (a source of acid insoluble ash [AIA]). We first mixed micro-ingredients and then slowly added them to the macro-ingredients to ensure a homogeneous mixture. We thoroughly mixed and steam-pelleted the ingredients using a California Pellet Mill, dried the pellets in a forced-air oven (22°C, 24 h), sieved them, and stored at -20°C. The proximate composition, energy, amino acid, and fatty acid profiles of the two test ingredients (microalgae) and three test diets used in the digestibility experiment were determined as described in Section 2.1.3 (results provided in Tables S2 and S3).

2.1.2. Fish, feeding and feces collection

Prior to the digestibility experiment, we randomly allocated juvenile rainbow trout (triploid, all female) with an average weight of 155.0 ± 2.5 g ($n = 3$) in twelve 150-L rectangular tanks (12 fish/tank, 3 tanks/diet, total of 108 fish for 9 tanks) in a freshwater recirculating system at the Laboratoire de Recherche en Sciences Aquatiques of Université Laval (Quebec, QC, Canada). We maintained the water temperature (12.5°C) and other parameters within limits recommended for rainbow trout by the National Research Council (NRC, 2011).

Prior to beginning the growth experiment (Section 2.2), we acclimated the fish to the feed for five days. During the experiment, we hand-fed the fish to apparent satiation twice daily on 2 days a week and, for the rest of each week, fed to a moderate feed-restriction ration (80% of satiety levels) to maximize feed efficiency and minimize feed waste (Bureau et al., 2006; Koko et al., 2010). The duration of the digestibility experiment was 4 weeks, during which feces were collected via a modified Guelph system based on Cho et al. (1982) once a day in the morning before feeding and stored at -20°C. Uneaten feed residues and feces were flushed out of the fecal collection column after each evening feeding. To collect feces, we sealed the bottom of the tank from the collector column by closing a valve, gently removed the column and then gently withdrew settled feces and surrounding water from the fecal collector. We allowed samples to settle in the collecting column before removing supernatant water with the pipette, and then transferred the feces in lidded aluminum dishes and froze them at -20°C. We pooled fecal samples by tank for the duration of the experiment. At the end of the experiment, we lyophilized, finely ground, and stored samples at -20°C for later proximate, amino acid, and fatty acid analyses.

2.1.3. Chemical analysis and calculations

We sent the three types of samples (pure microalgae, diets and feces) to New Jersey Feed Laboratory, Inc. (Ewing, NJ, USA) for the following types of analysis: moisture (Association of Official Analytical Chemists, AOAC, 1995, method 930.15), crude protein (AOAC 990.03), lipid (AOAC 920.39), ash (AOAC 942.05), crude fiber (AOAC 1978.10), energy (automated oxygen bomb calorimeter), amino acids (high-performance liquid chromatography analysis, via AOAC methods 994.12, 985.28, 988.15, and 994.12) and fatty acids (fatty acid methyl ester analysis, via AOAC method 963.22.). In addition, we analyzed AIA in feed and feces according to the methods of Naumann and Bassler (1976).

We calculated apparent digestibility coefficients (ADCs) for macro nutrients, amino acids, fatty acids and energy of the test and the reference diets using the standard method as described by Cho *et al.* (1982):

$$\text{ADC} = 1 - (F/D \times D_i/F_i)$$

where D = % nutrient (or kJ g^{-1} energy) of diet; F = % nutrient (or kJ g^{-1} energy) of feces; D_i = % digestion indicator (AIA) of diet; and F_i = % digestion indicator (AIA) of feces. We calculated the apparent digestibility of the microalgae as test ingredients using the equation proposed by Forster (1999), mathematically simplified by Bureau and Hua (2006) and recently documented in NRC (2011):

$$\text{ADC}_{\text{test ingredient}} = \text{ADC}_{\text{test diet}} + \left[(\text{ADC}_{\text{test diet}} - \text{ADC}_{\text{ref. diet}}) \times \left(0.7 \times D_{\text{ref}} / 0.3 \times D_{\text{ingredient}} \right) \right]$$

where D_{ref} is the percentage of nutrient or kJ g^{-1} energy in the reference diet, and $D_{\text{ingredient}}$ is the percentage of nutrient or kJ g^{-1} energy in the ingredient.

2.2. Growth experiment

2.2.1. Dietary design

We formulated four iso-nitrogenous (54% crude protein), iso-energetic (16 kJ g^{-1}) and iso-lipidic (17% lipid) experimental diets, following the requirements for optimum growth of juvenile rainbow trout (Table S4). The reference feed, which mimics the current commercial feed, contained 7.5% fishmeal and 13.5% fish oil. The three fish-free diets differed from each other in the specific combinations and relative amounts of dried whole cells of three microalgae spp. combined with canola oil to fully substitute fishmeal and fish oil. We formulated these fish-free test feeds by combining *Nannochloropsis* sp. (7%) and *Isochrysis* sp. (2.4%) with canola oil (13%) in the first case (NI); *Nannochloropsis* sp. (7%) and *Schizochytrium* sp. (2.5%) with canola oil (12%) in the second case (NS); and *Nannochloropsis* sp. (7%), *Isochrysis* sp. (2.4%), and *Schizochytrium* sp. (3.2%) with canola oil (11%) in the third case (NIS) (Table S4). We first mixed micro-ingredients and then slowly added them to the macro-ingredients to ensure a homogeneous mixture. We thoroughly mixed the ingredients and steam pelleted using a California Pellet Mill. Pellets were then dried in a forced-air oven

(22°C, 24 h), sieved and stored at −20°C. The initial size of the pellet was 2.0 mm, which was increased to 4.0 mm as the fish grew larger throughout the experiment. We obtained dried *Nannochloropsis* sp. and *Isochrysis* sp. from Reed Mariculture, Inc., Pasadena CA, USA, and dried *Schizochytrium* sp. from Algamac™, Aquafauna Bio-Marine, Inc., CA, USA. The proximate composition, energy, amino acid and fatty acid profiles of the experimental diets were determined as in Section 2.1.3 (results provided in Tables S4 and S5).

