Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

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Effects on growth and gut health in Atlantic salmon (*Salmo salar*) fed diets containing the macroalgae *Porphyra amplissima*

Master's thesis in Ocean Resources
Supervisor: Kjell Inge Reitan
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Christian Baohua Cheung
Trondheim, June 2021

Abstract

Morphological changes and inflammations in the digestive system of Atlantic salmon (*Salmo salar*) in aquaculture has been linked to the global trend of increasing use of plant-based ingredients in fish feed. Previous research has shown that adding small amounts of algae to feed may counteract these negative effects and even positively impact growth and welfare of fish kept in aquaculture.

This study investigated the effect of including 5% and 10% of the local Norwegian algae *Porphyra amplissima* to feed given to Atlantic salmon fry (*Salmo salar*). At an inclusion level of 5%, significant increase in wet weight and condition factor was observed. There were some indications of significant increase in fork length when use of the experimental diets, but insufficient statistical power limited to determine whether it originates from the diet with 5% or 10% inclusion levels. There was no statistically significant differences in specific growth rates (SGR) or histological characteristics of the digestive system, between groups given different diets. The significant positive effects on growth at 5% inclusion level and absence of any negative effects on growth or gut health at inclusion levels up to 10% indicate the exciting potential of using *P. amplissima* as a feed ingredient for Atlantic salmon (*S. salar*).

Sammendrag

Morfologiske endringer og betennelse i fordøyelsessystemet til Atlanterhavslaks (*Salmo salar*) i akvakultur har blitt knyttet til den globale tendensen til å øke andelen plantebaserte ingredienser i fiskefôr. Tidligere forskning har vist at tilsetningen av små mengder alger i fiskefôr kan bidra til å motvirke de negative effektene og positivt påvirke vekst og velferd til fisk i oppdrett.

Denne masteroppgaven undersøkte effekten av å tilsette 5% og 10% av den lokale norske rødalgen *Porphyra amplissima* i fôret gitt til atlanterhavslaks (*Salmo salar*). Ved en tilsetningsandel på 5%, ble det observert signifikant økning i våtvekt og kondisjonsfaktor. Det er indikasjoner på signifikant økning i kroppslengde ved bruk av de eksperimentelle diettene, men utilstrekkelig statistisk kraft begrenset muligheten til å fastslå om det stammer fra dietten med 5% eller 10% tilsetningsgrad. Det var ingen statistisk signifikante forskjeller i spesifikk vekstrate (SGR) eller histologiske karakteristikker av fordøyelsessystemet, for grupper som ble gitt forskjellige dietter. Den signifikante positive effekten på vekst ved en 5% tilsetningsgrad, og fraværet av negative effekter på vekst og tarmhelse ved en tilsetningsgrad opp til 10% viser til et spennende potensial for å bruke *P. amplissima* som fôringrediens til atlanterhavslaks (*S. salar*).

Contents

1. Introduction

1.1 Trends in aquaculture and feed

Feed is the largest component of cost for cultivation of Atlantic salmon (*Salmo salar*) in Norway (1), and generally, the largest component of cost in finfish aquaculture globally (2). The main sources of protein and lipids in finfish feed have traditionally been fish meal and fish oil (3). However, the supply of these ingredients has not been keeping up with the volumes demanded by the tremendous growth of the aquaculture industry in recent years (4, 5). Consequently, there has been a trend of increasing the use of plant-based ingredients in salmon diets with arguments originating from both cost-savings aspects and potential for further growth of the industry (6).

Studies has found mounting evidence that indiscriminate increases in plant-based components may negatively impact fish growth rates and fish welfare (7). Antinutritive factors and challenges in digesting plant-ingredients are believed to lead to detrimental effects in the digestive systems of fish (8). Recent studies demonstrate how substitution of fish oil with vegetable oils in fish feed is associated with persistent inflammation, and significantly shorter folds in the mid- and distal intestines in the fish (9, 10). Attempts to replace marine proteins with plant-based protein sources, including pea protein concentrate and soybean meal, has been linked to the occurrence of enteropathy and abnormalities in the intestines, including changes in goblet cells, absorptive vacuoles and microvilli (4, 11, 12). These changes in the digestive system have in turn been associated with negative effects on growth, mortality, feed intake, carcass composition, susceptibility to diseases and stress in the cultivated fish (8).

1.2 Algae as a sustainable feed ingredient

Previous research have suggested that adding small amounts of algae to feed may counteract some of the negative effects associated with plant-based ingredients and positively impact a number of key areas for cultivated fish species (13). The inclusion of small amounts of macroalgae species, including *Porphyra* spp. and *Ascophyllum nodosum* have been associated with increased growth rates, survival rate and food-efficiency (13, 14, 15). Studies have also found that the addition of dried algae could counteract inflammations in the intestines of fish that were fed diets rich on soybeans, improve the fatty acid composition in the tissue, as well as improve liver function (13, 16, 17). A key concern in intensive fish aquaculture systems is the prevalence of diseases (18). The presence of complex carbohydrates in macroalgae are believed to act as immunostimulants, as improved immunological

responses to diseases have been observed in a range of fish species given feed including algae (19, 20, 21, 22).

In addition to directly contributing to fish welfare and growth, utilising algae in feed enables interesting opportunities regarding the sustainability of the aquaculture industry. Concepts that recycles nutrients include Integrated Multi-trophic Aquaculture (IMTA) systems, where the waste from one species is used to grow another species (23). Using algae as one of these species could reduce both local and global pollutions and emissions and prevent harmful eutrophication events, as well as generating valuable biomass (24).

1.3 Specific species investigated in this study

The aquaculture of Atlantic salmon (*S. salar*) is a significant industry in Norway today, accounting for more than 94 percent of the total aquaculture production (25). Following the trend of other aquaculture industries, the ingredients have been increasingly shifted towards plant based origins, with a recent review finding over 71% of the feed given to this carnivorous fish originating from plants (26). These high inclusion levels have been associated with the respective negative effects on growth and welfare.

Species from the family of red macroalgae genus *Porphyra* have attracted interest as feed ingredients, as analyses show that they contain high amounts of protein, pigments, fatty acids and may stimulate the appetite of marine fish species (27). Previous studies of different Asian and American *Porphyra* species have indicated these macroalgae confer positive effects on finfish and are suitable candidates as feed ingredients (14, 15, 19). Amongst a number of *Porphyra* species investigated, Carmona et al. (2006) found that *Porphyra amplissima* achieved the highest growth rates when exposed to nutrient-rich waste streams, suggesting the potential for biomass production (24). In Norway, *P. amplissima* grows natively along the coast, which is a requirement for possible future sea-based aquaculture, according to Norwegian law (28). The possibilities of co-producing local *P. amplissima* as a feed ingredient for farmed Atlantic salmon could enable cost-savings, improved fish growth and welfare, improved nutrient utilisation and increased sustainability of the industry. The motivation of this study is to explore the possibility of such positive effects on farmed Atlantic salmon (*S. salar*) given feed containing small amounts of the native *P. amplissima*.

1.4 Growth and welfare parameters

1.4.1 Growth parameters

Common parameters to monitor growth includes wet weights, specific growth rate, fork lengths, and condition factor (29, 30). Wet weight is the weight of each individual measured with excess surface water removed. Fork length is the length of the fish measured from the snout to the shortest, or median caudal fin ray (31). Specific growth rate (SGR) describes the growth of the fish over time and is calculated based on the measured wet weight. Condition factors have been developed to standardise and enable broad comparisons of fish health, with the assumption that the heavier a fish is for a given length, the healthier it is (32). The condition factor used in this study is the commonly used Fulton's condition factor.

1.4.2 Welfare parameter for the digestive system

By both national and international law, farmed fish are required to have good welfare. However, no consensus exists on how welfare is defined or monitored (33). Experiments involving experimental diets generally focus on welfare indicators tied to the digestive system, including histological investigations of its morphology (34). The mid intestine, distal intestine and pyloric caeca are generally analysed, as significant dietary effects are commonly observable here (10). Symptoms of inflammation in the digestive system may include shortening of mucosal folds, changes in vacuolisation and reduction in tissue weight (34). Even in the absence of inflammation, significant changes in morphology of the mid and intestinal folds may occur in response to dietary changes (10).

The folding of the gastrointestinal tract of the fish is described as a method to enable a smaller tract diameter and increased inner surface area. Inflammations in and shortening of these folds could indicate reduced welfare (34, 35). Histological analyses of wild Atlantic salmon in Norway reveal that healthy individuals have long primary folds with complex secondary folding in the mid intestines, while the distal intestines have smaller irregular folds (35). The pyloric caeca is characterised by parallel folds along their lengths. Assuming inferior plant-based feed induce inflammations and shortening of folding in the digestive system, while the inclusion of algae counteracts this effect, there should be a difference in the relative inner surface area of the digestive systems between fish given normal feed and those given feed including algae.

1.5 Experimental aim and hypotheses

The aim of the study was to experimentally examine the effects on growth and gut health in Atlantic salmon (*Salmo salar*) fed diets containing the macroalgae *Porphyra amplissima*.

The hypotheses of this study was that, by adding small amounts of *P. amplissima* to feed given to Atlantic salmon (*S. salar*), compared with use of a control diet, one would observe:

1. Higher wet weight
2. Larger fork length
3. Higher condition factor
4. Higher Specific Growth Rate
5. Improved welfare indicators of the digestive system

Considerations of the findings to these sub-hypotheses would together answer the overall aim of the study.

2. Materials and methods

The experiment consisted of three main stages. The first stage involved collecting the required *P. amplissima* and manufacturing experimental feed based on the collected algae. The second stage involved setting up the facilities and rearing the Atlantic salmon. The final stage consisted of collecting and analysing samples and processing the collected data.

2.1 Preparing the experimental feed

2.1.1 Collecting *P. amplissima*

Approximately 1.7 kg fresh *P. amplissima* were collected on 16th and 23rd May 2020 by divers at Hammarvika, Frøya (63°41'49.6"N 8°49'21.8"E). The macroalgae were collected at 4-5 meters depth, with the substrate primarily consisting of coarse-grained sand.

The collected *P. amplissima* were carefully inspected and placed in tanks with flow-through seawater in NTNU Sealab until 26th May 2020. The macroalgae were then removed from the tanks, rinsed in fresh water and had the majority of their surface water removed by mechanical means (lightly shaking in a metallic basket so that the water would run off), before being frozen at -21 °C.

On 16th June 2020, the frozen *P. amplissima* were cut into chunks of around 300 grams, and put into a freeze-drier (Alpha 1-4 LSCplus, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) which was left to run until 23rd June 2020. The resulting freeze-dried algae were measured to weigh around 120 grams, and were kept stored at -21 °C.

2.1.2 Manufacturing experimental feed

The freeze-dried algae were sent from NTNU to a feed producer in Portugal (Sparos Lda) on 26th August 2020, using normal packaging (room temperature shipping) and was confirmed arrived and stored at the storage facilities of the feed producer on 1st September 2020.

The feed producer used the freeze-dried algae in a cold-extrusion manufacturing process to produce diets that aimed to be isocaloric, isoproteic and isolipidic with a 0.4 mm pellet size. One diet contained 10% freeze-dried algae, one diet contained 5% freeze-dried algae, and one diet contained no algae and acted as a control diet (see appendix for detailed compositions). This was achieved by adjusting two ingredients against the addition of the freeze dried algae: Dextrine, an indigestible carbohydrate, and wheat gluten, a high protein ingredient. The exact composition of *Porphyra* is known to vary across

species, local conditions and seasons. As pre-analyses of the composition of the harvested freeze-dried *Porphyra* was unavailable, it was assumed from typical values from literature on *Porphyra* (**Table 1**).

Table 1 Summary of typical composition values for *Porphyra spp.*, from literature.

