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Marine Coastal Development
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Co-supervisors: Jussi Evertsen, Frøya VGS
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Trondheim, May 2017

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Abstract

Organic waste released from a fish farm, is one of the main challenges facing aquaculture today, and to increase fish, production it is important to minimize the impact this have on the environment. The aim of this study was to elucidate the carotenoid Astaxanthin as a biological tracer from pen raised Atlantic salmon (*Salmo salar*) feed causing organic waste to the surrounding ecosystem, as to the author knowledge this is not done before. By using target organisms Blue mussel (*Mytilus edulis*), Whelk (*Buccinum undatum*), and seawater, seabed sediments and *in situ* video survey. *In vitro* absorbance and HPLC isolation of pigment extracts where the main methods used. The findings show increased Astaxanthin concentration in Atlantic salmon muscle tissue with increased size of fish at performed feeding program. High Astaxanthin content was found in fish feces at poor feed utilization. No abnormal high values of Astaxanthin were observed in water surface, Blue mussel and Whelk food uptake or tissue content. Zooplankton reveals Atlantic salmon's natural source of Astaxanthin. Seabed sediments showed presence of Astaxanthin, but in low concentration (<1.2 µg g^{-1}), which seems to be normal. Quick dilution of organic waste was observed, but also presence of *Beggiatoa* sp. reveals low oxygen level on the seabed and sedimentation of organic matter. For future research, measure Astaxanthin concentrations at sites with more accumulated organic waste on the seabed to conclude whether if this is a good method for future monitoring programs or not.

Key words: Astaxanthin, HPLC, ROV, *in vitro* absorbance, Light microscopy, Microphytobenthos, *Beggiatoa* sp., IMTA.

Sammendrag

Organisk avfall frigitt fra et oppdrettsanlegg, er en av hovedutfordringene oppdrettsnæringen har i dag, og for å øke produksjon av fisk, er det viktig å minimere virkningen dette har på miljøet. Målet med denne studien var å belyse karotenoidet Astaxanthin som en biologisk indikator fra Atlanterhavslaks (*Salmo salar*) oppdretts fôr som forårsaker organisk avfall til det omkringliggende økosystemet, med hensyn til forfatterens kunnskap, er dette ikke gjort før. Ved å bruke mål organismer som Blåskjell (*Mytilus edulis*), Whelk (*Buccinum undatum*) og sjøvann, havbunnsedimenter og *in situ* videoundersøkelse. *In vitro* absorbans og HPLC isolering av pigmentekstrakter var de viktigste metodene brukt. Resultatene viser økt Astaxanthin konsentrasjon med økt fiskestørrelse i Atlanterhavslaks muskelvev, ved utført fôringsprogram. Høyt Astaxanthininnhold ble funnet i avføring ved dårlig matutnyttelse. Ingen unormale høye verdier av Astaxanthin ble observert i vannoverflate, Blåskjell og Konksnegl matopptak eller vevsinnhold. Dyreplankton avslører laksens naturlige kilde til Astaxanthin. Havbunnsedimenter viste tilstedeværelse av Astaxanthin, men i lav konsentrasjon (<1,2 μg g^{-1}), som synes å være normal. Det ble observert rask fortynning av organisk avfall, men også tilstedeværelse av *Beggiatoa* sp. avslører lavt oksygenivå på havbunnen og sedimentering av organisk materiale. For fremtidig forskning, er forslaget å måle Astaxanthin konsentrasjoner på steder med mer akkumulert organisk avfall på havbunnen for å konkludere om dette er en god metode for fremtidige overvåkingsprogrammer eller ikke.

1. Introduction

1.1 Organic loading from a fish farm

Organic loading from the aquaculture industry are either food spill from excess feed pellets not eaten by Atlantic salmon, or organic waste released as feces from the fish. This particulate and organic matter sinks rapidly to the seabed and accumulates (Wang et al. 2012). Organic waste released from a fish farm is one of the main challenges facing aquaculture today, and to increase fish production it is important to minimize the impact this have on the environment (Ervik et al. 1997). Integrated multi-trophic aquaculture (IMTA) is one of the solutions that can help mitigate for environmental impacts, using the organic waste as food for other species at lower trophic level placed next to the farm, and thereby also increase the value of fish feed used for production (Wang et al. 2013). To reduce accumulation of organic loadings on the seabed, fish farms are also moved to more exposed areas from enclosed fjord systems (KILDE). To let ocean current and waves dilute emissions over bigger areas.

Aquaculture industry is dependent on good water quality and good environmental conditions on the seabed and in surrounding areas to make good quality seafood (NS 9410:16). To monitor this, it is required from Norwegian laws to do a pilot study before installation of a fish farm. Thereafter simultaneous with production do B-survey of construction zone, defined as the area with the greatest presence of organic matter, normally no more than 25 – 30 m from the installation, and C-survey of the transition zone, defined as the area where smaller particles and resuspended organic material sediments, normally no more than 500 m from the installation. Called a MOM survey, and examine benthic fauna, chemistry in sediments and sensory observations as e.g. smell, color and consistency (Hansen et al. 2001; NS 9410:16). For hard bottom substrates *in situ* video survey is required (NS-EN ISO 19493, 2007).

Big organic loads from a fish farm will change benthic fauna composition towards groups of organisms that is more tolerant to high organic loads and O_2 depletion of seabed sediments (Kutti et al. 2008). This can lead to a decrease in diversity, but an increase in productivity, also known as the paradox of enrichment (Rosenzweig 1971).

Polychaeta for example that are tolerant to high organic loadings are frequently used as indicator organisms for polluted areas (Rygg 2002).

1.2 Pigments

Pigments absorbs light at different wavelengths within photosynthetically active radiation (PAR 400 – 700 nm), and the reflected or transmitted light are seen as the color of the pigment (Sakshaug et al 2009). Primary producers produce pigments to use for photosynthesis, and in the marine environment they can be found in the euphotic zone, either as pelagic plankton, or at the seabed, then called microphytobenthos. Characteristic to photosynthesizing organisms are that all contains Chlorophyll *a*, and can serve as an indication of biomass of primary producers (Sakshaug et al 2009). Chlorophylls include several different pigments, and the degraded forms will here sometimes be called, Chlorophyll and its derivatives.

Photosynthetic pigments can be divided into two major groups, light-harvesting pigments (LHP), or photo protective carotenoids (PPC). LHP includes chlorophylls (Chl) and photosynthetic carotenoids (e.g. Chl *a*, Chl *b* and Chl c_1, c_2, and c_3, Fucoxanthin, Peridinin). PPC includes carotenes (β,β-carotene, β,e-carotene) and Xantophylls (e.g. Zeaxanthin, Diatoxanthin, Lutein) (Brunet et al. 2011; Rodríguez et al. 2006). Phytoplankton can be divided into three major pigment taxa, based on 13 different pigment groups (PG). The first pigment taxa consist of Chl *c*-containing Chromophytes that can be found within PG 1-5 and 10-12. Second pigment taxa are Chl *b*-containing Chlorophytes found within PG 6-9. The third pigment taxa are cyanobacteria in PG 13 (Johnsen and Sakshaug 2007). The PG pigment-specific markers can be used for taxonomic identification of phytoplankton classes and makes it possible to identify presence of different phytoplankton in a mixed sample or a sample with unknown content (Table 1, Johnsen and Sakshaug 2007).

Table 1: Phytoplankton groups with pigment markers, pigment groups (PG) and corresponding classes (Johnsen and Sakshaug 2007).

Phytoplankton class	**Pigment markers**	**PG**	**Phylum**
Bacillariophyceae	Fucoxanthin, Chl c_1, + c_2	1	Chromophyta
Dinophyceae I	Peridinin, Chl c_2	2	
Dinophyceae II	Acyl-ocyl-fucoxanthins, gyroxanthin-diester, Chl c_3	3	
Coccolithophycae	Acyl-ocyl-fucoxanthins, Chl c_3	4	
Pavlovophyceae	Fucoxanthin, Chl c_1, + c_2	5	
Prasinophyceae I	Prasinoxanthin, Mg 3,8 divinyl-phaeoporphyrin a_5 monomethyl	6	Chlorophyta
Prasinophyceae II	Lutein, Chl *b*	7	
Euglenophyceae	Neoxanthin, Chl *b*	8	
Chlorophyceae	Lutein, Chl *b*	9	
Chrysophyceae	Fucoxanthin, Chl c_1, + c_2	10	Chromophyta
Raphidophyceae	Violaxanthin, Chl c_1, + c_2	11	
Cryptophyceae	Phycobiliprotein*, alloxanthin, Chl c_2	12	
Cyanophyceae	Phycobiliproteins, zeaxanthin	13	Cyanobacteria

*Phycobiliprotein will not be examined in this study, due they are water-soluble and will not extract in organic solvent.

