Evaluation of *Schizochytrium* sp. as a source of DHA and *Haematococcus pluvialis* as a source of astaxanthin pigment in diets for farmed salmonids

by

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Dalhousie University is located in Mi'kma'ki,
the ancestral and unceded territory of the Mi'kmaq.
We are all Treaty people.

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ABSTRACT

Two studies were conducted to evaluate the digestibility of docosahexaenoic acid (DHA)-rich *Schizochytrium* sp. (Sc) in diets for Atlantic salmon (*Salmo salar*) and to assess the fillet pigmenting efficacy of a weakened whole cell *Haematococcus pluvialis* (Hp) in diets for rainbow trout (*Oncorhynchus mykiss*). Apparent digestibility coefficients (ADCs) for Sc by Atlantic salmon for DHA, lipid, protein, and energy, lipid, and DHA were 96.3%, 67.1%, 93.9%, and 70%, respectively. Pigmentation of rainbow trout by Hp biomass (0.31% Ax by dry weight) showed no significant ($p>0.05$) differences in growth, Ax digestibility (76.7%), fillet Ax content (8.86 mg/kg), retention (15.4%), *L*a*b**, or SalmoFan™ readings between diets containing Hp and synthetic Ax. Sc is a highly digestible source of polyunsaturated fatty acids and DHA, and the weakened-cell Hp ingredient is a bioavailable source of Ax that can achieve flesh coloration levels comparable to that of synthetic Ax in diets for farmed salmonids.

LIST OF ABBREVIATIONS USED

AA	Amino acid
ADC	Apparent digestibility coefficient
ALA	α-linolenic acid, 18:3n-3
ANOVA	Analysis of variance
ARA	Arachidonic acid, 20:4n-6
Ax	Astaxanthin
CP	Crude protein
DHA	Docosahexaenoic acid, 22:6n-3
DE	Digestible energy
DP	Digestible protein
DW	Dry weight
EAA	Essential amino acid
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid, 20:5n-3
FA	Fatty acid
FAME	Fatty acid methyl ester
FO	Fish oil
FM	Fish meal
FCR	Feed conversion ratio
HP	*Haematococcus pluvialis*
HPLC	High performance liquid chromatograph
K	Condition factor
LC-PUFA	Long chain polyunsaturated fatty acid
LNA	Linoleic acid, 18:2n-6
NAR	Net apparent retention
NEAA	Non-essential amino acid
n-3	Omega-3
n-6	Omega-6
MS-222	Tricaine methanesulfonate
MUFA	Monounsaturated fatty acid
PIT	Passive integrated transponder
PUFA	Polyunsaturated fatty acid
RM-ANOVA	Repeated measures analysis of variance
SC	*Schizochytrium* sp.
SD	Standard deviation
SFA	Saturated fatty acid
SGR	Specific growth rate
TAG	Triacylglycerol
TFA	Total fatty acids

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CHAPTER 1.0

INTRODUCTION

Aquaculture now accounts for more than 50% of seafood consumption by humans (FAO 2020), and the aquafeed industry is developing novel nutritional and functional ingredients to improve the sustainability, economics, and social acceptance of aquaculture. Since 2009, salmonid production has grown by 30% globally, and in 2018 production totalled 3.6 million tonnes; of which Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) accounted for 69% and 24% of production, respectively (FAO 2018). In 2018, salmonid production in Canada was approximately 135 000 metric tonnes and valued just under one billion dollars (CAD). Atlantic salmon comprise the majority (65%) of aquaculture production, and rainbow trout (5% of production) are becoming an increasingly relevant species to Canadian aquaculture with a 93% increase in production since 2011 (FAO 2018).

Finfish aquaculture is highly dependent on capture fisheries as a source of protein and essential fatty acids (EFAs) in commercial fish feeds. Nineteen percent of capture fishery production was reduced to fish meal (FM) and fish oil (FO) for aquafeeds in 2018 (FAO 2020). The finite supply of these ingredients coupled with the growth of salmonid farming have made finding alternative sustainable sources of protein and oil, particularly the two conditionally essential long-chain polyunsaturated fatty acids (LC-PUFAs) docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) a major challenge and priority for the aquaculture industry (Jones et al. 2015).

Salmonids require a dietary source of the omega-3 EFAs, EPA and DHA for healthy growth and development (Tocher 2003). The growing industry and global fish consumption combined with a plateau in wild fish stocks has led to an increase in cost of FM and FO and a decline in their use in aquafeeds. As a result, a substantial proportion (60% of FM and FO combined; Hodar et al. 2020) of these ingredients have successfully been replaced by cheaper terrestrial animal and plant ingredients such as poultry fat and soybean meal and oil (Hodar et al. 2020). The consumption of fatty fish by humans has a wide range of health benefits directly related to the consumption of EPA and DHA (Swanson et al. 2012). However, this decline in omega 3-rich ingredient inclusion in aquafeeds has significantly influenced the fatty acid (FA) profile of farmed salmonids by reducing the EPA and DHA content of the fillets (Sissener 2018). Consequently, this lowers the nutritional benefit to the human consumer and was deemed cause for concern by the International Fish Meal and Fish Oil Organization (Sprague and Tocher 2016). For the last 20 years, the aquafeed industry has sought ingredients rich in EPA and/or DHA to supplement increasingly plant-based aquafeeds to restore the fatty acid (FA) profile of farmed fish and increase the nutritional benefits to the consumer.

Farmed Atlantic salmon are a high-end product aimed at the mid-upper class whose sophisticated tastes motivate another aspect of aquafeed formulation: salmonid pigmentation. The color of salmonid fillets significantly influences consumer perception of product quality and value, and consumers are willing to pay more for deep pink/red colored fillet (Steine et al. 2005). Astaxanthin (Ax) is a carotenoid pigment responsible for the coloration of wild and farmed salmonids (Higuera-Ciapara et al. 2006). Salmonids are

not able to endogenously synthesize astaxanthin. In the wild, Ax is produced by single celled organisms at the base of the food wed and salmonid pigmentation is achieved from their prey via bioaccumulation, while in intensive farming, synthetic Ax beadlets are added to the feed (Lim et al. 2018). The global Ax market was estimated at $1.2 billion CAD in 2019, and synthetic Ax comprises >95% of the feed market with DSM (Dutch State Mines; Denmark) the global leading manufacturer (Lim et al. 2018 Stachowiak and Szulc 2021). The global Ax market is projected to reach $4.3 billion CAD by 2027, and despite algal Ax currently only comprising <1%, biological sources are gaining potential due to consumer demand for non-synthetic products (Stachowiak and Szule 2021). Growth of the aquaculture and Ax industries coupled with consumer demand for non-synthetic feed additives have led to a significant increase in research and development of Ax from non-synthetic sources such as crustacean by-products, yeasts, and microalgae as dietary pigments in farmed fish (Higuera-Ciapara et al. 2006; Zhang et al. 2020).

The global production of microbial products was valued at $6.7 billion CAD in 2017, and their use in aquafeeds has expanded over the last decade with various species of yeast, bacteria, microalgae and other microbes now being used as sources of protein, omega-3 FAs, and Ax in aquafeeds to reduce the industry's reliance on FO, FM and synthetic Ax (Tibbetts 2018; Jannathulla et al. 2021). The protist, *Schizochytrium* sp. (Sc) and microalgae, *Haematococcus pluvialis* (Hp) are two single-cell ingredients with high potential as sources of DHA and Ax, respectively (Lorenz and Cysewski 2000; Wang et al. 2021). Dietary Sc inclusion increases flesh DHA content in Atlantic salmon (Kousoulaki et al. 2015), but inclusion levels are negatively correlated with lipid and

energy diet digestibility (Zhang 2013; Kousoulaki et al. 2015), and Sc-specific digestibility has not been assessed for Atlantic salmon. This lack of digestibility data is a gap in the knowledge surrounding the utility of this novel feed ingredient (Glencross et al. 2020).

Researchers have not been able to achieve flesh pigmentation similar to that of synthetic Ax using Hp whole cells (Sommer et al. 1991;1992) without cell disruption (Young et al. 2016), or carotenoid extraction (Bowen et al. 2002) in salmonid diets. Hp cells possess a rigid cell wall that prevents the direct use of whole cells as an aquafeed pigment and requires pre-rupture or extraction in order to increase Ax bioavailability prior to feed formulation. Increased production costs, decreased product stability, and scalability challenges associated with cell-rupture and extraction significantly impact the ability for Hp to compete with synthetic Ax (Mendes-Pinto et al. 2001). For Hp to be a successful aquafeed pigment, a product with improved Ax bioavailability is needed.

This thesis aims to address these unanswered questions by: 1) Assessing digestibility of Sc whole cells in diets for Atlantic salmon (*Salmo salar),* and 2) Assessing the pigmenting efficacy of a novel form of Hp that is produced with a weakened cell wall structure in diets for rainbow trout (*Oncorhynchus mykiss*) which aims to overcome Ax availability challenges associated with the typical rigid cell form. Chapter 2 provides a review of the literature and necessary background information surrounding key topics of interest to this thesis. Chapter 3 reports the digestibility of Sc by Atlantic salmon. Chapter 4 reports the pigmenting ability of a novel weakened whole cell Hp product in rainbow trout, and

Chapter 5 summarizes the key findings, research significance, study limitations and suggestions for future research.

The results from Chapter 3 of this thesis have been submitted o *Aquaculture* for publishing as an independent manuscript and was published on November 17th, 2020.

CHAPTER 2.0

LITERATURE REVIEW

This chapter presents a comprehensive review of the literature pertaining to the role of the two species of interest in this thesis, *Schizochytrium* sp. and *H. pluvialis*. Focusing on lipids and proteins in salmonid nutrition, the use of and alternatives to FO, FM, and synthetic Ax in aquafeeds, and microbial ingredients and their role(s) in aquafeeds. The aim of this review is to establish the state of knowledge surrounding the use of Sc and Hp in salmonid feeds and identify current gaps in the literature which will provide the necessary rationale for the experimental work presented in Chapters 3 and 4 of this thesis.

2.1 SALMONID NUTRITION

2.1.1 LIPIDS AND FATTY ACIDS

Lipids are integral to proper health and development of all vertebrates and are fundamental for daily bodily functions and reproduction (De Silva et al. 2012). Lipids are the primary source of energy and EFAs for fish and are carriers of vitamins, carotenoids and precursors to hormones and enzyme co-factors (De Silva et al. 2012). Lipids are typically composed of FAs, which are carbon chains with a carboxylic acid group and methyl group on each end of the molecule. FA carbon chains are either completely saturated and termed saturated fatty acids (SFAs) or contain methylene double bonds between one or more of the carbon atoms and are termed unsaturated fatty acids. Monounsaturated FAs (MUFAs) contain only one double bond, while polyunsaturated FAs (PUFAs) contain at least two (Tocher 2003). PUFAs are further classified by their number and positioning of the double bonds as well as their chain length. PUFAs with a double bond in the third (n-3) position from the methyl

end of the chain are part of the omega-3 family of FAs, with the same nomenclature applying to the omega-6 FAs. PUFAs with a chain length greater than 20 carbons are termed long-chain PUFAs (LC-PUFAs) (Tocher 2003).

Fatty acids that are required for survival but cannot be synthesized within the body are considered "essential" (Sargent et al. 2003). Alpha-linolenic (ALA; 18:3n-3) and linoleic acids (LNA; 18:2n-6) are the precursors to all omega-3 and 6 fatty acids and cannot be synthesized by vertebrates so are therefore classified as EFAs (Brenna et al. 2009). Two important long-chain polyunsaturated FAs, EPA and DHA, are formed from the elongation and desaturation of ALA (Sargent et al. 2003; Brenna et al. 2009). Arachidonic acid (AA) is formed from the elongation and desaturation of LNA. The conversion of ALA to EPA and DHA in mammals and carnivorous fish is not enough to produce adequate amounts for healthy growth and development, therefore they are deemed "physiologically essential" (Tocher 2015). The total n-3 PUFA dietary requirement of salmonids including ALA, EPA and DHA can range from 1 to 2.5% of the diet and is influenced by the proportion of lipid in the diet, life stage, and stress level (NRC 2011; Huyben et al. 2021; 2021).

Lipids are both a source of energy and structural support within the cell. Lipids generate energy in the form of ATP via mitochondrial β-oxidation, a complex pathway where FAs are broken down via a series of four steps (dehydrogenation, hydration, oxidation, and thyolisis) within the mitochondrial matrix to produce energy (Calder 2016). Lipids are also the most abundant component of the plasma membrane and play a key role in cell structure and signalling by contributing to membrane rigidity and shape and acting as secondary

messengers (Sargent et al. 2003; Sunshine and Iruela-Arispe 2017). EPA and DHA are important for eye and brain development, lipid metabolism, and cardiovascular function (Calder 2014). DHA is an important FA in neural tissues, and EPA plays an important role in reducing inflammatory damage by dampening the effects of eicosanoid production by AA that is associated with a high intake of LNA, and a high n-6:n-3 ratio in the diet (Sargent et al. 2003). The ratio of DHA:EPA in FO ranges from 1.2-1.9 (NRC 2011) however, the n-3 PUFA "sparing" effect suggests a DHA: EPA >1 may enhance n-3 PUFA tissue deposition and minimize EPA loss to β-oxidation (Codabaccus et al 2012). DHA + EPA deficiency can cause reduced growth, fin erosion, impaired immune function and increased mortality (Sargent et al. 2003; Glencross et al. 2014; Huyben et al. 2021). Studies examining the discrete roles and essentiality of EPA and DHA in Atlantic salmon are lacking. Emery et al (2016) and Glencross et al. (2014) both observed preferential catabolism of EPA and consistent retention of DHA despite lower dietary EPA concentrations in Atlantic salmon. This indicates DHA alone can maintain fish health, but the synergistic effects of DHA+EPA in the diet result in benefits to growth, feed conversion, and health markers which reinforces the importance of both FAs in the diets of Atlantic salmon (Glencross and Rutherford 2011; Glencross et al. 2014).

The inclusion of EPA and DHA in salmonid feed serves two primary functions: to ensure adequate growth and development of the fish, and as a vector for the delivery of physiologically essential FAs to humans. In humans DHA and EPA intake can prevent cardiovascular disease, rheumatoid arthritis, and neurological disorders (Calder and Yaqoob 2009; Laye et al. 2018). Fish consumption is the main route of DHA and EPA

delivery to humans (Gladyshev et al. 2013), as the majority of EPA and DHA are produced by plants and single-celled organisms within the aquatic environment, with minimal contribution by terrestrial plants and algae. Over the last decade, inclusion of ingredients such as FM and FO that are high in EPA and DHA in salmonid aquafeeds has declined from 46 to <10%. From 2006 to 2015, EPA+DHA content of Scottish farmed salmon decreased from 2.8 g/100 g to 1.4 g/100 g which compromises the nutritional value to the consumer (Sprague and Tocher 2016; Sissener 2018; FAO 2020). Therefore, it is imperative that EPA and DHA are included in commercial diets for farmed salmonids and this thesis has contributed to the growing knowledge base of potential EPA+DHA rich aquafeed ingredients by investigating the digestibility of the DHA-rich *Schizochytrium* sp.

2.1.2 PROTEINS AND AMINO ACIDS

Proteins are essential macronutrients for the growth and development of all living organisms. They provide nitrogen and essential amino acids (EAAs) in the form of amino acid (AA) chains covalently joined by peptide bonds. Protein can account for up to 75% of fish tissues (on a dry matter basis), which are comprised of twenty-two unique amino acids (Wilson 2003). AAs are the building blocks for all proteins, with each protein having a unique number, sequence and configuration. When protein is consumed by the fish, it is broken down via digestive enzymes called proteases to release free amino acids which are absorbed into the blood stream and distributed throughout the body and utilized by organs and tissues to synthesize new proteins (Wilson and Halver 1986). Non-essential amino acids (NEAAs) can be synthesized adequately by the fish from metabolic intermediates, while conditionally essential AAs can be synthesized by the fish, but depending on dietary

supply of precursors, life stage, and overall health, rates of utilization can surpass rates of synthesis and supplementation is sometimes required (Lall and Anderson 2005; Li et al. 2009). Conditionally essential AAs for fish are cysteine, glutamine, hydroxyproline, proline, and taurine (Li et al. 2009). Arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine cannot be synthesized in sufficient quantities to support optimum growth and are deemed EAAs which must be included in the diet (Lall and Anderson 2005; NRC 2011). Additionally, conditionally essential amino acids

Dietary protein requirements for salmonid fish range from 40-50% for maintenance by replacing existing and/or lost proteins, and for growth which requires the building of new proteins (Lall and Anderson 2005; De Silva et al. 2012). This requirement is necessary for two reasons: to supply sufficient amounts of EAAs, and to supply NEAAs for growth and subsequent synthesis of other NEAAs (Lall and Anderson 2005). If these requirements are not met, protein catabolism can occur, which can result in weight loss, and withdrawal of protein from muscle tissue to sustain the function of vital tissues and organs. Contrarily, if too much protein is provided, only what is needed will be used for protein synthesis, and the remainder will be converted to energy or excreted as excess nitrogen if not digested (Peragon et al. 1994). Additionally, protein requirements are influenced by protein-to-energy balance. Excess energy from non-protein sources may limit protein consumption, and conversely, if insufficient energy is available, protein will be used to satisfy the energy requirement of the fish (Wilson 2003).

Requirements for each EAA differ between fish species, however, reduced growth coupled with symptoms of deficiency are generally common across species (Lall and Anderson 2005). For example, fin erosion, cataracts, and scoliosis are associated with lysine, methionine, and tryptophan deficiency, respectively (Poston et al. 1977; Walton et al. 1984a; NRC 2011). If all EAA requirements are not met, those that are limiting will restrict growth (Lall and Anderson 2005; De Silva et a. 2012). To avoid this, synthetic crystalline AAs are often added to the feed to increase the quantities of limiting EAAs. In salmonid feeds, the most common limiting EAAs are typically lysine and methionine (NRC 2011). When formulating aquafeeds it is important to consider protein quality, which is generally determined by AA composition and quantity as well as their digestibility and subsequent bioavailability to the fish (Boye et al. 2012). Understanding the quantity of digestible protein available from an ingredient to the target species is an essential step in understanding the effectiveness of a novel ingredient. Although this thesis primarily focuses on the role of Sc in aquafeeds as it pertains to DHA supplementation, whole cell algal biomass contains additional macronutrients such as protein which can add to the value of this novel ingredient.

2.2 ROLE OF FISH MEAL AND OIL IN AQUAFEEDS

The principal sustainability issue in the farming of carnivorous fish is the use of marine resources in aquafeeds. FM is a highly digestible source of protein, containing a well-balanced amino acid profile (Table 2.1), oil fraction that includes LC-PUFAs (Table 2.2), increases feed palatability, is free of anti-nutritional factors, and contains bioactive compounds such as flavonoids, nucleotides and water-soluble peptides that are known to

positively influence growth (Hardy 2010; Kousoulaki et al. 2015; Turchini et al. 2019). FO contains equal proportions of the three major FA groups (SFAs, MUFAs, and PUFAs), and is particularly high in essential LC-PUFAs, DHA (4.9-12.6% TFA) and EPA (8.4-17.0% TFA; Table 2.2) (Tocher 2015).

Of the 179 million tonnes of total fish production in 2018, 88% was utilized for direct human consumption while approximately 10% (18 million tonnes) of fish were reduced to FM and FO for aquafeeds. Furthermore, annual catches of anchoveta and jack mackerel (*Trachurus murphyi*), two common species reduced to FM and FO, have declined by 50% since 1994 (FAO 2020). A reduction in supply coupled with a growing aquaculture industry has drastically increased the prices of FM and FO over the last decade from $1500 to $1800 and $1000 to $2500 CAD/tonne, respectively (FAO 2020). Fish feed can account for up to 50% of carnivorous finfish production costs (Sarker et al. 2013), therefore rising ingredient costs are a major area of concern for the industry. Due to price increases and concerns over socially sustainable aquaculture, the inclusion of FM and FO in salmon feeds has declined from 46% in 2007 to as low as <10% in favour of cheaper, more available ingredients such as soybean meal ($440 CAD/tonne), wheat and corn gluten. and soybean oil ($1000 CAD/tonne; Tacon and Metian 2008; FAO 2020).