Source	(36)	(37)	(38)	(39)	(39)	(40)	Average values across all literature sources
Species	*Porphyra columbina*	*Porphyra spp.*	*Porphyra tenera*	*Porphyra tenera*	*Porphyra Haitanesis*	*Poprhyra spp.*	
Crude protein	24.61 ± 0.21 %	29.1 ± 0.2 %	33-47 %	36.88 ± 0.90 %	32.16±1.21	24 %	31,2%
Crude lipid	0.25 ± 0.06 %	0.1 ± 0.0 %		2.25 ± 0.29 %	1.96±0.4	1 %	1,1%
Crude ash	6.46 ± 0.09 %	10.9 ± 0.1 %		9.07 ± 0.29 %	8.78±0.12	19 %	10,8%
Moisture	12.79 ± 0.07 %	8.4 ± 0.5 %		3.66 ± 0.25 %	6.74±0.51		7,9%

The experimental diets were shipped back to Norway and received on 14th October 2020, see **Figure 1**.

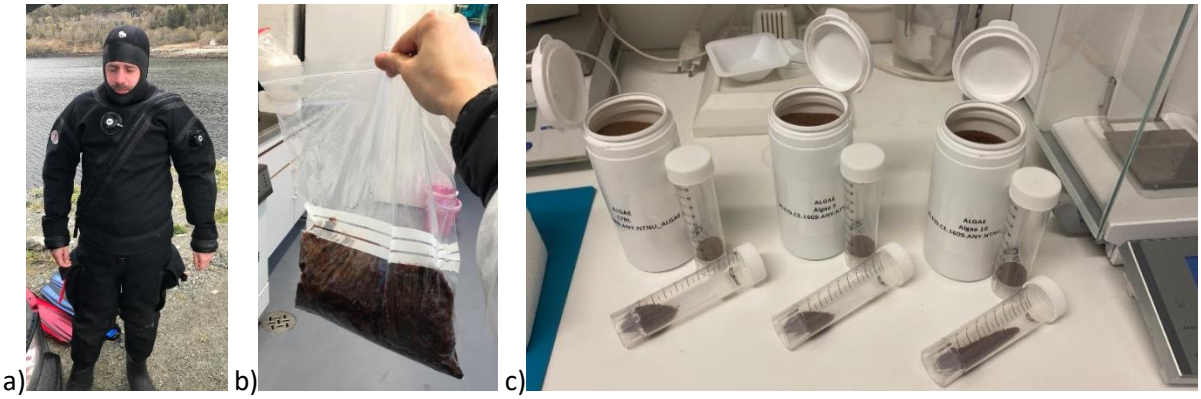

Figure 1 Steps during the manufacturing of the experimental feed. a) Diver preparing to collect *P. amplissima*. b) Fresh *P. amplissima* rinsed in fresh water. c) Manufactured experimental feed and control feed during control-weighing.

2.2 Feeding trials

The holding facilities were provided by NTNU Centre of Fisheries and Aquaculture (NTNU Sealab), located in Trondheim, Norway. The salmon fry originated from a university course at NTNU for 1st-year students at the Bachelor in Engineering, Aquaculture (BIHAV) programme. The BIHAV-students performed hatching and start-feeding of Atlantic salmon. They monitored water parameters and performed husbandry of the salmon yolk sac alevins and fry during the first 10 weeks, under supervision and support from technical and academic staff. After the 10 weeks, the ownership and

responsibility were transferred to this study. All fish were raised under similar conditions during the course.

2.2.1 Cooperation with BIHAV students

The BIHAV-students received around 12 000 fertilised salmon eggs from AquaGen on 16th September 2020. They were kept in specialised hatching containers in a flow-through system at NTNU SeaLab for the first phase, before they were transferred to start-feeding tanks. The salmon eggs hatched around two weeks after they had arrived, between 27th and 29th September 2020, and were given Astroturf to hide in. On 13th November 2020, the salmon yolk sac alevins were transferred from the hatching containers to nine start-feeding tanks, which were also set-up with flow-through. On 21st November 2020, first feeding was initialised and the salmon fry were given a commercial diet from AquaGen with a pellet-size of 0.15 mm, fed through 24-hour automatic feeders. Subsequently, the salmon fry would be given feed throughout the day, with the automatic feeders being topped up with a new ration every 24 hours.

The BIHAV students monitored and recorded the temperature, oxygen-levels and air-pressure constantly throughout the period. Dead eggs and individuals were removed and recorded daily. The temperature was initially measured at around 7 °C, and slowly raised to around 9 °C in the hatching containers. The water in the start-feeding tanks were constantly kept at 13 °C. The oxygen levels were consistently measured to be around 11-12 mg/L. The light regime was kept at 24 hours light (24:0 Day-Night cycle).

2.2.2 Master thesis

The BIHAV students finished their practical course-requirements on 27th November 2020. The ownership, responsibility and husbandry of the salmon fry were then transferred to this study. The fry were around 8 weeks old at the start of the experiment. For each of the nine tanks, the following protocol was executed on 27th November 2020:

a. The salmon fry was randomly fished out with a small net, counted, and transferred to a temporary container until a total of 200 individuals were fished out.
b. The remaining individuals in the tank was then fished out with a larger net. A fixed number were randomly sampled, the rest were euthanized.
c. The tanks were emptied, any necessary maintenance carried out, and then refilled with water and stocked with the 200 individuals that were initially fished out.

Each of the nine tanks were assigned a 0.4 mm experimental diet through a one-time randomised computer-function, with the options being “control”, 5% algae-diet” or “10% algae-diet”. Subsequent feed given to their respective automatic feeders would adhere to this diet-assignment for the duration of the experiment (**Figure 2**).

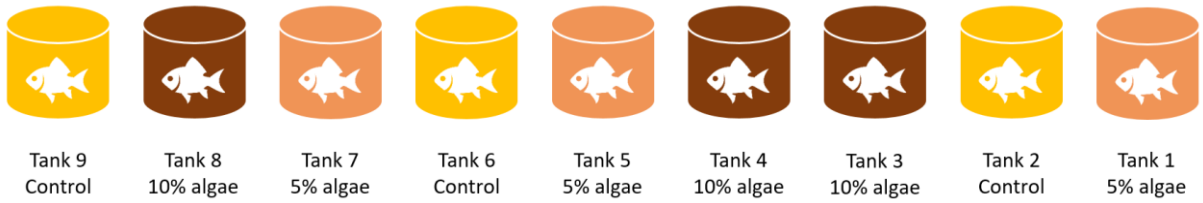

Figure 2 Overview of the diets assigned to each of the nine tanks, using a one-time randomised computer function. Three diets with three replicates each. Each tank contained 200 individuals, for a total of 1800 individuals, at the start of the feeding experiment, 27th November 2020.

To ensure the salmon fry would be fed to satiation, the salmon fry were given a daily amount of feed equivalent to 5% of their weight. The starting amount on 27th November 2020 would target a salmon fry weight around 5% above the grand average of the weights from the sampling (meaning, the first ration of feed would be 5% of 105% average weight = 5.25% of average weight). For each subsequent day, the feed would be increased assuming a food-conversion-ratio (FCR) of 1. The assumption would be that the weight of the salmon fry today would exactly equal the weight of the salmon fry yesterday plus all the feed provided yesterday. The same amount of feed was distributed to all the tanks, irrespective of diet.

The experimental procedure of this study consisted of two phases and three samplings, see **Figure 3**. The first phase lasted from 27th November 2020 to 21st December 2020, and the second phase lasted from 22nd December 2020 to 11th January 2021. The three samplings were conducted at the start of phase 1, between phase 1 and phase 2, and at the end of phase 2. During phase 1, 200 individual salmon fry were kept in each of the nine tanks, while during phase 2, 20 individual salmon fry were kept in each of the nine tanks. On 21st December 2020, during the second sampling and between phase 1 and phase 2, a similar procedure as was done on 27th November 2020 was carried out. For each tank, the number of salmon fry was reduced from 200 to 20 individuals, out of the 180 individuals taken out, a number were randomly sampled and the rest was euthanized.

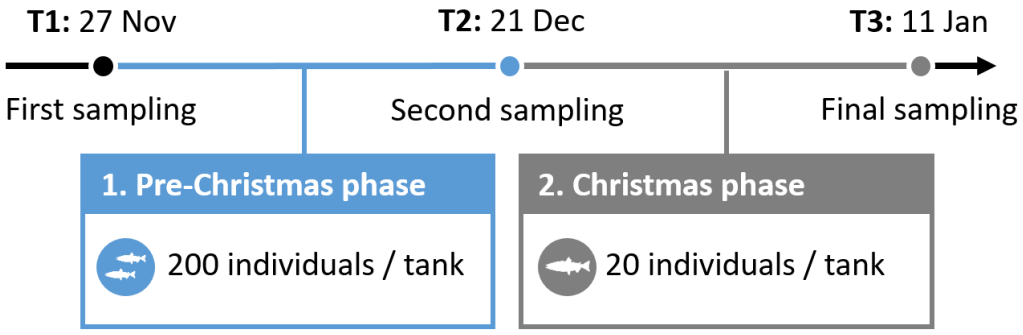

Figure 3 Graphical representation of the two phases and three samplings in the experimental procedure

The temperature and oxygen-levels in each tank were measured and dead individuals were fished out, recorded and weighed daily (see appendix for detailed overview). The water in the tanks were consistently measured to be around 13 °C. The oxygen levels were consistently measured to be around 10 mg/L. The detailed overview of all the measurements for all the tanks can be found in the appendix. The light regime was kept at 24 hours light (24:0 Day-Night cycle). The type of diet assigned to each tank were kept constant throughout the experiment, irrespective of sampling or phase.

2.3 Data collection

2.3.1 Sampling

The methodology for each of the three samplings followed a similar protocol, with the sampling size at the third sampling being the only difference. The required number of individuals were randomly selected and euthanized in excess buffered MS222 (0.5 g/L concentration). Then, the individuals were sampled according to four different categories:

Weight and Length

i. "Fork length" was measured for each individual, using a ruler. The fork length is defined as the distance from the snout to the shortest caudal fin ray, where the tail is splitting (31), see the illustration of the upper left corner of **Figure 8**.

ii. Surface water was removed by laying each individual on tissue paper, 3 seconds on each side.

iii. Each individual was put into pre-weighed plastic tubes and their caps were put on.

iv. Each plastic tube with salmon fry was weighed, and the wet weights were found by subtracting the weight of the empty plastic tubes.

v. For dry weight measurements, the cap of each plastic tube was removed, and the tubes with salmon fry were dried in an oven at 105 °C for 48 hours. The tubes with salmon fry were then weighed, and the dry weights were found by subtracting the weight of the empty plastic tubes.

Histological analyses of digestive system

i. The digestive system was exposed using a scalpel:
   - For the first and second sampling, the head was removed approximately 1 mm behind the gills, but the body was otherwise untouched.
   - For the third sampling, the digestive system was cut out of the body, retrieving the stomach, pylorus caeca, intestines, and discarding the body.

ii. The body or organs were put into a larger plastic tube with 30 ml formaldehyde buffered solution (4% buffered at pH 6.9) with one tube for each tank. The caps were put on.

iii. The plastic tubes with salmon fry were stored at 2 °C before further processing.

RNA-analyses

i. Similar steps as (i.) above

ii. The body or organs were put into a larger plastic tube with 30 ml RNAlater solution with one tube for each tank. The caps were put on.

iii. The plastic tubes with salmon fry were stored at 2 °C for 24 hours.

iv. The plastic tubes with salmon fry were then transferred to -20 °C for long-term storage before further processing.

Total protein- and lipid-analyses

i. Surface water was removed by laying each individual on tissue paper, 3 seconds on each side.

ii. The individuals were put into a larger plastic tube with one tube for each tank.

iii. The plastic tubes with salmon fry were filled with nitrogen gas, and the caps were put on.

iv. The plastic tubes with salmon fry were transferred to -80 °C for long-term storage before further processing.