Bacillariophyceae and Dinophyceae are hereafter called diatoms and dinoflagellates respectively.

Invertebrates contain many different carotenoids and are the most diverse group of pigments, which appear as color from yellow, to orange and red. Invertebrates can only synthetize pigments from a food source as phytoplankton or other animals, and can use the color directly or transform them to other useful pigment compounds through metabolism. Bivalves (Blue mussel) obtain their pigmentation through phytoplankton carotenoids, and many of them are metabolites with origin from fucoxanthin, alloxanthin, diadinoxanthin and diatoxanthin (Maoka 2011; Banaranayake 2006)

Astaxanthin is the most abundant carotenoid found in many marine organisms such as crustaceans (planktonic zooplankton) and salmonids (e.g. Atlantic salmon), and play an

important role as a powerful antioxidant and are of vital importance. Following Astaxanthin through the food web it is synthesized by Crustaceans that obtains precursor carotenoids (β-Carotene, Zeaxanthin and Lutein) from phytoplankton. Crustaceans are thereafter eaten by e.g. Atlatic salmon, bringing Astaxanthin further into the food web. Astaxanthin is taken up by the salmon and this is due to its red distinct color (Andersson M et al. 2003; Maoka 2011). Farm raised Atlantic salmon does not obtain the color of muscle tissue through Crustaceans, instead Ataxanthin is needed to be added in the fish feed (Tolasa S et al. 2005).

Added pigments in fish feed consist of 15 % of feed pellet costs, and is the most expensive constituent, and consist often of artificial produced Astaxanthin. Feed pellet costs consist thereafter of 50 % of total production costs (KILDE). Concentrations of pigmented muscle tissue are shown as the redness of the fish is due to consumers preferences (Alfnes et al. 2006).

1.3 Aim of this study

The main objective of this study is to elucidate the carotenoid Astaxanthin as a biological tracer from pen raised Atlantic salmon feed causing organic waste to the surrounding ecosystem.

The sub objectives are: A) Use Astaxanthin as a tracer for fish feed (pellets with artificial Astaxanthin) and trace [Astaxanthin] concentration (denoted [ ]) in salmon muscle (feed uptake) and feces (feed loss). B) Trace [Astaxanthin] in target organisms and substrates in the surrounding environment. The target organisms and substrate are: Phytoplankton and zooplankton (water masses), Blue mussel (filter feeder), Whelk (bottom feeder) and seabed sediments. C) *In situ* video survey for visual bottom and water column for biological information and substrate state.

To carry out this, pigment analysis, light microscopy, grab sampling for taxonomic and pigment examination, ROV (Remotely Operated Vehicle) with video camera for in situ monitoring was performed, to provide with detailed and broad information regarding pigmentation and general health state of the surrounding ecosystem. Methods that will be used for pigment analyses are spectrophotometer that is a quick and easy method, in combination with HPLC (High Pressure Liquid Chromatograph) which provides with more

extensive pigment information. The study was performed at the island Frøya in Sør Trøndelag, Norway.

The study will contribute to an enhanced knowledge based management of the Atlantic salmon farming industry for a better nature management and decisions.

2. Materials and Methods

Experimental material was collected at Frøya, an island located outside the Trondheimsfjord, in the middle part of Norway (Figure 1). The collection period was from March to October 2016. Laboratory work was done at Trondheim Biological Station (TBS), Department of Biology, NTNU from March to November 2016.

2.1 Description of location

The area used for pigment monitoring Astaxanthin from salmon feed (pellets, producing organic loads) was the salmon farm Måsøval AS (MA) at location Bukkholmen (Figure 1) located offshore of Sistranda (Frøya) sheltered by the island Inntian.

Lamøvågen (Figure 1), a sheltered bay on the island Inntian, was used for control sampling of Blue mussel and water samples, located 1.3 km northeast of Måsøval. Sampling sites included in this project are shown in figure 1. The map shows surrounding islands, water depths and specific features of the areas nearby. It is reasonable to believe that sampling site Måsøval has stronger water current and a higher exchange of water, when compared to Lamøvågen, because of their location in the surrounding ecosystem, with Måsøval being more exposed and at a greater depth. Lamøvågen is an isolated location, with minimal influence or pollution from humans, with a water depth less than 10 m at sampling location. Hellskjæret (Figure 1) 4.6 km north of the fish farm was used as a control for sediment sampling and *in situ* video survey.

General water current at Måsøval was measured during one week in August 2016, shows a dominant direction towards NNW at water depths of 10 and 22 m, with maximum current speed of ~0.14 and ~0.12 m/s respectively (Klebert et al. unpublished). The location is protected by islands from ocean swell, and water depth at Måsøval is 50-60 m.

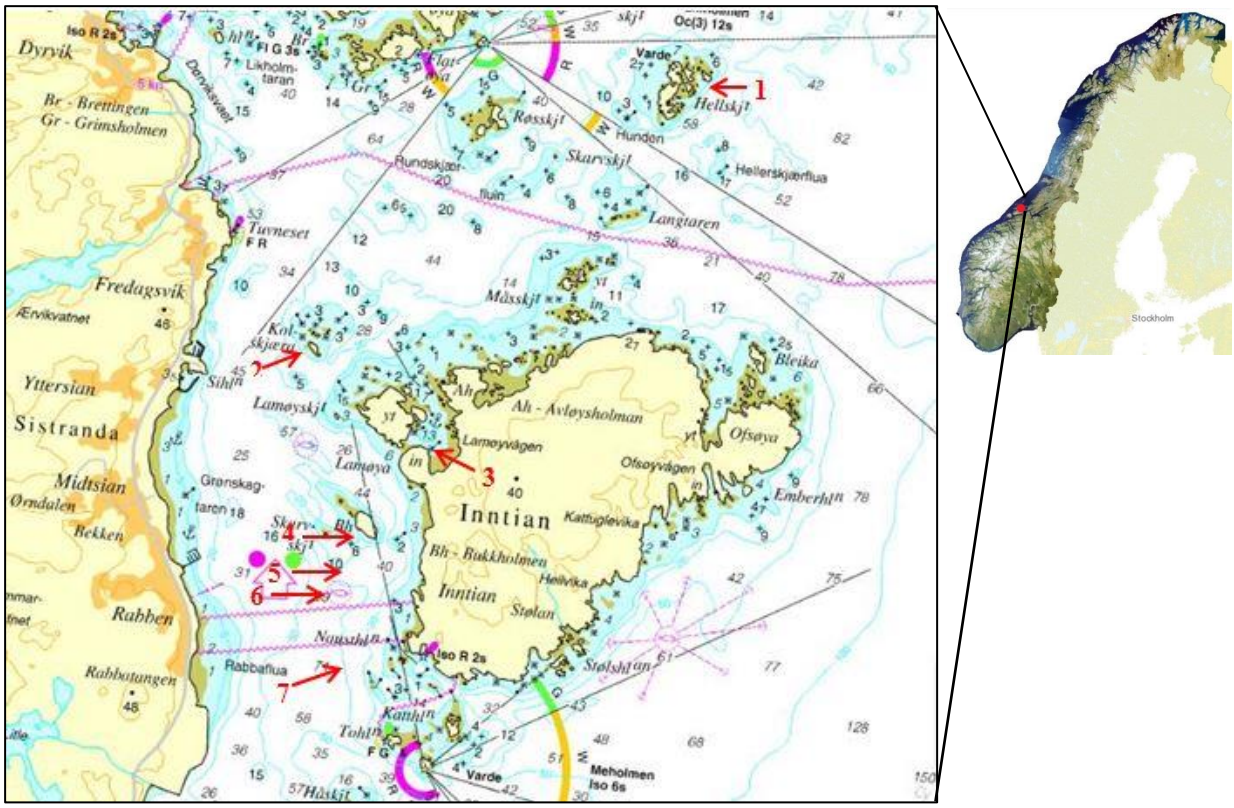

Figure 1: To the right, a map of Norway with the island Frøya marked with a red dot. To the left a map of sampling area located on eastern part of Frøya, offshore of Sistranda. Red arrows show specific sampling locations: Arrow no. 1, Hellskjæret used for control sediment samples and *in situ* video survey. No. 2, Kolskjæra collection of *B. undatum.* No. 3, Lamøyvågen collection of control *M. edulis* and water. No. 4, Bukkholmen collection of *B. undatum*. No. 5, sediment samples. No. 6, Måsøval AS, collection of food pellets, *S. salar*, water sampling, *M. edulis* and *in situ* video survey. No. 7, zooplankton net draw (Source: www.norgeskart.no).