2.2.2. Fish husbandry and feeding

All fish used in the growth experiment were female triploid trout with initial body weight of 30–50 g, stocked into 150-L recirculating culture system tanks system with 29 fish per tank. Three tanks of fish were randomly assigned to each dietary treatment. Fish were fed the experimental diets to apparent satiation twice daily (at 8:00 am and 3:30 pm), 6 days a week, for 12 weeks (Bélanger-Lamonde et al., 2018). During the experiment, the recirculating system environment was maintained at optimum levels for rainbow trout culture: 12.5°C for water temperature with other environmental parameters remaining within limits recommended for rainbow trout by the National Research Council (NRC, 2011). After 12 weeks of feeding, fish fasted for 24 h before tissue samples were collected for compositional analyses.

2.2.3. Biological sampling procedures, fillet preparations, and growth measurements

We bulk-weighed the fish at the beginning of the experiment, and again every three weeks until the end of the experiment (84 days). We stopped feeding the fish for 24 h prior to each bulk-weight sampling event. We sampled 3 fish per tank at day 42 (middle) and 84 (final) for fillet fatty acid composition. During middle sampling, we immediately filleted each fish from a standardized dorso-anterior landmark, weighed each fillet and liver, then packaged them in sterile polythene bags (Whirl-pak, Naso, Fort Atkinson, Wisconsin) and stored frozen (−20°C). We then freeze-dried and weighed each fillet and compared it to the respective fresh-fillet weight to calculate the loss of water/moisture, allowing us to express fatty acid data on a wet weight basis. During final sampling, the entire fish biomass of every tank was weighed. Three fish per tank were sacrificed for whole body proximate analysis. Three fish per tank were filleted from a standardized dorso-anterior landmark, packaged in sterile polythene bags (Whirl-pak, Naso, Fort Atkinson, Wisconsin), and stored frozen (−20°C) until fatty acid analysis.

We determined the dietary effects on growth by evaluating final weight, weight gain, feed conversion ratio (FCR), specific growth rate (SGR), protein efficiency ratio (PER), and survival rate. We calculated the indices as follows (Sarker et al., 2014): weight gain (g) = final weight – initial weight; weight gain (%) = [final weight – initial weight]/initial weight × 100%; FCR = feed intake/weight gain; SGR (% day⁻¹) = [ln final wet weight (g) – ln initial wet weight (g)]/time (days); PER = weight gain (g)/protein fed (g); and survival rate (%) = [final number of fish/initial number of fish] × 100%.

3. Statistical analysis

We conducted one-way analysis of variance (ANOVA) of apparent digestibility coefficients for macronutrients, fatty acids, and amino acids in the reference and test diets, as well as for test ingredients. We then conducted ANOVA of growth performance and feed utilization parameters, whole body proximate composition, and fillet fatty acid composition and, when significant differences were found, compared the treatment means using Tukey's test of multiple comparisons, with a 95% confidence interval. For all statistical analyses we used the IBM Statistical Package for the Social Sciences (SPSS) program for Windows (v. 21.0, Armonk, NY, USA).

4. Results

4.1. Digestibility of macronutrients, energy, amino acids, and fatty acids in diets

We determined the ADCs of macronutrients, energy, amino acids, and fatty acids in the test diets (**Table 1**) and the tested microalgae (**Table 2**), as well as of individual fatty acids in the diets (Table S6) and microalgae (Table S7). We found significantly higher ADC of dry matter in the *Isochrysis* sp. diet (76.6%) than *Nannochloropsis* sp. diet (70.3%), and that the former was not significantly different from ADC of dry matter in the reference diet (76.1%) (**Table 1**). The ADC of protein in the *Isochrysis* sp. diet (91.2%) was similar to that in the reference diet (92.9%) ($P > 0.05$); *Nannochloropsis* sp. had a significantly lower value (85.0%) than the reference and *Isochrysis* sp. diets. We detected the lowest ADC value of lipid in the *Isochrysis* sp. diet (76.7%) compared with the reference (87.3%) and *Nannochloropsis* sp. diets (80.2%), but there was no significant difference between the test diets. We found the highest ADC of energy in the reference diet (81.0%), which was not significantly different from that in the *Isochrysis* sp. diet (78.0%), and the lowest in the *Nannochloropsis* sp. diet (75.2%). We did not find significant differences between diets for the ADC of ash (which ranged from 51.5 to 62.0%). Also, the ADC of crude fiber was significantly higher in the *Isochrysis* sp. diet (94.2%) than in the *Nannochloropsis* sp. diet (77.2%), but the reference (93.9%) and *Isochrysis* sp. diets did not show any difference.

The ADCs of essential amino acids for the test diets are presented in **Table 1**. The ADCs of all of these amino acids in the *Isochrysis* sp. test diet were highly digestible (>90%) and did not differ significantly from the reference diet. The ADCs of all essential amino acids in the *Nannochloropsis* sp. diet were significantly lower than in the *Isochrysis* sp. and reference diets. The ADCs of essential amino acids in the *Nannochloropsis* sp. diet were >80% (up to 87%), except for that of tryptophan (68.5%). The lowest ADCs of all essential amino acid values were thus detected in the *Nannochloropsis* sp. diet.

The ADCs of most of the major fatty acid fractions among the test diets differed significantly (Table S6). The ADCs of the polyunsaturated fatty acids 20:5n3 EPA, 22:6n3 DHA, n6 PUFA, n3 PUFA, n6 LC PUFA, and n3 LC PUFA in the *Isochrysis* sp. and reference diets were highly digestible overall (>90%). ADCs for most of the individual

Table 1: Apparent digestibility coefficients (%; mean \pm standard error, n = 3) of nutrients in the reference diet and test diets for rainbow trout. DOI: <https://doi.org/10.1525/elementa.404.t1>