The weight samples for the third sampling were unfortunately destroyed after the sampling day and no dry-weight measurements were obtained for this sampling. Due to time constraints, for this study, it was decided to proceed with the data from the two first sample-categories only: “weight and length” and “histological analyses of digestive system”. The data for weight and length was collected on the day of sampling and was ready for data processing. The samples for histological analyses were further processed before measurements were taken.

2.3.2 Processing of histological samples

The aim of processing the histological samples was to obtain sections where the mid- and hind-intestines as well as the pyloric caeca would be clearly visible and easily analysed. A number of different histological samples were processed in different ways, with the parameters of variations being:

i. Different sampling number, e.g. from second sampling or third sampling.

ii. Different cut, e.g. where along the body/intestine the tissue were cut to produce tissue samples for further processing.

iii. Different pre-treatment baths, e.g. whether the samples were submerged in formaldehyde buffered solution (4% buffered at pH 6.9), phosphate buffered saline (PBS) or decalcifying solution (Osteosoft by Merck KGaA).

The final combination of parameters to produce sections for further processing included: samples from the second sampling only. Pre-treatment of formaldehyde buffered solution (4% buffered at pH 6.9) only. Specific cutting planes were determined, based on pre-cutting dissections of three individuals from the second sampling. The cutting planes were as follows, see **Figure 4**: A first vertical cut was conducted at the start of the dorsal fin. Sections of the mid-intestine and pyloric caeca were obtained by sectioning 2 mm from the first cut, towards the front of the body. Sections of the distal intestine were obtained by sectioning 3 mm from the first cut, towards the back of the body.

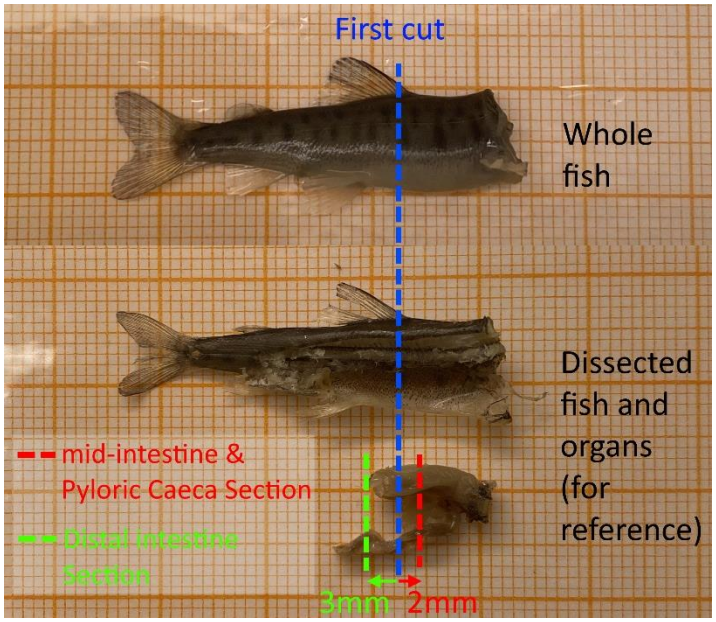

Figure 4 Photographs of fish carcass before and after dissection to illustrate the relative anatomy and positioning of the internal digestive organs of the fish. Superimposed are lines indicating the cutting planes: first a vertical cut at the start of the dorsal fin, the sections of the mid-intestine and pyloric caeca were obtained 2 mm from the first cut, the sections for the distal intestine were obtained 3 mm from the first cut.

Practically, the sections were obtained using the following protocol:

i. The first vertical cut at the start of the dorsal fin was conducted.

ii. Two more vertical cuts at approximately 5 mm at each side of the first vertical cut were conducted to produce two pieces of tissue.

iii. The tissue samples were contained in plastic cassettes and subjected to automatic dehydration through graded ethanol baths followed by infiltration with paraffin wax using an automatic tissue processor (Leica TP1020, Leica Microsystems, Nussloch GmbH, Germany), overnight.

iv. Tissue samples were retrieved from the machine and moulded into paraffin blocks with an orientation that enabled transverse sectioning, and with the plane of the first cut facing outwards. The paraffin blocks were mounted under cassettes to enable ease of manipulation in subsequent steps. The paraffin blocks were left in a refrigerator overnight to ensure they were sufficiently cooled down.

v. The paraffin blocks were mounted on a microtome (Leica RM2255, Leica Biosystems, Nussloch GmbH, Germany), for trimming and then sectioning:

   - With the plane of the first cut facing outwards, the paraffin blocks were repeatedly trimmed at 10 µm thickness, until the first sections containing tissues were produced (as opposed to sections of pure paraffin).
   - Then, the electronic counter of the microtome was reset, and the block was trimmed:
     - For “front blocks”, where we would like sections of the mid-intestine and pyloric caeca at 2 mm from the first cut, the trimming consisted of 200 sections × 10 µm thick.
     - For the “back blocks”, where we would like sections of the hind-intestine at 3 mm from the first cut, the trimming consisted of 300 sections × 10 µm thick.
   - The microtome was then switched to 4 µm thick sectioning mode. Sections of 4 µm were then obtained, floated in a 45 °C water bath for smoothing out, and transferred to glass slides.
   - The glass slides were mounted vertically in a basket and left at 37 °C in an oven overnight to ensure they were completely dry.

vi. The sections were subject to Haematoxylin and Eosin staining as per the standard protocol given (see appendix for detailed overview of protocol).

vii. The sections were then mounted with mounting medium (Neo-Mount, Merck KGaA, Germany) and glass cover slides and left under a fume hood overnight.

The sections were scanned in a slide scanner (Hamamatsu NanoZoomer-SQ, Hamamatsu photonics K.K. Japan) at 1-layered 40x magnification to obtain images for analysis. The images were opened in the graphics processing software ImageJ and traced using a drawing tablet (Cintiq 24HD, Wacom Co., Ltd, Japan), see **Figure 5**.

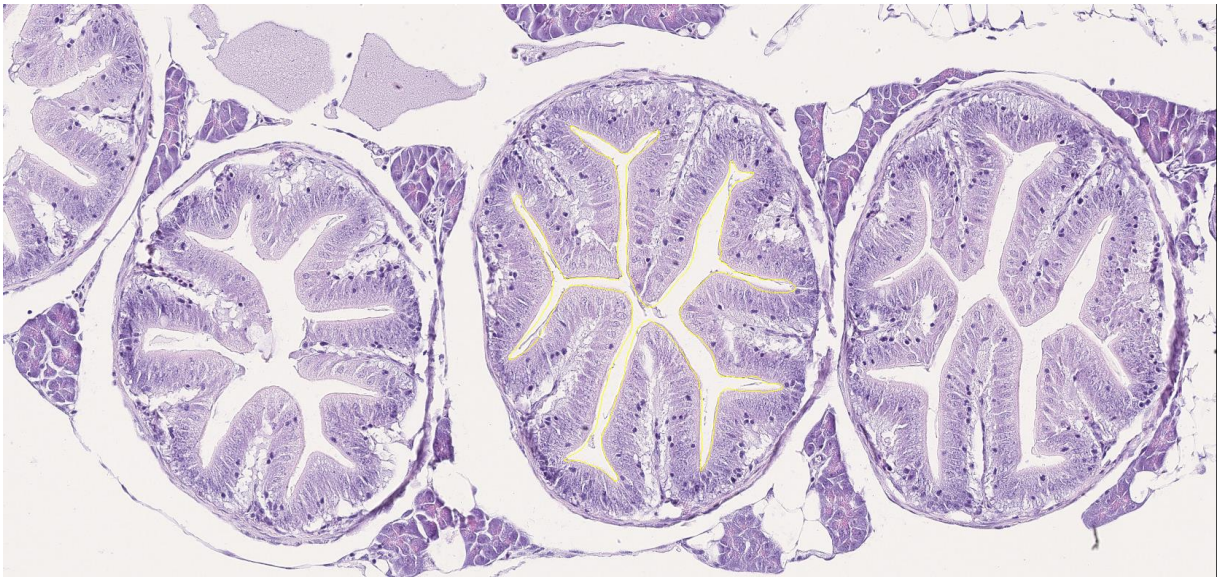

Figure 5 Screenshot from ImageJ displaying an example tracing, where the yellow line traces the inner diameter of the pyloric caeca and is automatically measured by the software.

During analysis of the collected histological samples, for a majority of the samples, the tissue suffered from both mechanical and disintegration damages. The primary causes were suspected to be the high digestive activity in the digestive system that had partially digested the tissue, as well as strong muscle spasms at the time of euthanizing. Due to damages and time constraints, histological samples for data processing were only obtained from the second sampling, for two diets: control diet and 10% algae diet.

Studies targeting morphology of the digestive system commonly measure standard parameters including the height and width of the folds and the thickness of the walls (10), with advantages being ease of comparisons across different studies. Disadvantages include the different sizes of the digestive systems, which corresponds to the sizes of the fish and its environmental factors, possible bias in the selection of the folds to measure, and the requirements to have pristine and clear sections. Young, developing digestive systems are commonly only qualitatively described, as quantitatively measuring the standard characteristics of developing folds is challenging (41).

In the study an index that consists of a ratio of inner to outer surface area was calculated and used. The inner surface area is a measure of advantageous morphological features including folding, while the outer surface area is a measure of the size of the digestive system. The ratio would thus act as an index on the relative development of the digestive system, reflecting the combined effects of both positive developments, e.g. complex folding, and negative developments, e.g. inflammation and shortening of intestinal folds due to dietary effects. Instead of using folding heights, widths and wall thickness as parameters to estimate the increased surface area due to well-developed folds, the proposed index directly measures the relative increase of surface area. A key advantage of using such an index is its robustness and ability to measure morphology data on developing digestive systems or when there are challenges with the sections, for instance in this thesis where several sections were damaged (**Figure 6**).

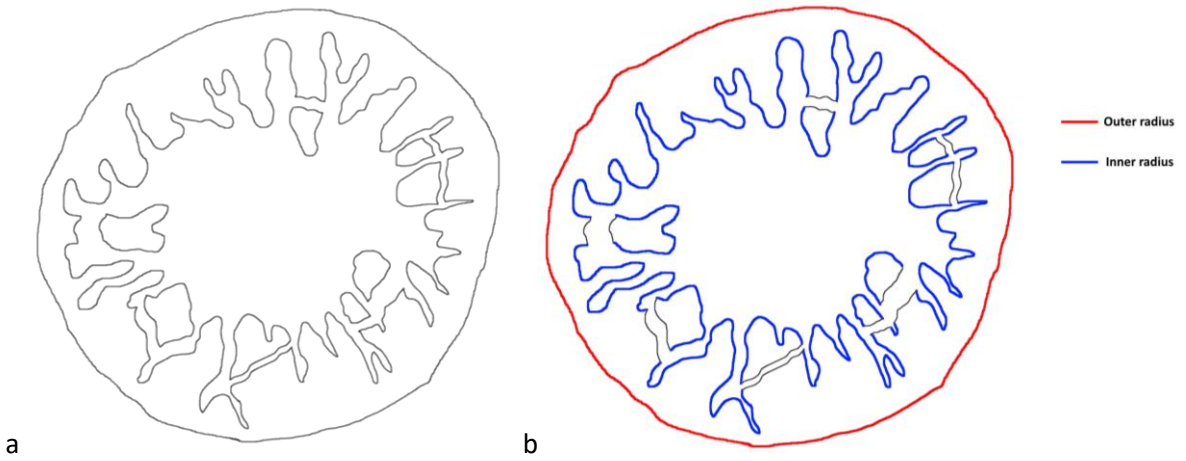

Figure 6 a. Illustration of typical mid-intestine section obtained in this study. Standard parameters including fold height, fold width and wall thickness is challenging to measure due to developing folds and damages to section. b. Illustration of how the inner and outer radius is measured for the proposed index. The outer ratio (coloured red) measures the relative size of the digestive system. The inner radius (coloured blue) measures the relative increase in surface area due to folding. Note that fracture surfaces are not included in the inner radius (left black)

2.4 Data processing

Statistical analyses were conducted using Rstudio version 1.2.5001. The significance level was set to 5% for all significance testing.