At Måsøval, pen no. 8 was used for collection of fish and fish feed during the whole sampling period. Size of the pen is 43 m in diameter, 27 m deep, with mesh size of 22.5 mm. The pen was equipped with a closed lice skirt reaching 10 m down the water column during whole sampling period. At the start of the production (March 1st 2016), 45 069 kg of Atlantic salmon smolt was put in the pen, and feeding started the following day. At the end of this experiment (October 3rd 2016), the biomass was 201 419 kg. Expected slaughtering is scheduled at beginning of May 2017. Before this production cycle, the fish farm was lying fallow in 3 months, and the location has been used for pen raised salmon approximately since 1998.

Within a few days after feeding start of the fish (March 8th 2016), 150 Blue mussels was collected at the control site (Lamøvågen) and divided into three crab pots with covered entrances. One used for sampling, and two as backup. The pots were placed at Måsøval, attached to three different buoys 31.5 m from pen no. 8, north to northwest (Figure 2). Collected material and approximate placing of each sampled material are shown in Figure 2.

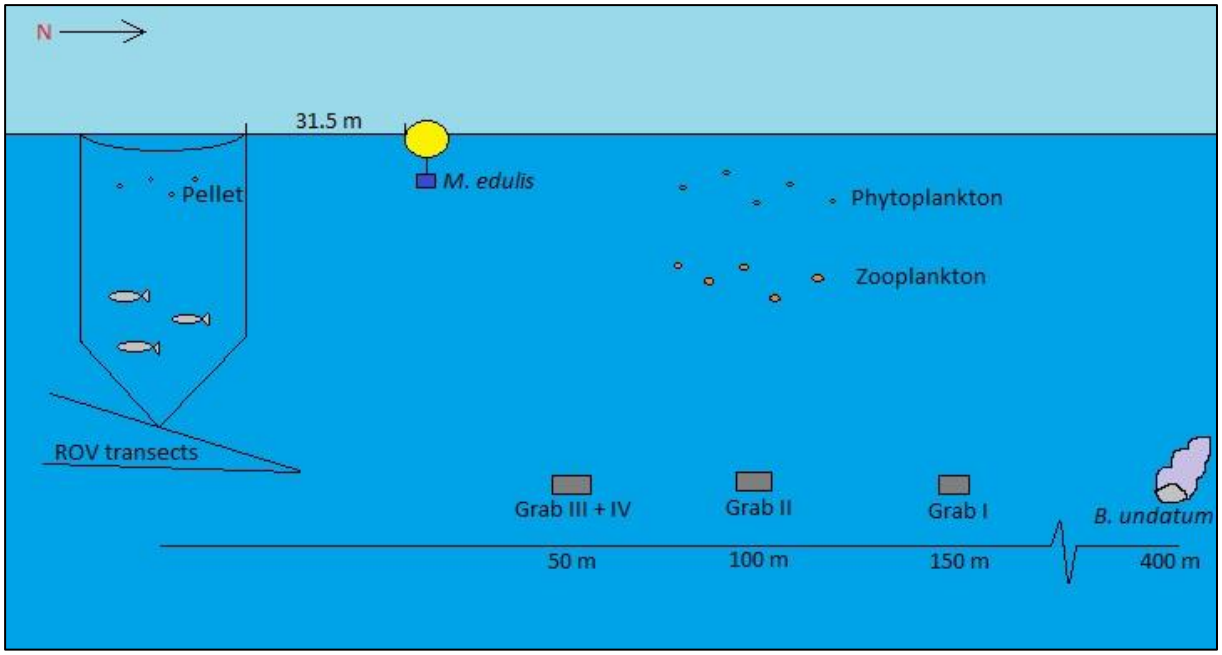

Figure 2: Overview of collected material at Måsøval. Atlantic salmon in pen no. 8, ROV transects with video recording under the pen. Location of grab samples, collection of Blue mussel (*Mytilus edulis*) and Whelk (*Buccinum undatum*). Direction of north is indicated (Image: Kamilla Sporsheim).

2.3 Collection of experimental material

Collection of fish was performed eight times, once a month from March through October 2016, for the rest of the material as often as possible within that period (approximately once a month). Three individuals of Atlantic salmon from pen no. 8 were collected each month. Feed pellets where collected 3 times during the sampling period, one at the beginning (March, Serial no: 1303469, Biomar AS), one halfway (June, 7304760), and one at the end (October, 1304170), and containing 40, 50 and 50 µg g^{-1} Astaxanthin respectively. For each sampling day, 7 l of surface water for phytoplankton examination was collected in dark bottles from each sample site, Måsøval and Lamøvågen (controls). Water samples were further used for spectrophotometry of pigment extracts, HPLC analyses (of pigments, including Astaxanthin)

and microscopy of phytoplankton. Zooplankton was collected using a net with a mesh size of 500µm, from 60m depth and up to the surface (vertical haul). Three individuals of Blue mussel were collected at Måsøval and three individuals from the control site. Three individuals of Whelk were caught in the wild, with custom-made pots, as often as possible during the sampling period. They were caught next to Kolskjæra, 1.7 km north of Måsøval, Bukkholmen, 300-400 m north of Måsøval or Hammerbergskjæret, located 2 km south of Måsøval (Figure 1). Sampled material of Atlantic salmon, feed pellets, Blue mussel, Whelk and sediment was put in a freezer at -20ºC immediately after collection until further work.

On the last sampling day (October 5th 2016), sediment and benthic fauna samples were collected with the use of a Van Veen Grab from the vessel R/V Gunnerus (NTNU). Grab samples were taken from four locations next to salmon pen no. 8 (Figure 3). One sample was taken at a distance of 150 m, one at 100 m, and two at 50 m from the pen, at depths of 17 m, 17 m, 19 m and 19 m respectively. Depth gradients and bottom topography at Måsøval are shown in Figure 3.

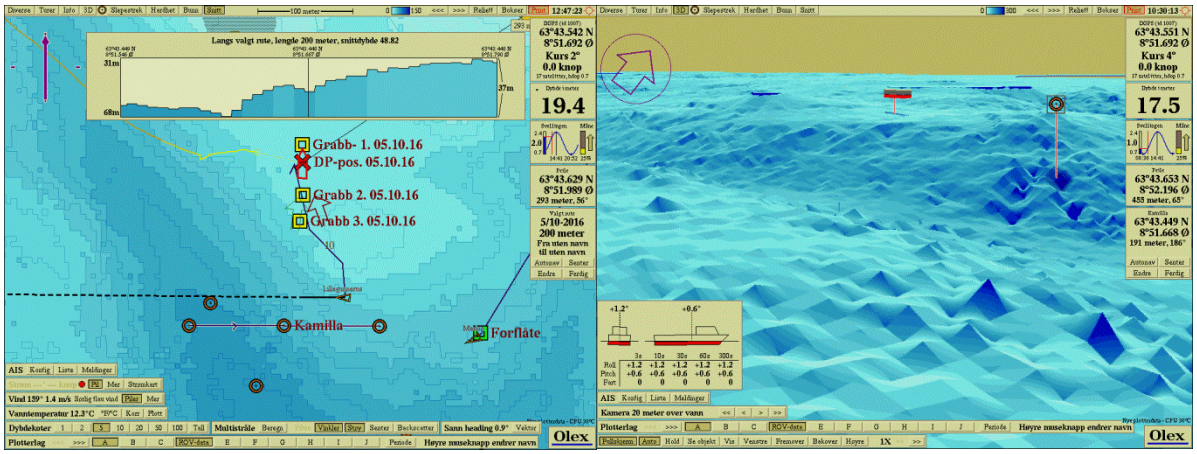

Figure 3: Olex maps showing Måsøval AS, location Bukkholmen at Frøya. Left: Round circles shows the different salmon pens. Pen named Kamilla is pen no. 8. Floating stage to the right, and yellow squares showing positions for grab samples operated from R/V Gunnerus. Grab I, 150 m from pen, grab II, 100 m from pen and grab III+IV, 50 m from pen performed twice on slightly different spots. Arrow in upper left corner indicates north. Right: Shows bottom topography, pen no. 8 marked as a circle, and position to R/V Gunnerus.

Controls for grab sampling and *in situ* video survey where taken twice next to Hellskjæret at 43 m depths (Figure 4). Depth gradients and bottom topography are shown in Figure 4. Species identification of benthic fauna from sediment samples, 150 m (Grab I), 100 m (Grab II), Control I and Control II was performed during a NTNU course in Marine Biodiversity (BI 2036). A small scoop (ca. 50 g) of each sediment sample was immediately after sampling put in freezer at -18ºC until spectrophotometry and HPLC analyses of pigment extract (1 month later).