The proportion EPA and DHA in salmon feeds has declined by approximately 50%, with an increase in terrestrial FAs oleic (18:1n-9) and linoleic (18:2n-6; Sprague and Tocher 2016). This has significantly altered the FA profile of Atlantic salmon, which may negatively impact fish health and growth, as well as reduce the nutritional benefits of fish

to consumers (Sissener 2018). For example, a study on Scottish farmed salmon determined that in 2015, two 130 g portions of salmon would be needed to obtain the same amount of EPA+DHA as one 130 g portion in 2006 (Sprague and Tocher 2016). It is clear that both economic and environmental sustainability concerns have driven the aquaculture industry to reduce its dependence on ocean-caught resources in aquafeeds by increasing the proportion of terrestrial ingredients. An important question remains that if the salmonid farming industry continues to expand, where will the EPA+DHA in aquafeeds come from?

2.2.1 FISH MEAL AND OIL ALTERNATIVES

The replacement of FM and FO in aquafeeds for carnivorous fish has been an important goal over the last decade (Naylor et al. 2009; Turchini et al. 2019). Alternative marine resources such as fishery by-products, by-catch, and lower trophic level organisms such as krill have been used in aquafeeds (Olsen et al. 2010), however, the dependency on any marine resource presents the same set of availability and environmental challenges associated with FM and FO. A wide range of ingredients of plant, terrestrial animal, and microbial origin that have the potential to supplement or reduce the industry's dependence on marine ingredients in aquafeeds (Gatlin et al. 2007). This ongoing research effort will continue to improve our understanding of how various existing and novel raw materials affect the health and growth of many farmed aquatic species. Additionally, this thesis will aid in formulating aquafeeds that contain a mixture of complementary ingredients in attempt to reduce the industries reliance on singular finite materials such as FM and FO.

2.2.1.1 PLANT ALTERNATIVES TO FISH MEAL AND OIL IN AQUAFEEDS

Common plant protein sources used in aquafeeds include soybean meal, wheat gluten meal, corn gluten meal, and soy or corn protein concentrates (Table 2.1) (Hardy 2010; NRC 2011). The majority of these ingredients are inexpensive and widely available, and their increased inclusion in salmonid feed formulations has significantly reduced the FM content of the diet (Olivia-Teles et al. 2015). However, these ingredients present several challenges when included at high levels in salmonid diets. The inclusion of plant-based proteins has nutritional limitations due to the presence of non-soluble carbohydrates and undesirable pigments or anti-nutritional factors such as phytic acid, saponins, protease inhibitors, and non-starch polysaccharides can cause mineral deficiencies, interfere with protein and lipid uptake, and decrease feed intake in soybean meal (Uran et al. 2009; Hardy et al. 2010). For example, the anti-nutritional factors present in soybean meal can cause intestinal enteritis as well as reduced palatability in salmonid feeds (Uran et al. 2009). Additionally, some plant proteins such as corn gluten meal possess carotenoid pigments which alter salmonid flesh color (Hardy 1996).

Current plant-based alternative lipid sources include canola oil, soybean oil, flaxseed oil, and palm oil (Table 2.2; Miller et al. 2008). Partial replacement of fish oil with plant oils has been widely studied for the past 20 years (Caballero et al. 2002; Turchini et al. 2019). Plant oils are a good source of digestible energy however, they lack DHA and EPA (Table 2.2), which can have a negative impact on fish health, and human health may also be impacted as a result of reduced DHA and EPA intake (Hixson et al. 2013). This lack of EPA and DHA in plant oils is a major factor limiting complete substitution of FO, as well as a major factor contributing to the decline in EPA and DHA content of farmed salmonids

(Shepherd et al. 2017). Transgenic plant oils may be a solution to this (Codabaccus et al. 2011; Lenihan-Geels et al. 2013), however the use of genetically modified organisms presents challenges in terms of consumer acceptance, ethics, and food safety (Osmond and Colombo 2019).

2.2.1.2 ANIMAL ALTERNATIVES TO FISH MEAL AND OIL IN AQUAFEEDS

Terrestrial animal meals and oils have been used to supplement FM and FO in salmonid feeds for decades (Kaushik 1990; Hodar et al. 2020). Such ingredients commonly include: poultry fat, poultry by-product meal, blood meal, feather meal (Table 2.1; Table 2.2; NRC 1993; FAO 2012; NRC 2011). Terrestrial by-products are readily available, economical, and have a more complete AA profile than most plant materials however, quality control and consumer acceptance is an issue (Naylor et al. 2009; Luthada-Raswiswi et al. 2021). For example, although non-ruminant processed animal protein is an approved aquafeed ingredient in most countries including Canada, a voluntary ban still exists in the UK due to fear of negative consumer reaction (Shepherd et al. 2017).

DHA and EPA are predominantly delivered to the food chain via primary production from aquatic organisms, this means fat sources from higher trophic level terrestrial animals will not be as concentrated in these EFAs as marine oils (Table 2.2; Gladyshev et al. 2013). This characteristic makes these ingredients a useful energy source, supplying the fish with lipids and FAs however, animal oils alone are not able to provide the fish with sufficient EPA and DHA they require for healthy growth and development. In contrast, single-celled organisms are a novel group of aquafeed ingredients that have potential as alternative protein and lipid sources, particularly EFAs.

2.2.1.3 MICROBIAL ALTERNATIVES TO FISH MEAL AND OIL IN AQUAFEEDS

Microbial ingredients includes biomass originating from single-cell sources including bacteria, yeast and fungi, protists, and microalgae (Becker et al. 2007). The use of microbes in aquaculture have historically been used as enrichment of live feeds such as rotifers and brine shrimp in production of larval teleosts and shellfish (Leger et al. 1986). More recently, microbial ingredients have been evaluated as sources of protein and lipid in aquafeeds for juvenile carnivorous fish, such as salmonids (Jones et al. 2020). Sustainability advantages of microalgae culture include their ability to utilize unique, inexpensive feedstock, convert waste gasses such as CO_2 and methane, high density production, and wastewater remediation capabilities (Ritala et al. 2017; Jones et al. 2020). The isolation and utilization of sole protein or lipid fractions from microbial ingredients is often not technically or economically feasible, therefore the efficacy of these ingredients must be considered based on the biochemical and proximate composition of the whole product (Becker et al. 2007). On one hand, this presents advantages, as microbial ingredients contain a broad spectrum of other nutrients, pigments, vitamins and minerals, and bioactive peptides that are lacking in the aforementioned animal and plant proteins and lipids (Ritala et al. 2017; Acquah et al. 2020). On the other hand, this presents challenges associated with whole cell culture, cell wall and subsequent nutrient digestibility, downstream processing, and concentration of target ingredients (Tibbetts 2018).

Chlorella vulgaris, Arthrospira maxima, and *Nannochloropsis gaditana,*and many others have been evaluated for their potential as protein sources in aquafeeds, as they can accumulate moderate to high levels of protein (14-75% dry weight) and possess favorable

and conserved AA profiles (Table 2.1; Acquah et al. 2020; Tibbetts 2018). Unruptured whole cell microalgae as a protein source can only make up 10-15% of the total crude protein requirement before growth and feed efficiency are compromised due to issues regarding nutrient digestibility associated with the cell wall (Skrede et al. 2011). In terms of lipid sources, focus has been on species from genera *Schizochytrium* (family *Thraustochytriaceae*) and *Crypthecodinium* (family *Crypthecodiniaceae*), specifically for their ability to accumulate high amounts (up to 68% total fatty acids, TFA) of DHA (Table 2.2). Of all potential DHA-rich alternatives, *Schizochytrium* sp. contains the highest % DHA (44% TFA; Table 2.2) and has been able to partially replace FO and increase DHA content of the fillets when included in diets for Atlantic salmon (Carter et al. 2003; Kousoulaki et al. 2016; Wang et al. 2021). However, if Sc is to become a common ingredient in commercial aquafeeds, more needs to be known surrounding its utility by Atlantic salmon. Specifically, digestibility data is scarce in the literature, which is critical information for aquafeed formulation.

2.3 *SCHIZOCHYTRUIM* SP.

Schizochytrium is one of 13 genera belonging to the *Thraustochytrid* family (Raghukumar 2002). Sc is a heterotrophic protist that is fast growing, simple to culture and able to produce high amounts of DHA. The cells are thin-walled and spherical or ovoid in shape and form large, pale yellow colonies due to the presence of β-carotene. Reproduction by continuous binary fission results in efficient biomass production rates in culture, increasing Sc's potential as an aquafeed ingredient (Yokoyama et al. 2007; Marchan et al. 2018). Sc cells can synthesize DHA using two separate pathways. Via the aerobic PUFA biosynthetic

pathway, which involves the synthesis of SFAs via the Fatty Acid Synthase (FAS) pathway which undergo a series of desaturation and elongation reactions to form PUFAs including docosapentaenoic acid (DPA; 22:5n-3), which is converted to DHA via the delta-4 desaturase. Or, Sc can utilize the anaerobic PUFA biosynthesis pathway, which involves the *de novo* synthesis of PUFAs using a polyketide synthase (PKS)-like enzyme (Metz et al. 2001; Wang et al. 2021). The use of strains (T18, S31, SR21) described as "*Schizochytrium* sp." is most common, and when specified, species include *S. aggregatum*, *S. limacinum*, *S. mangrovei*, *Schizochytrium* sp. T18 (Wang et al. 2021). Sc can contain as much as 71% lipid, of which up to 44% can be DHA (Tibbetts 2018). FA composition of Sc can vary widely depending on strain and production methods, and other dominant FAs in Sc are palmitic acid (16:0; up to 16-38%) and oleic acid (18:1n-9; <1- 27%). Protein content can vary between 12-39% of biomass, and due to the plant-like cell wall structure of most microbial organisms, the carbohydrate content of Sc tends to be high (17-39%) (Hong et al. 2011; Tibbetts 2018).

Sc is commonly cultured in closed fermenters. Heterotrophic cultivation requires a source of carbon (glucose as an example) and nitrogen (yeast extract or peptone for example), and parameters such as pH (5-8), temperature (variable), and salinity (2-50 g/L) can be manipulated to achieve desirable metabolite production within the cells (Wang et al. 2021). This form of production presents some advantages; cultures do not require constant light exposure or CO_2 delivery (such as photoautotrophic species) and can achieve higher (up to 400% DW/L greater) cell densities which combined can reduce production costs (Borowitzka 1999; Guedes et al. 2011; Kamalanathan et al. 2018). The proximate and

biochemical composition of Sc can vary depending on strain and culture method (Tibbetts 2018). For example, DHA production of Sc strain SR21 can vary between 26-47.39 g/L and DHA yield from Sc strain HX-308 grown using glucose and yeast as sources of carbon and nitrogen was 28.45 g/L while the same strain grown using cane molasses and algal-residue extract only yielded 15.22 g/L (Wang et al. 2021). Therefore, characterization of nutrient profile and the digestibility of macronutrients and FAs from Sc by target aquaculture species is integral to its development as a commercial aquafeed ingredient. Currently no data is available on the digestibility of Sc biomass by Atlantic salmon.

2.3.1 USE OF *SCHIZOCHYTRIUM* SP. IN AQUAFEEDS

The effects of Sc as a DHA supplement in salmonid feeds has been subject to Xover 15 published studies. Low inclusion levels (<5% of diet) of Sc meal (250 g/kg DHA) + yeast extract slightly improved weight gain (3%) and fillet DHA content of Atlantic salmon (9-10%; Kousoulaki et al. 2015). FO-free diets containing a mixture of 10% *Thraustochytrid* meal and 11.5% canola oil significantly increased muscle DHA content (19.43% TFA) when compared to a diet containing canola as the only oil source in Atlantic salmon (12.33% TFA; Carter et al. 2003). Due to the low EPA content of Sc biomass (1-16% TFA; Tibbetts 2018), EPA fillet content is negatively correlated with increasing Sc in inclusion in the diet (Sprague et al. 2015; Kousoulaki et al. 2015; 2016) which could impact fish health and fillet nutritional quality. Serrano et al. (2021) were successful in overcoming this by adding 4.5% each of Sc and *Nannochloropsis oceanica* meals in rainbow trout diets to achieve 50.69 mg/100g EPA and an EPA:DHA ratio of 1.71 compared to a fish oil control (56.27 mg/100g EPA; EPA: DHA = 1.57).

Protein was well (>80%) digested in diets containing 15% Sc whole cell meal (Kousoulaki et al. 2015), however, inclusion levels of Sc are negatively correlated with lipid digestibility in the diet for Atlantic salmon. For example, apparent digestibility coefficients (ADCs) were 95.8%, 96.0%, 93.9%, and 87.8% in diets containing 0, 1%, 6%, and 15% Sc meal (Kousoulaki et al. 2015), and Zhang et al. (2013) reported a >50% decrease in lipid digestibility from 95.5% in the control diet to 63.4% in diets containing 20% Sc. Two studies have investigated Sc-specific digestibility at 30% inclusion in diets for rainbow trout and reported ADCs of 91-98%, 88-91%, and 86% for DM, protein, and lipids, respectively (Zhang 2013; Belanger et al. 2021). Clearly ingredient digestibility is species-specific and no data exists on the nutrient digestibility of Sc by Atlantic salmon which limits our understanding of the availability of nutrients from Sc in this species.

When investigating any novel ingredient, it is imperative that the ingredient digestibility and subsequent nutrient availability be determined for the target species to ensure optimum feed formulation (Glencross 2007). To date, Sc digestibility in Atlantic following a standard, limited-inclusion digestibility design with 30% test ingredient and 70% reference diet has been conducted for rainbow trout, but not Atantic salmon (Cho et al. 1982; Glencross 2007; Belanger et al. 2021). The first objective of this thesis was to investigate the digestibility of Sc whole cells by Atlantic salmon.

2.4 ASTAXANTHIN IN AQUAFEEDS

Astaxanthin (Ax; 3,3′-dihydroxy-β,β-carotene-4,4′-dione) is a secondary xanthophyll carotenoid that is characteristically red in color, and is a common feed additive used to pigment the flesh of farmed salmonids (Higuera-Ciapara et al. 2006). Carotenoids are a family of >600 pigments, which are biosynthesized by various photosynthetic microorganisms, fungi, yeasts, and some higher plants (Goodwin 1980). Most carotenoids possess two cyclic end-groups joined by a 40-carbon polyene chain backbone which gives rise to their distinctive light-absorption and antioxidant characteristics (Britton 1995; Namitha and Negi 2010). Two major groups of carotenoids exist: the carotenes, whose structure contains only hydrocarbons; and xanthophylls, which are the oxygenated derivates of carotenes and possess oxygen-containing functional groups (Guedes et al. 2011). Xanthophylls can be further classified into primary and secondary xanthophylls. Primary xanthophylls are directly involved in photosynthesis and survival. Secondary xanthophylls, are typically contained within lipid bodies as a by-product of carotenogenesis and commonly produced by microalgae in response to environmental stress (Jin et al. 2003).

Groups of carotenoids have different functions and actions, which are determined by their specific molecular geometry (Britton et al. 2008). The structure of Ax is characterized by two benzoic rings each with a hydroxyl and keto group on either end of the molecule (Figure 2.1; Ambati et al. 2014). This structure results in three configurational isomers: two possible enantiomers and one mesomere, depending on the position of the hydroxyl groups (3R,3’R, OH groups attached above the plane of the molecule; 3S, 3’S, OH groups

attached below the plane of the molecule; and 3R,3'S) (Jackson et al. 2008). Due to the presence of conjugated double bonds within the chain, all-trans and cis- isomer forms of Ax are possible. In addition to optical and geometric isomers, one or both of the hydroxyl groups can react with fatty acids to form mono- and di-esters, but it may also be found free (unesterified; Higuera-Ciapara et al. 2006). Ax can therefore exist in multiple stereoisomer, geometric isomer, free, and esterified forms, and these chemical signatures can be used to identify the source of Ax in wild and farmed salmon (Turujman et al. 1997).

2.4.1 ASTAXANTHIN METABOLISM

Salmonids accumulate the majority of dietary Ax in muscle tissues. Flesh pigmentation depends not only on the inclusion of carotenoids in the feed, but also on the pharmacokinetics of the compound following ingestion. Absorption and retention of dietary Ax is similar among mammals and fish and involves the following steps: i) Disruption and release of Ax from the food; ii) uptake of Ax into lipid micelles; iii) uptake of Ax-containing lipid micelles by enterocytes; iv) incorporation of Ax into chylomicrons for transport; v) secretion of Ax within the systemic circulation; vi) Ax distribution, metabolism, and recycling (van het Hof et al. 2000; Lim et al. 2018). Due to the hydrophobic nature of the Ax molecule, salmonid pigmentation via Ax supplementation is generally viewed as an inefficient and highly variable process often associated with low digestibility and poor absorption by the GI tract. Apparent digestibility coefficients (ADCs) and net apparent retention (NAR) rates of Ax range from 30-90% and 2-22% in salmonids, respectively (Bjerkeng & Berge 2000; Ytrestoyl et al. 2005; Torrissen et al. 1989; Storebakken and No 1992).

Ax metabolism can differ depending on species and diet composition. For example, Atlantic salmon are less efficient at depositing Ax compared to rainbow trout (March and MacMillan 1996; Ytrestoyl et al. 2005). Fish size, feed intake, sex, and life stage can all influence Ax retention among fish of the same species (Bjerkeng 1992; Ytrestoyl et al. 2005). Ax deposition rates are slower in younger fish, and a positive correlation exists between body weight and level of pigmentation (Bjerkeng et al. 1992; Forsberg 1996). Flesh pigmentation in Atlantic salmon and rainbow trout follows a saturation curve, where carotenoids accumulate in the flesh in quantities of 6-20mg/kg until the onset of sexual maturation, where they are redistributed to the skin and eggs (Meyers 1994).

2.4.2 COMPARISON OF SYNTHETIC AND BIOLOGICAL ASTAXANTHIN

Biological Ax is synthesized from primary sources such as microalgae, yeast, and higher plants via the isoprenoid pathway similar to other lipid-soluble molecules such as sterols, hormones and some vitamins (Lorenz and Cysewski 2000). This pathway begins with acetyl-CoA and through a series of oxidative steps is converted to astaxanthin as the final product. Chemical synthesis of Ax is produced from petroleum feedstock via a Wittig condensation reaction of two, C15-phosphonium salts with a C10-dialdehyde (Widmer 1981).

Biological and synthetic Ax share the same chemical formula but should not be considered the same molecule due differences in esterification, stereochemistry, and carotenoid complex (Capelli et al. 2013). Biological Ax is comprised of 95% esterified molecules, and

synthetic Ax is exclusively “free”, meaning non-esterified. There are differences in shape between the two molecules, biological Ax is predominantly found in the 3S, 3’S and 3R, 3’R forms, while synthetic Ax contains a mixture of optical isomers at a ratio of 1:2:1 (3R, 3’R), (3R, 3’S), (3S, 3’S; Ambati et al. 2014). Synthetic Ax contains Ax as the sole carotenoid, whereas sources of biological Ax can contain additional carotenoids such as beta-carotene, canthaxanthin, and lutein as part of a natural carotenoid complex (Capelli et al. 2013).

These structural differences between synthetic and biological Ax result in functional differences in antioxidant capacity, with biological Ax exhibiting 20 and 55 times stronger free radical elimination and singlet oxygen quenching ability than synthetic (Capelli et al. 2013). Additionally, the aforementioned differences have led to their consideration as independent substances in terms of safety as feed and human supplements. Natural Ax has undergone human clinical trials and various products are approved for human consumption in approved doses of 2-12mg/day in Canada, USA, and Europe (Brendler and Williamson 2019). Synthetic Ax is lacking clinical trial data surrounding safety parameters, and benefits of synthetic Ax in humans, and the compound is not approved by the CFIA for human consumption. Synthetic Ax has raised safety concerns due to the presence of trace amounts of residual solvents and chemicals (Edwards et al. 2016), toxicity of other synthetic carotenoids such as beta-carotene and canthaxanthin (Capelli et al. 2013), and its potential carcinogenic effects on rats when fed at high doses of 200 and 1,000 mg/kg body weight/day (Brendler and Williamson 2019). Both natural and synthetic Ax have been

approved for inclusion in salmonid feeds in Canada and the USA in doses up to 80 mg/kg (Brendler and Williamson 2019; CFIA 2021).

Ax is included in commercial aquafeeds for juvenile salmonids at concentrations of 40-75 mg Ax/kg to achieve flesh pigmentation levels of 7-24 mg Ax/kg (Purser and Forteath 2012). Major synthetic Ax products are CAROPHYLL® Pink (11% Ax) and Lucantin® Pink produced by DSM (formerly Hoffman la Roche; Denmark) and BASF (Germany), respectively and cost approximately $2000 CAD/kg (Stachowiak and Szulc 2021). The lack of consumer acceptance of synthetic feed additives has sparked research into alternative sources of Ax and various animal, plant, and microbial sources have been identified and studied (Higuera-Ciapara et al. 2006). Thus far, few ingredients have been able to match the effectiveness of synthetic Ax in achieving industry-standard pigmentation, the following sections will review the specifics of these non-synthetic Ax alternatives.