2.4.1 Calculation of condition factor

The condition factor (CF) was calculated using the formula (32):

$$CF = 100 \times \frac{w}{l^3}$$

Where: w is the fish weight (g)

l is the fork length (cm)

The CF is commonly scaled with the factor 100 such that it gives values close to 1 for salmon species (32).

2.4.2 Calculation of specific growth rate

The specific growth rate (SGR) for each of the diets were calculated using the formula:

$$SGR = 100(e^{\frac{\ln(w_f/w_i)}{t}} - 1)$$

Where: w_f is the final weight (g)

w_i is the initial weight (g)

t is the time elapsed (days)

Amongst the other commonly used equations for SGR, this equation would be the one to correctly have the units "percent increase in weight per unit time" (42). In this case, since the time is measured in days, the units of SGR would be "$\%\ day^{-1}$".

The values for initial and final weights used in calculating the SGR was the average wet weight from each tank, for each diet. As each diet had three replicates, the sample size for each diet was three.

2.4.3 Processing of histology data

The ratio of inner to outer surface area was calculated using the formula:

$$r = \frac{d_{inner}}{d_{outer}}$$

Where: r is the calculated ratio

d_{inner} is the distance traced in the inner area (µm)

d_{outer} is the distance traced in the outer area (µm)

The simple model assumes the distances traced are representative for the surface areas (**Figure 6**). Each part of the digestive system were compared to the corresponding part in the diets. The mid-intestine was compared to the mid-intestine, and similarly the distal intestine was compared to the distal intestine and pyloric caeca was compared to pyloric caeca.

2.4.4 Statistical testing using one-way ANOVA

A one-way ANOVA test was employed to test for statistical significant differences between groups receiving different diets, for all parameters, at each sampling. To account for possible tank-effects within the groups receiving the same diets, a full ANOVA model including effects from both diets and tanks, and their interaction were first investigated. As there was found no significant interaction or stand-alone effects from tanks, the simplest one-way ANOVA model, including only diet as an independent variable, was used for all samplings.

A significant p-value from the ANOVA test indicates significant deviations between at least one pair of groups given different diets. For the tests that gave a significant p-value as determined by an ANOVA test, a post-hoc test involving Tukey Honestly Significant Difference (TukeyHSD) test was conducted on the data to identify which groups and diets that have significant differences.

3. Results

3.1 Wet weight

At start (the first sampling), there was a small statistically significant difference in wet weight means between the three different diets groups, as determined by the one-way ANOVA ($F(2,87) = 5.39$, $p = 0.00621$). The TukeyHSD post-hoc test found no significant differences in wet weight between the 5% algae diet group and the control diet group ($p = 0.591$), and no significant differences between the 10% algae diet and the 5% algae diet groups ($p = 0.0723$). The TukeyHSD test found a significant difference in wet weight between the 10% algae diet and the control diet groups($p = 0.00534$) (**Figure 7**).

At the second sampling (after 24 days feeding), there was no statistically significant difference in wet weight means between groups given different diets, as determined by the one-way ANOVA ($F(2,87) = 2.16$, $p = 0.121$). No further post-hoc test was pursued for the second sampling.

At the third sampling (after 45 days feeding), there was a statistically significant difference in wet weight means between groups given different diets, as determined by the one-way ANOVA ($F(2,167) = 8.83$, $p = 0.00621$). The TukeyHSD post-hoc test revealed no significant differences in wet weight between the groups given the 5% algae diet and the groups given the 10% algae diet ($p = 0.952$). The TukeyHSD test found a significant difference in wet weight between the groups given the 5% algae diet or 10% algae diet and the control diet ($p = 0.00170$ for the 5% algae diet, $p = 0.000660$ for the 10% algae diet, respectively).

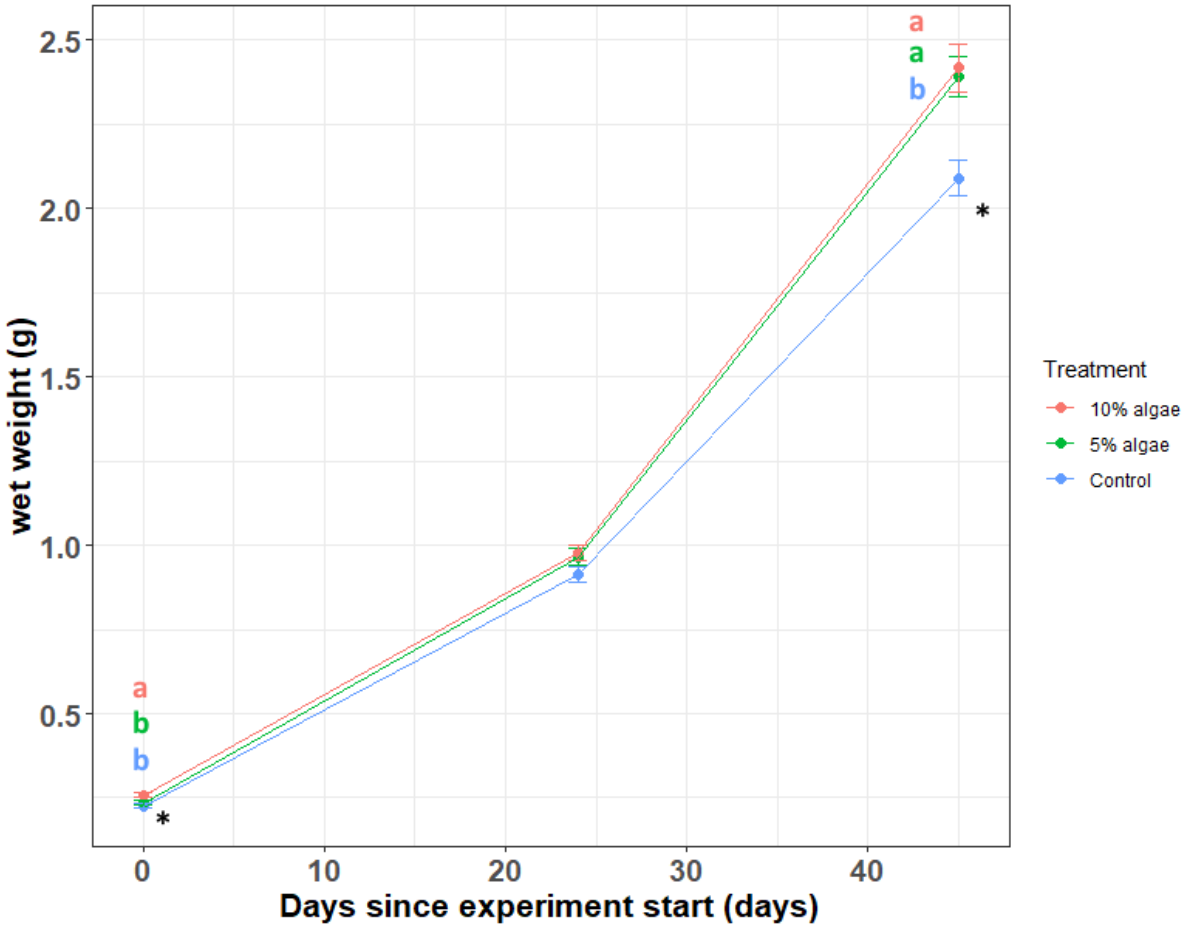

Figure 7 Summary plot of wet weight data for each of the diets at each of the samplings with standard error. Asterisks (*) denote statistically significant differences in means, as determined by a one-way ANOVA test. The letters (a, b) mark statistically significant differences as determined by a post-hoc Tukey HSD test. Different letters denote statistically significant differences between groups. At the first sampling, the group with 10% algae diet had significantly different wet weight than the groups of 5% or control diets. At the third sampling, the group given 5% or 10% algae diet had significantly different wet weights than the group given the control diet, but no statistically significant difference between each other.

3.2 Fork length

At the first sampling (the first sampling), there was no statistically significant difference in fork length means between groups given different diets, as determined by the one-way ANOVA ($F(2,87) = 2.23$, $p = 0.114$). At the second sampling (after 24 days feeding), there was no statistically significant difference in fork length means between groups given different diets, as determined by the one-way ANOVA ($F(2,87) = 1.44$, $p = 0.242$). No further post-hoc test were pursued for the first or second sampling.

At the third sampling (after 45 days feeding), there was a statistically significant difference in fork length means between groups given different diets, as determined by the one-way ANOVA ($F(2,167) = 3.06$, $p = 0.0494$). However, the TukeyHSD post-hoc test found no significant differences in fork length between the groups given the 10% algae diet and the 5% algae diet ($p = 0.940$) or the groups given the

10% algae diet and the control diet (p = 0.128). The TukeyHSD test also failed to find significant differences in fork length between the groups given the 5% algae diet and the control diet (p = 0.0585) (**Figure 8**).

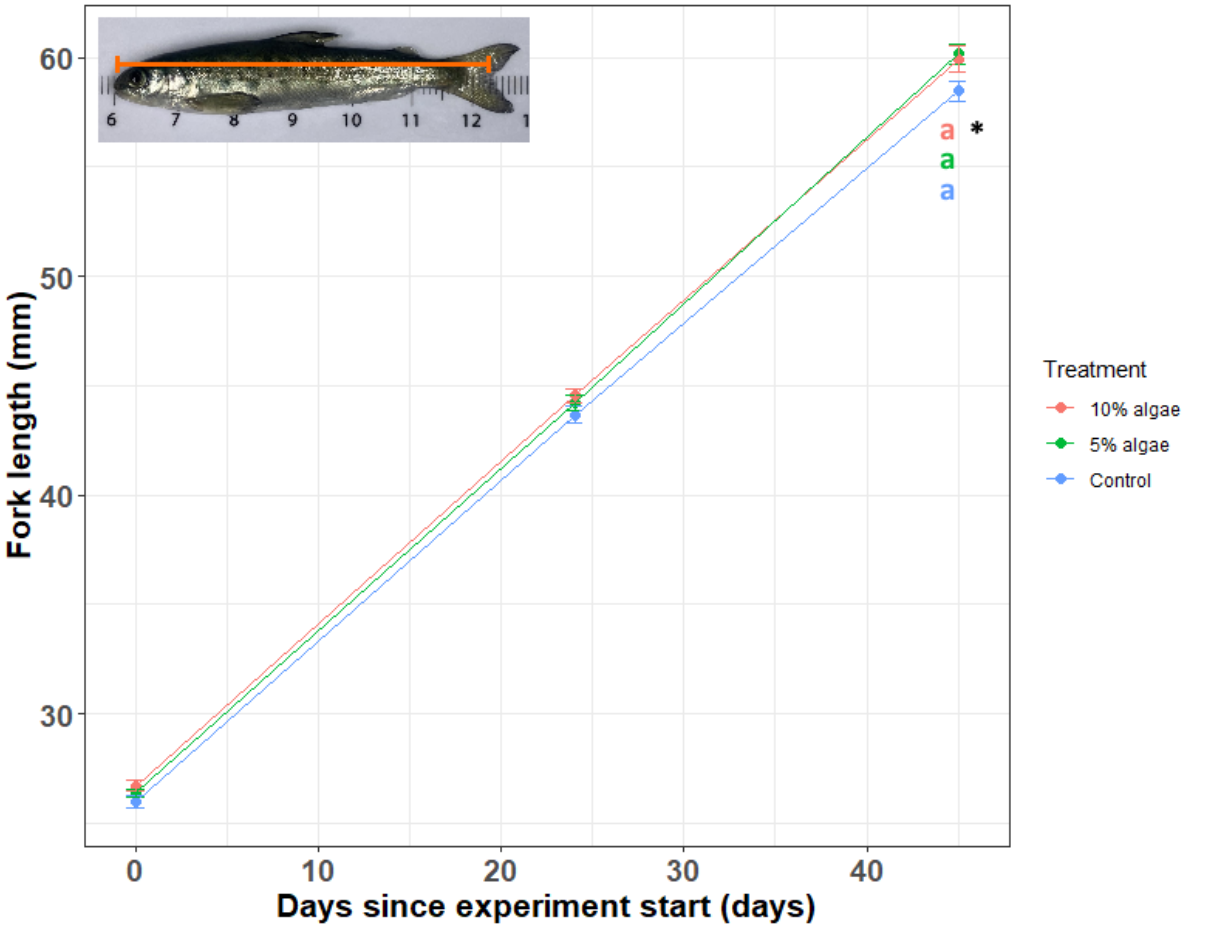

Figure 8 Summary plot of fork length data for each of the diets at each of the samplings with standard error. Asterisks (*) denote statistically significant differences in means, as determined by a one-way ANOVA test. The letters (a, b) mark statistically significant differences as determined by a post-hoc Tukey HSD test. In this case, only the third sampling had significantly different means, as determined by a one-way ANOVA test, but none of the differences were significant as determined by a Tukey HSD test, for the given significance level of 5%.