After the sediment sampling, a SeaBotix ROV (Remotely Operated Vehicle, Teledyne LBV200 HD, USA) was used for an *in situ* video survey, operated from a workboat (Polar Circle). Two cameras were mounted on the ROV, one filming in high-definition (680 line High resolution color camera – 0.1 Lux), and one standard-definition (lower resolution) with depth measurements. The ROV was equipped with light sources (700 Lumen 270Âº LED), and driven beneath pen no. 8, right above seabed at ~57 m depth, and next to Hellskjæret at ~40 m depth (Figure 4). Two different transects at each location were carried out. The video recording was running from surface, along the sea bed and up to the surface again. Information about particle concentration, pelagic organisms in the water column, health state of the seabed, benthic organisms and accumulated organic matter on the seabed was noted.

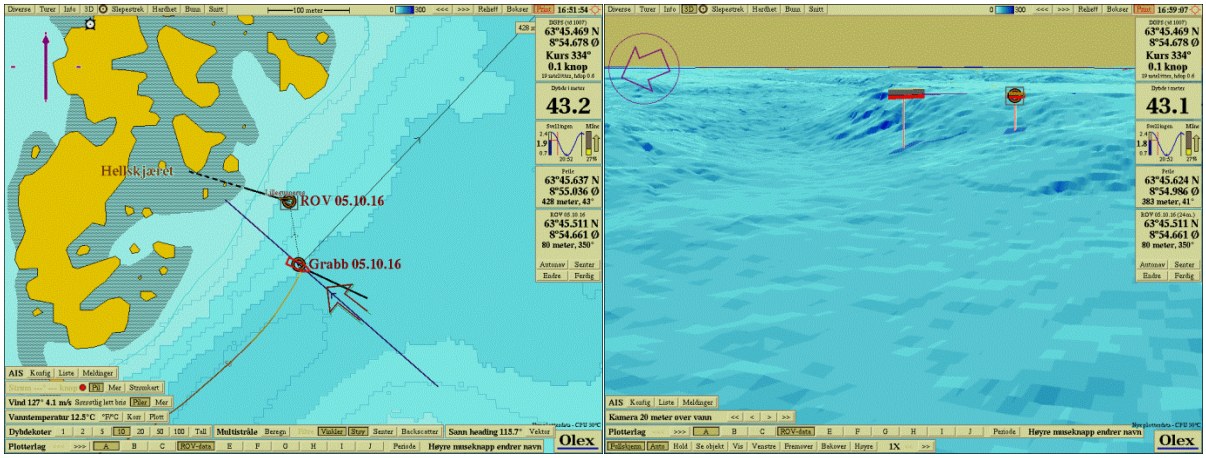

Figure 4: Olex maps showing Hellskjæret used for control grab sampling, and control *in situ* ROV survey, October 5th 2016. Left: Upper round circle show position of workboat operating the ROV. "Grabb 05.10.16" indicates the position for R/V Gunnerus during grab sampling. Two grab samples were taken at 43 m depth. Arrow in upper left corner indicates north. Right: Shows bottom topography, workboat operating the ROV (circle) and position to R/V Gunnerus (rectangle).

2.4 Preparation of samples for further analyses

Equipment for pigment analyses was washed laboratory soap, rinsed well afterwards with hot water, and washed with methanol before the experiment. Preparation of all tissue samples was done as cold as possible (ca. 4ºC), to prevent pigment degradation. For each species three individuals from the same sample day was used as replicates (n=3). All prepared samples stored in a -20ºC freezer until further measurement.

Atlantic salmon was measured for length and weight and pictures were taken of each whole individual. A scalpel was used to cut out the last 3 cm of the hind gut, feces was scraped off, wrapped in aluminum foil, marked and put in a -20ºC freezer, until pigment analyses (max 2 month). In front of the dorsal fin a 3 x 3 cm, 0.5 cm thick piece of skin and muscle tissue was cut out with a scalpel. Picture was taken of muscle tissue after melted, on an opaque white plastic plate. Approximately 1 g muscle tissue under the removed part was put in a -20ºC freezer, until further pigment analyses. Feed pellets were soaked in distilled water to get its wet weight.

Water samples were filtrated within 1 hour after collection with a Whatman GF/F glass fiber (Whatman Inc, USA, 25 mm diameter) filter with mesh size of 0.4 µm, by the use of a small filtrate setup, operated with a pressure pump. Three replicates from each samples was filtrated on each filter until coloration of the filter, normally 1500 – 2000 mL seawater was filtered. Zooplankton collected from the zooplankton net, was studied in a stereomicroscope (Leica EZ4, Germany, 8-35X magnification) within the same day of collection (kept alive and stored cold), and dominant taxa were noted. Water was removed by the use of a coffee filter, and put in a -20ºC freezer divided in three replicates for each sampling day.

Blue mussel was opened with the use of a knife. One half was put aside to melt, and pictures were taken of the mantle. Stomach and mantle was removed from other half for further analyses. A piece of Whelk foot was cut off and frozen. Shell put aside to melt, and cracked to find the stomach that were immediately put into a -20ºC freezer until further analyses. Sediment samples were divided in three different replicates from each sample, and kept frozen until further analyses.

The plan was to carry out HPLC of all samples, due to high fat content in pellet, salmon muscle tissue and feces, Blue mussel mantle and stomach, Whelk foot and stomach and zooplankton, this could not be performed due to risk of clogging HPLC column. The different samples prepared and experimental method used for each samples are shown in Table 1.

Table 1: Overview of collected material, further preparation of samples or tissue used, and experimental method used for each sample.

Material		**Sample**	**Method**			
			Spectro-photometer	**HPLC**	**Micro scopy**	**Pictures**
Pellet		Added water → ww	x			x
Pellet feeder	*S.salar*	Muscle tissue	x			x
		Feces	x			
Water samples	Phytoplankton	Filters + Lugol fixed	x	x	x	
	Zooplankton	Filtrated	x			
Filter feeder	*M. edulis*	Mantle	x			x
		Stomach	x			
Benthic feeder	*B. undatum*	Foot	x			
		Stomach	x			
Sediment		Removed water	x	x		
In situ video		Framegrab pictures				x

2.5 Measurement of bio-optical characteristics

***In vitro* pigment absorbance:**

A Unicam UV 500 spectrophotometer together with Thermo Vision PRO software (Thermo Fisher Scientific, USA) was used. Measuring spectral absorbance, also called optical density (OD, dimensionless) of a pigment extract at wavelengths between 350 and 800 nm, where used to detect pigment signatures (absorbance maxima or minimum), according to the method of Mitchell and Kiefer (1988). For all samples the organic solvent 100 % Methanol (MeOH) was used to extract pigments from tissue, kept in 4 ºC fridge, to prevent pigment degradation.

Samples from feed pellets, Atlantic salmon muscle tissue and feces, Astaxanthin standard, filtrated sea water containing phytoplankton, zooplankton, Blue mussel stomach and mantle,

Whelk stomach and foot, and sediment samples were extracted to measure *in vitro* absorbance.

Before extraction of pigments, pellets, all tissue samples, zooplankton and sediment samples were weighted with a Sauter AR 1014 weight with ±0.001 g accuracy before extraction in 5 – 10 mL MeOH (depending of tissue and pigment concentration). Sample and MeOH were grinded together with the use of a mortar, put in test tubes sealed with a cap, and stored 12 - 24 h in a dark -18 ºC freezer for pigment extraction. Test tubes were shaken several times within extraction period, to ensure good extraction of containing pigments. The following day extracted materials were filtrated through a 0.45µm filter attached to a syringe, to prevent debris and corresponding light scattering during spectrophotometer measurements. Extracts were put in a 1 cm cuvette that was placed in the spectrophotometer. The cuvette was rinsed with MeOH before every new measurement. Whatman GF/F extracts was first measured with HPLC, thereafter 1 mL MeOH added to have enough solution for the spectrophotometer measurements. Astaxanthin standard were measured separately, and together with extracts of Atlantic salmon muscle tissue, measured once, with one replicate. A blank sample with MeOH was measured for every 10-20 samples. Pigment signatures were studied with use of specific absorbance of chlorophylls, xantophylls and carotenes (Roy et al. 2011, see appendices).

***In vitro* pigment isolation:**

In vitro HPLC (High Pressure Liquid Chromatograph) measurements where performed using a Hewlett-Packard 1100 series HPLC system that isolates, identifies and quantifies pigment content in a solution from 350 to 700 nm, according to the method of Rodríguez et al. (2006). The instrument was equipped with a diode array detector, an HPLC column: from Waters Symmetry C8 (3.5µm, 4.6 x 150 mm, WAT200630), and eluents according to Zapata et al. (2000).