2.4.3 SYNTHETIC ASTAXANTHIN ALTERNATIVES

2.4.3.1 *PLANT AND ANIMAL ALTERNATIVES TO SYNTHETIC ASTAXANTHIN*

Crustacean meals and by-products contain 0.15% Ax and have been used to achieve pigmentation levels of 6.57 mg Ax/kg in brook trout (Saito and Regier 1971). However, low Ax content, seasonal variability, product instability, and poor digestibility due to their high chitin and ash content limit the feasibility of crustacean sources as an alternative to synthetic Ax (Garcia-Chavarria and Lara-Flores 2013).

Species of higher plants *Capsicum annum* (red pepper)*, Tagetes erecta* (marigold flower), and from the genus *Adonis* (*A. aestivalis* and *A. annua*) contain between 0.1-1% total carotenoids (Yanar et al. 2007; Stachowiak and Szulc 2021). Diler and Gokoglu (2004) achieved a carotenoid concentration of 5mg/kg in rainbow trout from feeding diets containing 7.6g/100g red pepper meal (60 mg/kg Ax) for 3 months. However, the fillets were inferior in terms of color and taste compared to synthetic Ax. Marigold flowers did not produce the desired pink/red color but rather a peach/yellow colored flesh in rainbow trout due to the presence of other carotenoids Yanar et al. (2007). Flowers from the *Adonis* genus contain moderate (1% DM) levels of Ax but are not a cost-effective source of pigment due to low flower yield per area of cultivation (Mawson et al. 1995).

In general, Ax sources from higher plants and crustaceans are not suitable alternatives to synthetic Ax due to their low Ax content, limited bioavailability, negative influence on fillet sensory properties, and lack of economic and logistical feasibility for large-scale production. Microbial synthesis of Ax has advantages over the aforementioned Ax sources due to ease of culture and ingredient availability that does not depend on ecological and environmental factors. However, production costs and Ax bioaccessibility of algal Ax are two persistent challenges for the industry.

2.4.3.2 MICROBIAL ALTERNATIVES TO SYNTHETIC ASTAXANTHIN

Microbial synthesis of Ax is an intensely developing research area, and the microalgae *Haematococcus pluvialis,* bacteria *Paracoccus carotinifaciens,* and yeast *Xanthophyllomyces dendrorhous*, are produced commercially and have received the most attention from the aquaculture industry as potential alternatives to synthetic Ax

(Rodriguez-Sifuentes et al. 2021). *P. carotinifaciens,* has an Ax content of 2.1% of biomass and a mix (1.4% DW) of other carotenoids that competitively inhibit Ax deposition (Hayashi et al. 2021). The addition of 50 mg/kg of *P. carotinifaciens* in diets for rainbow trout resulted in inferior Ax fillet content (3.6 mg/kg Ax) compared to synthetic Ax (6.1mg/kg) and altered flesh color to a darker red due to competitive inhibition of Ax by other carotenoids (Hayashi et al. 2021). *X. dendrorhous* (the anamorphic state of *Phaffia rhodozhyma*) has an Ax content of 0.2-1% of the cell (Mikyakawa 2021). The thick, polysaccharide-rich (20-25% DM) cell walls of the yeast reduce Ax availability and hamper pigmentation of salmonid flesh (Stachowiak and Szulc 2021). Ax flesh retention levels were 3.8%, 9.7%, and 17.5% in rainbow trout supplemented diets containing *X. dendrorhous* cells with 45%, 70% and 97% cell disruption (enzymatic disruption), respectively (Storebakken et al. 2004).

The microalgae *Haematococcus pluvialis* is considered the best source of natural Ax, due to its ability to accumulate up to 6% DW Ax (Mikyakawa 2021). However, similar to *X. dendrorhous*, the robust cell wall of Hp prevents the direct use of whole cells in feed formulations and requires additional processing to increase Ax bioavailability (Mendes-Pinto et al. 2001; Kuehnle and Shurr 2019). Processing methods include mechanical disruption, pressure fracture, enzyme, heat, or chemical treatment. Cellular homogenization and pressure fracture (500 psi) are two effective methods that recovered >85% of the carotenoid (Mendes-Pinto et al. 2001), and marketable fillet pigmentation levels (3-5 ug/g Ax) in rainbow trout (Young et al. 2016). Chemical and enzyme treatments are inefficient and can result in carotenoid losses of 20-35% (Mendes-Pinto et al. 2001;

Choubert et al. 2006). These processes can represent up to 80% of production costs, adding an additional step that poses challenges in terms of scalability, consistency, cost, and lowers product shelf life (Mendes-Pinto et al. 2001; Kuehnle and Shurr 2019). Cell-free Hp carotenoid extract can successfully pigment the flesh of rainbow trout (Bowen et al. 2002), however, is not economically feasible or practical on a large scale due to increased Ax oxidation (Mendes-Pinto et al. 2001).

For Hp to be economically competitive with synthetic Ax, there is a need for the development of products that require minimal disruption (Rodriguez-Sifuentes et al. 2021). The second objective of this thesis was to test the pigmenting ability of a novel Hp whole cell product in rainbow trout. This pre-commercial *H. pluvialis* whole cell product has been produced heterotrophically (Kuehnle Agrosystems, Honolulu, HI; US Patent # 11.034.968) with a weakened cell wall which aims to overcome Ax bioavailability challenges encountered in the literature by eliminating the need for cell rupturing.

2.5 *HAEMATOCOCCUS PLUVIALIS*

Haematococcus pluvialis (Hp) is a unicellular alga belonging to the *Haematococcaceae* family. It is ubiquitous in the freshwater environment and is common in stagnant waters globally (Guiry 2021). Hp is distinguished by its ability to produce high (6% of the cell amounts of astaxanthin; Mikyakawa 2021). This characteristic is associated with its unique life-cycle that gives it the ability to tolerate a wide variety of environmental conditions by utilizing the powerful antioxidant capabilities of Ax for protection from stressful conditions such as high temperatures or nutrient deprivation.

Hp has two main growth phases: a green vegetative phase and red cyst phase. The green phase is characterized by rapid growth of motile vegetative cells under favourable environmental conditions. During this phase, Hp is rich in protein (up to 40% DW; Boussiba and Vonshak 1991) and lutein is the dominant carotenoid (Harker et al. 1996). Environmental stressors such as intense heat or nutrient limitation induce the red phase, which involves the encystment of non-motile cells to form aplanospores with thick and rigid cell walls called haematocysts. As they mature, the haematocysts accumulate astaxanthin in lipid bodies to defend against oxidative stress (Hagen et al. 2002; Han et al. 2013). Due to the change in cell wall structure and formation of lipid bodies, lipid and carbohydrate levels are elevated in the red stage and protein levels decrease (Shah et al. 2016). Carotenoid content of haematocysts can reach up to 6% of total biomass, with Ax representing up to 95% of total carotenoids (Wayama et al. 2013). All Hp-derived Ax is in the 3S,3'S isomer form, and the majority is esterified and in the all-trans configuration (Renstrom and Liaaen-Jensen 1981; Capelli 2013).

Hp is industrially produced by photoautotrophic cultivation in open raceway ponds or closed photobioreactors (Jannel et al. 2020). This is a two-step process where the first step involves maximizing biomass production (0.8 g/L) of green-phase cells under optimal culture conditions (BG-11 or BBM growth medium, 20-28 °C, pH of 7-8, irradiation of 40-50 umol photons/m/s; 1.5-5 mM of nitrogen; Shah et al. 2016). The second step uses nitrogen limitation or the addition of NaCl (0.25-0.5% w/v) to induce stress and trigger haematocyst formation and Ax accumulation (Han et al. 2013). Hp can also be grown

heterotrophically and mixotrophically with the addition of acetate, and organic and inorganic sources of carbon (Shah et al. 2016). Cells are then harvested by sedimentation, spray-dried, and additional processing such as cellular disruption and Ax extraction using may be necessary, depending on the intended use (Jannel et al. 2020).

2.5.1 USE OF *H. PLUVIALIS* IN AQUAFEEDS

Hp has been evaluated as a dietary source of pigment for salmonids in whole cell, cell-ruptured and extracted forms at dietary concentrations ranging from 40-80 mg/kg Ax (Sommer et al. 1991, 1992; Bowen et al. 2002; Choubert et al. 2006; Young et al. 2017). Whole cell Hp does not effectively pigment salmonid flesh when compared to synthetic Ax. Flesh carotenoid content of rainbow trout fed diets containing whole cell Hp (2% Ax) were 6.2 mg/kg while synthetic Ax produced flesh carotenoid levels of 12.7 mg Ax/kg (Young et al. 2017). Sommer et al. (1991) determined homogenized Hp cells achieved significantly superior flesh pigmentation compared to whole cells in rainbow trout diets. Additionally, Ax deposition (4-6 mg/kg Ax) was not statistically different in rainbow trout fed diets containing 40 mg/kg of Ax extract from Hp cells and synthetic Ax (Bowen et al. 2002). Pigmentation of rainbow trout by pressure-disrupted (5000 psi) haematocysts (67 mg/kg dietary Ax) was equal to that of synthetic (58 mg/kg dietary Ax) Ax achieving flesh pigmentation between 3-4 mg/kg Ax (Young et al. 2017. These trials clearly indicate that the rigid cell wall of Hp is limiting Ax bioaccessibility to the fish. Although cell-rupture and Ax extraction methods may be sufficient in pigmenting the flesh of salmonids by increasing Ax bioavailability, the additional procedures required are still too costly to make

Hp-derived Ax economically competitive with its synthetic counterpart (Shah et al. 2016; Viera et al. 2018).

The pre-commercial Hp product used in this study was produced by Kuehnle Agrosystems (Honolulu, HI) using a novel fermentation culture method to obtain weakened whole cells (US Patent # 11.034.968). This method employs aerobic fermentation using organic acids and eliminates haematocyst formation associated with hypercarotenogenesis in photosynthetic production (Kuehnle and Shurr 2019). Heterotrophic fermentation in steel tanks removes the need for photon absorption and allows for higher cell densities (0.8 g/L vs 0.9-2.65 g/L; Jannel et al. 2020) and lower water usage (3700 L/kg of algal biomass with photoautotrophic production vs 40 L/kg algal biomass with heterotrophic production; Kuehnle and Shurr 2019) which can reduce cost of production. This technology could potentially allow for improved scalability, product consistency, and contaminant control which are all needed in order for single celled pigments to be competitive with synthetic Ax (Hu et al. 2018). This thesis will advance the state of knowledge surrounding the potential of Hp as an alternative dietary pigment to synthetic Ax by being the first to conduct an experiment using this novel Hp product in diets for farmed rainbow trout.

2.6 KNOWLEDGE GAPS AND OBJECTIVES

2.6.1 KNOWLEDGE GAPS

The current plant-based ingredients used to substitute FM and FO are devoid of EPA and DHA which poses potentially negative effects on fish and human health. The aquafeed industry needs omega-3 rich ingredients that are produced economically and sustainability,

and whose effects on the target species are thoroughly understood. Previous research has established that low levels (5% of diet) of *Schizochytrium* sp. biomass are capable of increasing fillet DHA content without negatively impacting growth in Atlantic salmon. An important knowledge gap is the digestibility of macronutrients and FAs of *Schizochytrium* sp. by Atlantic salmon. To fully understand the potential, and limitations of this ingredient, it is important to evaluate its effects on all industry relevant parameters including digestibility. Ingredient digestibility represents the first bottleneck for nutrient assimilation (Glencross et al. 2014) and therefore, understanding the precise percent digestibility of macronutrients and fatty acids by the target species is essential to determining the validity of a novel feed ingredient and this information is necessary to ensure accurate feed formulation.

Effective, natural forms of Ax are needed in salmonid aquafeeds, and *Haematococcus pluvialis* has the most potential as an alternative to synthetic Ax due to its high Ax content. The common two-step cultivation process of Hp for Ax production produces haematocysts with rigid cell walls that result in poor digestibility and low bio-accessibility of Ax and prevents the direct use of whole cells as dietary pigments in salmonid feeds. To achieve successful flesh pigment levels, haematocysts must be ruptured prior to feed formulation which represents a significant increase in operation cost as well as a reduced product shelf life. A solution is needed that allows for increased Ax bioavailability from Hp cells that does not involve the use of additional processing. The Hp product used in this trial was manufactured such that the cell wall of the haematocyst is weakened to improve

digestibility and eliminates the need for cell rupture but its ability to pigment the flesh of salmonids has not yet been evaluated.

2.6.2 RESEARCH OBJECTIVES

The main objectives of this thesis were to evaluate the effects of two whole cell microbial ingredients by assessing digestibility of *Schizochytrium* sp. (Chapter 3) and the pigmenting ability of *Haematococcus pluvialis* (Chapter 4) when supplemented in diets for farmed salmonids. To achieve this, two experiments were conducted over separate time periods: a two-week trial feeding a diet containing 30% Sc biomass with fecal collection, and a 10-week experiment feeding a diet containing Hp cells as the source of dietary pigment with the following objectives:

Objective 1: To determine the digestibility of protein, lipid, gross energy, and fatty acids from Sc by Atlantic salmon? This objective aims to address the knowledge gap surrounding the absence of Sc-specific digestibility data by Atlantic salmon and contribute data which will aid in future feed formulations containing this ingredient.

Objective 2: To determine if the novel weakened whole cell Hp is capable of replacing synthetic Ax in aquafeeds for rainbow trout? This objective aims to fulfill the aquafeed industry's need for a digestible, effective, non-synthetic feed pigment.

2.7 TABLES AND FIGURES

Table 2.1 Crude protein content (%; as-fed) and amino acid composition (%) of various plant, animal, and microbial protein sources in aquafeeds.

Source	Crude Protein	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Tyr	Val
Fish meal (anchovy)[1]	65.4	3.7	1.6	3.1	5.0	5.1	2.0	2.7	2.8	2.2	3.5
Fish meal (herring)[1]	72.0	3.7	1.5	3.6	4.7	7.3	2.2	2.7	2.5	2.1	3.3
Krill meal[1]	58.8	3.5	1.2	2.8	4.4	4.2	1.8	2.5	2.5	-	2.8
Plant sources											
Soybean meal[1]	48.5	3.6	1.3	2.6	3.8	2.2	0.7	2.7	2.0	1.3	2.7
Soy protein concentrate[1]	63.6	4.6	1.6	2.9	4.9	3.9	0.8	3.3	2.4	2.3	3.1
Wheat gluten meal[1]	80.7	3.8	2.0	3.7	6.3	4.9	1.6	4.5	1.6	-	4.0
Corn gluten meal[1]	63.7	1.9	1.2	2.3	9.4	1.1	1.9	2.8	2.0	0.9	2.7
Corn protein concentrate[1]	75.0	3.0	2.1	4.1	16.7	1.1	2.3	6.2	3.0	-	4.5
Animal sources											
Poultry by product meal[1]	55.9	4.3	1.1	2.3	4.3	3.3	1.3	1.7	2.1	1.2	3.7
Feather meal[1]	83.3	5.8	0.7	4.2	6.9	1,8	0.5	4.1	3.9	2.0	4.6
Black soldier fly meal[2]	36.2	8.2	5.3	5.8	6.9	7.6	1.5	6.9	5.4	6.4	6.3
Microbial sources											
Sc meal[3]	12.3	8.0	5.3	3.7	7.0	3.0	12.0	4.0	4.0	2.0	6.0
C.vulgaris[4]	31.5	1.7	0.5	0.9	2.1	1.4	0.5	1.4	1.3	0.02	1.5
N. granulate (g/100g protein)[5]		7.3	2.1	5.0	9.2	6.9	2.5	5.5	4.7	-	6.1
A.Platensis (g/100g protein)[6]		6.0	1.5	3.4	8.4	5.4	2.5	6.2	5.5	4.3	6.9

[1] NRC 2011, [2] Barroso et al. 2014, [3] Sprague et al. 2016, [4] Tibbetts et al. 2017, [5] Tibbetts et al. 2015, [6]Andreeva et al. 2021.

Table 2.2 Fatty acid composition (% total fatty acids) of fish, plant, animal, and microbial lipid sources used in salmonid aquafeeds.

Oil/fat	SFA	MUFA	LA	AA	ALA	EPA	DHA	n-3 PUFA	n-6 PUFA	PUFA	n-3: n-6
Fish oils[1]											
Anchovy oil	28.8	24.9	1.2	0.1	0.8	17.0	8.8	31.2	1.3	32.5	24.0
Herring oil	20.0	56.4	1.1	0.3	0.6	8.4	4.9	17.8	1.4	19.2	12.7
Plant oils[1]											
Soybean oil	14.2	23.2	51.0	-	6.8	-	-	6.8	51.0	57.8	0.1
Rapeseed oil	4.6	62.3	20.2	-	12.0	-	-	12.0	20.2	32.2	0.6
Linseed oil	9.4	20.2	12.7	-	53.3	-	-	53.3	12.7	66.0	4.2
Animal fats											
Poultry fat	28.5	43.1	19.5	-	1.0	-	-	1.0	19.5	20.5	0.0
Beef tallow	47.5	40.5	3.1	0.4	0.6	-	-	0.6	3.1	3.7	0.2
Microbial oils											
Sc oil[2]	44.1	1.3	0.3	0.8	0.2	0.8	44.0	45.9	8.7	54.6	5.3
C. cohnii[3]	40.2	36.2	7.4	0.1	0.9	0.7	14.0	15.9	7.4	23.5	2.0

[1] Turchini et al. 2008, [2] Sprague et al. 2016, [3] Ganuza et al. 2008.

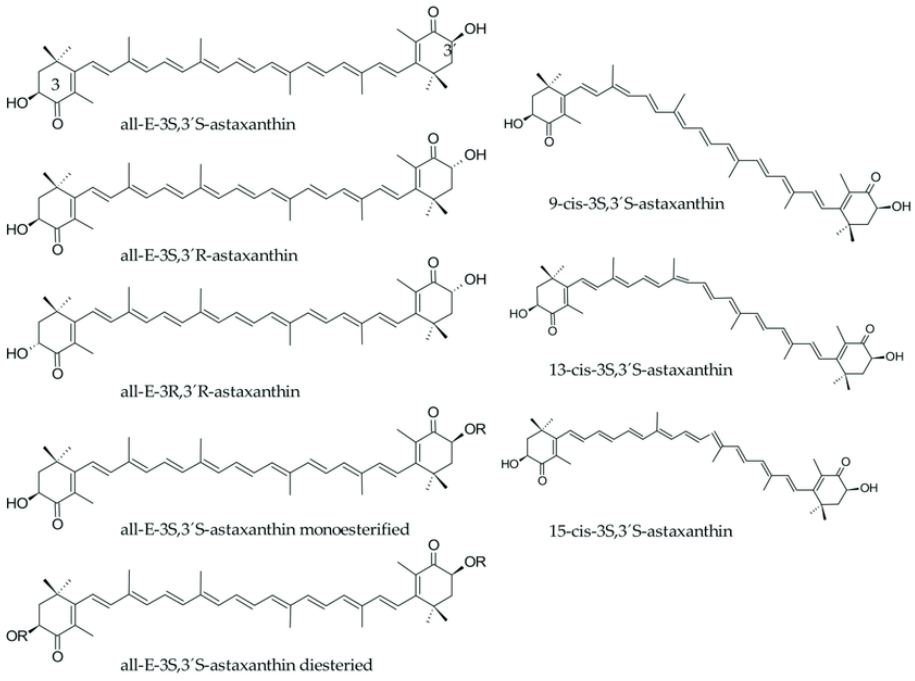

Figure 2.1 Structural arrangement of astaxanthin isomers from Viera et al. 2018.