Nutrient	Diet ingredients ^a			P value
	Reference	70% Ref + 30% <i>Nanno</i>	70% Ref + 30% <i>Is</i>	
Proximate composition				
Dry matter	76.1 \pm 1.0 [a]	70.3 \pm 0.7 [b]	76.6 \pm 1.0 [a]	<0.01
Crude protein	92.9 \pm 0.6 [a]	85.0 \pm 0.3 [b]	91.2 \pm 0.3 [a]	<0.01
Lipid	87.3 \pm 1.6 [a]	80.2 \pm 1.2 [ab]	76.7 \pm 0.8 [b]	<0.01
Ash	51.5 \pm 1.2	55.2 \pm 1.6	62.0 \pm 2.0	0.26
Crude fiber	93.9 \pm 0.3 [a]	77.2 \pm 1.4 [b]	94.2 \pm 0.2 [a]	<0.01
Energy	81.0 \pm 1.3 [a]	75.2 \pm 0.6 [b]	78.0 \pm 0.85 [a]	0.01
Essential amino acids				
Arginine	94.5 \pm 0.3 [a]	86.9 \pm 0.2 [b]	94.9 \pm 0.2 [a]	<0.01
Lysine	94.4 \pm 0.3 [a]	85.6 \pm 0.1 [b]	94.9 \pm 0.2 [a]	<0.01
Isoleucine	91.8 \pm 0.4 [a]	85.2 \pm 0.1 [b]	91.9 \pm 0.3 [a]	<0.01
Leucine	91.8 \pm 0.4 [a]	84.8 \pm 0.2 [b]	92.6 \pm 0.2 [a]	<0.01
Histidine	92.3 \pm 0.4 [a]	86.2 \pm 0.2 [b]	92.6 \pm 0.3 [a]	<0.01
Methionine	95.2 \pm 0.4 [a]	87.2 \pm 0.2 [b]	95.0 \pm 0.1 [a]	<0.01
Phenylalanine	91.9 \pm 0.4 [a]	81.8 \pm 0.2 [b]	92.7 \pm 0.2 [a]	<0.01
Threonine	92.4 \pm 0.3 [a]	80.7 \pm 1.2 [b]	92.5 \pm 0.2 [a]	<0.01
Tryptophan	95.3 \pm 0.3 [a]	68.5 \pm 1.1 [b]	92.7 \pm 0.3 [a]	<0.01
Valine	92.7 \pm 0.4 [a]	81.0 \pm 0.2 [b]	93.1 \pm 0.25 [a]	<0.01
Fatty acid fractions ^b				
Total SFA	71.7 \pm 0.8 [a]	65.8 \pm 0.7 [b]	66.9 \pm 1.6 [b]	0.04
Total MUFA	72.3 \pm 1.2	69.6 \pm 0.6	73.3 \pm 0.8	0.18
Total PUFA	90.7 \pm 0.6 [a]	76.3 \pm 0.4 [b]	91.7 \pm 0.4 [a]	<0.01
20:5n3 EPA	96.9 \pm 1.1 [a]	81.1 \pm 0.4 [b]	94.5 \pm 0.2 [a]	<0.01
22:6n3 DHA	92.4 \pm 0.5 [a]	88.3 \pm 0.2 [b]	91.8 \pm 0.4 [a]	<0.01
Total n3 PUFA	94.1 \pm 0.5 [a]	78.1 \pm 0.4 [b]	93.3 \pm 0.3 [a]	<0.01
Total n6 PUFA	92.1 \pm 0.4 [a]	85.8 \pm 0.2 [b]	93.8 \pm 0.3 [a]	<0.01

^a Ref refers to reference diet; *Nanno*, *Nannochloropsis* sp.; *Is*, *Isochrysis* sp.; mean values not sharing a common bracketed letter in the same row differ significantly as determined by Tukey's HSD test, P < 0.05; both letters appearing together means no difference.

^b SFA refers to saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

and major fatty acid fractions were significantly higher in the *Isochrysis* sp. diet than in the *Nannochloropsis* sp. diet. However, ADCs did not differ significantly among C14:0, C15:0, C17:0, C18:1n9, C20:n9, saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and C18:3n3 alpha linoleic acid (ALA). With the exception of SFAs, none of the individual and major fatty acid fractions differed significantly between the *Isochrysis* sp. and reference diets. The ADC of 18:3n3 ALA was significantly higher in fish fed the *Nannochloropsis* sp. diet (98.1%) than the *Isochrysis* sp. diet (90.6%) or reference diet (93.7%).

4.2. Digestibility of amino acids in test microalgal ingredients

In terms of the calculated digestibility of the microalgal ingredients (**Table 2**), significantly higher ADC values for dry matter, crude protein, ash, crude fiber, and energy were detected for the *Isochrysis* sp. compared to the *Nannochloropsis* sp. The greatest difference was observed in the ADC of crude fiber, which was much lower in the *Nannochloropsis* sp. (38.1%) than the *Isochrysis* sp. (96.0%). We did not detect significant differences in the ADC of lipid between the two microalgal ingredients, but the ADCs of all ten of the individual essential amino acids

Table 2: Apparent digestibility coefficients (%; mean \pm standard error, n = 3) of nutrients in test ingredients *Nannochloropsis* sp. and *Isochrysis* sp. for rainbow trout. DOI: <https://doi.org/10.1525/elementa.404.t2>