Astaxanthin standard, filtrated seawater on Whatman GF/F filters and sediment samples were prepared for *in vitro* HPLC measurements. Weighted, extracted and stored as the same procedure as for absorbance measurements. Filters were added 1.6 mL MeOH, the following day the filter samples were mixed with a Vortex mixer. HPLC pigment extracts were filtrated

using a 0.2µm filter attached to a syringe. Samples were then ready to put in the HPLC for measurements. The HPLC software Hewlett Packard "Chem32" was used to control the HPLC, obtain chromatograms and spectral signatures of pigments to obtain quantitative and qualitative information of chlorophylls, xanthophylls and carotenoids (Rodríguez et al. 2006, Roy et al. 2011). Measurements were taken from 440 nm readings of the chromatograms, since all pigments absorbs light at this wavelength (Roy et al. 2011, see appendices for chlorohylls, xanthophylls and carotenoids). The pigment separation as a function of time is related to polarity of a given pigment where the most polar compound is separated first. Pigment peaks with retention time where compared with a pigment library of standards (isolated by Kjersti Andresen, TBS NTNU) according to Rodríguez et al. (2006) using international HPLC "method II" (Roy et al. 2011).

2.5.1 Calculations and data analysis

***In vitro* pigment absorbance:**

Results obtained from spectrophotometer, the absorbance (OD) of a solution were corrected for a blank sample with MeOH, to subtract the extraction medium form the sample. Thereafter the average from 750 to 800 nm was subtracted, adjusting for light scattering. All samples were corrected for its dilution factor, mL MeOH used for extraction divided on grams wet weight of sample. Average values was calculated from three replicates (n=3), and standard deviation (±SD) calculated. From this the coefficient of variation (±CV) was calculated of average value to compare data sets, by using Equation 1.

$$\text{CV}\ (\pm\%) = \frac{\sigma}{\bar{x}} \times 100 \qquad (1)$$

Where σ is standard deviation and $\bar{x}$ is the average value from the replicates.

Pigment concentration: was calculated for Chlorophyll *a* in phytoplankton and Astaxanthin in fish feed, Atlantic salmon muscle tissue and feces, obtained from absorbance values, using Equation 2.

$$[\text{Pigment}](\mu g L^{-1}) = \frac{\left(OD(\lambda_{max}) - OD(\lambda_{min})\right) \times\ mL\ MeOH\ extraction}{abs.coeff \times\ L\ sea\ water\ filtrated} \times\ 1000 \qquad (2)$$

Chlorophyll *a* OD λ_{max} are absorbance at 665 nm (Chlorophyll *a* red peak), Astaxanthin OD λ_{max} are absorbance at 474 nm. OD λ_{min}, are absorbance at 750 nm.

74.5 g L^{-1} cm^{-1} is the absorption coefficient (abs. coeff) for 1 g pure Chlorophyll *a* dissolved in 1 L MeOH at 665 nm. Astaxanthin absorption coefficient is 206 g L^{-1} cm^{-1} (MacKinney 1941; Roy et al. 2011).

***In vitro* pigment isolation:**

Peaks from the HPLC-isolated pigments shown in chromatograms at 440 nm were integrated, and the integrated areal from each different pigment signature was used in Equation 3, to get the quantitative amount of each pigments per grams in the sample according to Rodríguez et al. (2006):

$$\frac{\mu g\, pigment}{g_{wet\ weight}} = \frac{area_{\lambda} \times\ Rsf_{\lambda}\ \times V_e}{V_i\ \times\ g_{tissue}} \qquad (3)$$

Where $area_{\lambda,}$ is the integrated area under each curve (area) in in the chromatograms at 440 nm, Rsf_{λ} is the response factor (ng/area) at 440 nm calculated from pigment standards. V_e is extraction volume in mL, and V_i is injection volume in µL to the HPLC.

2.6 Microscopy of water samples

Subsamples from collected surface water at Måsøval and Lamøvågen where put in 300 mL glass bottles, and fixed with 1% neutral Lugol, within one hour after sampling. This was done every sampling day, and used further for microscopy of phytoplankton species, to supplement with taxonomic information to the pigment analysis. Preparation of microscopy samples was done with the Utermöhl sedimentation cell counting technique (Utermöhl 1958). Sedimentation chambers were used to sediment microscopic particles for 24 h. Each sample was going through systematic with magnification of 200X, then the same sample was repeated at 400X for smaller taxa, with the use of a Leica DM IRB light microscope (Leica Microsystems Wetzlar GmbH, Germany). Microscopic organisms were counted and identified. Detected to genus if possible, otherwise the lowest level of taxonomic level identified.

3. Results

Major findings from pigment monitoring of Astaxanthin as a biomarker from organic loads from a salmon farm in surrounding ecosystem is described below or when appropriate visualized with figures, tables or pictures. Qualitative and quantitative pigment information, based on absorbance measurement (pigment extracts) and High Pressure Liquid Chromatograph (HPLC, isolated pigments) are shown to provide, as good as possible, information of Astaxanthin as a biomarker. To make it easier to see if Astaxanthin are present in a pigment extract, all absorbance figures in this section show the absorbance signature of pigment extracts with Astaxanthin standard, for comparison.

3.1 Farm raised Atlantic salmon

To be able to trace Astaxanthin as a biomarker, the source of the pigment in the fish feed will first be presented. Then follow the same path that Astaxanthin is taking in chronological order from fish feed to the salmon muscle tissue or feces and out in surrounding ecosystem using phytoplankton, zooplankton, Blue mussel and Whelk as target organisms.

3.1.1 Feed pellet

Pigment composition given to Atlantic salmon through feed pellets are shown as *in vitro* pigment absorbance in wet weight (Figure 5), from a selection of three different types of feed given to the fish in pen no. 8 within the sampling period. Feed PW1 (Pellet Wet weight, type 1) given in March, PW2 in June, and PW3 in October. The different types of pellets contained 40, 50 and 50 µg g^{-1} Astaxanthin respectively. From readings of the graph there is a small variation in pigment concentration. The shapes of the absorption spectra of extracts from feed pellets are the same, indicating the same pigment composition (Figure 5). Common spectral characteristic is a shoulder at 420 nm, and two distinct peaks at 449 and 471 nm, respectively. The Astaxanthin standard was characterized with one absorbance peak at 474 nm, showing Astaxanthin is present in the fish feed.

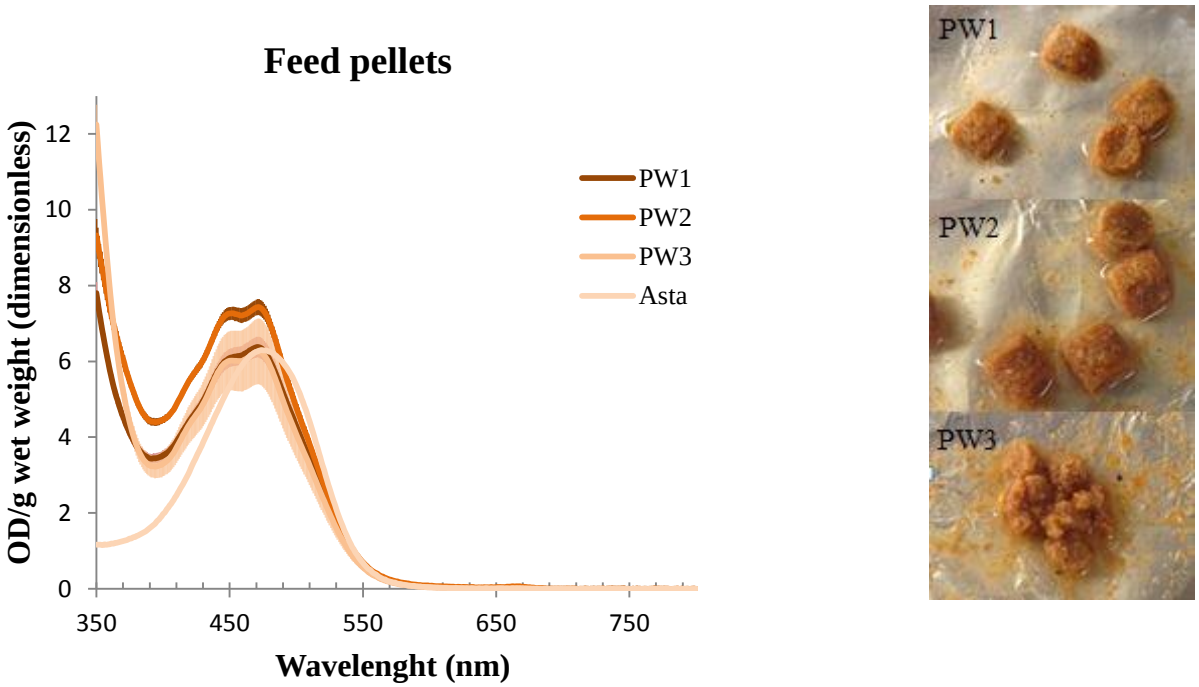

Figure 5: Left: *In vitro* absorbance spectra ((OD (λ)) of feed pellets containing Astaxanthin g^{-1} wet weight (n=3). PW1 fed in March, PW2 in June and PW3 in October 2016. ±SD is shown as shaded areas for each colored line. For size fractions of pellet (PW1-3), see material and methods. **Right:** Pictures of soaked feed pellets with distilled water, PW1, PW2 and PW3. For PW3, only one pellet is shown.