CHAPTER 3.0

DIGESTIBILITY OF *SCHIZOCHYTRIUM* SP. WHOLE CELL BIOMASS BY ATLANTIC SALMON (*SALMO SALAR*)

3.1 ABSTRACT

Schizochytrium sp. (Sc) is a promising, nutritious, and sustainable source of polyunsaturated fatty acids (PUFAs) in aquafeeds for carnivorous fish. This study investigated the apparent digestibility coefficients (ADCs) of PUFAs, along with macronutrients, other major fatty acids (FAs) and gross energy of Sc whole cells when included in diets for Atlantic salmon (*Salmo salar*). Thirty fish (27.9±1g) were randomly allocated to eight-120L tanks and fed a reference or test (70% reference diet and 30% Sc biomass) diet. Over three weeks, fish feces were collected by sedimentation using fecal collection columns and analyzed for digestible dry matter, gross energy, macronutrients, and FAs. ADCs of protein, lipid, and gross energy of Sc were 93.9%, 67.1% and 70% respectively. Major FAs in the Sc ingredient were palmitic acid (44.7% FAME), docosahexaenoic acid (33.2% FAME) and docosapentaenoic acid (10.1% FAME). SFAs and PUFAs in the test diet were 15% and 7.4% higher than the reference diet, which was reflective of Sc's FA composition. Dry matter, total lipid, gross energy and SFAs were significantly ($p<0.001$) more digestible in the reference diet. PUFAs and DHA were well digested (>95%) in both diets but significantly ($p=0.038$ and $p<0.001$, respectively) more digestible in the test diet. This study shows that whole cell Sc offers a source of highly digestible (98%) PUFAs and protein with no need for oil extraction or cell disruption.

3.2 INTRODUCTION

Salmonid farming is an efficient animal protein production system (1.2 g feed per g gain) compared to terrestrial farming (1.8-6.3 g feed per g gain), but is disproportionately reliant on the forage fishery for aquafeed production (FAO 2020). The continued use of fish meal (FM) and fish oil (FO) obtained from forage fisheries to provide farmed salmonids with

the necessary essential fatty acids (EFAs) and essential amino acids (EAAs) is not economically sustainable and poses a threat to both marine biodiversity and human food security (Naylor et al. 2000; Naylor et al. 2009). Finding alternative sources of protein and oil for aquaculture that are sustainable, technically and economically feasible, and nutritious for both the fish and the end consumer has been deemed necessary to facilitate the expansion of sustainable aquaculture (Jones et al. 2015). As aquaculture production rises, with producing over half of the world's seafood (FAO 2020), the global demand for aquafeed also increases; therefore, it is crucial the aquaculture sector identifies additional supplemental ingredients to FM and FO that satisfy these qualities. Terrestrial plant and animal-based ingredients such as canola oil, soybean meal, poultry fat, and blood meal are currently used in aquafeeds to alleviate the economic and environmental costs associated with FM and FO (Turchini et al. 2009; NRC 2011).

Marine FO has high concentrations of the omega-3 (n-3) long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFAs), namely eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). Bioconversion of the polyunsaturated fatty acid (PUFA), alpha-linolenic acid (ALA; 18:3n-3), commonly obtained from terrestrial oil seeds such as canola in commercial salmonid diets, to EPA and DHA in fish or humans is not efficient enough to meet dietary requirements, thus it is essential to include sources of these n-3 LC-PUFA in the diet (Bou et al. 2017; Saini and Keum 2018). DHA is integral for the maintenance, structure, and fluidity of cell membranes as well as cell signalling (Stillwell and Wassall 2003). EPA is involved in the anti-inflammatory response via eicosanoid production (Sargent et al. 2003; Emery et al. 2016). The inclusion of EPA and

DHA in salmonid feed is two-fold: to ensure adequate growth and development of the fish, and as a vector for the delivery of these EFAs to humans, which prevent cardiovascular disease, rheumatoid arthritis, and neurological disorders (Rennie et al. 2003; Calder and Yaqoob 2009; Siscovick et al. 2017; Laye et al. 2018).

Terrestrial animal and plant-based feed ingredients have been heavily investigated and are widely used as sources of abundant and economically-friendly protein and lipid in aquafeeds (Turchini et al. 2009; NRC 2011; Hartviksen et al. 2014). Despite the recent popularity of these ingredients, their use in aquafeeds has several limitations. Terrestrial feed ingredients do not supply all the EAA and EFA that are required by fish, which can impact fish health, as well as human health, as consumers of farmed fish fillets (Sprague et al. 2016). Terrestrial plants do not generally produce n-3 LC-PUFAs in any valuable quantity and plant ingredients often contain anti-nutritional factors that can impede digestion and nutrient absorption (Hardwood 1996; Francis et al. 2001; NRC 2011). Additionally, their production is becoming increasingly ecologically unsustainable (large land footprint, the use of chemical pesticides, irrigation and polluting run-off) (Pahlow et al. 2015; Fry et al. 2016). If the aquaculture sector transfers its reliance on the forage fishery to terrestrial agriculture, it risks simply shifting current sustainability challenges from oceans to farmlands.

The use of microalgae in the aquaculture industry that is primarily for live feed supplementation is not novel; however, their use in formulated grow-out diets is more recent. Microalgae naturally produce EPA and DHA in the marine food web, and possess

a relatively conserved (little variation between species and cultivation method), high-quality (complete and bioavailable) amino acid profile (Becker 2007; Acquah et al. 2020). They can grow under a variety of autotrophic, heterotrophic and mixotrophic conditions and are capable of high production rates, require simple nutrient inputs and accumulate functional metabolites such as the n-3 LC-PUFA and carotenoids (Borowitzka 2013; Winwood 2013; Hardwood 2019). Furthermore, the use of lower trophic level feed ingredients can improve the sustainability of aquaculture through the "Blue Revolution" by reducing its environmental footprint in terms of water and land use, CO_2 conversion, nutrient recycling, and wastewater remediation (Tibbetts 2018; Yarnold et al. 2019). These single-celled organisms can utilize sustainable media derived from waste streams, simple "low cost" ingredients and industry by-products (Pleissner et al. 2013; Patil and Gogate 2015; Song et al. 2015; Yin et al. 2018). Integrating sustainable algal production systems into aquaculture will shift the industry towards a circular bioeconomic approach by creating ecofriendly value chains with reduced environmental impact while contributing nutritious products to aquaculture and subsequently humanity (Subhadra and Grinson-George 2011; Yarnold et al. 2019).

Schizochytrium sp. (referred to as Sc here forward) is a genus of single-celled heterotrophic protists belonging to the *Thraustochytrid* family that behaves much like, and is commonly referred to for simplicity as, microalgae (Leyland et al. 2017). This genus is ubiquitous in the marine environment, but is most commonly found in environments containing decomposing organic matter, which explains its ability to thrive on various waste inputs (Yokoyama and Honda 2007; Leyland et al. 2017; Marchan et al. 2018). Its high production

rate, tolerability of a wide range of conditions and ability to accumulate large amounts of lipid (>70%) and DHA (>50% total fatty acids) make it a promising FO alternative (Lewis et al. 1999; Tibbetts 2018). Several Sc DHA-rich products (*life*sDHA, DHAgold, Algamac, PureOne) are now commercially available in oils, powders, or capsules for nutraceuticals, bio-ingredients, and aquaculture and animal feeds (Marchan et al. 2018). Inclusion of Sc oil and meal in aquafeeds has been studied on farmed Atlantic salmon, rainbow trout, Nile tilapia, catfish, giant grouper and longfin yellowtail (Kousoulaki et al. 2015; 2020; García-Ortega et al. 2016; Kissinger et al. 2016; Sarker et al. 2016; Bélanger-Lamonde et al. 2018; Tibbetts et al. 2020). Sc whole cell biomass is ideal over extracted oil from an economic standpoint because it eliminates the extraction process which is a costly additional processing step. Additionally, the lipids are less susceptible to oxidation in whole cell form which can affect product quality and shelf life (Balduyck et al. 2017). In previous studies, Sc biomass or oil was included in the diet at levels varying from 0-30% as either a supplement or replacement to FO, often paired with a terrestrial oil source. Results from these studies commonly indicate normal growth and development and an increase in DHA levels of the fillets. Macronutrient digestibility was negatively impacted in some trials, but few studies reported digestibility values for the novel ingredient. To date, digestibility has been measured for Sc in Atlantic salmon feed at inclusion levels of <15% for whole cell biomass (Carter et al. 2003; Zhang 2013; Kousoulaki et al. 2015; Kousoulaki et al. 2016; Mizambwa 2017), and 2.6-13% for extracted oil (Miller et al. 2007; Tibbetts et al. 2020). There are no results in the literature reporting the digestibility values for the Sc biomass specifically by Atlantic salmon at inclusion levels >15% of the diet.

Digestibility is a key step (following composition characterization) in evaluating the potential of an ingredient for aquafeeds and is the first bottleneck for nutrient assimilation by the fish (Glencross et al. 2007). Since nutrient and energy digestibility for single-celled ingredients is highly variable depending on the species/strain, cultivation strategy and harvest/processing methods, it is important to evaluate any novel ingredient on the target aquaculture species consuming it (Acquah et al. 2020). In general, species-specific digestibility data for microalgae and microbial ingredients in the aquafeed industry is lacking, which curtails the accuracy and optimisation of feed formulations to meet the nutritional needs and growth potential for the species being farmed. The objective of this study is to determine the digestibility of protein, lipid, energy, and fatty acids (FA) from Sc whole cell biomass by Atlantic salmon at a high (30%) inclusion level, employing a classic digestibility trial design using fecal collection columns (Cho and Slinger 1979; Cho et al. 1982; Glencross et al. 2007).

3.3 MATERIALS AND METHODS

3.3.1 DIET PREPARATION

A basal diet was formulated that satisfied the nutritional requirements of Atlantic salmon (NRC 2011). Sc biomass was grown by heterotrophic fermentation and obtained from Kuehnle AgroSystems (Honolulu, HI, USA). A reference diet (Table 3.1) was produced that contained 99.45% basal diet with no test feed ingredient. A test diet was produced containing 69.62% basal diet and 29.83% test ingredient (Sc biomass). Both diets contained 0.5% chromic oxide (Cr_2O_3) and 0.05% cholestane ($C_{27}H_{48}$) as inert digestion indicators for protein and lipids, and fatty acids respectively (Williams et al. 1962; Sigurgisladottir et

al. 1992). Dry ingredients for both diets were mixed in an industrial 19 L Hobart mixer (Model A-200-T) for five minutes, and then oils were added and mixed for an additional five minutes. After mixing, the diets were steam pelleted into 3 mm pellets using a laboratory scale pelleting mill (California Pellet Mill, San Francisco, Ca, USA) at the Dalhousie Agricultural Campus (Truro, NS, Canada). The pellets were dried in a JWP ST series industrial cabinet oven at 80°C for four hours and sieved to remove fine particles. Diets were stored in fibreglass feed bags and kept frozen at -20°C until needed.

3.3.2 EXPERIMENTAL DESIGN

Atlantic salmon were obtained from Cape D'Or Sustainable Seafoods (initial weight 27.9 g ± 1 g; Wentworth, NS, Canada). The fish were randomly distributed (30 fish per tank) into a digestibility system consisting of eight tanks (120 L), with four replicates per treatment. Each tank was equipped with a fecal collection column which was a modification of the Guelph system (Cho et al. 1982) designed by Tibbetts et al. (2006). Freshwater was supplied to each tank (3 L/min) and maintained at 14°C in a continuously aerated (14 mg/L dissolved oxygen, >90% gas saturation) flow-through system. Diets were hand-fed to apparent satiation twice daily at 0900 h and 1400 h. The fish were acclimated in the system and fed a commercial-grade diet (EWOS Canada) for one week prior to feeding the experimental diets. Experimental diets were fed for four days prior to beginning fecal collection. Ethical treatment of fish was carried out according to the Canadian Council on Animal Care (CCAC) guidelines (institutional animal care protocol 2018-042).

3.3.3 FECAL COLLECTION

The fecal collection system consisted of eight cone-shaped circular tanks outfitted with a removable fecal collector on each tank to allow quick settling of fecal matter and easy cleaning. Following afternoon feeding the tanks and collection columns were cleaned to remove any residual particles. Fecal matter settled overnight and was collected prior to feeding the following morning (0800 h). Fecal matter was collected in plastic containers and funneled into two-50 mL plastic tubes per tank and centrifuged (4000 rpm; 2750 × g) for 10 minutes at 4°C. The supernatant was decanted, and the remaining fecal matter was weighed, and spooned into a collection bottle that was kept frozen at -20°C. Fish were fed routinely until 20 g of fecal matter was collected, this was considered one collection phase. This was repeated three times (three phases, 60 g feces total), in order to monitor any changes in digestibility over time. The three collection phases were consolidated into two by splitting 50% of feces collected in phase two between phases one and three. This was to achieve sufficient sample volume for the desired analyses following freeze drying, the authors acknowledge this could dilute potential differences in digestibility between phases and is recognized as a shortcoming in this study.

3.3.4 ANALYTICAL METHODS

Diet samples were sent to the Nova Scotia Department of Agriculture Analytical Lab (NSDA, Bible Hill, NS, Canada) for proximate composition analysis. Diet samples and feces were freeze dried for 72 hours and ground into homogenous samples in preparation for analyses. Fecal and diet samples were sent to the Canadian Feed Research Centre, at the University of Saskatchewan (Saskatoon, SK, Canada) for chromic oxide analysis.

Crude protein content of fecal samples was analyzed by the Dumas method (% nitrogen × 6.25) (Ebeling 1968) using a Leco protein/N analyzer (Model FP-528, Leco Corp., St Joseph, MI, USA). Crude lipid content of the fecal samples was determined using an ANKOM XT15 extraction system (ANKOM Technology, Macedon, NY, USA; AOCS 2005). Gross energy content of fecal samples was determined using a Parr Isoperibol Calorimeter (Model 6300, Parr Instrument Co., Moline, IL, USA).

Total lipid was extracted using a modified Folch method (Folch et al. 1957), as in Hixson et al. (2017). Freeze-dried fecal samples were subsampled and individually ground to a fine powder in liquid nitrogen using a mortar and pestle, and the resulting powder was weighed to the nearest microgram. Each sample was extracted three times, using 2 mL of chloroform/methanol (2:1; v/v) and then pooled (total 6 mL). Polar impurities were removed by adding 1.6 mL KCl solution (0.9% w/v). The organic layer was removed using a lipid-cleaned glass pipette and pooled. The resulting lipid-containing solvent was concentrated to 2 mL by evaporating with nitrogen gas. The lipid extract was then prepared for gas chromatography by derivatizing into fatty acid methyl esters (FAME) using the Hildich reagent (sulfuric acid) as the catalyst (Christie 2003). FAMEs were extracted twice using hexane:diethyl ether (1:1; v/v), then dried under a gentle stream of nitrogen. The dry FAME extract was re-dissolved in hexane and individual FAME were separated using a gas chromatograph (GC). All solvents used in the extraction and FAME derivatization procedures were of high purity HPLC grade (> 99%). A known concentration of 5 alpha-cholestane (C8003, Sigma-Aldrich, St. Louis, Missouri) was added to each sample prior to extraction to act as the internal standard to estimate extraction and instrument recovery

efficiency. FAMES were analyzed by gas chromatography equipped with a flame ionization detector at the Marine Lipids Lab at Dalhousie University (Halifax, NS, Canada). FAMES and cholestane were identified by comparison of retention times with those of known standards (PUFA No. 3; Sigma-Aldrich, St. Louis, MI, US). Carbohydrate was calculated by difference [100-(protein+lipid+moisture+ash)].

3.3.5 DIGESTIBILITY CALCULATIONS

To determine macronutrient and FA digestibility, chromic oxide and cholestane were used as digestibility markers. The proportion of chromic oxide and macronutrients in the feed were compared directly to that in the feces, and the proportion of cholestane and FA in the feed was compared directly to that in the feces (areas obtained by peaks from gas chromatograph data). The following calculations were used to determine digestibility of diets and nutrients from Sc (Maynard et al.1979; Forster 1999; Tibbetts et al. 2006):

Dry matter digestibility (%) = $100 - (100 \times [Cr_2O_3 \text{ diet} / Cr_2O_3 \text{ feces}])$

Macronutrient digestibility (%) = $100 - (100 \times [Cr_2O_3 \text{ in diet} / Cr_2O_3 \text{ in feces}] \times [\text{nutrient in feces/nutrient in diet}])$

Fatty acid digestibility (%) = $100 - [100 \times (\text{area cholestane-feed}) / (\text{area cholestane-feces})] \times [(\text{area FA-feces}) / (\text{area FA-feed})]$

Sc macronutrient digestibility (%) = ADC (%) = $([a+b] \times ADCNt - [a] \times ADCNr) \times b^{-1}$

Where: a = nutrient contribution of reference diet to nutrient content of test diet

b = nutrient contribution of test ingredient to nutrient content of test diet

ADCNt = apparent digestibility coefficient of the nutrient in the test diet

ADCNr = apparent digestibility coefficient of nutrient in the reference diet

3.3.6 STATISTICAL ANALYSIS

Mean DM, protein, lipid, energy, and FA ADC ± standard deviation (SD) were calculated from the average of four tanks receiving each experimental diet for each collection phase. Grubbs test was used to determine outlier removal in the data set before running additional statistical analyses. A repeated measures analysis of variance (RM-ANOVA) with 95% confidence was used (Minitab19 Statistical Software) to compare ADCs of DM, protein, lipids, phases. Once it was determined that there were no differences in ADC among collection periods within a tank, the collection periods were pooled into a single mean. The pooled mean was used in a one-way ANOVA to determine treatment differences in ADC. Each replicate tank was an experimental unit and ADC values were compared within and between treatments. Tests for normality and homogeneity of variance were completed, and data was transformed before statistical analysis if necessary.

3.4 RESULTS

3.4.1 BIOMASS AND DIET COMPOSITION

The whole cell Sc biomass used in this study contained 11.9% crude protein and 63.6% crude lipid (Table 3.2). The major FAs (>90% total FAME) were 14:0 (4.4% FAME), 16:0 (44.7% FAME), 22:5n-6 (docosapentaenoic acid-6, DPA-6; 10.1% FAME) and DHA (33.2% FAME). Other physiologically relevant FAs (e.g., linoleic acid; LNA, ALA, EPA and arachidonic acid; ARA) were all contained in trace (<1%) amounts. The n-3/n-6 ratio reported for the Sc ingredient was 3.0.

The test diet contained approximately 13% less protein and 12% more lipid than the reference diet (Table 3.3). The addition of 30% Sc biomass increased dietary lipids from 20.4% in the reference diet to 32.5% in the test diet. Gross energy was 5.5% higher in the test diet compared to the reference diet. Moisture and ash levels were comparable between diets.

Overall, saturated fatty acids (SFA) were 15% higher in the test diet than the control, with 16:0 accounting for most of this difference (16.6% higher than control, Table 3.3). The test diet contained 21.6% less total MUFA than the reference diet, with the most notable difference in 18:1n-9 (26% in the reference diet vs. 12% in the test diet). Dietary PUFAs were higher in the test diet (40%) compared to the reference diet (32%), with the most substantial difference in DHA (19.8% in the test diet vs. 4.6% in the reference diet), while EPA was lower in the test diet (4.0%) compared to the reference diet (7.6%). The n-3/n-6 ratio was 1.5 in the reference diet compared to 2.4 in the test diet (Table 3.3).

3.4.2 DIGESTIBILITY

There were no significant differences in apparent digestibility coefficients (ADCs) between fecal collection phases for either dietary treatment for macronutrients or FAs (see Appendix: Table 3.1); therefore, all reported results are pooled averages from both collection phases for each tank. Protein digestibility was similar between diets (89%), however ADC of DM and lipid were lower (2% and 9.9%, respectively) in the test diet (Table 3.4). ADCs for gross energy were lower in the test diet compared with the reference

diet but given that gross energy content of the test diet was higher in the test diet initially, total digestible energy (DE) available from each diet was similar around 20 MJ/kg for both treatments. Digestibility of total SFA was significantly ($p<0.001$) lower in the test diet (69%) compared to the reference diet (84%). The largest difference in FA digestibility between treatments was reported for 16:0, where the ADC was significantly ($p<0.001$) lower in test diet. Of all FA groups, the highest ADC values were reported for the PUFAs with EPA and DHA showing the highest ADC values (98.1% and 96.8% respectively). PUFA digestibility was significantly ($p=0.032$) higher in the test diet. ADCs for MUFAs did not differ ($p=0.797$) between the two dietary treatments (Table 3.4).

ADCs for macronutrients and FAs in the Sc biomass alone are reported in Table 3.5. Protein, lipid, and energy ADCs were 93.9%, 67.1%, and 70% respectively. Overall, total SFAs were poorly (57.4%) digested, namely 14:0 and 16:0 as these two SFAs were most abundant. Contrastingly, PUFAs in the Sc ingredient were highly digestible (98%), with both DHA and EPA reporting ADCs >98%. Monounsaturated FAs in Sc were also well (94.2%) digested (Table 3.5).