3.3 Condition factor

At the start of the experiment (the first sampling), there was a statistically significant difference in condition factor means between the different diet groups, as determined by the one-way ANOVA ($F(2,87) = 4.58$, $p = 0.0129$) (**Figure 9**). The TukeyHSD post-hoc test found no significant differences in condition factor between the 5% algae diet and the control diet groups ($p = 1.00$). The TukeyHSD test revealed significant differences in condition factor between the 10% algae diet group when compared to either the 5% algae diet or the control diet groups ($p = 0.0277$ compared to the 5% algae diet, $p = 0.0277$ compared to the control diet, respectively).

At the second sampling (after 24 days feeding), there was no statistically significant difference in condition factor means between groups given different diets, determined by the one-way ANOVA ($F(2,87) = 1.27$, $p = 0.329$). No further post-hoc test were pursued for the second sampling.

At the third sampling (after 45 days feeding), there was a statistically significant difference in condition factor means between groups given different diets, as determined by the one-way ANOVA ($F(2,167) = 28.26$, $p < 1 \times 10^{-7}$). The TukeyHSD post-hoc test found significant differences in condition factors when comparing the groups given either the 5% algae diet or the 10% alga diet to the control diet ($p = 5 \times 10^{-7}$ for the 5% algae diet, $p < 1 \times 10^{-7}$for the 10% algae diet, respectively). The TukeyHSD test failed to find significant differences in condition factor between the groups given the 5% algae diet and the 10% algae diet ($p = 0.167$).

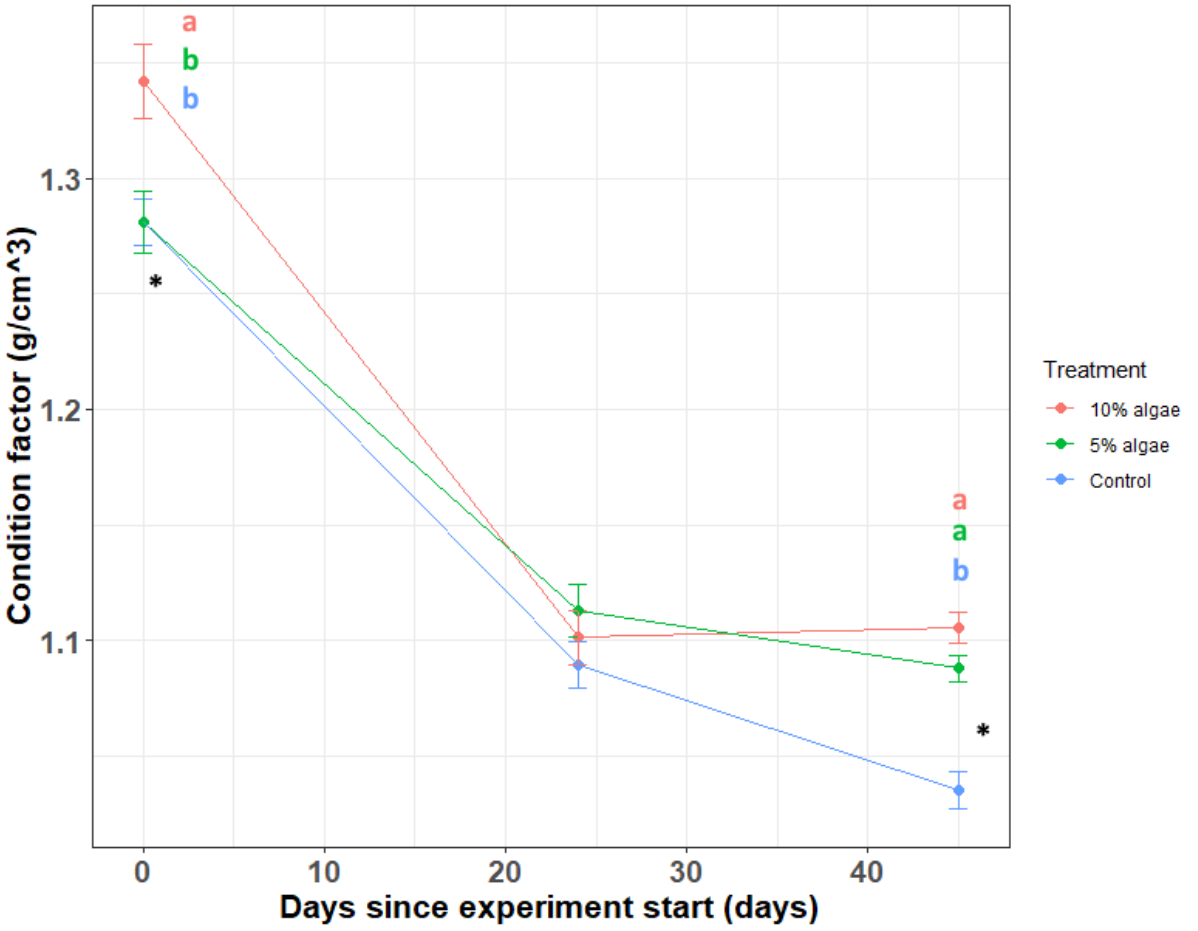

Figure 9 Summary plot of condition factor for each of the diets at each of the samplings with standard error. Asterisks (*) denote statistically significant differences in means, as determined by a one-way ANOVA test. The letters (a, b) mark statistically significant differences as determined by a post-hoc Tukey HSD test. Different letters denote statistically significant differences between groups. At the first sampling, the 10% algae diet group had significantly different condition factor than the 5% or control diet groups. At the third sampling, the group given 5% or 10% algae diet had significantly different condition factor than the group given the control diet, but not significantly from each other.

3.4 Specific Growth Rate

The SGR was calculated from sampling 1 to sampling 2 (SGR1-2) and from sampling 1 to sampling 3 (SGR1-3) for each group given different diets (**Table 2**). There was no statistically significant difference in SGR1-2 between groups given different diets, determined by the one-way ANOVA ($F(2,6) = 0.508$, $p = 0.626$). There was no statistically significant difference either in SGR1-3 between groups given different diets, determined by the one-way ANOVA ($F(2,6) = 1.51$, $p = 0.295$). No further post-hoc tests were pursued for the SGR data.

Table 2 *S*ampling period, diet, mean Specific Growth Rate (SGR), and results from ANOVA test. Sample size n = 3 for each diet at each sampling, as each diet had 3 replicates. No significant p-values were found for any of the tests.

Sampling # (from, to)	Diet	Mean SGR (± standard error) (% day^{-1})	ANOVA test results
From Sampling 1 to Sampling 2 (SGR1-2)	Control	5.98 ± 0.40	$F(2,6) = 0.508$
	5% algae	6.05 ± 0.01	$p = 0.626$
	10% algae	5.72 ± 0.12	
From Sampling 1 to Sampling 3 (SGR1-3)	Control	5.07 ± 0.14	$F(2,6) = 1.51$
	5% algae	5.27 ± 0.06	$p = 0.295$
	10% algae	5.11 ± 0.05	

3.5 Histological analyses

The intestinal distances (mid intestine, distal intestine, pyloric caeca) in the control and 10% algae groups had no significant differences in inner to outer ratios (**Figure 10**). The ratios for the mid intestine (± SE, n = 5) were 2.11 ± 0.24 for the group and 2.19 ± 0.24 for the 10% algae diet (p=0.808). The ratios for the distal intestine (± SE, n = 5) were found to be 1.82 ± 0.08 for the group given the control diet and 1.70 ± 0.13 for the group given the 10% algae diet (p=0.441). The ratios for the pyloric caeca (± SE, n = 5) were 1.27 ± 0.16 for the group given the control diet, and 1.08 ± 0.14 for the group given the 10% algae diet (p=0.365).

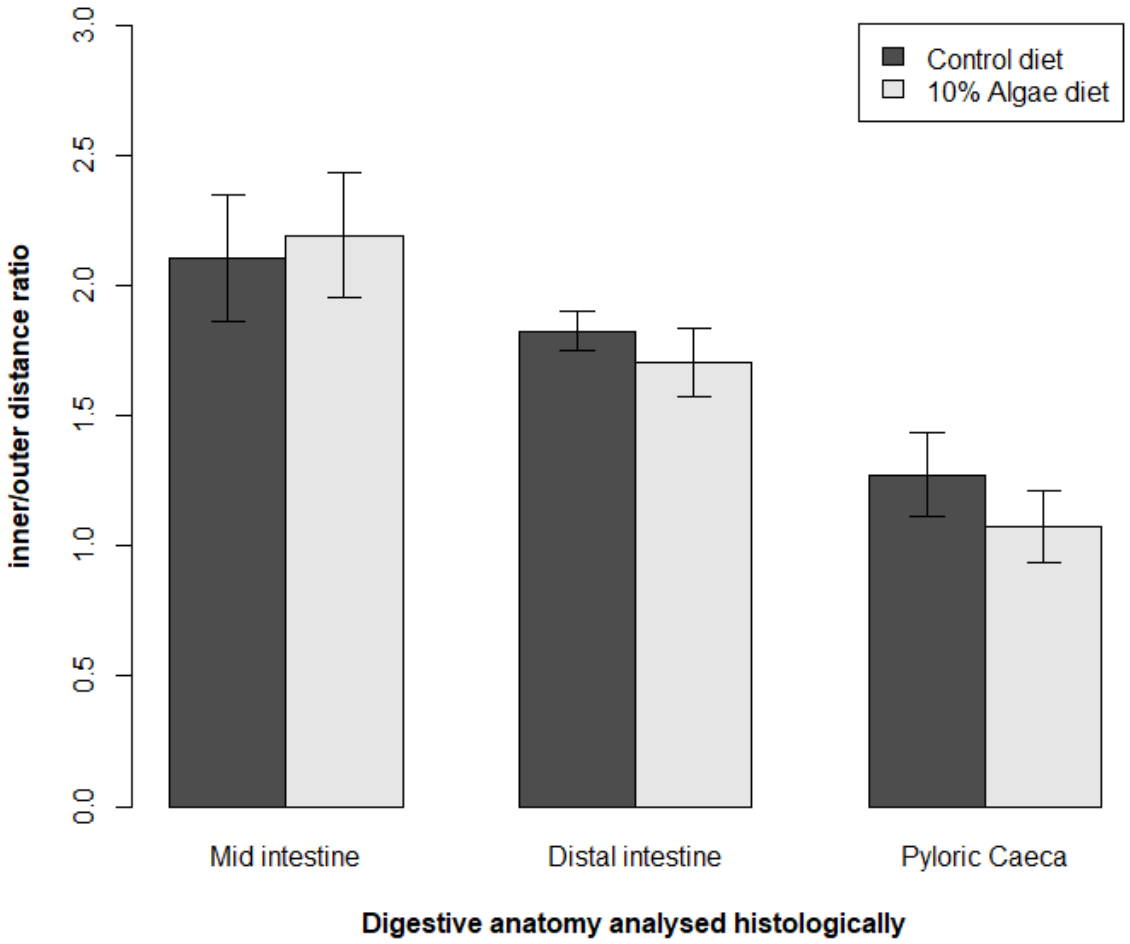

Figure 10 Summary plot of ratio of outer and inner distance for the three areas of digestive anatomy for each diet, with standard errors.