Calculated Astaxanthin concentration [Asta], of feed pellets (wet weight) was done with the use of the absorbance measurements and Asta's specific absorption coefficient to compare with the given value from Biamar AS. Pellet samples (n=3) analyzed for [Asta] in this project showed lower pigment concentrations: PW1 contained 30.96±1.30 (SD), PW2 36.07±1.02 and PW3 30.42±4.20 µg [Asta] g^{-1} wet weight, respectively. This indicates an underestimation of [Asta], especially for PW3 (see discussion).

3.1.3 Astaxanthin standard

An Astaxanthin pigment standard (Asta std.) was prepared for absorbance measurements in spectrophotometer (pigment extracts) and corresponding isolation of pigments using HPLC. This was done to identify the *in vitro* absorbance (OD (λ)) characteristics of Astaxanthin. The Asta std. absorbance spectra were compared with Atlantic salmon muscle tissue pigment extract, and one sample with a mixture of them both (Figure 6) shows all the same absorbance maximum peak at 474 nm, this verifies that it is Astaxanthin in Atlantic salmon extract.

Atlantic salmon has also a small peak at 415 nm as shown in Figure 7A. For Asta std. measured with HPLC see section 3.2.5 Figure 24.

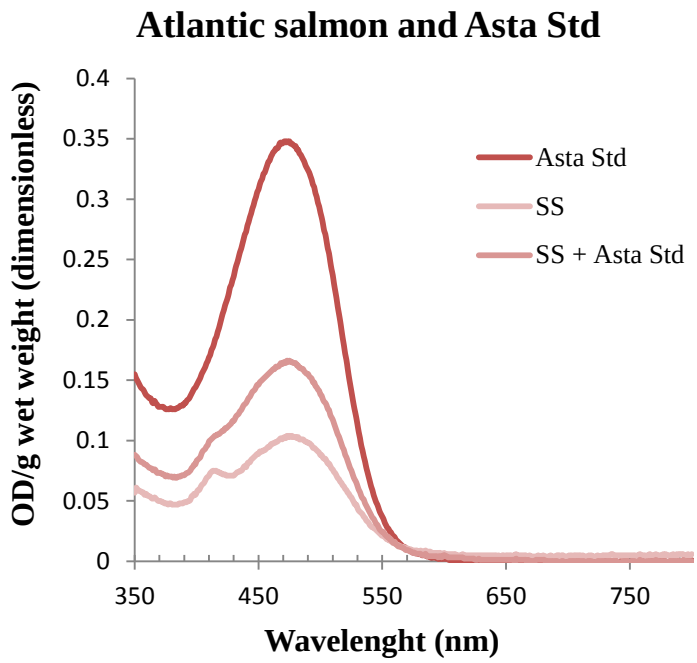

Figure 6: *In vitro* absorbance spectra ((OD (λ)) of Astaxanthin g^{-1} wet weight. Dark red line shows Astaxanthin standard (Asta Std.), light pink line Atlantic salmon muscle tissue (SS) and pink line SS and Asta Std**.** together.

3.1.2 Atlantic salmon

In vitro absorbance (OD (λ)) of pigment extracts from Atlantic salmon muscle tissue (Figure 7A) indicated efficiency in food uptake (using Astaxanthin as a tracer), and non-digested food in feces (Astaxanthin) (Figure 7B) from March to October 2016. Muscle tissue showed absorbance maxima at 409-416 nm, a small shoulder at 449 nm and a major absorbance maximum at 473±3 nm (Figure 7A). Muscle tissue from March, appeared white by eye, indicate low concentration of Astaxanthin, and verified with no absorbance of pigments. Absorption (491-476 nm) of Astaxanthin in pigment extracts from muscle tissue increased as a function of time, i.e. increase in coloration of tissue with age of fish (Figure 8).

Indication of non-digested food in pigment extracts from Atlantic salmon feces sampled in March obtained no absorbance maxima peak indicating low Astaxanthin content (Figure 7B). Feces from April, in contrast, obtained the highest pigments absorbance value, which means

high Astaxanthin content in feces, and an indication of over feeding, together with high pigment content in extracts from May, June and July. Low absorbance from pigment extracts from August to October indicated that most of the feed had been utilized in the gut. All absorbance signatures, except Mars and September, had distinct absorbance maximum peaks at 446±1 nm, and 468±2 nm, and smaller maximum peaks at 400 nm and 414 nm. Feces from September have a distinct maximum absorbance peak at 400 nm and two less distinct at 446 and 470 nm. Both figures show a high ±SD which means a high biological variation between the replicates.

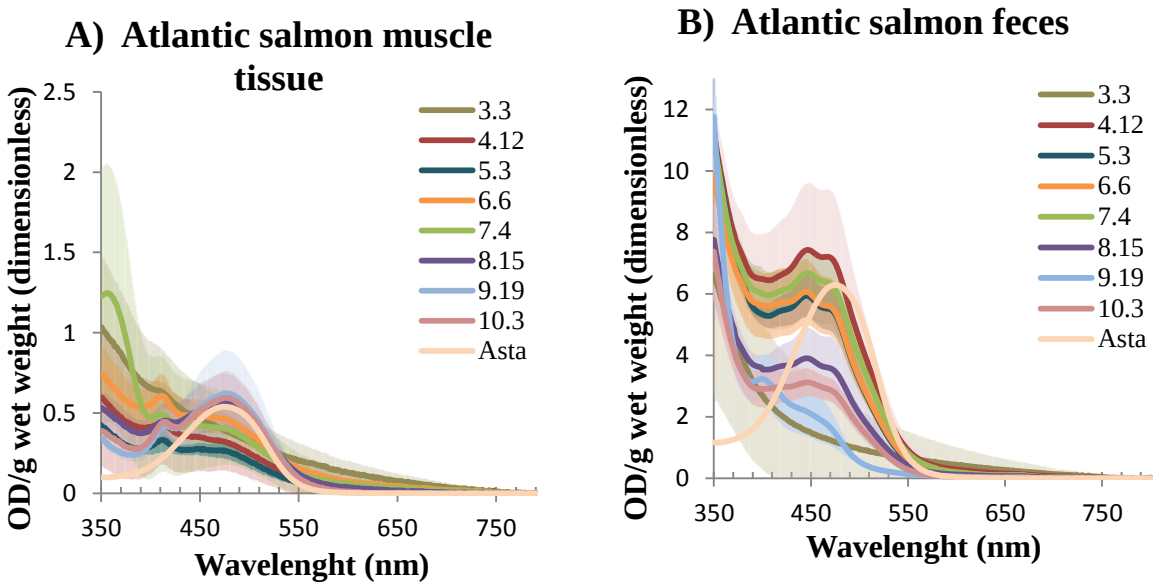

Figure 7: *In vitro* absorbance spectra (OD (λ)) of Atlantic salmon **A)** muscle tissue containing Astaxanthin and **B)** feces containing Astaxanthin, g^{-1} wet weight (n=3) from March to October. Dates of collected samples are shown to the right of the graphs with corresponding color of the line, and ±SD is shown as shaded areas. Note different y-axis.

Monthly change in coloration of Atlantic salmon muscle tissue from March to October, are shown in Figure 8. One replicate from each month are shown, and chosen as the representative average between three replicates. All replicates from all sample days can be found in Appendix 2. The pictures make it easier to see the increase in coloration of muscle tissue with time, and are in agreement with absorption spectra of pigment extracts in Figure 7A.