3.5 DISCUSSION

Due to its nutrient composition and efficient growth characteristics, Sc biomass has shown potential as a high-quality, digestible source of DHA and protein for salmonids. This study aimed to evaluate the digestibility of macronutrients, energy, and FAs of a whole cell Sc biomass product at a high (30%) inclusion level in Atlantic salmon diets. This allowed for the determination of digestibility values of the test diet and the Sc biomass specifically. In

general, the results align with those observed in previous studies; DHA and protein digestibility were high, while SFA, lipid, and energy digestibility were lower in the test diet containing 30% Sc compared to the reference diet.

The composition of the whole cell Sc biomass used in this study was similar to values reported in other studies with ranges of 1.6-3.5% (moisture), 11.9-20.7% (CP), 53.6-61.4% (lipid), 3.7-9.0% (ash) (Carter et al. 2003; Zhang 2013; Kousoulaki et al. 2015; Sarker et al. 2016). The FA profile of the Sc biomass was predominantly SFA (51.7%) and PUFA (46.7%) with a very small (<1%) MUFA proportion. The two most abundant FAs were palmitic acid (16:0) and DHA, comprising 28% and 21% of the total biomass, respectively. The remaining major FAs were myristic (14:0; 4.4%) and n-6 docosapentaenoic acid (DPA-6; 22:5n-6; 10.1%). Together these four FAs represented >90% total FAs. This FA profile is characteristic among *Thraustochytrid* strains and within the range of those reported by Tibbetts et al. (2020) and observed by other studies (Song et al. 2015; Sarker et al. 2016; Sprague et al. 2016).

The proximate and biochemical composition of both experimental diets were within nutritional guidelines for Atlantic salmon, with the exception of digestible protein in the test diet which was 2% below the recommended 36% (Table 3.3) (NRC 2011). Moisture, carbohydrate, ash, and energy content of the two diets were similar, and lipid and protein content varied considerably between the reference and test diet, due to the high inclusion of Sc at 30%. Compared to the reference diet, the test diet contained considerably more lipid and less protein, which was reflective of Sc's composition. Similar changes in diet

composition were observed by Sarker et al. (2016), who conducted a digestibility trial (reference diet + 30% *Schizochytrium* biomass) with Nile tilapia.

The difference in FA profiles between the reference and test diets were reflective of the FA profile of the Sc biomass, which was expected given that Sc contains predominantly lipid (63%) and was included in a relatively high (30%) proportion in the test diet. Palmitic acid and DPA-6 more than doubled and DHA was nearly four times greater in the test diet (19.8% total fatty acids) compared to reference (4.6% total fatty acids). Conversely, EPA levels in the test diet decreased by 90% (from 7.6% in the reference diet to 4.0% in the test diet) along with many MUFAs (16:1n-7, 18:1n-9, 22:1n-11) resulting in a reduction of just over 50% in total MUFAs compared to the reference diet. The n-3/n-6 ratio increased with Sc inclusion (1.5 in the reference diet compared to 2.4 in the test diet), which was expected given the substantial increase observed for n-3 FAs (largely DHA) and slight decrease in n-6 FAs (largely LNA) in the test diet. These changes in dietary FA composition align with the only other study evaluating Sc biomass at 30% inclusion for tilapia (Sarker et al. 2016) and are on trend with studies that evaluated Sc at lower inclusion levels when FAs groups were not intentionally balanced (Zhang 2013; Kousoulaki et al. 2015).

Protein digestibility did not differ between treatment diets, while all other macronutrients and gross energy were significantly lower in the test diet. The digestibility values for the reference diet are comparable to those from other digestibility and growth trials using a FO-based reference diet with salmonids (Kousoulaki et al. 2015; Kousoulaki et al. 2016; Bélanger-Lamonde et al. 2018; Tibbetts et al. 2020). Results from this trial are most

comparable to those of Kousoulaki et al. (2015), where Sc biomass was supplemented at 15% of the diet and dietary lipids were not balanced. Apparent digestibility coefficients for protein, lipid, and gross energy in that trial were 87.1%, 87.8% and 79.7%, which are comparable to those observed in the test diet for this study (Table 3.4).

The positive relationship between FA carbon chain length, unsaturation, and digestibility in salmonids is well-established (Austreng 1978; Sigurgisladottir et al. 1992) and was observed for both diets in this study resulting in AD values of n-3>n-6>PUFA>MUFA>SFA. This is due to the increased affinity of lipolytic enzymes for PUFAs (Caballero et al. 2002; Menoyo et al. 2007; Miller et al. 2007; Colombo-Hixson et al. 2011; Tibbetts et al. 2020). Similar trials evaluating Sc biomass with dietary inclusion levels ranging from 2.5-15% reported SFA digestibility values between 67-80% (Kousoulaki et al. 2015; 2016; 2020). Two out of the three trials included vegetable oils in the reference diets to balance the SFA content of Sc in the test diets, and in all cases, SFA digestibility decreased with increasing Sc inclusion. Studies that evaluated Sc extracted oil at inclusion levels between 2-13% (33-100% FO replacement) reported SFA ADCs between 71-76% (Miller et al. 2007; Tibbetts et al. 2020). When dietary SFAs were balanced among treatments, Tibbetts et al. (2020) reported higher SFA digestibility with increasing Sc oil inclusion (from 2-9% of the diet), as well as improved SFA digestibility compared to Miller et al. (2007), where dietary SFAs were not balanced. When compared to Kousoulaki et al. (2015) (a trial using Sc biomass) lipid and FA digestibility was higher at 15% Sc inclusion (compared to the 30% used in this study) for all FA groups except SFAs. In all trials using Sc biomass, MUFA and PUFA digestibility was very good (>98%)

(Kousoulaki et al. 2015; 2016; 2020). These collective findings indicate that SFAs in Sc may be more digestible than SFAs from other sources and when SFAs are not equated, observed decreases in SFA digestibility compared to reference diets may be due to formulation imbalances in SFA rather than the Sc ingredient specifically. Additionally, high MUFA and PUFA digestibility indicate that the cell wall is unlikely the main factor contributing to observed decreases in lipid and energy digestibility in the test diet. However, it is important to note that Tibbetts et al. (2020) and Miller et al. (2007) used Sc extracted oils which have different properties than whole cell meals and therefore strict comparisons cannot be drawn.

Lipid digestibility was significantly lower in the test diet compared to reference (Table 3.4). The ADC of PUFAs was very high (>95%) in both diets, but higher in the test diet. The observed decrease in lipid digestibility of the test diet can largely be attributed to the ADC of SFAs, particularly palmitic acid. It is well known that Atlantic salmon have a limited ability to digest SFAs and may also preferentially absorb PUFAs (Johnsen et al. 2000; Huyben et al. 2021; 2021). Several factors could be contributing to this result: a critical threshold for SFA absorption above which emulsion or micelle formation is impaired, surplus of PUFAs (particularly DHA) which were preferentially absorbed over other SFAs, formulation imbalances, or the position of the FAs on the microbial TAG (Caballero et al. 2002; Menoyo et al. 2003; Bogevik et al. 2018). Where FAs were not balanced in the test diet (SFA and PUFA levels were in excess of typical diet formulations), ADCs for lipid and FAs may be attributed to formulation imbalances in total SFA and PUFA, as opposed to Sc directly and the dietary threshold for efficient SFA absorption

could have been exceeded. Alternatively, the observed decrease in ADC of SFAs in the test diet may be directly related to the FA profile of Sc, the position of the SFAs on the microbial TAG or cell-wall components hindering bioavailability of nutrients. The larger difference in gross energy vs digestible energy observed in the test diet is likely a result of low energy availability due to decreased lipid digestibility (on account of lower SFA digestibility).

Apparent digestibility coefficients of protein, PUFAs and MUFAs from Sc were high (>90% for protein and MUFAs, >98% for PUFAs**)**, and ADC of DM, energy and lipid were in the lower range (67-70%). Previous research using Sc in aquafeeds for salmonids indicates that the recalcitrant cell wall of Sc could be partly responsible for hindering macronutrient digestibility, as salmonids have limited ability to digest some plant biomass due to the cell wall structure of plants and presence of indigestible carbohydrates (e.g., starches, polysaccharides, and cellulose; Colombo 2020), although this may not be the case for all microbial ingredients (Burr et al. 2011; Kousoulaki et al. 2015). However, given that some macronutrients were very well digested (for protein and PUFA, ADC was >90%) there is likely another factor contributing to the observed ADC values for energy and SFAs.

It is well-established that salmonids have limited ability to digest SFAs, which is further impacted by low water temperatures and increasing dietary levels (Johnsen et al. 2000; Menoyo et al. 2003; Ng et al. 2003). Tripalmitin is a triacylglycerol that has palmitic acid (16:0) in all three ester positions (Bogevik et al. 2018). Its inclusion can have a negative effect on utilization of lipids by slowing lipid hydrolysis and absorption (Bogevik et al.

2018; Kousoulaki et al. 2020; Miller et al. 2007). It is possible that the tripalmitin content of Sc used in this study may be responsible for the relatively low AD of lipids and SFAs observed in the test diet. Additionally, FA digestibility can depend on its position in the microbial TAG and regiospecific lipase activity for SFAs esterified in the sn-1, 2, or 3 position (Sargent et al. 2003; Mu and Høy 2004; Bogevik et al. 2018). The ADC for PUFAs, n-3 PUFAs and n-6 PUFAs was very high (>97%), with DHA being nearly completely digestible. This indicates that feeding high DHA (6.4% of diet) did not affect its digestibility. A selective absorption of DHA over SFA may have contributed to the observed ADC values for many SFAs (Johnsen et al. 2020). Furthermore, although carbohydrate digestibility was not calculated in this study it cannot be ruled out as a factor contributing to the observed ADC values for the test diet or Sc ingredient.

Digestibility, bioavailability, and composition of amino acids are the most important factors when assessing protein quality of an ingredient (Boye et al. 2012). Microbial protein is generally considered of high quality due to their broadly conserved and complete EAA profile, and a high EAA index (a calculation used to compare a novel protein source to a known balanced source) (Tibbetts 2018). Although not analyzed in this study, the EAA profile and protein efficiency ratio of Sc reported in the literature were summarized by Tibbetts (2018) and Acquah et al. (2020). *Schizochytrium* was reported to have a protein efficiency ratio (PER; amount of body weight gain given the amount of protein consumed) of 2.7-2.8 g/g when fed to Atlantic salmon and is most abundant in arginine, leucine and methionine (<1-12 g 100g protein $^{-1}$), two of which are the most common limiting EAAs in aquafeeds (NRC 2011; Yamamoto et al. 2012; Kousoulaki et al. 2015; Tibbetts 2018;

Acquah et al. 2020). Additionally, protein productive value (PPV; daily protein deposition rate given the amount of protein consumed) and nitrogen retention efficiency for *Thraustochytrium* (related genus) were 53% and 37-41% respectively (Carter et al. 2003; Betiku et al. 2016; Acquah et al. 2020) when fed to Atlantic salmon and rainbow trout. Given that protein digestibility was high (89% in the diet and 94% in Sc), and assuming all amino acids were digested equally it can be inferred that AA digestibility was also high. Although the Sc biomass used in this study is relatively low (12%) in protein, it can still be considered a high-quality protein source which furthers its usefulness in whole cell form, as opposed to an extracted oil, which could only be used as a source of lipids and EFAs.

It is also important to note that the diets used in this trial were produced by steam pelleting, where many related studies (and indeed commercial salmonid aquafeeds) used extruded diets, this may have played a role in any observed differences in AD values between studies. Gong et al. (2016) compared macronutrient digestibility of Atlantic salmon diets containing microbial ingredients between steam and extrusion pelleting methods and concluded that nutrient digestibility of microbial biomass-included diets was lower than reference diets and that extrusion pelleting improved digestibility of DM, protein and ash.

Based on the compiled results from this trial, and existing literature, it appears that the inclusion of the Sc biomass at very high levels (30% of the diet) in Atlantic salmon feed does seem to negatively affect overall energy, lipid and SFA digestibility, while the high inclusion levels do not effect (or in some cases increase) protein and PUFA digestibility.

It is a challenge to make strict comparisons between this study and that of Kousoulaki et al. (2015) and others due to differences in fish size, water temperature, fecal collection method, feed formulation, Sc strain and nutrient composition, which are all capable of altering digestibility of a feed and its ingredients (Ng et al. 2003; Glencross et al. 2007; NRC 2011). This effect could be in part due to dietary formulation imbalances or microbial cell wall components; however, the most widely reported consensus is the high SFA content and fully saturated TAGs present in the Sc ingredient negatively affected SFA digestibility, and subsequently lowered the overall lipid and energy digestibility of the diets (Bogevik et al. 2018). Nonetheless, this trial shows that Sc biomass can be an excellent source of digestible PUFAs, particularly DHA and protein. Additionally, metabolism and retention of DHA may be more efficient when dietary SFA are high due to a sparing effect (Codabaccus et al. 2012). Generally, DHA has been deemed more physiologically important in terms of fish health, while EPA has been considered more dispensable (Watanabe 1993; Copeman et al. 2002; Trushenski et al. 2012). DHA comprises 21% of Sc biomass used in this study, of which, is nearly 100% is digestible. Conversely, the Sc ingredient used in this trial was almost completely devoid of EPA, which is characteristic of previous research and *Thraustochytrid* DHA-rich products (Carter et al. 2003; Miller et al. 2007; Ganuza et al. 2008; Eryalçin et al. 2013).

The n-3/n-6 ratio of this ingredient is 3, which is much higher than those reported for vegetable and terrestrial animal oils (NRC 2011). This ratio has become increasingly relevant as n-6 rich plant and animal materials gain popularity in the aquafeed sector. Over the last decade the n-3 LC-PUFA content of salmon feeds has declined drastically, which

has translated in some cases to a 60% decline in EPA+DHA content of farmed Atlantic salmon tissue (Sprague et al. 2016). The dietary requirement of salmonids for essential n-3 LC-PUFAs is typically expressed as a combined amount of "DHA+EPA" and although these two LC-PUFAs are often coupled, they play several independent physiological roles and may not be equivalent in satisfying n-3 LC-PUFA demand (Codabaccus et al. 2012; Glencross et al. 2014; Horn et al. 2019). Low EPA may impact the n-3/n-6 and EPA/ARA ratio which could have a range of implications related to smolting ability, disease pathogenesis, cell membrane fluidity, impaired anti-inflammatory response and immune function (Bell et al. 1996; Simopoulos 2002; Stillwell and Wassall 2003; Miller et al. 2007; Norambuena et al. 2016)**.** However, some studies suggest that most of n-3 LC-PUFA requirement could be met by DHA alone based on observed disproportionate rates of β-oxidization and bioconversion of EPA → DHA and lower tissue retention rates of EPA compared to DHA (Codabbacus et al. 2012; Glencross et al 2014; Emery et al. 2016). Other studies suggest Atlantic salmon are able to maintain EPA tissue levels even when the diet is lacking by increased EPA preservation, reduced β-oxidation of EPA, retro-conversion of DHA → EPA and/or increased desaturation and elongation of shorter chain FAs from plant oils (Kousoulaki et al. 2015; Belanger-Lamonde et al. 2018). However, given that the EPA+DHA requirement of Atlantic salmon was recently re-evaluated by Qian et al. (2020) and found to be 0.5% EPA+DHA (lower than the widely accepted 2%) it is possible that these experiments all met or exceeded this requirement, even in "low-EPA" or negative control diet formulations. Furthermore, it is difficult to determine the essentiality of EPA solely based on how it is metabolized; most trials are conducted under standard conditions with minimal stressors where the true effects of EPA deficiency may not be apparent. More

research needs to be conducted on the discrete roles and dietary requirements of these two fatty acids by Atlantic salmon.

3.5.1 CONCLUSIONS

In addition to low EPA content, several other factors including protein and carbohydrate content could limit the potential of Sc biomass as a complete replacement for FM and FO (Tocher et al. 2019). However, as production costs improve, Sc biomass has major potential as a high-quality source of digestible DHA and protein as a FM and FO supplement and can increase the efficiency of finishing diets while further reducing organic pollutants associated with marine-derived ingredients (Betiku et al. 2015; Sprague et al. 2015; Belanger-Lamonde et al**.** 2018). Added benefits associated with the use of Sc biomass versus extracted oil include reduced cost (minimal downstream processing), improved product stability (cell wall protects lipids from oxidation), and bioactive compounds such as β-glucans, carotenoids, flavonoids and peptides (may improve intestinal health, growth or feed efficiency) (Miller et al. 2007; Borowitzka 2013; Winwood 2013; Singh et al. 2015; Lyons et al. 2017; Kousoulaki et al. 2020). Furthermore, the well-managed sustainable harvest of marine ingredients, although finite, are still a useful resource for the aquafeed industry if research continues to alleviate dependence on these ingredients (Tacon and Metian 2008).

Future research should focus on the effects of Sc biomass in nutritionally balanced diets, with or without EPA supplementation from FO. This would eliminate effects due to dietary imbalances and determine impacts, if any, of feeding diets supplemented with n-3 LC-

PUFAs from Sc biomass. Focus should be on measuring diet digestibility, growth parameters, fish health (intestinal and immune), tissue composition, and fillet quality.

3.6 TABLES AND FIGURES

Table 3.1 Formulation and proximate composition (as-fed basis) of the reference diet.

	% of diet
Ingredient[a]	
Fish meal (74.7% CP)	25.0
Ground wheat (14% CP)	16.0
Poultry byproduct meal (62% CP)	20.0
Blood meal (90% CP)	8.00
Empryeal® (75% CP)	12.0
Fish oil	10.0
Canola oil	5.00
Vitamin/Mineral mix (14.6% CP)[b,c]	0.20
Dicalcium phosphate	2.00
Pigment mix[d]	0.25
Lysine HCl	0.50
Choline chloride	1.05
Chromic oxide	0.50
Cholestane	0.05
Proximate composition[e]	
Moisture (%)	3.60
Ash (%)	9.13
Crude protein (%)	51.2
Lipid (%)	20.4
Carbohydrate (%)[f]	19.3
Gross energy (MJ/kg^{-1})	23.9

[a] All ingredients were supplied by Northeast Nutrition (Truro, NS, Canada).
[b] Vitamin mixture (IU or g/kg of premix): vitamin A, 900 000 IU; vitamin D_3, 400 000 IU; vitamin E (dl-alpha tocopheryl acetate), 25 000 IU; vitamin K (menadione sodium bisulphate), 3.0 g thiamin, 3.0 g; riboflavin, 4.0 g; pantothemic acid (as d-calcium pantothenate), 12.0 g; biotin, 0.1 g; folic acid, 1.0 g; vitamin B_{12}, 0.003 g; niacin, 15.0 g; pyridoxine, 4.0 g ascorbic acid, 30.0 g; carrier (ground wheat).
[c] Mineral mixture (g/kg of premix): manganous oxide, 23.0 g; zinc oxide, 70.0 g; cooper sulfate, 6.0 g; potassium iodide, 2.0 g; carrier (ground wheat).
[d] Pigment mix contains (mg/kg): selenium, 0.22 mg; vitamin E, 250 IU; vitamin C, 200 mg; astaxanthin, 60 mg; wheat shorts, 1988 mg.
[e] n=3.
[f] Calculated as [100 - (crude protein+lipid+ash)].

Table 3.2 Biochemical and proximate composition (dry matter basis) of *Schizochytrium* biomass.

	% of biomass
Proximate composition	
DM	97.8
Ash	8.60
Protein[a]	11.9
Lipid	63.6
Carbohydrate[b]	13.7
Fibre	3.80
Fatty acids (% of FAME)	
12:0	0.15
14:0	4.44
15:0	0.48
16:0	44.7
16:1n-7	0.06
17:0	0.29
18:0	1.19
18:1n-9	0.71
18:1n-7	0.08
18:2n-6	0.37
18:3n-6	0.10
18:3n-3 (ALA)	0.17
18:4n-3	0.18
20:0	0.18
20:3n-6	0.23
20:3n-3	0.09
20:4n-6	0.80
20:4n-3	0.52
20:5n-3 (EPA)	0.70
22:0	0.10
22:5n-6 (DPA)	10.1
22:5n-3	0.24
22:6n-3 (DHA)	33.2
24:0	0.13
∑SFA	51.7
∑MUFA	0.80
∑PUFA	46.7
∑n-3	35.1
∑n-6	11.6
n-3:n-6	3.03

[a] Crude protein (N x 4)

[b] Calculated as [100 - (moisture+crude protein+lipid+ash)].