Nutrient	Test ingredients ^a		P value
	<i>Nannochloropsis</i> sp.	<i>Isochrysis</i> sp.	
Proximate composition			
Dry matter	56.7 \pm 1.3 [b]	77.1 \pm 1.9 [a]	0.01
Crude protein	69.3 \pm 1.5 [a]	86.5 \pm 1.7 [a]	0.01
Lipid	60.1 \pm 0.6	62.8 \pm 0.7	0.20
Ash	63.0 \pm 4.3 [b]	71.1 \pm 2.5 [a]	0.01
Crude fiber	38.1 \pm 4.3 [b]	96.0 \pm 3.7 [a]	0.01
Energy	62.1 \pm 1.4 [b]	72.6 \pm 1.3 [a]	0.01
Essential amino acids			
Arginine	74.5 \pm 0.2 [b]	99.2 \pm 4.4 [a]	0.01
Lysine	72.6 \pm 0.9 [b]	101.4 \pm 0.0 [a]	0.01
Isoleucine	63.1 \pm 1.0 [b]	92.1 \pm 0.9 [a]	0.01
Leucine	71.8 \pm 1.5 [b]	94.5 \pm 1.2 [a]	0.01
Histidine	74.1 \pm 0.8 [b]	93.2 \pm 1.1 [a]	0.01
Methionine	69.8 \pm 0.7 [b]	94.9 \pm 0.7 [a]	0.01
Phenylalanine	64.8 \pm 1.4 [b]	94.4 \pm 0.9 [a]	0.01
Threonine	67.4 \pm 4.8 [b]	94.0 \pm 0.6 [a]	0.01
Tryptophan	11.8 \pm 2.8 [b]	84.4 \pm 2.4 [a]	0.01
Valine	58.9 \pm 1.4 [b]	98.5 \pm 0.1 [a]	0.01
Fatty acid fractions ^b			
Total SFA	55.9 \pm 1.2	58.9 \pm 2.6	0.41
Total MUFA	44.7 \pm 5.0 [b]	72.2 \pm 6.3 [a]	<0.01
Total PUFA	61.8 \pm 0.7 [b]	91.7 \pm 0.8 [a]	<0.01
20:5n3 EPA	69.4 \pm 0.8 [b]	87.7 \pm 2.2 [a]	<0.01
22:6n3 DHA	ND ^c	91.0 \pm 1.4	NA ^c
Total n3 PUFA	63.9 \pm 0.7 [b]	92.6 \pm 0.4 [a]	<0.01
Total n6 PUFA	76.2 \pm 0.7 [b]	98.4 \pm 1.1 [a]	<0.01

^a Mean values not sharing a common bracketed letter in the same row differ significantly as determined by Tukey's HSD test, P < 0.05; both letters appearing together means no difference.

^b SFA refers to saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

^c ND refers to not detectable (<1% of total fatty acids); NA, not applicable.

were significantly higher in the *Isochrysis* sp. (all > 90.0%) than in the *Nannochloropsis* sp. (Table 2).

With the exception of the fatty acid fractions C14:0, C15:0, and C16:0, the ADCs of all individual major fatty acid fractions between the two microalgal ingredients were significantly different (Table S7). The ADCs of these fatty acid fractions were significantly higher in the *Isochrysis* sp. than the *Nannochloropsis* sp. with the exception of C18:3n3 ALA, which was significantly higher in the *Nannochloropsis* sp. (98.3% compared to 90.3%). The ADC of EPA was significantly higher in the *Isochrysis* sp. (87.7% compared to 69.4%), and though DHA was not detectable

in *Nannochloropsis* sp., the ADC of DHA in *Isochrysis* sp. was high (91.0%).

4.3. Growth experiment

Final feed intake of the reference diet containing fishmeal and fish oil was observed to be significantly higher than that of the other diets (Table 3) where fishmeal and fish oil were fully replaced by the tested combinations of marine microalgae (and canola oil). With the exception of the reference diet, no significant differences in final weight gain and SGR were observed among trout in the treatment groups that received the fish-free diets containing the

tested combinations of marine microalgae. Trout fed the reference diet (0.92) had significantly lower (improved) FCR (0.92) that did not differ when fishmeal and fish oil were fully replaced in the NS diet (0.98); however, fish fed the NI and NIS diets had significantly higher (poorer) FCR. The protein efficiency ratio of the reference-fed fish did not differ when fishmeal and fish oil were fully replaced in the NI, NS, and NIS diets (Table 3). Trout appeared healthy at the end of the experiment and showed no difference in survival among the diets. The whole body proximate composition of trout did not differ among dietary treatments, except for crude protein which was highest in the NS diet and lowest in the reference diet (data not shown).

The fillet fatty acid composition (% of total fatty acids) of rainbow trout reflected the fatty acid composition of

the different diets (Tables 4 and S5). One fraction of the saturated fatty acids, palmitic acid (16:0), had the highest concentration in the fillet irrespective of dietary treatment, as well as significantly higher amounts deposited in the flesh of trout fed the reference, NS, or NIS diet compared to fish fed the NI diet (Table S8). The concentration of total SFAs was affected significantly by dietary treatments and was higher in the fish fed the reference or NS diet compared to those fed the NI or NIS diet.

All fractions of total monounsaturated fatty acids in the fillets were significantly affected by dietary treatments (Tables 4 and S5). Fish fed the reference diet displayed the highest MUFA amount, which was reflected the MUFA content in the experimental diet. Irrespective of the diet, oleic acid (18:1n9) was the most abundant MUFA in the

Table 3: Growth indices (mean \pm standard error, n = 3) of rainbow trout fed experimental diets for 84 days. DOI: <https://doi.org/10.1525/elementa.404.t3>

Index	Diet ^a				ANOVA	
	Reference	NI	NS	NIS	F value	P value
Initial weight (g)	41.9 \pm 0.6	42.5 \pm 0.5	41.4 \pm 0.7	41.7 \pm 0.4	0.68	0.58
Final weight (g)	207.5 \pm 2.5 [a]	178.0 \pm 2.1 [b]	185.9 \pm 0.9 [b]	184.6 \pm 4.7 [b]	18.7	0.00
FCR ^b (ratio)	0.92 \pm 0.0 [a]	1.0 \pm 0.0 [b]	0.98 \pm 0.0 [a]	1.0 \pm 0.0 [b]	5.24	0.02
SGR ^c (%/day)	1.9 \pm 0.0 [a]	1.7 \pm 0.0 [b]	1.8 \pm 0.0 [ab]	1.8 \pm 0.1 [ab]	11.7	0.00
PER ^d (ratio)	2.1 \pm 0.0	1.9 \pm 0.1	2.0 \pm 0.0	1.9 \pm 0.0	4.46	0.53
Feed intake (g/fish)	138.9 \pm 4.2 [a]	119.5 \pm 4.9 [b]	122.6 \pm 2.1 [b]	124.7 \pm 1.6 [b]	5.87	0.02
Survival rate ^e (%)	97.7 \pm 2.3	100.0 \pm 0.0	100.0 \pm 0.0	97.7 \pm 1.1	0.98	0.44

^a NI refers to *Nannochloropsis* sp. + *Isochrysis* sp.; NS, *Nannochloropsis* sp. + *Schizochytrium* sp; NIS, *Nannochloropsis* sp. + *Isochrysis* sp + *Schizochytrium* sp.; mean values not sharing a common bracketed letter in the same row differ significantly (P < 0.05); both letters appearing together means no difference.