Qualitatively, during analysis of the histological sections, it was consistently noted high amounts of plant fibres in the intestines of all individuals given the 10% algae diet. These were absent in the control group.

4. Discussion

The experimental results found significant increases in wet weight and condition factor at 5% inclusion of *P. amplissima*, and no statistically significant differences in individual fork length, SGR or morphological changes in the digestive system, which shows that inclusion of *P. amplissima* can be considered as a possible solution for future feed for Atlantic salmon. Many earlier fish feeding experiments with inclusion of algae do not reveal any positive effects. In a review of more than 51 different studies, where different species and amounts of macroalgae were included into the diets of different fish species, only 10 studies found some positive effects (43). The majority of cases reported no significant effects or negative effects from the inclusion of macroalgae.

There are few studies on experimental feed given to the early developmental stages of Atlantic salmon at similar conditions (41). To my knowledge, only Sahlmann et al. (2015) have published detailed weight and length data of Atlantic salmon reared under similar conditions during the first stages of its life, being fed diets including soybean meal. In the study, Sahlmann et al. (2015) found no significant differences in growth, histomorphological development or gene expressions in individuals given the experimental or control diet, indicating his findings may be used as a baseline literature value to compare against.

In general, the wet weight and length data found in this study agree with the findings of Sahlmann et al. (2015) for the first two samples, giving slightly larger values at the third sampling. When comparing data, linear extrapolation was used to match similar ages. At the first sampling, the experimental data indicate means of all groups for wet weight and fork length of 0.24 ± 0.01 g, and 2.63 ± 1.44 cm respectively, which is in close agreement with the findings by Sahlmann et al. (2015) of 0.23 g, and 2.69 cm. At the second sampling, the experimental data indicate means for wet weight and fork length of 0.95 ± 0.01 g and 4.42 ± 0.21 cm respectively, which is slightly different to the findings by Sahlmann et al. (2015) of 1.01 g and 4.19 cm. At the third sampling, the experimental data indicate means for all groups for wet weight and fork length of 2.30 ± 0.04 g and 5.95 ± 0.30 cm respectively, which are both larger than the findings by Sahlmann et al. (2015) of 2.23 g and 5.23 cm.

The general agreement for the first two samplings with the findings of Sahlmann et al. (2015) give support to the validity of the experimental results, while the positive difference at the third sampling indicate superior performance of the diets in this experiment. Sahlmann et al. (2015) experimented with two different diets and both diets had higher gross energy density (21.6 MJ/kg for fishmeal diet and 21.3 MJ/kg for soybean diet) than the diets in this experiment (20.6 MJ/kg for all three diets). Sub-

dividing according to diets at the third sampling, it is clear that the groups given the control diet had lower wet weight (2.09 ± 0.05 g) while the groups given the 5% and 10% *P. amplissima* diets had higher wet weight (2.39 ± 0.06 g and 2.42 ± 0.07 g, respectively) when comparing to the findings of Sahlmann et al.(2015). This satisfies the expectation that a diet with lower gross energy density (control diet) performs worse than the diet with higher gross energy density, while indicating that inclusion of *P. amplissima* has a positive effect on wet weight, despite the lower gross energy density.

The increasing difference in fork length compared with the findings by Sahlmann et al. (2015) at the second and third sampling indicate that the fork length deviate over time. Even though the one-way ANOVA test indicate a marginally statistically significant difference in fork length at the third sampling, the post-hoc TukeyHSD test finds no statistically significant difference in fork length between the groups given different diets (the smallest p-value found was for the 5% algae diet at $p = 0.0585$). This indicates that the 5% and 10% algae diets performed better than the control diet at the third sampling, but does not provide definite statistical support for which diet.

A key factor when comparing differences in growth at the third sampling is the difference in temperature, as Atlantic salmon parr is known to grow faster with higher temperature up to a given upper limit (44). Sahlmann et al. (2015) kept their salmon fry at 11-12 °C, while the salmon fry in this experiment were kept at 12-13 °C. An experiment on juvenile Atlantic Salmon reared in varying temperatures no higher than 10 °C in Tromsø observed significantly lower growth (45). However, the close agreement between the experimental wet weight and fork length, and the findings of Sahlmann et al. (2015) at the first two samplings indicate other factors drive the differences at the third sampling, with diet performances being the likely key factor.

Surveys of farmed Atlantic salmon found the typical condition factor to be within the range of 1.05 to 1.50, varying over time and life cycle (46, 47, 48). Though these values are for older individuals, they are consistent with condition factors calculated from weight and length data observed by Sahlmann et al. (2015) on very young salmon fry. The condition factors found in this experiment generally agree with the values from literature (with one exception), supporting the validity of the experimental results.

Higher condition factors are generally associated with healthier individuals (32). The condition factors observed in this experiment generally decreased from the first to second sampling. This would be expected due to the transition from commercial-grade diets at the first sampling, to less energy intensive experimental diets at later samplings. The condition factors for the groups given 5% or 10% algae diets were observed to be 1.10 ± 0.01 at both the second and third samplings, indicating a

stabilisation of the parameter. The condition factor for the group given the control diet was observed to follow a consistently declining trend from the first to the third sampling, with a mean of 1.03 ± 0.01 at the third sampling, indicating that the performance of the control diet was poor. Possible explanations could be negative effects from the indigestible ingredients, including Dextrine.

Even though every effort was taken to randomise the data collection, by randomly assigning diets to each tank and randomly sampling individuals, the results from the first sampling still indicate a statistically significant higher wet weight and condition factor for the individuals assigned the 10% algae diet. This makes it challenging to conclude on the effects of the 10% algae diet on wet weight and condition factor at the third sampling when comparing to the other diets, knowing that the starting point (first sampling) was also statistically significant. The individuals that were assigned the 5% algae diet did not have any statistically significant difference compared to the control group in wet weight and condition factor at the first or second sampling, which enables us to place greater confidence on the statistically significant increases at the third sampling.

The statistical analyses of the wet weight data for all diets at all three samples show a pattern of similar wet weight at the first and second sampling (apart for the 10% algae diet group at the first sampling), followed by a statistically significant deviation at the third sampling. Given consistently different diet performance at all points in time, one would generally expect to see deviations in wet weight and condition factor at the second sampling, followed by an even larger deviation at the third sampling. One explanation for the lack of deviations between the groups given different diets at the second sampling could be that the salmon fry required a period of time to get used to the newly introduced diets. As the new diets were introduced right after the first sampling, the salmon fry could have eaten poorly for a while, leading to similar wet weights measured at the second sampling. After the second sampling, as the individuals had gotten familiar with their diets and were eating properly, the differences in diet performance became apparent as diverging wet weights at the third sampling.

Specific growth rate (SGR) accounts for differences in starting weight, enabling the effects of the 10% algae diet to be included. The calculated SGR of the Atlantic salmon from the first to the second sampling were all similar within statistical uncertainties, with 5.98 ± 0.40 $\%day^{-1}$ for the control diet, 6.05 ± 0.01 $\%day^{-1}$ for the 5% algae diet and 5.72 ± 0.12 $\%day^{-1}$ for the 10% algae diet. The calculated SGR of the Atlantic salmon from the first to the third sampling were also all similar within statistical uncertainties, with 5.07 ± 0.14 $\%day^{-1}$ for the control diet, 5.27 ± 0.06 $\%day^{-1}$ for the 5% algae diet and 5.11 ± 0.05 $\%day^{-1}$ for the 10% algae diet. SGR is known to depend on both age and temperature (49), with young individuals exposed to hot temperatures (within acceptable ranges) having the highest SGR

(50). Literature estimates on Atlantic salmon fry of similar age and exposed to similar temperature ranges suggest SGR in the range of 3.83 to 5.35 (41, 50, 51). The SGR found in this experiment were generally found to be higher than literature values at the second sampling, and agreeing with literature values at the third sampling. The lack of statistically significant differences in SGR between the three diets in this study indicate that factors other than the feeds themselves are causing higher SGR than expected. Possible factors include method of feed distribution, stress, fish health and other environmental factors, but without detailed comparisons with literature sources, the key factor(s) is challenging to pinpoint. In general, the relatively high means for SGR in this experiment indicate satisfactory feed intake and growth.

While inflammation in the intestines of older Atlantic salmon as a response to plant-based ingredients is well documented (4, 11, 12), fry and young parr seem to lack this inflammation response, even at high inclusion levels (41, 52). Possible explanations include under-developed adaptive immune system that is unable to mount a response at such a young age. The results from the histological analyses in this experiment agrees with literature, with no statistically significant differences in histological characteristics found in any of the parts of the digestive system, when comparing individuals given control diet or 10% algae diet. This implies no negative effects or negative immune responses on the digestive system of Atlantic salmon fry when given feed with high inclusion levels of *P. amplissima*.

The lack of negative inflammation response to novel feed ingredients open exciting questions regarding the age when the immune response develops, and whether it is possible to modify or exploit the initial lack of immune response to plant-based ingredients. Future studies could explore whether it is possible to delay or supress immune responses through specialised early diets or whether even higher inclusion levels could be introduced without impact on welfare during the first start-feeding phase.

4.1 Summary

From the increase in wet weight and condition factor, there is clear evidence that 5% *P. amplissima* in the diet leads to statistically significant increased biomass of Atlantic salmon fry (*S. salar*). This is supported by a significant positive effect on fork length at the last sampling point, when considering both experimental diets (both 5% and 10% *P. amplissima*). The SGR were not observed to have any statistically significant difference, suggesting that the positive differences in wet weight, condition factor and fork length deviated over time. There was no significant differences observed in the morphology of the digestive system, indicating that the presence of *P. amplissima* neither stimulated

nor impeded the development of the digestive system, agreeing with literature (41). The general agreement with literature values for several parameters support the validity of the experimental results.

4.2 Lessons for future research

Future research on including macroalgae into feed for Atlantic salmon could benefit from both learnings and results obtained in this study. Challenges encountered generally fall into two categories: Sample damage and sampling capacity. The results indicate an optimum inclusion level of *P. amplissima* in Atlantic salmon feed, which is similar to inclusion levels of other algae species (43).

To minimise damages and disintegration of tissue for histological analyses, future histological sampling should adhere to three principles: the fish should be starved prior to sampling, to reduce digestive activities in the system. The organs should be sufficiently exposed to fixating medium, extraction of organs should be preferred. If impractical, larger cuts along the body is possible. Simple decapitation should only be utilised if the previous steps prove highly impractical.

Sufficient capacity should be prepared during sampling days for future experiments. The daily rearing of the fish was distributed amongst a number of trained volunteering BIHAV- and master-students and had a manageable work-load. Capacity requirements for sampling days that involve 8-10 additional man-hours on top of the daily activities should not be underestimated. The key requirement should be sufficient capacity during sampling procedures so that the time between administering euthanasia and submerging the body or organs in fixating medium is as short as possible.

Based on the results from this study, future research involving *P. amplissima* could narrow down their targeted inclusion levels. From the results on wet weight, indications found in the SGR, and the histological analyses, there seems to be some evidence that 5% inclusion level of *P. amplissima* may yield more positive impact than an inclusion level of 10%. Further research could therefore first target a 5% inclusion level and focus on broader sets of research questions of interest, instead of testing a range of different inclusion levels. As sample damage and time constraints limited this study to the histological effects of a 10% algae inclusion diet, an open research question concerns the histological effects of 5% inclusion.

The low condition factors measured at the third sampling for the group given the control diet indicate poor performance by the control diet. This effect could originate from the indigestible ingredients used

in the control diet, including Dextrine, to achieve isocaloric, isoproteic and isolipidic diets. Future experiments involving algae as a feed ingredient should consider testing other indigestible ingredients in the control diet to ensure a higher condition factor for the control group.