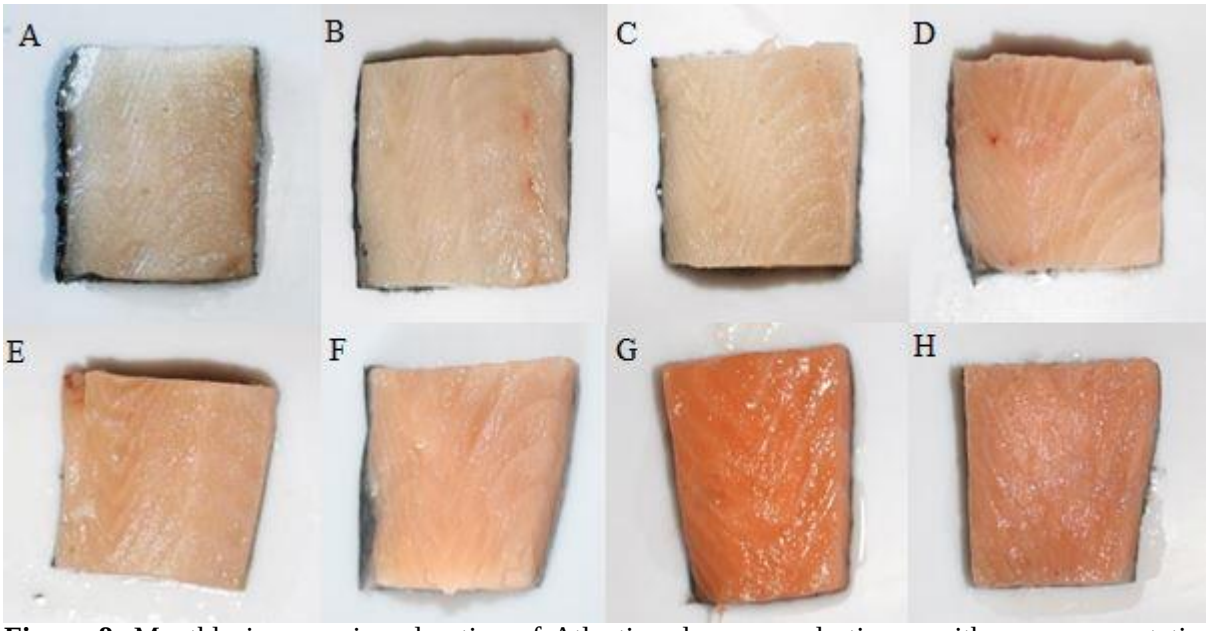

Figure 8: Monthly increase in coloration of Atlantic salmon muscle tissue, with one representative replicate from March to October. **A:** March, **B:** April, **C:** May, **D:** June, **E:** July, **F:** August, **G:** September, **H:** October. All pictures have the same opaque white plastc plate as background to make it easier to compare colors (Photo: Kamilla Sporsheim).

Calculated [Asta] in Atlantic salmon muscle tissue, obtained from absorption peak (Figure 7A) are shown in Figure 9. Each measurement is from the same spot of the fish as the tissue samples in Figure 8, and makes therefore the pictures and [Asta] comparable. Calculated [Asta] as a function of whole fish weight (kg) (Figure 9A), and fish length (cm) (Figure 9B). The trend line between the samples makes it easier to see that the two graphs have similar shapes, and that [Asta] is generally increasing with both weight and length of whole fish.

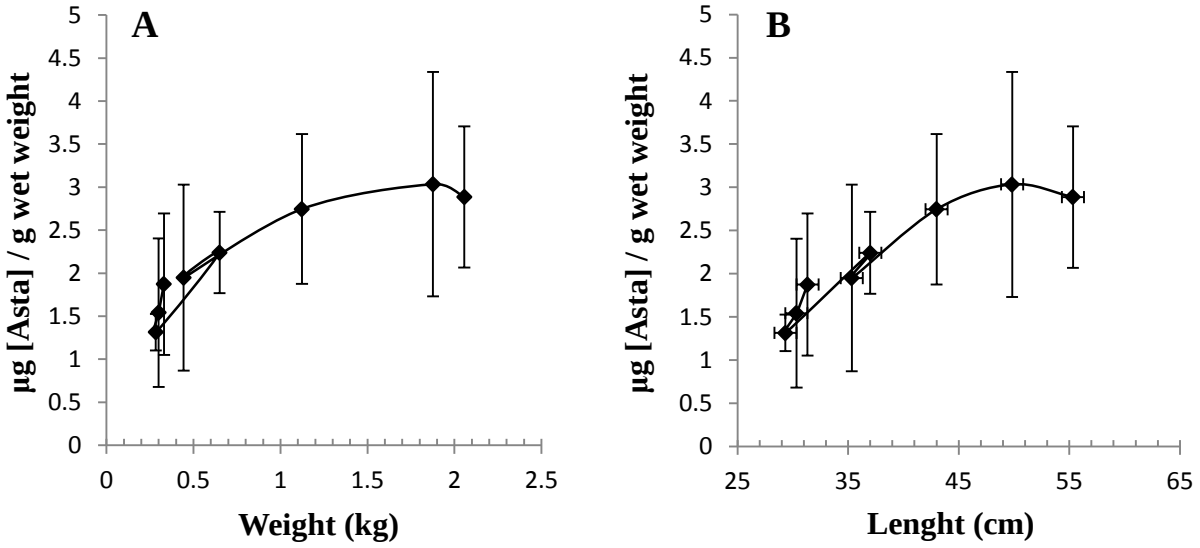

Figure 9: Concentration of Astaxanthin ([Asta]) in Atlantic salmon muscle tissue (µg g^{-1} wet weight) as a function of **A)** weight in kilograms, and **B)** length in centimeters of whole fish from March to October. Each sample day are marked with a dot, with a trend line between data points. Each data point comprises average values (n=3) of [Asta] and average values (n=3) of weight/length from the replicates, and ±SD shown as error bars, calculated from [Asta] only.

Calculated [Asta] in feces of Atlantic salmon, obtained from absorption peak (Figure 7B) are shown in Figure 10. Samples from March 3rd and September 19th contains small amount of [Asta] compared to the other samples. April 12th, May 3rd, June 6th and July 4th contained the highest [Asta] values, indicating higher amount of undigested fish feed. When comparing Figure 10 with Figure 7B they share clear similar patterns in pigment concentration.

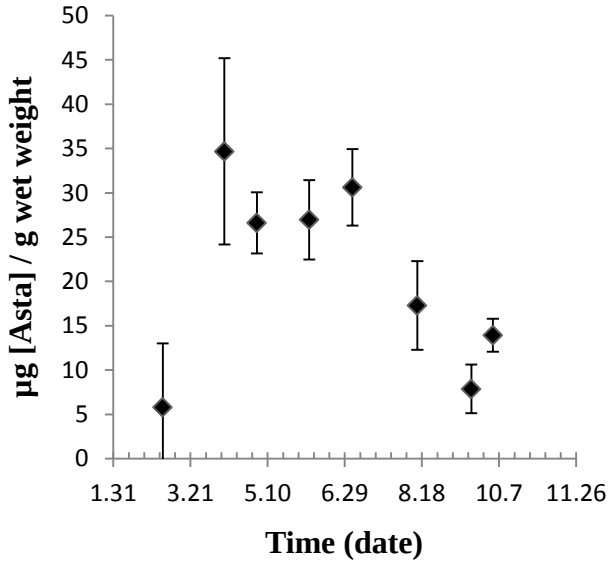

Figure 10: Calculated Astaxanthin concentration [Asta] in Atlantic salmon feces (µg g^{-1} wet weight) as a function of time in dates of sampling. Each data point comprises average values (n=3) and ±SD shown as error bars. Feeding started March 2nd 2016, and sampling dates for feces was March 3rd, April 12th, May 3rd, June 6th, July 4th, August 15th, September 19th and October 3rd.

3.2 Surrounding habitat of the salmon farm

The presence of Astaxanthin was used as a biomarker for fish feed (feed pellets) in surrounding habitat, e.g. in natural occurring phytoplankton and zooplankton, Blue mussel (filter feeder), Whelk living on the seabed feeding on settled organic matter, and seabed sediments was used to investigate the presence of Astxanthin with its origin from Atlantic salmon fish feed. *In situ* video survey was performed to get a better overview, and a picture of how it looks like below the fish farm.

3.2.1 Phytoplankton

To obtain background information of what is present in the water surface, and its pigment composition, *in vitro* absorbance (OD (λ)) of pigment extracts from filtrated seawater containing phytoplankton was measured. Collected at the fish farm (Figure 11A), and control site (Figure 11B), from April to October. All absorbance spectra shows a shoulder at 419 nm, a maximum peak variating between 434 and 439 nm, a shoulder at 475 nm, and three small but not distinct peaks at 585 nm, 615 nm and 638 nm. All spectra have a clear maximum peak at 665 nm, except one with the peak at 666 nm. This indicates the signatures of chlorophylls and the small shoulder indicates carotenoids.