Table 3.3 Biochemical and proximate composition (as-fed basis) of reference and test diets.

	Reference	Test
Proximate composition (% of biomass)		
Moisture	3.60	4.00
Ash	9.10	8.70
Protein[a]	51.1	38.6
Lipid	20.4	32.5
Carbohydrate[b]	15.7	16.1
Gross energy (MJ/kg^{-1})	24.0	25.4
DP (g/kg^{-1})	45.5	34.3
DE (MJ/kg^{-1})	20.1	20.2
DP/DE ratio (g DP/MJ DE^{-1})	22.6	17.0
FAs (% of FAME)		
14:0	4.20	4.60
16:0	15.8	32.4
16:1n-7	5.10	2.40
18:0	3.50	2.20
18:1n-9	26.0	12.0
18:2n-6	10.80	5.20
18:3n-3 (ALA)	2.65	1.30
20:4n-6	0.50	0.40
20:5n-3 (EPA)	7.60	4.00
22:1n-11	2.64	1.20
22:5n-6 (DPA)	0.16	0.50
22:6n-3 (DHA)	4.60	19.8
∑SFA	26.9	41.9
∑MUFA	40.4	18.8
∑PUFA	32.7	40.2
∑n-3	17.8	27.1
∑n-6	12.1	11.5
n-3/n-6 ratio	1.50	2.40

[a] Crude protein (N x 6.25).
[b] Calculated as [100 – (crude protein+lipid+ash)].

Table 3.4 Apparent digestibility coefficients (% ± SD) of DM, macronutrients, energy and major fatty acids in the reference and test diets.

	Reference	Test	F-stat	P-value
DM	76.9 ± 0.8[a]	74.9 ± 0.8[b]	23.26	<0.001
Protein	89.0 ± 0.6	89.2 ± 0.5	0.38	0.548
Lipid	92.8 ± 1.6[a]	82.9 ± 1.4[b]	247.35	<0.001
Energy	83.8 ± 0.6[a]	79.7 ± 0.4[b]	148.27	<0.001
14:0	90.6 ± 1.0[a]	78.5 ± 2.1[b]	184.03	<0.001
16:0	82.7 ± 1.8[a]	66.3 ± 3.4[b]	125.41	<0.001
16:1n-7	95.8 ± 0.5[a]	95.0 ± 0.8[b]	5.31	0.037
18:1n-9	94.6 ± 0.7	94.9 ± 1.0	0.51	0.485
18:2n-6	95.4 ± 0.6	96.0 ± 0.9	1.95	0.185
20:4n-6	94.6 ± 0.5[a]	96.1 ± 0.7[b]	21.64	<0.001
20:5n-3	97.8 ± 0.3	98.1 ± 0.6	1.09	0.313
22:5n-6	92.1 ± 3.6[a]	95.9 ± 0.5[b]	7.48	0.018
22:6n-3	93.1 ± 1.5[a]	96.3 ± 0.7[b]	25.18	<0.001
∑SFA	84.0 ± 1.4[a]	69.3± 3.1[b]	134.62	<0.001
∑MUFA	93.5 ± 0.8	93.6 ± 1.1	0.07	0.797
∑PUFA	96.0 ± 0.5[a]	96.7 ± 0.6[b]	5.75	0.032
∑n-3	96.3 ± 0.6	96.5 ± 0.5	0.33	0.577
∑n-6	94.9 ± 0.7[a]	95.9 ± 0.5[b]	9.74	0.008

Different superscripts indicate significant difference ($p<0.05$) between values.

Table 3.5 Apparent digestibility coefficients (ADC; % ± SD) of DM, macronutrients, energy and FAs from *Schizochytrium* biomass when fed to Atlantic salmon

	ADC (%)
Proximate composition	
DM	70.0 ± 3.8
Protein	93.9 ± 7.0
Lipid	67.1 ± 3.6
Gross energy (MJ/kg^{-1})	70.0 ± 2.7
Fatty acids (% of FAME)	
14:0	66.3 ± 10.7
16:0	56.9 ± 12.3
16:1n-7	91.9 ± 4.4
18:1n-9	96.1 ± 5.1
18:2n-6	97.7 ± 4.2
18:3n-3 (ALA[1])	97.9 ± 3.4
20:4n-6	99.4 ± 2.5
20:5n-3 (EPA[1])	98.8 ± 2.4
22:5n-6 (DPA[1])	96.4 ± 1.0
22:6n-3 (DHA[1])	98.9 ± 1.9
∑SFA[2]	57.4 ± 12.2
∑MUFA[2]	94.2 ± 5.9
∑PUFA[2]	98.0 ± 1.2
∑n-3	97.8 ± 2.2
∑n-6	97.5 ± 1.5

[1] ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosahexaenoic acid; DHA, docosahexaenoic acid.

[2] SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids

CHAPTER 4.0

H. PLUVIALIS IN A WEAKENED WHOLE CELL FORM IMPROVES FILLET PIGMENTING EFFICIENCY IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

4.1 ABSTRACT

Fillet color is an important quality indicator for consumers and can influence the market value of salmonid products. To achieve flesh coloration in farmed salmonids, synthetic astaxanthin (Ax) pigment is added to the feed. Cost, safety, and consumer preference are increasing research efforts for non-synthetic Ax alternatives. *Haematococcus pluvialis* (Hp) is an efficient producer of Ax but hasn't been effective in pigmenting salmonid flesh due to its rigid cell wall structure, which limits bioavailability of the Ax compound. A novel form of Hp that has been cultivated heterotrophically to possess a weakened cell wall structure might be the solution to increasing Ax bioavailability from Hp whole cells. To assess pigmenting ability, Ax digestibility, tissue content, and fillet coloration were measured in rainbow trout (*Onchorhynchus mykiss*) fed diets containing this pre-commercial Hp product. Sixty rainbow trout (992 g ± 177.24 g) were PIT (passive integrated transponder)-tagged, distributed among six tanks, and fed one of three experimental diets, namely No Ax (N-Ax), synthetic Ax (S-Ax; 80 mg/kg Ax), or Ax from Hp whole cells (Hp-Ax; 60 mg/kg Ax). After 10 weeks, Ax digestibility, tissue Ax content, and flesh color were assessed. No significant ($p>0.05$) differences were evident in either growth metrics, Ax digestibility, fillet Ax content, retention or coloration were observed between S-Ax and Hp-Ax treatments. Ax digestibility and retention was 76.7 ± 2.68% and 15.4 ± 0.23%, respectively, for fish fed the Hp diet. SalmoFan™ color readings of S-Ax and Hp-Ax fillets ranged from 28-34 and were significantly higher than N-Ax fillets ($p<0.001$). This novel whole cell *Haematococcus pluvialis* was equally efficient at pigmenting the flesh of rainbow trout compared to synthetic Ax. This product has potential as a replacement for synthetic astaxanthin in salmonid feeds.

4.2 INTRODUCTION

Carotenoids are a group of lipid-soluble pigments produced by algae, plants, and some species of fungi and bacteria (Lorenz and Cysewski 2000). Astaxanthin is a carotenoid most notably responsible for the pinkish-red coloration of aquatic species such as salmonids, shrimp, and other crustaceans (Young et al. 2017). This coloration is achieved through bioaccumulation, where microbes that synthesize the carotenoids via primary production are ingested by planktonic species and small crustaceans that are then ingested by higher organisms (Lorenz and Cysewski 2000). Consumers consider a deep pink flesh color an important quality indicator of salmonid fillets (Viera et al. 2018).

The salmonid aquaculture industry is a major market for astaxanthin, where global production of farmed salmonids alone is estimated to be over 2.86 million tonnes, and the addition of dietary pigment can account for up to 15% of feed costs (FAO 2020; Jannel et al. 2020). Rainbow trout are a valuable aquaculture species in North America, with a combined production of over 31 000 tonnes between Canada and USA and are a model species in salmonid pigmentation research (FAO, 2018). This market is dominated by synthetically derived astaxanthin (>95%), however consumer preference is shifting toward more natural, sustainable ingredients; hence the need for alternative sources of astaxanthin with similar pigmenting efficacy as the synthetic version (Shah et al. 2016; Young et al. 2017; Viera et al. 2018).

Several sources of astaxanthin have been investigated as alternatives to the synthetic pigment in salmonid feeds, such as animal materials (crustacean meals and wastes) and

higher plants (red pepper and marigold flowers) (Diler and Gokoglu 2004; Yanar et al. 2007; Garcia-Chavarria and Lara-Flores 2013). These ingredients are cheap and accessible; however, they have limitations that inhibit their ability to compete practically with synthetic astaxanthin in the aquafeed sector. Animal materials contain ash and chitin that hinder digestibility, thus inclusion rates, and higher plants contain a mix of carotenoids in addition to astaxanthin that can result in undesirable flesh coloration and taste (Yanar et al. 2007; Garcia-Chavarria and Lara-Flores 2013). Furthermore, many of these potential sources are low in astaxanthin relative to synthetic sources (Shahidi and Synowiecki 1991).

The use of microorganisms as feed additives has advantages in terms of ingredient availability, ease of culture manipulation, and production sustainability. The fast-growing red yeast *Xanthophyllomyces dendrorhous* (anamorph of the *Phaffia rhodozhyma*) accumulates about 0.4% to 0.5% dry weight (DW) natural astaxanthin and inclusion of 15% *Phaffia* biomass in the diet for rainbow trout has achieved flesh Ax concentrations as high as 10ug/g (Johnson et al. 1977). However, the tough cell wall of the ingredient combined with relatively low Ax content has led to a decline in interest from the aquafeed industry in favour of other hyperproducing algal sources such as *Haematococcus pluvialis* (Bhosale and Bernstein 2005). Of all microbial ingredients, the microalga *Haematococcus pluvialis* (Hp) has shown the most potential as a naturally derived source of astaxanthin for aquafeeds (Bowen et al. 2002; Moretti et al. 2006; Young et al. 2017).

Hp is a unicellular alga that is ubiquitous in freshwater environments (Shah et al. 2016). It can produce high (up to 6% DW) amounts of astaxanthin within its cell (Han et al. 2013).

Hp has two main growth phases, a green vegetative phase and a red cyst phase. The green phase is characterized by rapid growth of motile cells under favourable conditions. During this phase, Hp contains a high amount of protein, and lutein and β-carotene are the dominant carotenoids. Environmental stressors induce the red phase which is characterized by the encystment of non-motile cells to form haematocysts. As the haematocysts mature, the cells accumulate astaxanthin in lipid bodies to defend against oxidative stress (Hagen et al. 2002; Han et al. 2013).

Hp is conventionally produced through two-step batch photoautotrophic cultivation where the first step prioritizes biomass production under optimal conditions in the green phase and the second step focuses on astaxanthin production in the stress-induced red phase (Han et al. 2013). Downstream processes like harvesting, mechanical cell wall disruption, drying and in some cases, carotenoid extraction, are all often necessary to produce an effective final product (Mendes-Pinto et al. 2001; Young et al. 2017). Photoautotrophic production of Hp lends to low biomass productivity, reduced bioaccessibility of astaxanthin due to the structure of the haematocysts, and the added expense of cell wall disruption required to mitigate this problem (Sommer et al. 1991; Mendes-Pinto et al. 2001; Choubert et al. 2006; Liu et al. 2014) which is why Hp is still generally considered an inferior product to synthetic astaxanthin. Extraction of astaxanthin from Hp cells can drastically increase Ax bioaccessibility, however these processes are expensive, labor intensive, and can affect the stability and reduce the shelf life of the compound (Han et al. 2013). Other culture methods have been developed using heterotrophic, mixotrophic, or a combination of methods in attempt to increase biomass productivity, cell density or astaxanthin accumulation. Some

of these methods have been successful on a laboratory scale but are often not feasible for large-scale production (Han et al. 2013; Shah et al. 2016). The Hp product used in this trial was produced by Kuehnle Agrosystems (Honolulu, Hi, USA) using a novel fermentation culture method to obtain weakened whole cells (US Patent # 11.034.968; Kuehnle and Shurr, 2019).

The flesh-pigmenting ability of Hp has been evaluated for salmonid feeds in whole cell, cell-ruptured, and extracted forms at inclusion levels ranging from 40-80 mg/kg Ax (Sommer et al. 1991; Sommer et al. 1992; Choubert and Heinrich 1993; Bowen et al. 2002; White et al. 2002; Choubert et al. 2006; Moretti et al. 2006; Young et al. 2017). These studies indicate that Hp is capable of pigmenting the flesh of salmonids (Moretti et al. 2006; Young et al. 2017) although tissue pigmentation levels of fish fed Hp are generally inferior to those fed synthetic Ax (Choubert et al. 2006). These results are attributed to poor cell rupture rates, and the resulting decrease in availability of Ax, due to the poor digestibility of the Hp cell wall (Sommer et al. 1991; Choubert et al. 1993). Single-celled organisms often have robust cell walls that require additional processing such as cellular disruption via bead milling, or treatment with heat, chemicals or enzymes (Mendes-Pinto et al. 2001) and carotenoid extraction (White et al. 2003) to increase the bioavailability of astaxanthin. These processing methods can help achieve sufficient pigmentation of salmonid flesh but can also decrease the stability of the pigment and increase the cost of production (Storebakken et al. 2004; Tibbetts 2018). These additional procedures are too costly to make Hp-derived Ax economically competitive with its synthetic counterpart in animal and aquafeeds (Shah et al. 2016; Viera et al. 2018).

The objective of this study was to assess the pigmenting efficacy of a novel form of *Haematococcus pluvialis* clonally selected (non-GMO, non-mutated) and produced to possess a weakened cell wall and a null hypothesis that there will be no difference in pigmentation of rainbow trout fed either Hp-derived or synthetic Ax (Kuehnle and Schurr 2019). The Hp used in this trial is grown heterotrophically with the addition of organic acids to produce vegetative motile pigmented macrozooids rather than haematocysts. This eliminates the rigid cyst structure associated with hypercarotenogenesis during photosynthetic production and the subsequent need for carotenoid extraction (Kuehnle and Schurr 2019). Furthermore, there are advantages to supplementing with a whole cell Ax source (as opposed to disrupted cells, extracts, or synthetic Ax), such as enhanced product stability, superior antioxidant defense, and the addition of nutritionally beneficial amino acids, fatty acids, vitamins and minerals contained within the cell (Liu et al. 2014; Shah et al. 2016; Tibbetts 2018). Additionally, a heterotrophic growth strategy presents several advantages, such as scalability, higher growth rates and increased cell density (Lewis et al. 1999; Guedes et al. 2011; Gladyshev et al. 2013). A 10-week feeding trial was conducted to compare the flesh coloration of rainbow trout fed diets containing no added pigment, synthetic pigment, or whole cell Hp biomass.

4.3 MATERIALS AND METHODS

4.3.1 TEST INGREDIENT AND DIET PREPARATION

Proximate, fatty acid (FA), and Ax composition of the Hp biomass is presented in Table 4.1. The Hp biomass contained predominantly protein (49%) and lipid (24%), with the

major FAs being palmitic (16:0), vaccenic (18:1n-7), linoleic (18:2n-6), alpha-linolenic (18:3n-3) acids, accounting for >67% total FAs. Total Ax content of the Hp ingredient was 3089 ug/g (0.31% of biomass), and the all-trans isomer was dominant comprising 86% of total Ax (Table 4.1).

Three treatment diets were formulated commercial grade without pigment; N-Ax, commercial grade with synthetic pigment (ASTA; Divi's Laboratories Ltd, India); S-Ax and test feed with pigment from *Haematococcus pluvialis* (Kuehnle Agrosystems, Honoulu, HI, USA); Hp-Ax (Table 2). N-Ax containing no added pigment, and S-Ax containing 80 mg/kg synthetic Ax were used as negative and positive controls respectively (Table 4.2). Total Ax content of the N-Ax, S-Ax and Hp-Ax diets was 16.9 mg/kg, 80.5 mg/kg, and 60.6 mg/kg respectively. Ax content of the Hp-Ax diet was lower than the target concentration of 80 mg/kg Ax due to the Hp biomass containing 0.31% DW Ax, which was lower than the 0.5% Ax value used in the feed formulation.

All diets contained the same baseline formulation of 46% protein and 23% lipid, and satisfied the nutrient requirements of rainbow trout (NRC 2011). All diets contained 0.5% chromic oxide (Cr_2O_3) as an inert digestibility marker. Diets were prepared in the Chute Animal Nutrition Centre at the Dalhousie University Agricultural campus (Truro, NS). Dry ingredients for both diets were mixed in an industrial 19 L Hobart mixer (Model A-200-T) for five minutes (SOP #SAMP7). After mixing, diets were extruded into 8 mm pellets using a single screw KAHL extruder with mixing conditioner (Amandus Kahl GmbH & Co. KG; Type OEE8). The pellets were dried for eight hours at 60°C and sieved

to remove fine particles. Pellets were then vacuum coated with dietary oils (Dinnissen Process Technology). All diets were stored in fiberglass feed bags at -20°C until needed.

4.3.2 EXPERIMENTAL DESIGN

Rainbow trout were originally obtained from Fraser Mills Hatchery (Triploid, all female eggs; Antigonish, NS) in 2018. All fish were fed commercial, non-pigmented feed prior to the start of the trial. Sixty fish containing PIT (passive integrated transponder) – tags inserted into the dorsal musculature following CCAC guidelines and were distributed into six-500 L fiberglass tanks (10 fish per tank, initial mean weight ± SD of 992 g ± 177.24 g. Diets were fed in duplicate where individual fish were considered the experimental unit (n=20 for each treatment). A closed, recirculating system supplied continuously aerated (14 mg/L dissolved oxygen, >90% gas saturation) freshwater to each tank and was maintained at 12°C in a recirculating system (11.8 L/min; pH = 7.6; alkalinity = 124mg/L $CaCO_3$ eqv.). Fish were fed at a rate of 1% body weight at 1000 h daily and re-weighed half-way (five weeks) through the trial to account for increases in body weight and ensure feed rate was maintained. Fish were cared for and handled in accordance with the guidelines of the Canadian Council of Animal Care (Institutional approved protocol #2019-034).

4.3.3 SAMPLE COLLECTION

At Week 0 all fish were anesthetized with tricaine methane sulfonate dissolved in water at 150 mg/L (MS222; Syndel, Ferndale, WA, US) and fork length, body weight and PIT tag number were recorded from each fish, and fillet samples were taken from six fish belonging

to the same cohort as the 60 fish used in this trial. At Week 6, fish were anesthetized, weighed and PIT tags were scanned. The feed ration (1% body weight/fish/day) was adjusted to account for fish growth. At Week 10, all fish were euthanized by a combination of a captive bolt stunner, followed immediately by exsanguination by a slit through the ventral aorta and placed in an ice bath for 15 minutes. After bleeding, fork length and body weight were recorded from all fish. Fecal samples were collected 24-hours post-feeding by intestinal dissection and pooled by tank for digestibility analysis. The digestive tract was removed from the euthanized fish and fecal matter was collected from the posterior distal portion of the intestine. Following stripping, the intestine was inverted to ensure all feces were collected. Fillets were taken from both sides of each fish for carotenoid analysis. Fillet color was assessed immediately post-sampling using a SalmoFan™ (Hoffmann-La Roche Basel, Switzerland) and a HunterLab MiniScan EZ 45/0 LAV colorimeter (Hunter Associates Lab, Inc., Reston, VA, USA) with EZMQC-OPT EasyMatch Quality Control Software. Remaining fillets were frozen at -20°C until needed for tissue analysis. A subsample of all diets was taken for proximate and biochemical composition analysis.

4.3.4 ANALYTICAL METHODS

Diet, and fecal samples were freeze dried for 72 hours and ground into homogenous samples using a mortar and pestle. Diet samples were analyzed for proximate composition by the Nova Scotia Department of Agriculture Analytical Lab (NSDA, Bible Hill, NS, Canada). Carbohydrate content of the diets was calculated by difference [100 (protein+lipid+moisture+ash)]. Diets and fecal samples were analyzed for chromic oxide content at the Canadian Feed Research Centre (University of Saskatchewan, Saskatoon,

SK). Apparent digestibility coefficients (ADCs) for astaxanthin from the three experimental diets were measured using the indirect digestibility determination method (NRC 2011). Whole, skinless fillets were ground using a commercial grade meat grinder and freeze-dried at the National Research Council Marine Research Station (Ketch Harbour, NS, Canada). Ax content of diet and tissue samples were analyzed by an external lab (Valorēs, Shippagan, NB) according to Folch et al. (1957) and Yuan and Chen (1997). Tissue samples were analyzed for crude protein and lipid content at the Dalhousie University Agricultural Campus (Truro, NS, Can). Crude protein in tissue samples was analyzed by the Dumas method (% nitrogen × 6.25) (Ebeling 1968) using a Leco protein/N analyzer (Model FP-528, Leco Corp., St Joseph, MI, USA). Crude lipid content of the tissue samples was determined using an ANKOM XT15 extraction system (ANKOM Technology, Macedon, NY, USA; AOCS 2005).