The presence of plant fibres in the intestines of individuals given the 10% algae diet indicate incomplete digestion of the plant structures such as cell walls. As the *P. amplissima* was added without additional treatment apart from freeze-drying, this could indicate incomplete access to all of the nutrition in the algae, agreeing with previous studies (43). To reveal the full potential of adding *P. amplissima* to the fish diet, some additional treatment of the algae prior to inclusion into feed production should be considered for future experiments involving *P. amplissima*.

The lack of inflammation responses in the digestive system of the Atlantic salmon fry even at high inclusion levels of *P. amplissima* agree with studies showing a lack of response at young ages (41, 52). This opens the exciting opportunity for future studies to determine at what age the inflammation response starts to develop in Atlantic salmon and whether it is possible to manipulate its development.

5. Conclusions

Including 5% *P. amplissima* in feed given to Atlantic salmon (*S. salar*) fry resulted in significant higher wet weight and condition factor compared to the control group. Including 10% *P. amplissima* in feed given to Atlantic salmon (*S. salar*) fry also resulted in significantly high wet weight and condition factor compared to the control group, but due to differences in wet weights at the start of the feeding experiment, these results were only taken as indicative. There is insufficient evidence to draw a statistically significant conclusion from the measured SGR, though the high means for all diet groups compared to literature values indicate satisfactory growth for all diets.

One-way ANOVA tests indicate no statistically significant difference in fork length at the first or second sampling, but a statistically significant difference at the third sampling. As a post-hoc TukeyHSD test found no statistically significant difference in fork length between the groups given different diets, this indicates that there is a significant difference in the mean of the fork length between the groups given different diets, but not enough statistical power to determine which group is significant.

There was found no significant differences in the histological analysis between the control group and the group given 10% algae diet. The histological characteristic used was a ratio that described the relationship between the inner and outer surface areas of the mid intestine, distal intestine and pyloric caeca, respectively. Due to sample damages and time constraints, analyses for the 5% algae diet group was not pursued.

To conclude, this study found significant evidence that including 5% *P. amplissima* to the diets given to Atlantic salmon (*S. salar*) fry had significant positive effects on growth of the fish. Combining the results from the weight, length and histological analyses, there is evidence that inclusion levels up to 10% *P. amplissima* in feed does not impart any negative effects on growth or gut health, which opens up for further research on *P. amplissima* as a feed ingredient for Atlantic salmon (*S. salar*) .

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7. Appendices

7.1 Composition of experimental feeds

The experimental feed manufactured followed the final composition given in the table below (**Table 7.1.A**).

Table 7.1.A Final composition of experimental feed manufactured

Ingredients	CTRL	ALGAE 5	ALGAE 10
	%	%	%
Fishmeal LT70	45,000	45,000	45,000
Fish protein hydrolysate	5,000	5,000	5,000
Squid meal	5,000	5,000	5,000
Krill meal	14,800	14,800	14,800
Fish gelatin	3,000	3,000	3,000
NTNU - ALGAE		**5,000**	**10,000**
Dextrine	6,100	3,100	
Wheat gluten	3,900	1,900	
Wheat meal	4,000	4,000	4,000
Vit & Min Premix	1,500	1,500	1,500
Vitamin C35	0,100	0,100	0,100
Vitamin E50	0,100	0,100	0,100
Betaine HCl	0,500	0,500	0,500
Antioxidant	0,200	0,200	0,200
MSP (Monosodium phosphate)	1,000	1,000	1,000
L-Taurine	0,300	0,300	0,300
Soy lecithin	5,000	5,000	5,000
Fish oil	2,400	2,400	2,400
Algae oil	2,100	2,100	2,100
Total	**100,000**	**100,000**	**100,000**

As fed basis	CTRL	ALGAE 5	ALGAE 10
Crude protein, % feed	55,0	55,0	55,0
Crude fat, % feed	15,0	15,0	15,0
Crude fat (no oils)	12,3	12,7	12,4
Fiber, % feed	0,2	0,2	0,2
Starch, % feed	8,6	5,5	2,4
Ash, % feed	12,0	12,5	13,1
Gross Energy, MJ/kg feed	20,6	20,6	20,6
Arg, % feed	3,58	3,53	3,47
His, % feed	1,31	1,28	1,25
Ile, % feed	2,25	2,19	2,14
Leu, % feed	3,92	3,80	3,69
Lys, % feed	3,92	3,89	3,87
Thr, % feed	2,32	2,28	2,24
Trp, % feed	0,57	0,56	0,54
Val, % feed	2,58	2,52	2,46
Met, % feed	1,34	1,31	1,29
Cys, % feed	0,45	0,42	0,40

Met + Cys, % feed	1,79	1,74	1,69
Phe, % feed	2,22	2,13	2,05
Tyr, % feed	1,77	1,71	1,66
Phe + Tyr, % feed	3,98	3,84	3,71
Tau, % feed	0,68	0,68	0,68
Asx, % feed	4,77	4,72	4,67
Glx, % feed	7,80	7,18	6,59
Ala, % feed	3,23	3,19	3,15
Gly, % feed	4,10	4,05	4,00
Pro, % feed	2,87	2,66	2,47
Ser, % feed	2,41	2,32	2,24
Total P, % feed	1,82	1,82	1,82
Ca, % feed	2,0	2,0	2,0
Ca/P	1,1	1,1	1,1
Na, % feed	1,3	1,3	1,3
Vit A, IU/kg	43259,7	43259,7	43259,7
Vit D3, IU/kg	3732,2	3732,2	3732,2
Vit E, mg/kg	650,6	650,6	650,6
Vit K3, mg/kg	68,5	68,5	68,5
Vit B1, mg/kg	45,5	45,5	45,4
Vit B2, mg/kg	50,0	50,0	49,9
Vit B3, mg/kg	493,7	493,6	493,5
Vit B5, mg/kg	155,3	155,3	155,3
Vit B6, mg/kg	30,2	30,2	30,2
Vit B9, mg/kg	23,0	23,0	23,0
Vit B12, mg/kg	0,2	0,2	0,2
Vit C, mg/kg	1850,3	1850,3	1850,3
Choline, mg/kg	2820,0	2820,0	2820,0
Inositol, mg/kg	750,2	750,2	750,2
Betaine, mg/kg	6188,4	6188,4	6188,4
C14, % feed	0,7	0,7	0,7
C16, % feed	3,2	3,2	3,2
C18, % feed	0,5	0,5	0,5
C18:1n9, % feed	1,9	1,9	1,9
LNA (C18:2n6), % feed	1,8	1,8	1,8
ALA (C18:3n3), % feed	0,2	0,2	0,2
ARA, % feed	0,1	0,1	0,1
EPA, % feed	1,63	1,63	1,63
DHA, % feed	1,69	1,69	1,69
EPA+DHA, % feed	3,33	3,33	3,33
ARA/EPA, % feed	0,1	0,1	0,1
Total phospholipids, % feed	4,51	4,51	4,51

7.2 Haematoxylin and Eosin staining protocol

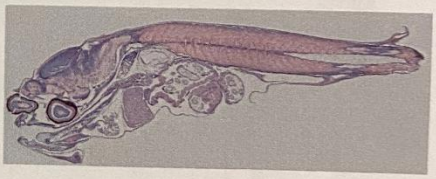
The Haematoxylin and Eosin staining procedure followed a standard protocol given (**Figure 7.2.A**).

Hematoxylin and Eosin (H&E) staining

•Place slides containing paraffin sections in a slide holder
•Deparaffinize and rehydrate sections:

TissueClear	5 min	
TissueClear	5 min	
TissueClear	5 min	
100 % etanol	2 min	
100 % etanol	2 min	
96 % etanol	2 min	
70 % etanol	2-3 min	
dist.H_2O	5 min	
Mayers Hem.	3 min	(2-5 min)
Tap water running	3 min	(to allow stain to develop)
1% HCl i 70 % etanol (acid alcohol 4 ml 6M HCl + 200 ml 70 % etanol)	Dip 5x (fast)	(to destain)
Tap water running	3 min	
0,5% eosin	2 min	
Tap water	dip	
dist.H_2O	dip	

Air dry or proceed to dehydration.



Dehydration:

70 % etanol	dip
100 % etanol	30 sek
100 % etanol	2 x 2 min
TissueClear	5 min
TissueClear	5 min
TissueClear	5 min

•Coverslip slides using NeoMount

Nuclei - blue
Cytoplasm, connective tissue, muscle etc. - varying shades of pink

Figure 7.2.A Standard Haematoxylin and Eosin staining protocol employed in this study

7.3 Data collected on conditions during rearing

The oxygen saturation, temperature and mortality for each of the nine tanks were monitored and recorded for the duration of the experiment (**Tables 7.3.A** and **7.3.B**).

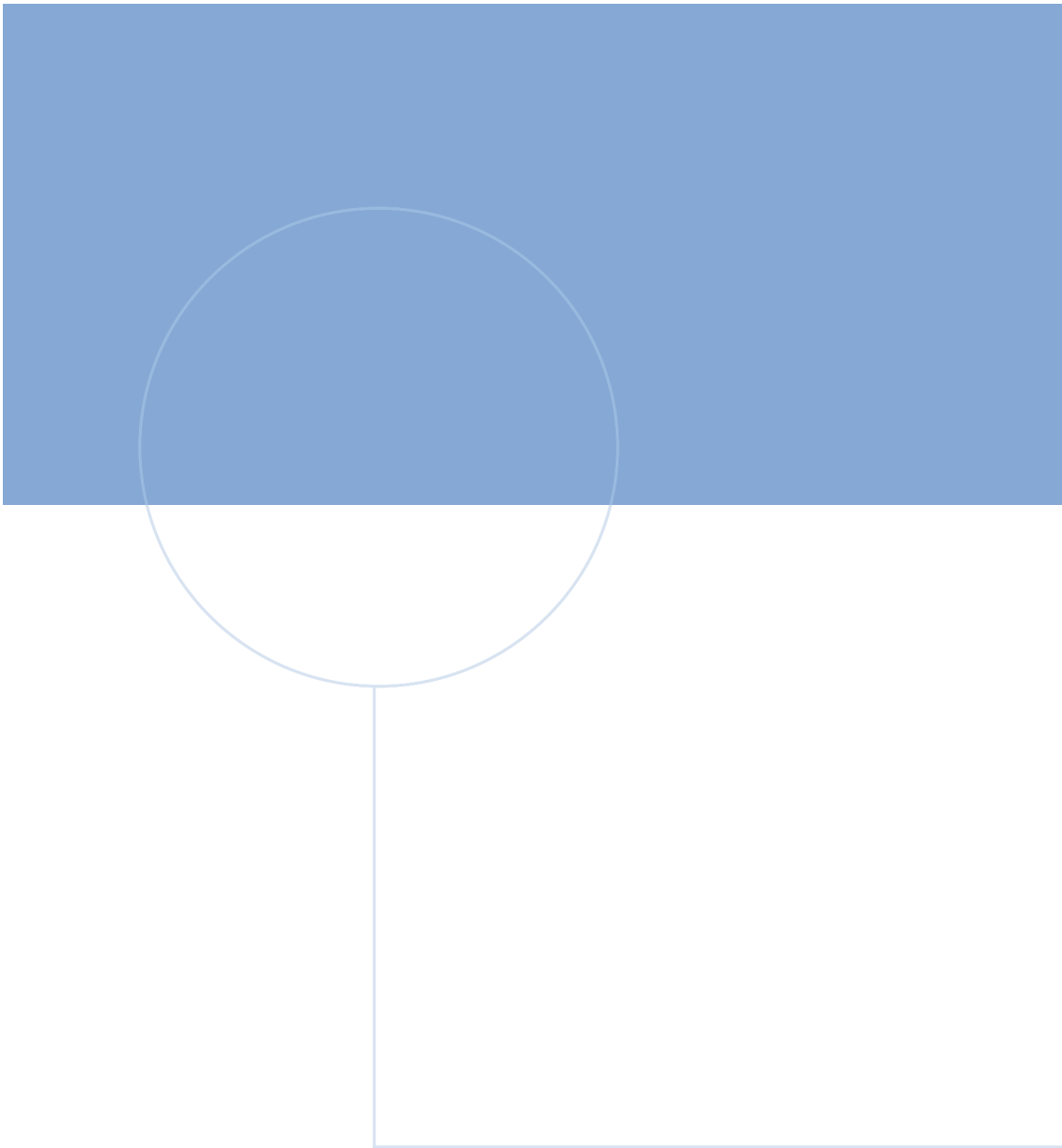
Table 7.3.A Recorded oxygen saturation and temperature for each of the nine tanks for the duration of the experiment.