^b Feed conversion ratio = feed intake (g)/weight gain (g), where weight gain (%) = [final wet weight (g) – initial wet weight (g)]/initial wet weight (g) \times 100%.

^c Specific growth rate (% day⁻¹) = 100 \times [ln final wet weight (g) – ln initial wet weight (g)]/time (days).

^d Protein efficiency ratio = weight gain (g)/protein fed (g).

^e Survival rate (%) = [final number of fish/initial number of fish] \times 100.

Table 4: Major fatty acid fractions (mean \pm standard error, n = 9) of fillets from rainbow trout fed experimental diets for 84 days. DOI: <https://doi.org/10.1525/elementa.404.t4>

Composition ^b (% of total fatty acids)	Fillet ^a				ANOVA	
	Reference	NI	NS	NIS	F value	P value
Total SFA	24.4 \pm 0.2 [a]	20.7 \pm 0.23 [ab]	24.1 \pm 1.9 [ab]	22.4 \pm 0.2 [b]	5.78	0.02
Total MUFA	52.0 \pm 0.2 [a]	40.5 \pm 0.21 [b]	43.1 \pm 3.8 [b]	38.6 \pm 0.4 [b]	23.33	<0.01
Total PUFA	23.6 \pm 0.3 [b]	38.8 \pm 0.3 [a]	41.6 \pm 3.7 [a]	39.0 \pm 0.6 [a]	5.25	0.03
20:5n3 EPA	2.2 \pm 0.0 [a]	0.8 \pm 0.0 [b]	0.8 \pm 0.0 [b]	0.8 \pm 0.0 [b]	43.76	<0.01
22:6n3 DHA	7.9 \pm 0.1 [a]	4.8 \pm 0.6 [b]	6.4 \pm 0.2 [b]	7.2 \pm 0.3 [ab]	39.85	<0.01
Total n3 PUFA	12.9 \pm 0.2 [a]	10.3 \pm 0.2 [b]	12.2 \pm 1.1 [a]	12.2 \pm 0.4 [a]	9.49	0.01
Total n6 PUFA	9.9 \pm 0.2 [b]	28.4 \pm 0.4 [a]	29.4 \pm 2.6 [a]	26.8 \pm 0.2 [a]	84.67	<0.01

^a Nine fish per diet (three fish/replicate with three replicates/diet); mean values not sharing a common bracketed letter in the same row differ significantly as determined by Tukey's HSD test, P < 0.05; both letters appearing together means no difference.

^b SFA refers to saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

fillets, with significantly higher amounts deposited in the flesh of trout fed the NI, NS, and NIS diet compared to fish fed the reference diet.

All individual polyunsaturated fatty acids varied greatly among the four dietary treatments (Tables 4 and S5). Total PUFA and n6 PUFA were significantly lower in the fish fed the reference diet compared to fish fed the NI, NS, or NIS diets. Rainbow trout fed the NI, NIS, or NIS diets had significantly higher concentrations of the total n6 LC PUFA compared to those fed the reference diet, due to the higher supply of the component in the NI, NIS, and NIS diets compared to the reference diet. With respect to the n3 fatty acids, rainbow trout fed the reference diet had the highest amount of 22:6n3 DHA in the fillet lipids, which was not significantly different from the fish fed the NIS diet (Table 4), reflecting the higher 22:6n3 DHA supplied by these diets (Table S5). Total SFA contents in the fish liver were not significantly different among the four dietary treatments (Table S9), even though the level of total SFAs in the reference diet was much higher than in the other diets (Table S5). The content of DHA in the liver was not significantly different among the dietary treatments.

Figure 1 shows the amounts of DHA deposition in the fish fillet (mg/g fillet). The amounts of 22:6n3 DHA deposited in the fish fillet (mg/g fillet) significantly increased in fish fed the reference or NIS diet compared to the NI and NS diets.

5. Discussion

In this study, we determined the digestibility of nutrients in two marine microalgal species and test diets to evaluate feasibility of using them in aquafeed for rainbow trout. This study also explored, for the first time, full substitution of dietary fishmeal and fish oil in rainbow trout aquafeed by combining biomass from two or three microalgal

species with canola oil. Our overall results represent encouraging steps towards developing formulations that combine microalgal biomass to achieve fish-free aquafeeds for salmonids.

5.1. Importance of digestibility analysis

The digestibility of feed ingredients is key information for formulating sustainable feeds, but publicly available information on digestibility for specific microalgal species in aquafeeds is virtually non-existent (Barone et al., 2018). This lack of information often leads researchers and practitioners to extrapolate the nutritive value of microalgae from their chemical composition. Conducting quantitative digestibility experiments to identify highly digestible microalgae can reduce feed costs, minimize negative environmental impacts (including phosphorus and nitrogen eutrophication emissions), and improve the feed conversion ratio of aquafeeds (Glencross et al., 2007; Sarker et al., 2013, 2016, 2018). The aquafeeds industry can be expected to use microalgae as a nutritional source only if it gets specific information on parameters such as nutrient and anti-nutrient composition, palatability and digestibility, all of which are critical factors given that highly digestible ingredients will both improve FCRs and reduce nutrient loads in fish culture effluents.