Quantitative color measurements were taken in duplicate from each fillet (two fillets per fish). Fillets with skin on were covered with plastic wrap and color values were measured from the center of each fillet just above the lateral line. Color was measured within the L^* (lightness), a^* (redness), and b^* (yellowness), color space in accordance with the Commission Internationale de l-Eclairage (CIE, 1976). a^* and b^* values were then transformed to chroma (C^*) and hue ($H(^\circ)_{ab}$) to reflect the three-dimensional characteristics of color within the $L^* C^* H(^\circ)_{ab}$ color space per Wyszecki and Stiles (1967). Chroma denotes the degree of color saturation in terms of intensity and clarity (Christiansen et al. 1995; Nickell and Bromage 1998) and hue angle describes the relationship between redness and yellowness, where the angular measurement indicates the

predominant color ($H(^o)_{ab}$ = 0 indicates red hue and $H(^o)_{ab}$ = 90 indicates a yellow hue) (Choubert et al. 1997; Nickell and Bromage 1998). Values were generated using daylight setting (D65, CIE L, a, b). Additionally, each fillet was measured 'blind' using a SalmoFan™ by un-biased third party. Light, sampling surface, and temperature remained constant throughout the color measurement process. An average of each value measured via colorimeter or Salmofan™ was taken and later analyzed for statistical differences in fillet pigmentation between diet treatments.

4.3.5 CALCULATIONS

4.3.5.1 GROWTH

Growth metrics were calculated based on length and weight measurements from initial (week 0) and final (week 10) samplings as well as feed consumption based on a feed rate of 1% body weight per day. The following parameters were calculated to determine the efficacy of all diets:

Condition factor (K) = 100 x (Final body weight (g) / Final fork length $(cm)^3$)

Weight gain (g/fish) = (Final weight fish n_i (g) – Initial weight fish n_i (g))

where n_i = fish with specific PIT tag, and subscript $_i$ denotes individual number

Specific growth rate (SGR) = 100 x [ln(Final body weight (g))-ln(Initial body weight (g))]/Number of days

Feed conversion ratio (FCR) = Feed intake (dry; g)/Wet weight gain (g)

4.3.5.2 DIGESTIBILITY

To determine astaxanthin digestibility of the experimental diets, chromic oxide was used as an inert digestibility marker in the feed and ADCs were calculated by the indirect digestibility determination method. The proportion of chromic oxide to nutrient/ingredient (i.e., astaxanthin) in the feed was compared directly to that in the feces. The following equation was used to calculate astaxanthin digestibility of the three treatment diets (Maynard et al.1979; Forster 1999; Tibbetts et al. 2006):

Ax digestibility (%) = 100 – (100 x [Cr_2O_3 in diet / Cr_2O_3 in feces] x [Ax in feces/Ax in diet])

The following calculation was used to determine net apparent retention (NAR) of astaxanthin from the experimental diets (Wathne et al. 1998; Bjerkeng et al. 1999):

$NAR = [100 \times 0.61 \times ((Wgt_f \times Ax_f)-(Wgt_i \times Ax_i))] / ((Wgt_f - Wgti) \times FCR \times Ax_d)$

Where subscripts $_i$ and $_f$ denote initial and final values respectively and;

Wgt = individual fish weight (g)

Ax = astaxanthin concentration of the tissue (mg/kg)

Ax_d = astaxanthin concentration of the diet (mg/kg)

FCR = feed conversion ratio for the respective tank

Factor 0.61 represents the average flesh percentage in the whole fish

4.3.5.3 COLORIMETER

The following calculations were used to transform the $L^*a^*b^*$ color values to reflect the three-dimensional characteristics of color within the $L^* C^* H(^\circ)_{ab}$ color space (Wyszecki and Stiles 1967; Pavlidis et al. 2006):

$$H(^\circ)_{ab} = \arctan(b^*/a^*)$$

$$C^* = (a^{*2}+b^{*2})^{1/2}$$

4.3.6 STATISTICAL ANALYSIS

Tests for normality and homogeneity of variance of the residuals were completed, and Grubb's test was used to determine the presence/absence of outliers in the data set before running additional statistical analyses. One-way ANOVA with 95% confidence was used (Minitab19 Statistical Software) to compare differences in weight, length, tissue Ax, ADC and NAR of Ax, and color metrics across treatments. Tank effects were assessed prior to pooling treatment data. Individual fish represented an experimental unit since fish were PIT-tagged and measured as individuals throughout the study, therefore n=20 for each treatment. A Tukey HSD post-hoc test was used for multiple comparisons if ANOVA indicated significant differences (alpha = 0.05) among treatment means. Correlation analysis was conducted to assess the relationship between NAR and dietary Ax concentration.

4.4 RESULTS

4.4.1 GROWTH PERFORMANCE

All treatment tanks exhibited 100% survival after the ten-week trial. No significant differences in growth, condition factor, SGR or FCR were observed between treatments (Table 4.3). Average weight gain ± standard deviation (SD) per fish was 927 ± 172.49g, 909 ± 136.86g, and 896 ± 201.37g for N-Ax, S-Ax, and Hp-Ax treatments respectively. FCR and K ranged from 1.02-1.08 and 1.75-1.79 respectively and were both highest (non-significant) in the Hp-Ax fish (Table 4.3).

4.4.2 TISSUE COMPOSITION

No significant differences in lipid or protein content of fish muscle tissues were observed between diet treatments (Table 4.4). Total lipid in the tissues ranged from 19-24%, total protein ranged from 74-78% and did not differ significantly ($p=0.231$ and $p=0.106$ respectively) between dietary treatments. Total Ax content of tissues was lowest in the N-Ax treatment (5.48 μg/g) and highest in S-Ax (9.11 μg/g) and Hp-Ax (8.86 μg/g) treatments, S-Ax and H-Ax treatments did not differ significantly from each other (Table 4.4). In all treatments, Trans Ax was the dominant isomer form and accounted for >90% in fish tissues. Across all treatments, fish tissues contained <5% 13-cis Ax and trace amounts (<0.3 μg/g) 9- and di-cis Ax (Table 4.4).

4.4.3 DIGESTIBILITY

ADCs of total Ax for all diets were between 53-77% (Table 4.5). All forms (except di-cis) of Ax were less digestible in the N-Ax diet compared to that of the S-Ax and Hp-Ax diets.

Astaxanthin was the most digestible (76.8%) in the Hp-Ax diet for all isomer forms except di-cis (Table 4.5). Digestibility of all isomers varied between diets. NAR of astaxanthin ranged from 13-15% for the pigmented experimental diets and was highest (although not significant) in the Hp-Ax treatment group. NAR was significantly higher ($p=0.001$) in trout fed the N-Ax diet (34.8%) compared to the other treatment groups (Table 4.4). Correlation analysis revealed a strong negative correlation ($r = -0.971$, $p = 0.001$) between NAR and dietary Ax concentration.

4.4.4 COLOR METRICS

*L*a*b** and SalmoFan™ readings of muscle from trout fed the three treatment diets are displayed in Figure 4.1. All fillets showed a significant decrease in *L** when compared to baseline following 10 weeks of feeding. Hp-Ax fillets showed intermediary values (41.03 ± 2.8; Figure 4.1) and did not differ significantly from either N-Ax or S-Ax treatments (Figure 4.1). A significant increase in *a** ($p<0.001$) and *b** ($p=0.006$) readings was observed between Week 0 and Week 10 fillet samples across all diets (Figure 4.1). Redness (*a**) was significantly ($p<0.001$) higher in trout muscle fed S-Ax and Hp-Ax diets at Week 10 compared to the negative control (N-Ax; Figure 4.1). Yellowness (*b**) increased from Week 0 and did not differ significantly between diet treatments at Week 12. Hue decreased ($p<0.001$) in all treatment groups from baseline and values were not statistically significant between Hp-Ax and S-Ax fish on Week 10 (Table 4.6). An increase in chroma was observed in all treatments from Week 0, again Hp-Ax fillets showed intermediary values (42.87 ± 4.4) and did not differ significantly from either N-Ax or S-Ax treatments (Table

4.6). SalmoFan™ readings increased in all fillets from Week 0, and final readings in Hp-Ax fish (30.69 ± 1.8) did not differ significantly from S-Ax fish (32.4±1.6; Figure 4.1).

4.5 DISCUSSION

Through novel production techniques, digestible thin-walled Hp whole cells were produced with the goal of increasing Ax bioavailability to the fish without the need for additional processing. The objective of this experiment was to assess the pigmenting ability of this pre-commercial Hp product by measuring Ax digestibility, tissue content, and fillet coloration in rainbow trout. Overall, our results showed no significant differences in growth, tissue Ax content and retention, Ax digestibility, and color in rainbow trout fed treatments containing either synthetic or microalgal Ax. In general, these results confirm our null hypothesis that there would be no difference in pigmenting efficacy between synthetic Ax and the Hp product being tested in this trial.

4.5.1 GROWTH PERFORMANCE

Feed conversion ratios and condition factor were between 1.02-1.08 and 1.75-1.79, respectively. These values are within the upper range of those reported for rainbow trout of this size (Baxter 1998; Okumus and Mazlum 2002; NRC 2011; Shah et al. 2011). Muscle lipid and protein content were within range of values reported in other studies on rainbow trout using diets with similar compositions (Dumas et al. 2007). These results indicate that Hp was palatable, yielded good growth performance, and had no apparent negative effects on health compared to the synthetic or negative control treatments.

4.5.2 INGREDIENT AND DIET COMPOSITION

The proximate and biochemical composition of the Hp biomass (48.7% protein, 24.4% lipid, and 19% carbohydrate) was within range of that reported in the literature for protein (3-48%) and lipids (7-67%), while carbohydrate content was lower than that summarized in Tibbetts (2018). N-Ax and S-Ax diets varied considerably in protein and lipid content, the cause of this variation is unknown but may be a result of human error in feed formulation or analysis of a non-homogenous sample. The trans Ax isomer was the dominant isomer in all diets, which is common for these ingredients (Young et al. 2016; Bjerkeng 2020; Yu and Liu 2020). The Ax content of the Hp-Ax diet was 25% lower than the target formulation of 80mg/kg Ax due to a discrepancy in the theoretical (0.5% DW) versus actual (0.31% DW) Ax content of the Hp biomass used (Table 4.2). Degradation of the algal carotenoids during processing and storage is possible but storage and production procedures were identical across all diets, and algal and synthetic Ax both remain relatively stable (2-9% total carotenoid loss) during and following feed processing (Gouveia and Empis 2003; Jintasataporn and Yuangsoi 2012). Furthermore, cis-isomer content, an indicator of sample degradation, of the Hp-Ax diet was low, indicating the astaxanthin molecules were minimally affected by extrusion and storage procedures (Bjerkeng 2000; Britton et al. 2008). Due to being mostly esterified, quantification of algal carotenoids is more difficult and subject to error than free form synthetic Ax (Yuan et al. 1996; Lorenz and Cysewski 2000). All samples containing microalgal carotenoids in this experiment were analyzed using saponification as per Folch et al. (1957) and Yuan and Chen (1997), therefore we are confident the analytical methods and reported Ax contents of the diets are reliable. The reason for the observed discrepancy in Ax concentration between S-Ax and

Hp-Ax diets despite both sharing the same theoretical Ax formulation was likely an overestimation of the Ax concentration of the Hp biomass. This presented some challenges when comparing results from the two pigmented diets and is recognized as a limitation of this study.

The N-Ax diet, which did not contain added pigment, was shown to contain a low level of Ax (17 mg/kg) as compared with 81 mg/kg and 61 mg/kg for S-Ax and Hp-Ax, respectively, which was reflected in the fillet Ax content and colorimeter measurements when compared to Week 0 samples. Several dietary ingredients may be responsible for these results. Empyreal®, a corn protein concentrate, contains xanthophyll pigments which can deposit in the muscle tissue of rainbow trout resulting in a yellow flesh color (Park et al. 1997; Skonberg et al. 1998; Gatlin et al. 2007; Li et al. 2007). Fish meal and oils also contain astaxanthin and other carotenoids which could have contributed to the total Ax content of the diet (Matsuno 2001; Maoka 2011). Nonetheless, all diets contained an identical baseline formulation with minor adjustments to balance the nutrient composition of the Hp biomass in the Hp-Ax diet therefore, any incidental dietary carotenoids would be consistent across all treatments.

4.5.3 DIGESTIBILITY

Astaxanthin (total) digestibility was highest (although not statistically higher than S-Ax) for trout fed the Hp-Ax diet. The ADCs for astaxanthin in the current study were within range (50-70%) of those previously reported in the literature for rainbow trout (Foss et al. 1987; No and Storebakken 1991; Bjerkeng et al. 1997; White et al. 2003; Young et al.

2016). Astaxanthin digestibility can vary significantly among fish, depending on factors such as diet composition, dietary carotenoid content, carotenoid source, level of esterification, water temperature and ration size; these factors are important to consider when comparing ADCs across studies (Ytrestøyl et al. 2006; Britton et al. 2008). Additionally, a negative dose response pattern has been observed for Ax digestibility meaning, as dietary Ax content increases ADC for Ax tends to decrease (Choubery and Storebakken 1996; Wieruszewski 2000). Since the Hp-Ax and S-Ax diets did not contain the same amount of Ax, it is difficult to conclude whether Ax from Hp cells was more digestible than synthetic Ax, or the higher ADCs of the Hp-Ax diet were in-part due to the lower dietary concentration.

Generally, digestibility of Ax from Hp in previous studies ranged from 59-62% for disrupted cells, and 59.7% for cell-free Ax extracts (White et al. 2003; Young et al. 2016). Astaxanthin ADCs from whole Hp cells in this trial were higher than those reported for ruptured cells by Young et al. (2016), which suggests the cell wall of Hp in this study did not hinder Ax digestibility, as previously reported (Sommer et al. 1991; Sommer et al. 1992; Tibbetts 2018) and the novel weakened-cell pre-commercial product used in this study was bioavailable to fish without the need for cell processing. Astaxanthin digestibility of the Hp-Ax diet in this study was 76.8%, and to our knowledge, this is the highest digestibility value ever reported for astaxanthin from Hp whole cell biomass in salmonid feeds. In terms of digestibility of individual geometric isomers, it is well documented that all-trans Ax is better digested by fish than cis- isomers, which was the

case for all treatments in this study (Bjerkeng et al. 1997; Higuera-Ciapara et al. 2006; Britton et al. 2008).

ADCs from this experiment were highly variable, which may be due to individual variation, or the fecal collection method used (lethal stripping), which only provides a snapshot of how the ingredient is digested. Future research should complement these results by increasing the number of experimental units, and also by measuring ADCs using the sedimentation method to monitor digestibility overtime, which may help to reduce variability. Additionally, carotenoid breakdown and/or incomplete extraction during analysis can result in an over or underestimation of astaxanthin ADCs respectively (Britton et al. 2008). Therefore, Ax digestibility alone may not be a reliable indicator of Ax pigmenting efficacy and ideally should be utilized in conjunction with other metrics such as tissue or serum Ax concentration and quantitative or qualitative color measurements.

NAR of Ax in the S-Ax and Hp-Ax diets did not differ significantly and corroborated the research of Bowen et al. (2002) for rainbow trout fed diets containing 38-44 mg/kg of synthetic Ax (Carophyll Pink) or Hp carotenoid extract. NAR was negatively correlated with dietary Ax concentration. This correlation has been observed by other researchers, where low (<25 mg/kg) levels of Ax seem to have the largest retention and as levels increase further a negative dose response is observed (Torrissen 1985; Smith et al. 1992; Bjerkeng et al. 1999; Wieruszewski 2000). This may explain the high NAR values and subsequent flesh pigmentation observed for the N-Ax treatment group, where the diet contained 17 mg/kg Ax.

4.5.4 TISSUE COMPOSITION

Despite differences in dietary Ax concentration, yet similar Ax digestibility between fish receiving either pigmented treatment (S-Ax or Hp-Ax), both groups incorporated similar quantities of Ax into their tissues. Salmonids exhibit a positive dose response pattern for dietary Ax up to 50-70 mg/kg, and above which, a decreasing dose response occurs. This indicates that a threshold for Ax exists, and that including more dietary Ax does not necessarily increase Ax flesh content (Storebakken and No 1992; Torrissen 1995; Schiedt 1998; Bjerkeng 2000). This phenomenon may in part explain why Hp-Ax and S-Ax fish incorporated similar quantities of Ax in their tissues despite the S-Ax diet containing substantially more Ax. Contrarily, other studies have suggested there is no cap for Ax absorption and a positive relationship should exist between dietary and tissue Ax content, regardless of dietary Ax concentration (Choubert et al. 1994). Nonetheless, these results indicate that Ax from the whole cell Hp biomass used in this study is as efficiently deposited as synthetic Ax.

The isomer ratios observed for fish tissues in this study agree with those of Young et al. (2016). Fish muscle tissue was almost exclusively trans Ax, with trace amounts of the 13-cis isomer in fish fed Hp-Ax and N-Ax diets. Optical isomer composition of diets and ingredients was not measured in this study. However, it is generally accepted that most algae, including *Haematococcus pluvialis*, contain predominantly monoesterified Ax in the 3S3'S form (Lorenz and Cysewski 2000; Moretti et al. 2006; Holtin et al. 2009). Many studies have been conducted that examine the utilization of natural Ax esters by salmonids

(Schiedt and Leunenberger 1981, Schiedt 1985, Choubert and Heinrich 1993, Barbosa 1999, White 2002). Prior to absorption, Ax esters must be hydrolyzed to free Ax, and is suggested in the literature that this might be a limiting factor affecting Ax deposition rates from natural ingredients (Torrissen 1989; Grung 1992; Higuera-Capara et al. 2006). Contrarily, there are studies indicating this is not the case, and esterified Ax may be better absorbed than synthetic (free) Ax (Barbosa 1999; Bowen et al. 2002; White et al. 2003; Moretti et al. 2006). Digestibility, NAR, and tissue composition data from this study support the latter theory, that cleavage of Ax esters is not a limiting step for microalgae Ax absorption and esterified Ax from algae may be better absorbed than synthetic (free) Ax.

4.5.5 COLOR METRICS

A decrease in fillet lightness (L^*) and increase in redness (a^*) and yellowness (b^*) was observed for all treatments in this study after 10 weeks of feeding. These color changes are consistent with the addition of Ax to the feed of farmed salmonids (Yesilayer 2020). There were no differences in fillet $L^*a^*b^*$ readings between S-Ax and Hp-Ax fish, which indicates the Ax from Hp biomass was as effective as synthetic Ax in pigmenting the flesh of the rainbow trout. An increase in chroma and decrease in hue values towards 0º of trout muscle following dietary pigment supplementation indicated an increase in muscle coloration, specifically muscle redness which agrees with other studies (Christiansen et al. 1995; Nickell and Bromage 1998; Choubert et al. 2006).

Based on the SalmoFan™ readings, fillets from all three treatment groups (including the N-Ax) scored >27 which is considered marketable (Forsberg and Guttormsen 2006; Steine

2005 from Yesilayer 2020). SalmoFan™ readings from S-Ax and Hp-Ax fillets were not significantly different from one another and exceed the 26-29 range most reported in the literature for salmonids (Bowen 2002; Storebakken 2004; Forsberg 2006; Yesilayer 2020). According to Anderson (2000) and Steine (2005), consumers are willing to spend more money on fillets that score between 30-33 and perceive it as a fresher, better tasting, premium product compared to fillets scoring below 27. These color metrics indicate the pre-commercial Hp biomass in this study can produce pigmented fillets that surpassed market standard and could be considered premium quality.