Date	Tank 1		Tank 2		Tank 3		Tank 4		Tank 5		Tank 6		Tank 7		Tank 8		Tank 9	
	O_2 (mg/L)	Temp (°C)	O_2 (mg/L)	Temp (°C)	O_2 (mg/L)	Temp (°C)	O_2 (mg/L)	Temp (°C)	O_2 (mg/L)	Temp (°C)	O_2 (mg/L)	Temp (°C)	O_2 (mg/L)	Temp (°C)	O_2 (mg/L)	Temp (°C)	O_2 (mg/L)	Temp (°C)
28.11	10.61	12.7	10.62	12.7	10,59	12.8	10,59	12.8	10,53	12.8	10,58	12.8	10,59	12.8	10,57	12.8	10.56	12.9
29.11	10.64	12.6	10.61	12.7	10,58	12.8	10.62	12.7	10.61	12.7	10,59	12.8	10,54	12.8	10.61	12.7	10,57	12.8
30.11	10,59	12.8	10,59	12.8	10.52	12.9	10,57	12.9	10,60	12.8	10.53	12.9	10.64	12.7	10,59	12.8	10,57	12.8
01.12	10.61	12.7	10,60	12.8	10,59	12.8	10,59	12.8	10,57	12.8	10,56	12.8	10,57	12.8	10,59	12.8	10,59	12.8
02.12	10.61	12.7	10,59	12.8	10.61	12.7	10.61	12.7	10,57	12.8	10,59	12.9	10.52	12.9	10.55	12.9	10.52	12.9
03.12	10.52	12.9	10,59	12.8	10,61	12.8	10,59	12.8	10,59	12.8	10,59	12.8	10,58	12.8	10,60	12.8	10.56	12.9
04.12	10.53	12.9	10.51	12.9	10.49	12.9	10.55	12.9	10.55	12.9	10,60	12.8	10.52	12.9	10.49	12.9	10.49	12.9
05.12	10.55	12.9	10.52	12.9	10.53	12.9	10.54	12.9	10.53	12.9	10.51	12.9	10.52	12.9	10.52	12.9	10.52	12.9
06.12	10.56	12.9	10.53	12.9	10.54	12.9	10.54	12.9	10.56	12.9	10.52	12.9	10.52	12.9	10.54	12.9	10.52	12.9
07.12	10.55	12.9	10.52	12.9	10.52	12.9	10.52	12.9	10.52	12.9	10.55	12.9	10.53	12.9	10.49	12.9	10.39	13.0
08.12	10.49	12.9	10.55	12.9	10.53	12.9	10.56	12.9	10.51	12.9	10.52	12.9	10.53	12.9	10.52	12.9	10.40	13.0
09.12	10.54	12.9	10.52	12.9	10.54	12.9	10.52	12.9	10.51	12.9	10.52	12.9	10.54	12.9	10,59	12.8	10.38	13.0
10.12	10,50	12.8	10,53	12.8	10,59	12.8	10,59	12.8	10.52	12.9	10,59	12.8	10.52	12.9	10.52	12.9	10.52	12.9
11.12	10.52	12.9	10.51	12.9	10.53	12.9	10.55	12.9	10.52	12.9	10.55	12.9	10.56	12.9	10.55	12.9	10.49	12.9
12.12	10.53	12.9	10.52	12.9	10.56	12.9	10.52	12.9	10.53	12.9	10.54	12.9	10.49	12.9	10.52	12.9	10.52	12.9
13.12	10.55	12.9	10.53	13.0	10.52	12.9	10.55	12.9	10.51	12.9	10.54	12.9	10.56	12.9	10.54	12.9	10.49	12.9
14.12	9.96	12.9	10.51	12.9	10.53	12.9	10.52	12.9	10.56	12.8	10.55	12.9	10.54	12.9	10.52	12.9	10.52	12.9
15.12	10.55	12.9	10,59	12.8	10.61	12.7	10,59	12.8	10.62	12.7	10,58	12.8	10,57	12.8	10,59	12.8	10,59	12.8
16.12	10,57	12.8	10,57	12.8	10,57	12.8	10,56	12.8	10,57	12.8	10,57	12.8	10,57	12.8	10,55	12.8	10,59	12.8
17.12	10.60	12.7	10.61	12.7	10.63	12.6	10.64	12.6	10.63	12.6	10.65	12.6	10.64	12.6	10.34	12.6	10.60	12.7
18.12	9.94	12.9	9.87	12.9	9.87	12.9	0.85	12.9	9.88	12.9	9.96	12.9	9.84	12.9	9.89	12.9	9.85	13.0
19.12	9.87	12.9	9.83	12.9	9.81	12.9	9.77	12.9	9.81	12.9	9.90	12.9	9.76	12.9	9.81	12.9	9.81	13.0
20.12	9.96	12.8	9.97	12.8	9.95	12.8	9.94	12.8	10.00	12.8	10.07	12.8	10.00	12.8	9.99	12.8	9.86	12.9
21.12	10.36	12.9	10.36	12.9	10.35	12.9	10.36	12.9	10.36	12.9	10.36	12.8	10.36	12.9	10.36	12.9	10.46	12.9
22.12	10.27	12.8	10.26	12.8	10.27	12.8	10.27	12.8	10.28	12.8	10.28	12.8	10.30	12.8	10.27	12.8	10.28	12.9
23.12	10.31	12.9	10.31	12.9	10.32	12.9	10.32	12.9	10.33	12.9	10.33	12.9	10.32	12.9	10.32	12.9	10.32	13.0
24.12	10.37	12.9	10.37	12.9	10.39	12.9	10.38	12.9	10.40	12.9	10.40	12.9	10.39	12.9	10.40	12.9	10.39	13.0
25.12	10.23	12.9	10.23	12.9	10.25	12.9	10.25	12.9	10.27	12.9	10.25	12.9	10.28	12.9	10.28	12.9	10.28	13.0
26.12	10.09	12.9	10.08	12.9	10.10.	12.9	10.11	12.9	10.12	12.9	10.13	12.9	10.12	12.9	10.12	12.9	10.12	13.0
27.12	9.94	12.9	9.94	12.9	9.95	12.9	9.96	12.9	9.97	12.9	9.99	12.9	9.97	12.9	9.97	12.9	9.97	13.0
28.12	10.10	12.8	10.11	12.8	10.12	12.8	10.12	12.8	10.13	12.8	10.13	12.8	10.13	12.8	10.14	12.8	10.13	12.9
29.12	10.18	12.9	10.17	12.9	10.18	12.9	10.17	12.8	10.22	12.8	10.20	12.8	10.18	12.8	10.19	12.8	10.20	12.9
30.12	10.25	12.7	10.27	12.8	10.27	12.8	10.26	12.8	10.27	12.8	10.28	12.8	10.26	12.8	10.26	12.8	10.26	12.9
31.12	10.35	12.8	10.35	12.8	10.34	12.8	10.33	12.8	10.33	12.8	10.34	12.8	10.32	12.8	10.32	12.8	10.32	12.9
01.01	10.34	12.9	10.36	12.9	10.37	12.9	10.37	12.9	10.38	12.9	10.38	12.8	10.37	12.8	10.37	12.8	10.38	12.9
02.01	10.42	12.8	10.42	12.8	10.42	12.8	10.42	12.8	10.43	12.8	10.42	12.8	10.42	12.8	10.41	12.8	10.42	12.9
03.01	10.60	12.9	10.61	12.9	10.59	12.9	10.59	12.9	10.60	12.8	10.60	12.8	10.59	12.9	10.60	12.9	10.60	12.9
04.01	10.65	12.8	10.67	12.8	10.65	12.8	10.65	12.8	10.65	12.8	10.65	12.8	10.65	12.8	10.65	12.8	10.63	12.9
05.01	10.64	12.9	10.64	12.9	10.62	12.8	10.63	12.8	10.63	12.8	10.63	12.8	10.62	12.8	10.62	12.8	10.63	12.9
06.01	10.66	12.7	10.67	12.7	10.66	12.7	10.66	12.7	10.75	12.6	10.77	12.6	10.73	12.7	10.76	12.7	10.58	12.8
07.01	10.53	12.8	10.53	12.8	10.53	12.8	10.53	12.7	10.58	12.7	10.62	12.6	10.58	12.7	10.59	12.7	10.49	12.8
08.01	10.50	12.7	10.47	12.8	10.58	12.7	10.39	12.8	10.58	12.8	10.62	12.7	10.60	12.7	10.58	12.8	10.40	12.8
09.01	10.56	12.9	10.56	12.8	10.56	12.8	10.52	12.7	10.53	12.7	10.53	12.7	10.50	12.7	10.50	12.7	10.16	12.9
10.01	10.53	12.8	10.53	12.8	10.52	12.8	10.52	12.8	10.51	12.7	10.53	12.7	10.51	12.7	10.51	12.7	9.95	12.8
11.01	10.37	12.8	10.38	12.8	10.39	12.8	10.41	12.8	10.39	12.7	10.41	12.7	10.38	12.7	10.40	12.7	10.34	12.8

Table 7.3.B Recorded mortalities for each of the nine tanks for the duration of the experiment. Empty fields indicate no recorded mortality for the given tank, for the given date. Whenever mortalities were observed, the number of mortalities and their average wet weight were recorded.

Date	Tank 1		Tank 2		Tank 3		Tank 4		Tank 5		Tank 6		Tank 7		Tank 8		Tank 9	
	No.	Ave w. weight (g)	No.	Ave w. weight (g)	No.	Ave w. weight (g)	No.	Ave w. weight (g)	No.	Ave w. weight (g)	No.	Ave w. weight (g)	No.	Ave w. weight (g)	No.	Ave w. weight (g)	No.	Ave w. weight (g)
28.11	1	0.382	1	0.344														
29.11																		
30.11																		
01.12																		
02.12																		
03.12																		
04.12																		
05.12																		
06.12																		
07.12																		
08.12																		
09.12																		
10.12															1	0.2887		
11.12																		
12.12			1	0.2419														
13.12																		
14.12											1	0.2762						
15.12																		

Date	Tank 1		Tank 2		Tank 3		Tank 4		Tank 5		Tank 6		Tank 7		Tank 8		Tank 9	
	No.	Ave w. weight (g)	No.	Ave w. weight (g)	No.	Ave w. weight (g)	No.	Ave w. weight (g)	No.	Ave w. weight (g)	No.	Ave w. weight (g)	No.	Ave w. weight (g)	No.	Ave w. weight (g)	No.	Ave w. weight (g)
16.12																		
17.12											1	0.572						
18.12											1	0.555						
19.12											2	0.60825						
20.12											1	1.0922						
21.12					1	0.7036					2	0.6543						
22.12											1	0.5879						
23.12																		
24.12																		
25.12																		
26.12																		
27.12																		
28.12							1	1.3924										
29.12																		
30.12																		
31.12																		
01.01																		
02.01																	1	1.1194
03.01					1	1.2428			2	1.0717							1	1.4057
04.01			1	1.1552														
05.01																		
06.01																		
07.01																		
08.01																		
09.01																		
10.01																		
11.01																		



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