5.2. Digestibility differences between *Isochrysis* sp. and *Nannochloropsis* sp.

The results of our digestibility experiment suggest that *Isochrysis* sp. is an excellent source of digestible protein, amino acids, lipid and fatty acids for rainbow trout, and could be an alternative to replace fishmeal and fish oil or be used as a health-promoting long chain PUFA supplement in rainbow trout diets. We detected that the crude protein digestibility of the *Isochrysis* sp. ingredient was

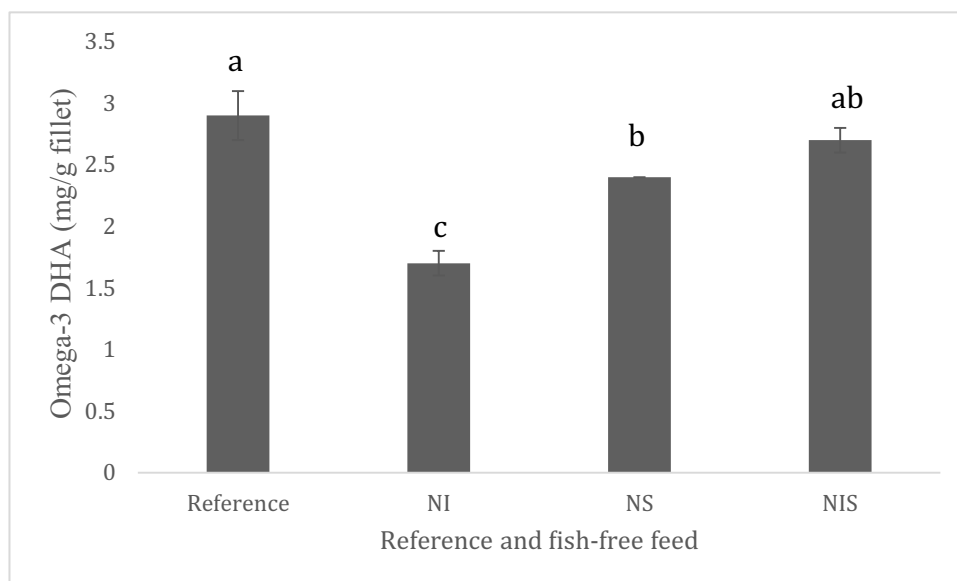


Figure 1: Amounts of DHA in the fillet of rainbow trout fed experimental diets for 84 days. NI refers to feed containing *Nannochloropsis* sp. + *Isochrysis* sp.; NS, *Nannochloropsis* sp. + *Schizochytrium* sp.; NIS, *Nannochloropsis* sp. + *Isochrysis* sp + *Schizochytrium* sp. Mean values not sharing a common letter above the bars differ significantly as determined by Tukey's HSD test, $P < 0.05$; both letters appearing together means no difference. Error bars indicate standard error of the mean, $n = 3$. DOI: <https://doi.org/10.1525/elementa.404.f1>

close to 90% and the essential amino acids in the *Isochrysis* sp. was highly digestible (ADC > 92%) for rainbow trout. The ADC value was higher than the protein digestibility of *Spirulina* sp. (84.7%) fed to Atlantic salmon, reported by Burr et al. (2011). The observed ADC of crude protein in the *Isochrysis* sp. was similar to the protein digestibility of other alternative protein ingredients, including canola protein concentrates and Indian Mustard concentrate (89–90%; Thiessen et al., 2004; Chowdhury et al., 2012).

The ADCs of crude protein (69%) and essential amino acids (59–75%) in *Nannochloropsis* sp. were depressed compared to *Isochrysis* sp. when considered as individual ingredients. The ADC of protein in whole cells of *Nannochloropsis* sp. was consistent with protein digestibility (72%) of *Nannochloropsis* sp. co-product (left-over algal meal from commercially grown algal biomass after extraction of oils for nutraceutical, chemical, or fuel applications) fed to Atlantic salmon (Gong et al., 2018). The values were, however, slightly lower than those for protein (81.1%) and essential amino acids (67–88%) of whole cells of *Nannochloropsis* sp. fed to Nile tilapia (Sarker et al., 2018). We found that the ADCs of crude protein (87%) and four essential amino acids, isoleucine (63%), threonine (67%), tryptophan (12%), and valine (59%), in the *Nannochloropsis* sp. were much lower ($P < 0.01$) than their ADCs in the *Isochrysis* sp. ingredient for rainbow trout.

The DHA-rich *Isochrysis* sp. showed an extremely high ADC of total n3 and n6 fatty acids both in test diets and individual ingredients. These results fit with previous studies, which reported ADCs of total n3 fatty acids including DHA > 99.4% in rainbow trout and tilapia when fish were fed another DHA-rich microalga *Scizochytrium* sp. (Sarker et al., 2016; Belange-Lamonde et al., 2018). Digestibility of SFA from the algal biomass that we used varied in the range formerly detected, between 60 and 99% (Caballero et al., 2002; Ng et al., 2010; Bélanger-Lamonde et al., 2018). Atlantic salmon fed varying levels of n3 and SFA were reported to have ADCs for total SFA in the above range (Menoyo et al., 2003). Similarly, in our experiment, the lowest ADC of total SFA was found in both the *Nannochloropsis* sp. and *Isochrysis* sp. test diets and the individual ingredients, which may have been due to higher total PUFA and n3 PUFA in the *Nannochloropsis* sp. and *Isochrysis* sp. In general, fish intestines show preferential digestion of PUFA and n3 PUFA over SFA. However, with the exception of C18:3n3 ALA, the ADC of all fatty acids was lower in *Nannochloropsis* sp., which may be attributable to known resistance of the complex cellulosic algal cell wall structure (including higher fiber content) of this alga to digestive enzymes, potentially inhibiting digestion by trout (Rodehutsord et al., 2000; Scholz et al., 2014). Despite the high digestibility of C18:3n3 ALA, the lower ADC of all fatty acids and the overall reduction in digestibility of protein, amino acids, and energy in the *Nannochloropsis* sp. versus *Isochrysis* sp. may explain the observed growth retardation in fish fed diets using only *Nannochloropsis* sp. to substitute for fishmeal and fish oil (NI, NS, and NIS diets).

Differences in cell wall structure may partially explain the ADC differences between the two microalgal species.