Despite L^* values being comparable to those in the literature for salmonids fed diets containing similar levels of Ax, a^* and b^* values of fillets from all groups in this study exceeded those documented for salmonids in the literature, which commonly range from 4-15 for a^* and 6-18 for b^* (Choubert et al. 2006; Moretti et al, 2006; Rahman et al. 2016; Yesilayer 2020). Conversely, Quevedo et al. (2010) calculated $L^*a^*b^*$ ranges for each color level in the SalmoFan™ card and all $L^*a^*b^*$ values were substantially higher than those in this study with the same SalmoFan™ reading. Variation in color measurements between studies may be due to species, size and gender of the fish, source and concentration of carotenoid, proximate composition of the diet, environmental conditions, and differences in color measurement methodologies (Arai 1987; Skrede 1989; Hatlen 1998; Britton et al. 2000; Forsberg and Guttormsen 2006; Ingle De La Mora 2006; Yesilayer 2020). Fish size influences carotenoid saturation point, where larger (>2 kg) salmonids have a higher tissue Ax saturation point (Bjerkeng 1992; Forsberg and Guttormsen 2006). Final mean fish weights in this trial were within range of market size (1875g ± 358.48g),

which is larger than most other studies investigating the effects of dietary pigments on rainbow trout (Bowen et al. 2002; White et al; 2002; Choubert 2010; Young et al. 2016). Additionally, variation in methodologies and experimental design between studies make comparing both quantitative and qualitative measurements of color difficult. Color readings can be influenced by illumination of the room, the table surface, and location on the fillet being measured, and SalmoFan™ readings depend on an individual's ability to distinguish colors (Skrede et al. 1990; Hatlen et al. 1998; Bjerkeng 2000; Yesilayer 2020). Nonetheless, the *L*a*b* readings obtained in this trial are proportional and consistent with little variation among measurements and the colorimeter was standardized prior to use.

4.5.6 CONCLUSIONS

Flesh pigment is one of, if not the most, important preference indicator by consumers of salmonid products. Additionally, consumer demand for naturally-derived consumables is increasing. *Haematococcus pluvialis* has great potential in satisfying this demand as an alternative to synthetic salmonid pigments. After 10 weeks, fillets from fish fed S-Ax and Hp-Ax did not differ in color or flesh Ax content and the Salmofan™ readings observed from both pigmented diets exceeded those suggested by industry. In some cases, the Hp ingredient was slightly superior to synthetic Ax in terms of net apparent retention, Ax digestibility, and tissue content, suggesting that Hp is capable of pigmenting adult rainbow trout as, if not more, efficiently than synthetic Ax. Furthermore, there are potential advantages to supplementing with a whole cell Ax source (as opposed to disrupted cells, extracts, or synthetic Ax) such as enhanced product stability, superior antioxidant defense, and the addition of beneficial nutrients.

To further assess the adequacy of algal Ax, future studies should also consider examining serum Ax and prolonged fecal collection via sedimentation to observe Ax absorption over time. Furthermore, the effects of extrusion processing on algal Ax remains largely unknown. This information would be crucial if/when these ingredients start being used in large-scale commercial settings. Additionally, future studies should assess the suitability of this natural Hp biomass as a pigment source for other commercially relevant salmonids such as Atlantic salmon and Arctic charr as significant variability in astaxanthin metabolism has been observed between salmonid species (March and MacMillan 1996).

4.6 TABLES AND FIGURES

Table 4.1 Biochemical and proximate composition of *Haematococcus pluvialis* biomass.

Proximate composition (% of biomass)	
Moisture	2.92
Ash	4.96
Protein	48.7
Lipid	24.4
Carbohydrate[1]	19.0
Fatty acids (mg/g)	
12:0	0.04
14:0	1.00
15:0	0.27
16:0	32.5
16:1n-7	1.15
17:0	0.21
18:0	0.40
18:1n-9	6.61
18:1n-7	13.8
18:2n-6 cis	28.7
18:3n-6	1.13
18:3n-3 (ALA)[2]	41.2
18:4n-3	3.21
20:0	0.16
20:3n-6	0.30
20:3n-3	0.30
20:4n-6	3.08
20:4n-3	0.47
20:5n-3 (EPA)	5.68
22:0	<0.50
22:5n-6 (DPA)[2]	<0.09
22:5n-3	<0.01
22:6n-3 (DHA)	0.07
24:0	0.72
∑SFA[2]	35.4
∑MUFA	29.9
∑PUFA	108.7
∑n-3	50.9
∑n-6	33.3
n-3:n-6	1.53

Astaxanthin (ug/g)	
Total	3089
All-trans	2654
9-cis	246
13-cis	154
di-cis	35

[1] Calculated as [100 - (moisture+protein+lipid+ash)].
[2] ALA, alpha-linolenic acid; DPA, docosapentaenoic acid; SFA, saturated fatty acid.

Table 4.2 Ingredients, proximate composition (as-fed basis) and carotenoid analysis of experimental diets (no astaxanthin; N-Ax, synthetic astaxanthin; S-Ax, and *Haematococcus pluvialis*; Hp-Ax).

	N-Ax	S-Ax	Hp-Ax
Ingredient[1] (%)			
Fish meal (74.7% CP)	20.0	20.0	20.0
Ground wheat (14% CP)	18.5	18.4	18.1
Soy protein concentrate (83.3% CP)	9.00	9.00	9.00
Poultry byproduct meal (62% CP)	15.0	15.0	15.0
Blood meal (90% CP)	10.0	10.0	9.30
Empryeal® (75% CP)	4.00	4.00	4.00
Fish oil	7.50	7.50	7.50
Canola oil	5.50	5.50	5.25
Poultry fat	5.50	5.50	5.25
Vitamin/Mineral mix[2,3] (14.6% CP)	0.20	0.20	0.20
Special pre-mix[4]	2.00	2.00	2.00
Haematococcus pluvialis biomass[5] (48.7%CP)	0.00	0.00	1.60
Synthetic astaxanthin	0.00	0.08	0.00
Dicalcium phosphate	2.30	2.30	2.30
Chromic oxide	0.50	0.50	0.50
Proximate composition[6] (%)			
Moisture	9.04	5.94	8.14
Ash	8.34	8.93	8.65
Crude protein	42.2	49.7	43.7
Lipid	27.2	20.1	22.7
Carbohydrate[7]	13.3	15.4	16.8
Astaxanthin content[8] (mg/kg)[h]			
Total Ax	16.9	80.5	60.6
Trans	16.9	67.7	52.4
9-cis	<2.1	5.5	4.67
13-cis	<2.1	7.3	3.47
di-cis	<2.1	<2.1	<2.1

[1] All ingredients were supplied by Northeast Nutrition (Truro, NS, Canada).
[2] Vitamin mixture (IU or g/kg of premix): vitamin A, 900 000 IU; vitamin D_3, 400 000 IU; vitamin E (dl-alpha tocopherol acetate), 25 000 IU; vitamin K (menadione sodium bisulphate), 3.0 g thiamin, 3.0 g; riboflavin, 4.0 g; pantothenic acid (as d-calcium pantothenate), 12.0 g; biotin, 0.1 g; folic acid, 1.0 g; vitamin B_{12}, 0.003 g; niacin, 15.0 g; pyridoxine, 4.0 g ascorbic acid, 30.0 g; carrier (ground wheat).
[3] Mineral mixture (g/kg of premix): manganous oxide, 23.0 g; zinc oxide, 70.0 g; cooper sulfate, 6.0 g; potassium iodide, 2.0 g; carrier (ground wheat).
[4] Special pre-mix (mg/kg): selenium, 0.22 mg; vitamin E, 250 IU; vitamin C, 200 mg; wheat shorts; 1988 mg.

[5] *Haematococcus pluvialis* biomass kindly supplied by Kuehnle AgroSystems (Honolulu, HI, USA).
[6] Calculated as [100 - (crude protein+lipid+ash+moisture)].
[7] n=2.
[8] n=3.

Table 4.3 Growth, feeding performance, and biometrics of rainbow trout fed one of three experimental diets (no astaxanthin; N-Ax, synthetic astaxanthin; S-Ax, and *Haematococcus pluvialis*; Hp-Ax).[1]

	N-Ax	S-Ax	Hp-Ax
Initial weight (g)	999 ± 163.63	1034 ± 202.43	943 ± 159.33
Final weight (g)	1925 ± 270.70	1942 ± 269.55	1839 ± 330.84
K[2]	1.77 ± 0.15	1.75 ± 0.14	1.79 ± 0.13
Weight gain (g/fish)	927 ± 172.49	909 ± 136.86	896 ± 201.37
SGR[3] (%/day)	0.89 ± 0.17	0.89 ± 0.17	0.89 ± 0.23
FCR[4]	1.02 ± 0.20	1.03 ± 0.16	1.08 ± 0.27

[1] Values are mean (n=20) ± SD. Within a row, superscripts indicate significant difference (<0.05) between values.

[2] K; Condition factor = 100 x (weight/length^3).

[3] SGR; Specific growth rate = 100 x [ln (final body weight) – ln (initial body weight)] / days.

[4] FCR; Feed conversion ratio = feed intake (dry matter) / wet weight gain.

Table 4.4 Astaxanthin (total and geometric isomers; mg/kg), net apparent retention (NAR; %) of Ax, lipid and protein (% of biomass) content of rainbow trout muscle tissue fed one of three experimental diets (no astaxanthin; N-Ax, synthetic astaxanthin; S-Ax, and *Haematococcus pluvialis*; Hp-Ax).[1]

	N-Ax	S-Ax	Hp-Ax
Ax content (mg/kg)			
Total Ax	5.48 ± 1.6^{a}	9.11 ± 1.5^{b}	8.86 ± 2.0^{b}
Trans	5.07 ± 1.5^{a}	8.58 ± 1.4^{b}	8.37 ± 1.9^{b}
9-cis	$<0.3 \pm 0.0$	$<0.3 \pm 0.0$	$<0.3 \pm 0.0$
13-cis	0.40 ± 0.1^{a}	0.53 ± 0.1^{b}	0.49 ± 0.1^{b}
di-cis	$<0.3 \pm 0.0$	$<0.3 \pm 0.0$	$<0.3 \pm 0.0$
Net Apparent Retention (%)	34.8 ± 2.6^{a}	13.2 ± 0.55^{b}	15.4 ± 0.23^{b}
Total lipid (%)	21.2 ± 2.5	24.4 ± 0.6	19.5 ± 0.8
Total protein (%)	74.8 ± 5.2	76.6 ± 4.6	77.9 ± 4.0

[1] All values are mean (n=20) ± SD. Within a row, superscripts indicate significant difference ($p<0.05$) between values.

Table 4.5 Apparent digestibility coefficients (ADC; % ± SD) of Ax from rainbow trout one of three experimental diets (no astaxanthin; N-Ax, synthetic astaxanthin; S-Ax, and *Haematococcus pluvialis*; Hp-Ax).

	N-Ax[1]	S-Ax[2]	Hp-Ax[2]
Total Ax	52.9	70.3 ± 16[a]	76.8 ± 2.7[a]
Trans	66.0	72.1 ± 9.9[a]	77.9 ± 1.6[a]
9-cis	41.0	63.9 ± 9.2[a]	80.4 ± 2.9[a]
13-cis	58.5	61.4 ± 35[a]	78.8 ± 4.6[a]
di-cis	94.6	90.0 ± 9.1[a]	57.3 ± 4.4[a]

[1] n=1, Data from this treatment are excluded from statistical analysis.
[2] n=2**,** Treatments that do not share the same letter are significantly ($p<0.05$) different.

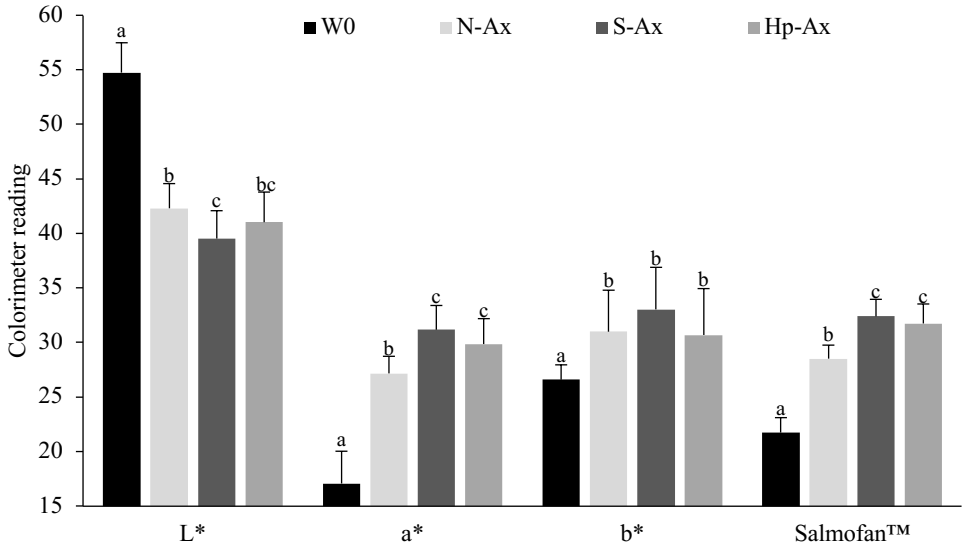

Figure 4.1 Mean (+SD) colorimeter (Lightness; *L**, redness; *a**, and yellowness; *b**) and Salmofan™ readings of rainbow trout fillets from baseline (W0; n=6), and after feeding one of three treatment diets (no astaxanthin; N-Ax, synthetic astaxanthin; S-Ax, and *Haematococcus pluvialis*; Hp-Ax; n=20) for 10 weeks. For each parameter measured, treatments that do not share the same letter are significantly ($p<0.05$) different.

Table 4.6 Hue ($H(°)_{ab}$) and Chroma (C^*) values for rainbow trout fillets at baseline (W0) and following feeding one of three experimental diets (no astaxanthin; N-Ax, synthetic astaxanthin; S-Ax, and *Haematococcus pluvialis*; Hp-Ax) for 10 weeks.[1]

	W0[4]	N-Ax[5]	S-Ax[5]	Hp-Ax[5]
$H(°)_{ab}$	1.00 ± 0.08^{a}	0.85 ± 0.04^{b}	0.81 ± 0.04^{c}	0.80 ± 0.05^{c}
C^*	31.69 ± 2.0^{a}	41.26 ± 3.7^{b}	45.45 ± 4.2^{c}	42.87 ± 4.4^{bc}

[1] Values are mean ± SD. Within a row, superscripts indicate significant difference (<0.05) between values.
[2] $H(°)_{ab}$; Hue = arctan (b^*/a^*).
[3] C^*; Chroma = $(a^{*2}+b^{*2})^{1/2}$.
[4] n=6.
[5] n=20.

CHAPTER 5.0

CONCLUSION

The use of conventional marine ingredients to supply essential fatty acids in diets for farmed salmonids is becoming increasingly economically and environmentally costly as the industry continues to grow. The use of synthetic astaxanthin as a dietary flesh pigment in salmonid aquafeeds is becoming undesirable as consumer preference is increasingly shifting towards naturally derived food additives. Microalgae and single celled organisms represent an exciting source of essential fatty acids and pigments for the aquafeed industry. This thesis investigated the functional and nutritional properties of two species of single celled organisms in feeds for farmed salmonids. Specifically, the digestibility of *Schizochytrium* sp. by Atlantic salmon and the pigmenting efficiency of a weakened cell *Haematococcus pluvialis* in diets for rainbow trout.

Digestibility values of dry matter, energy, protein, lipid, and fatty acids for *Schizochytrium* sp. by Atlantic salmon were determined by feeding a diet containing 70% basal mix and 30% Sc meal. The proximate and biochemical composition, and apparent digestibility values determined for the Sc meal indicate that it is an excellent source of DHA in aquafeeds for Atlantic salmon. Protein was extremely well (>90%) digested, and PUFAs, EPA, and DHA, were >98% digestible; this indicates the recalcitrant cell wall of Sc did not hinder nutrient bioavailability as shown for other microbial feed ingredients. Despite high DHA content (33% TFAs), the high SFA content of Sc negatively impacted lipid and energy digestibility. This trial indicated Sc is a highly digestible source of DHA and protein for Atlantic salmon however complete replacement of fish oil with Sc may be unrealistic

due to low SFA digestibility and low EPA content of the Sc ingredient, but partial substitution may help restore fillet DHA content. Future research should focus on the next steps in assessing the suitability of a novel feed ingredient including palatability, nutrient utilization and/or interference, and ingredient functionality.

Pigmenting ability of a diet containing 60 mg/kg Ax from a pre-commercial weakened whole cell *Haematococcus pluvialis* product was compared to a positive control diet containing 80 mg/kg of synthetic Ax in rainbow trout. Overall, Hp was successful in achieving flesh coloration that surpassed industry standard and did not differ significantly from that of synthetic Ax. Additionally, the digestibility and retention values for Ax from the Hp-Ax diet were equal to those in the S-Ax diet. The Hp-Ax and S-Ax diets in this trial did not contain the same amount of Ax as formulated, this concentration difference is regarded as a limitation of the study as it prohibits strict comparison between the two pigmented diets. Nonetheless, this novel whole cell *Haematococcus pluvialis* was equally efficient at pigmenting the flesh of rainbow trout compared to synthetic Ax. Results from this experiment indicate that this product has potential as a replacement for synthetic astaxanthin in salmonid feeds.

This research is significant as it contributes to the growing knowledge base of microbial ingredients in finfish aquafeeds. This thesis indicates that the microbial ingredients *Schizochytrium* sp. and *H. pluvialis* have major potential as sources of DHA and astaxanthin in aquafeeds, and the data obtained from these experiments will aid in the formulation of more sustainable and functional aquafeeds. More broadly, integrating

sustainable algal production systems into aquaculture will shift the industry towards a circular bioeconomic approach by creating ecofriendly value chains with reduced environmental impact while contributing nutritious products to aquaculture and humanity.

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APPENDIX

CHAPTER 3 SUPPLEMENTARY DATA

Table 3.5 Mean (± SD) apparent digestibility coefficients (ADCs) for DM, macronutrients, energy and FAs from collection phase 1 and 2 for both reference and test treatment diets fed to Atlantic salmon *(Salmo salar*).

	Reference		Test			
	Phase 1	Phase 2	Phase 1	Phase 2	F-stat	P-value
DM	76.8 ± 0.7	77.0 ± 1.0	75.1 ± 1.1	74.8 ± 0.5	0.00	0.988
Protein	88.6 ± 0.4	89.3 ± 0.6	89.6 ± 0.4	88.8 ± 0.2	0.01	0.922
Lipid	93.7 ± 1.1	91.8 ± 1.7	83.2 ± 1.5	82.5 ± 1.5	3.22	0.098
Energy	83.4 ± 0.6	84.1 ± 0.2	79.8 ± 0.6	79.7 ± 0.3	1.59	0.231
14:0	90.4 ± 1.1	91.0 ± 1.1	79.2 ± 2.6	77.5 ± 0.7	0.36	0.561
16:0	82.8 ± 1.6	82.7 ± 2.3	67.9 ± 3.8	64.0 ± 0.8	2.00	0.185
16:1n-7	95.6 ± 0.5	96.0 ± 0.6	95.2 ± 1.1	94.7 ± 0.6	0.02	0.878
18:1n-9	94.3 ± 0.6	94.8 ± 0.8	95.3 ± 1.0	94.4 ± 0.8	0.17	0.683
18:2n-6	95.3 ± 0.5	95.6 ± 0.6	96.3 ± 0.9	95.6 ± 0.8	0.14	0.711
20:4n-6	94.4 ± 0.5	94.8 ± 0.4	96.1 ± 1.0	96.0 ± 0.5	0.41	0.535
20:5n-3	97.7 ± 0.3	97.9 ± 0.2	98.1 ± 0.8	98.0 ± 0.3	0.06	0.814
22:5n-6	93.1 ± 3.1	90.8 ± 4.4	95.9 ± 0.7	95.8 ± 0.1	0.70	0.421
22:6n-3	93.4 ± 1.8	92.7 ± 1.4	96.3 ± 0.5	96.3 ± 0.9	0.23	0.638
∑SFA	83.9 ± 1.3	84.2 ± 1.8	70.6 ± 3.6	67.5 ± 0.9	1.17	0.303
∑MUFA	93.1 ± 0.6	93.9 ± 0.8	94.1 ± 1.1	93.2 ± 1.0	0.02	0.882
∑PUFA	95.9 ± 0.6	96.1 ± 0.7	96.8 ± 0.7	96.5 ± 0.4	0.11	0.749
∑n-3	96.3 ± 0.7	96.3 ± 0.6	96.2 ± 0.3	96.7 ± 0.6	0.60	0.456
∑n-6	95.1 ± 0.5	94.8 ± 0.9	95.9 ± 0.7	95.9 ± 0.2	0.16	0.697