We visually observed that the *Isochrysis* sp. diet produced compact feces, while the *Nannochloropsis* sp. diet resulted in feces that were mostly disintegrated. The differences observed between these two diets, both in digestibility and feces texture, may have resulted from differences in the cell wall structure of each of these microalgae. Nuño et al. (2013) reported that the cell wall of *Nannochloropsis* sp. is thicker and more rigid than that of *Isochrysis* sp., causing difficulty in digestion with modified nutrient absorption and feces texture in diabetic rats. The lower ADCs of nutrients and energy in the *Nannochloropsis* sp. compared to *Isochrysis* sp. ingredient could be attributed to the relatively non-starch polysaccharides, trypsin inhibitor, and fiber content. In the present study, the ADC of fiber in the *Nannochloropsis* sp. ingredient was significantly lower (38%) than the ADC of fiber in the *Isochrysis* sp. (96%), suggesting that rainbow trout did not effectively digest the whole cells of *Nannochloropsis* sp. due to its high content of complex polysaccharides (Scholz et al., 2014). We have also reported the depressed ADC of dietary nutrients and energy in tilapia due to these anti-nutritional factors (Sarker et al. 2016b, 2018). We speculated that high content of anti-nutrients and fiber might inhibit proteolytic enzymatic activity and induction of amylase activity, which eventually affects the efficiency of protein and amino acid utilization (Rodehutsord et al. 2000; Encarnaç o et al., 2004). Several studies have revealed that non-starch polysaccharides have a negative influence on digestion and absorption by modulating digesta viscosity and rate of passage and by altering gut physiology and morphology in fish (Glencross et al., 2008; Brinker and Reiter, 2011; Norambuena et al., 2015).

Microalgae-based diets are currently in the preliminary stage of development for rainbow trout and other fish species. Further processing of the microalgal ingredient into concentrates, disruption of cell walls, and extrusion pelleting may be needed to increase both the protein and the energy digestibility of certain microalgal species. Exogenous proteases may also augment endogenous peptidases by increasing protein digestibility and hydrolyzing proteinaceous anti-nutritional factors such as lectins, trypsin inhibitors and antigenic proteins (Douglas et al., 2000; Cowieson et al., 2008).

5.3. Effects of microalgae replacing fish ingredients on feed intake and growth

Our results present the first attempt to fully replace dietary fishmeal and fish oil in rainbow trout diet with a combination of microalgal biomass and canola oil. The fish fed the 100% fish-free, microalgae-containing diets displayed no major health or mortality problems during the 12-week experiment (juvenile life phase), but they did exhibit lower growth compared to fish fed the reference diet containing fishmeal and fish oil. This depressed growth was due to a significantly lower feed intake. This result is consistent with findings from previous studies which showed that lower growth performance in rainbow trout fed diets containing increased levels of plant ingredients was linked to reduction in feed intake (Panserat et al., 2009; Lazzarotto et al., 2018). Decreased growth was also

observed in Atlantic salmon fed a near-completely plant-based diet, compared to fish that were fed a (plant-free) diet containing fishmeal and fish oil (Torstensen et al., 2008). The authors of these studies suggested that the lower growth observed was related to the substitution of fishmeal, rather than the replacement of fish oil. Several other studies have demonstrated the optimal proportions (partial or full) for the substitution of fish oil by plant oil without compromising fish growth or health status (Refstie et al. 1998, 2000; Menoyo et al., 2005; Torstensen et al., 2005; Espe et al., 2006, 2007; Drew et al., 2007; Turchini et al. 2010; Hixson et al., 2014). In a number of studies, the growth of rainbow trout and Atlantic salmon fed a 100% vegetable oil-based diet was comparable to growth when fed a 100% fish oil diet (Menoyo et al., 2005; Drew et al., 2007; Hixson et al., 2014).

In our study, one of the factors affecting feed intake may have been the low palatability of microalgae diets. The significantly lower feed intake of fish fed microalgae diets was a primary cause of the poor weight gain of fish in these treatments, due to reduced nutrient intake relative to fish fed the reference diets. Walker and Berlinsky (2011) also reported that replacement of 15 and 30% of fishmeal protein with a microalgae mix of *Nannochloropsis* sp. and *Isochrysis* sp. caused a significant growth reduction in Atlantic cod. The authors concluded that reduced palatability of the algal meal caused the deterioration in cod growth.

5.4. Effects of microalgae replacing fish oil on fatty acid profile of rainbow trout

Dietary fatty acid composition has an obvious effect on the fatty acid profile of fish fillet and liver (Tan et al., 2009; Aliyu-Paiko et al., 2010; Ramezani-Fard et al., 2012). Farmed salmonid species are often marketed for their health-promoting properties, primarily their high n3 LC-PUFA content. However, this benefit has declined as current feeds have lower levels of n3 LC PUFA (DHA and EPA) because they contain vegetable oils as substitutes for fish oil. Over the past decade, the use of terrestrial sources oil in aquafeeds has significantly lowered levels of the beneficial DHA in the farmed salmonids (Berntssen et al., 2011; Sprague et al., 2016). We detected an equal amount of the deposition of DHA (essential key fatty acid for human) in fillets of trout fed fish-free feed with the combination of *Nannochloropsis* sp., *Isochrysis* sp., and *Schizochytrium* sp. (NIS diet) as in trout fed the reference diet. We also found that the EPA level was reduced in the fillet and liver of fish fed all microalgae diets. These DHA and EPA results suggest that the DHA content of the whole-cell biomass of *Isochrysis* sp. and *Schizochytrium* sp. was digested and absorbed well, whereas the biomass of EPA-rich *Nannochloropsis* sp. was not digested or absorbed as well as the fish oil. Similar results were also reported in earlier studies (Miller et al., 2007), suggesting that dietary fish oil totally replaced with *Schizochytrium* oil significantly increased the amount of DHA in juvenile Atlantic salmon muscle but also significantly reduced the level of EPA. Furthermore, we detected that the DHA content of the liver of fish fed all microalgae-combining diets (NI, NS, NIS) did not differ

significantly from that of fish fed the reference diet. Given the crucial importance of DHA in salmonids, our results indicate that DHA levels in farmed trout can be manipulated and tailored by altering the DHA composition of their diets by combining DHA-rich whole cells of microalgae (*Isochrysis* sp. and *Schizochytrium* sp.) with canola oil. The present study also suggests that whole-cell biomass of *Isochrysis* sp. and *Schizochytrium* sp. would be good candidates for DHA supplementation in trout diet formulated with vegetable oil. We recently reported replacing 75% of total fish oil in rainbow trout feed by combining whole cells of *Schizochytrium* sp. with canola oil as an efficient way to maximize the total fish oil replacement and also increase deposition of DHA and reduce exposure to persistent organic pollutants in fish fillet (Bélanger-Lamonde et al., 2018).

6. Conclusion

Our results for ADC of crude protein, amino acids, lipid, and fatty acids suggest that *Isochrysis* sp. is a potential substitute for fishmeal and fish oil or as a supplement for long-chain PUFA. Our results also point to further research to explore freeing the amino acids and fatty acids from *Nannochloropsis* sp. by enzymatic or extrusion processing to improve its digestibility. From a study of *in-vitro* digestibility we have shown that adding NSP enzymes to tilapia feed enhanced digestibility of protein supplied by *Nannochloropsis* co-product (left over biomass after oil extraction) ingredients (unpublished data). We are also examining extrusion processing, including whether the inclusion of one or more non-starch polysaccharide and protease enzymes in a microalgae diet enhances nutrient digestibility and retention/absorption and growth in fish and reduces nutrient loading in effluent. Finally, the reduced palatability that resulted in lower weight gain in this study may be possible to mitigate. Incorporating feeding stimulants (that promote ingestion and continuation of feeding; for example, taurine) in *Nannochloropsis* sp.-based diets for rainbow trout might improve palatability and growth performance (Gaylord et al., 2008; El-Sayed and Abdel-F, 2014), making possible the achievement of higher replacement levels of fishmeal and fish oil in trout feed.

Marine microalgae are recognized as among the most prominent future sustainable sources of n3 LC-PUFA to add to vegetable oils and replace fish oil in aquaculture feeds. Mainly heterotrophic DHA-rich *Schizochytrium* sp. have been investigated for this purpose in aquafeeds (Wang et al. 2017; Bélanger-Lamonde et al 2018). We acknowledge that DHA-rich microalgae-based oil, especially from *Schizochytrium* sp., needs to become cost-competitive with sources of fish oil for aquaculture feeds, as pure algal oil costs twice as much as fish oil in the present market (20 April 2017; Terazono, 2017). However, *Schizochytrium* sp. still offers potential as an alternative to fish oil, due to both production volume requirements and the economics of producing heterotrophic microalgae when compared to phototrophic microalgae. In contrast to phototrophic microalgal production, the production of heterotrophic microalgae is much easier via fermentation,

which utilizes sugars and other carbon sources (e.g., corn) for energy, is short-term (a few days) and, therefore, can be tailored to meet market requirements.

Several leading aquafeed companies recently have started including DHA-rich oil from *Schizochytrium* sp. into salmon feeds (Tocher et al., 2019). For example, within the last year, many agribusiness giants and animal nutrition companies (Corbion, BioMar, Archer Daniels Midland, ADM, and Veramaris) began developing fish oil substitutes with heterotrophic *Schizochytrium* sp., presumably due to the high production volumes and favorable economics of producing *Schizochytrium* sp. biomass. Veramaris (commercially using pure *Schizochytrium* oil) recently officially joined the future of fish feed/fish-free feed (F3) Fish Oil Challenge, a global challenge to sell the most “fish-free” fish oil for aquafeed in order to reduce demand pressures on wild-caught stocks (Under Current News, 2019). UK retailer Tesco has given feed ingredients like natural marine algal oil the thumbs-up for use in its salmon supply chain (Under Current News, 2019). However, the full potential of the environmental impact of phototrophic microalgae and heterotrophic microalgae in aquaculture remains to be realized. Towards this end, we are focusing our ongoing research on life cycle environmental analysis of microalgae-based (phototrophic and heterotrophic) fish-free feed for aquaculture.

Data Accessibility Statement

We have presented extensive data generated by this study in the tables and supplemental materials. Contact the corresponding author for further requests.

Supplemental files

The supplemental files for this article can be found as follows:

- **Table S1.** Ingredient composition of the reference diet and microalgae test diets for digestibility experiment. DOI: <https://doi.org/10.1525/elementa.404.s1>
- **Table S2.** Proximate chemical composition, energy, essential amino acid and fatty acid profiles of *Nannochloropsis* sp whole cells and *Isochrysis* sp whole cells as test ingredients. DOI: <https://doi.org/10.1525/elementa.404.s2>
- **Table S3.** Proximate, energy, amino acid and fatty acid profiles of the reference and test diets. DOI: <https://doi.org/10.1525/elementa.404.s3>
- **Table S4.** Formulation, proximate composition, and essential amino acids of four experimental diets for juvenile rainbow trout. DOI: <https://doi.org/10.1525/elementa.404.s4>
- **Table S5.** Fatty acid profiles of the experimental diets for nutritional feeding experiment. DOI: <https://doi.org/10.1525/elementa.404.s5>
- **Table S6.** Apparent digestibility coefficients of individual fatty acids in the reference diet and test diets for rainbow trout. DOI: <https://doi.org/10.1525/elementa.404.s6>
- **Table S7.** Test ingredient apparent digestibility coefficients of individual fatty acids in *Nannochloropsis* sp.

and *Isochrysis* sp. for rainbow trout. DOI: <https://doi.org/10.1525/elementa.404.s7>

- **Table S8.** Individual fatty acid content of fillets from rainbow trout fed experimental diets for 84 days. DOI: <https://doi.org/10.1525/elementa.404.s8>
- **Table S9.** Individual fatty acid content of liver from rainbow trout fed experimental diets for 84 days. DOI: <https://doi.org/10.1525/elementa.404.s9>

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Competing interests

No competing interests. Anne R. Kapuscinski is one of Elementa's Editors-in-Chief. She was not involved in the review process of this manuscript.

Author contributions

Conceived and designed the experiments: Pallab K. Sarker, Anne R. Kapuscinski; Performed the experiments: Emilie Proulx, Grant, W. Vandenberg; Analyzed the data: Pallab K. Sarker; Contributed reagents/materials/analysis tools: Pallab K. Sarker, Alex Sitek; Wrote- original draft: Pallab K. Sarker; Wrote- review & editing: Pallab K. Sarker, Anne R. Kapuscinski, Grant, W. Vandenberg, Emilie Proulx, Alexander J. Sitek.

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