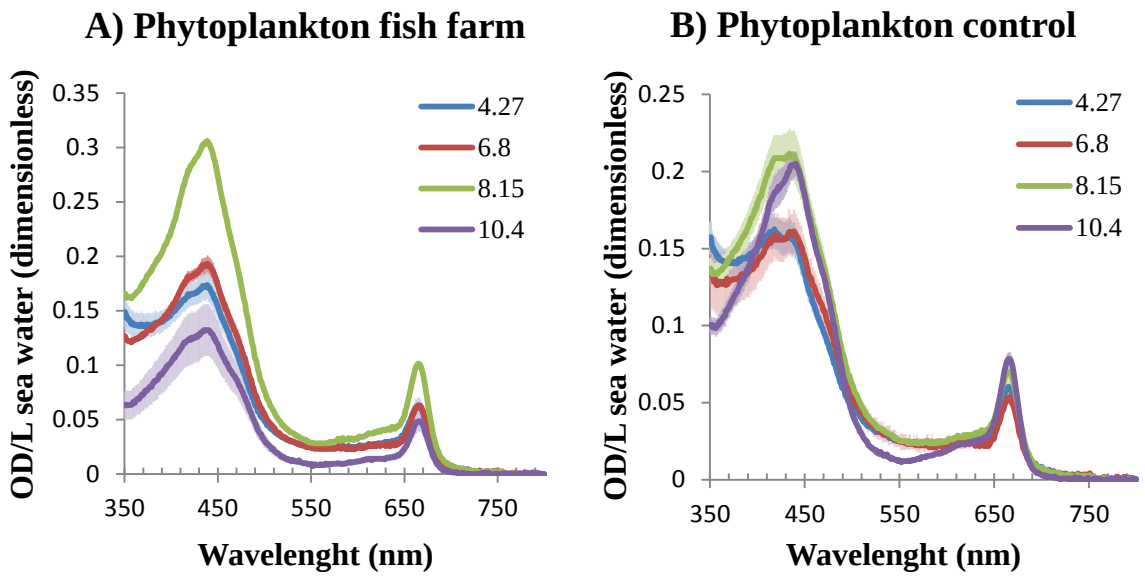

Figure 11: *In vitro* absorbance spectra (OD (λ)) of phytoplankton containing chlorophylls, and smaller amount of carotenoids L^{-1} filtrated sea water (n=3) from **A)** fish farm, and **B)** control site, from April to October. Dates of collected samples are shown to the right of the graphs with correspondingly color of the line, and ± SD is shown as shaded areas. Note different y-axis.

HPLC isolation of phytoplankton pigment markers were also done from the filtrated sea water extracts (Figure 12 and 13) to estimate the relative distribution of each phytoplankton class. A big variety of pigment groups are present in the samples, and this indicates different groups of phytoplankton present (see introduction). Chlorophyll *a* includes all photosynthetic algae. Phytoplankton present on the basis of marker pigments are dominated by Chromophyta (Fucoxanthin and Chlorophyll c_1 + c_2), then followed by Chlorophyta (Chlorophyll *b*). The main phytoplankton groups that can be detected due to their pigment markers are dinoflagellates (Dinophyceae I, Peridinin), Prymnesiophyceae (Coccolithophyceae, Chlorophyll c_3) and Prasinophyceae (Prasinoxanthin, Lutein).

Water samples from the fish farm (Figure 12), shows a peak in pigment concentration at August 15th, and a smaller peak at March 31st. Water samples from the control site (Figure 13), shows a peak at October 4th and a smaller peak at March 31st. There are small differences between different pigments present and concentrations at the two sites. Main differences are that the fish farm has a high Fucoxanthin concentration for March 31st, more Peridinin present

and an overall slightly higher pigment content. The control site contain Lutein, and that is absent for the fish farm.

Chlorophyll a was ranging between 0.14 and 0.94 µg L^{-1} for both sites. Little degraded chlorophyll was present, indicating healthy and happy phytoplankton cells.

When comparing HPLC isolation (Figure 12+13), with absorbance spectra (OD) (Figure 11), they show clear similarities in time of peak pigment concentration and in pigment content, as clear chlorophyll signature peaks, and carotenoids shoulders. Results from HPLC measurements helps distinguish between the different pigment groups that Figure 11 shows all combined.

Coefficient of variation in percentage (±CV %) of HPLC isolated pigments (n=3), is the ratio of the standard deviation (±SD) to the average, and are shown in Appendix 3. ±CV % is between 0.53 % and 173.21 % for fish farm samples and control samples. The reason for the high values is due to absence of pigment in one or two of the replicates and ±SD are higher than the average pigment content of the samples. Samples with pigment content in all three replicates were not more than 32.84 %, and relatively low ±CV, with most of the samples below 16 %.

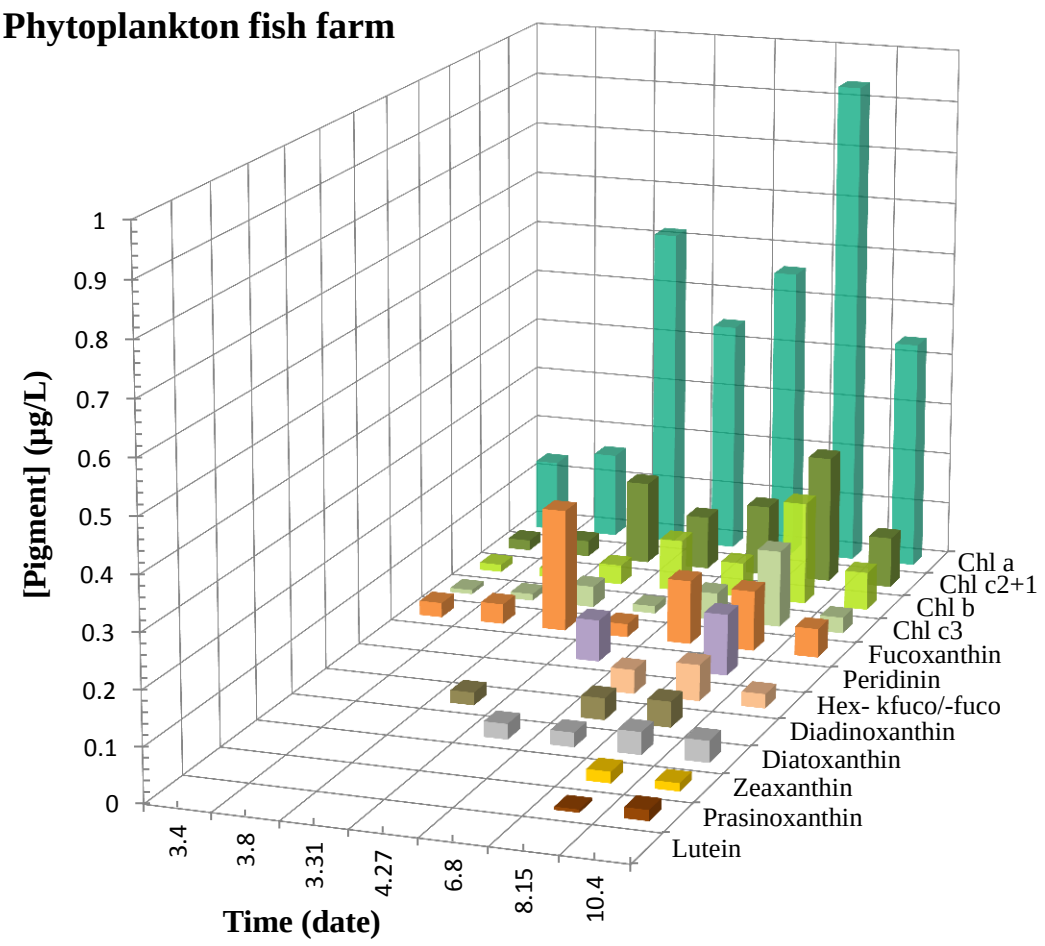

Figure 12: HPLC isolated pigments from phytoplankton extracts obtained from filtrated sea water that is identified and quantified and given as pigment concentration ([Pigment]) (µg L^{-1} filtrated sea water). Water samples collected at the fish farm (Måsøval), March 4^{rt}, 8th and 31st, April 27th, June 8th, August 15th and October 4th 2016. Each column shows the average (n=3), and the pigments representing the columns are shown to the right. (Hex-kfuco/-fuco = 19'-Hexanoyloxy-4-ketofucoxanthin/19'-Hexanoyloxyfucoxanthin).

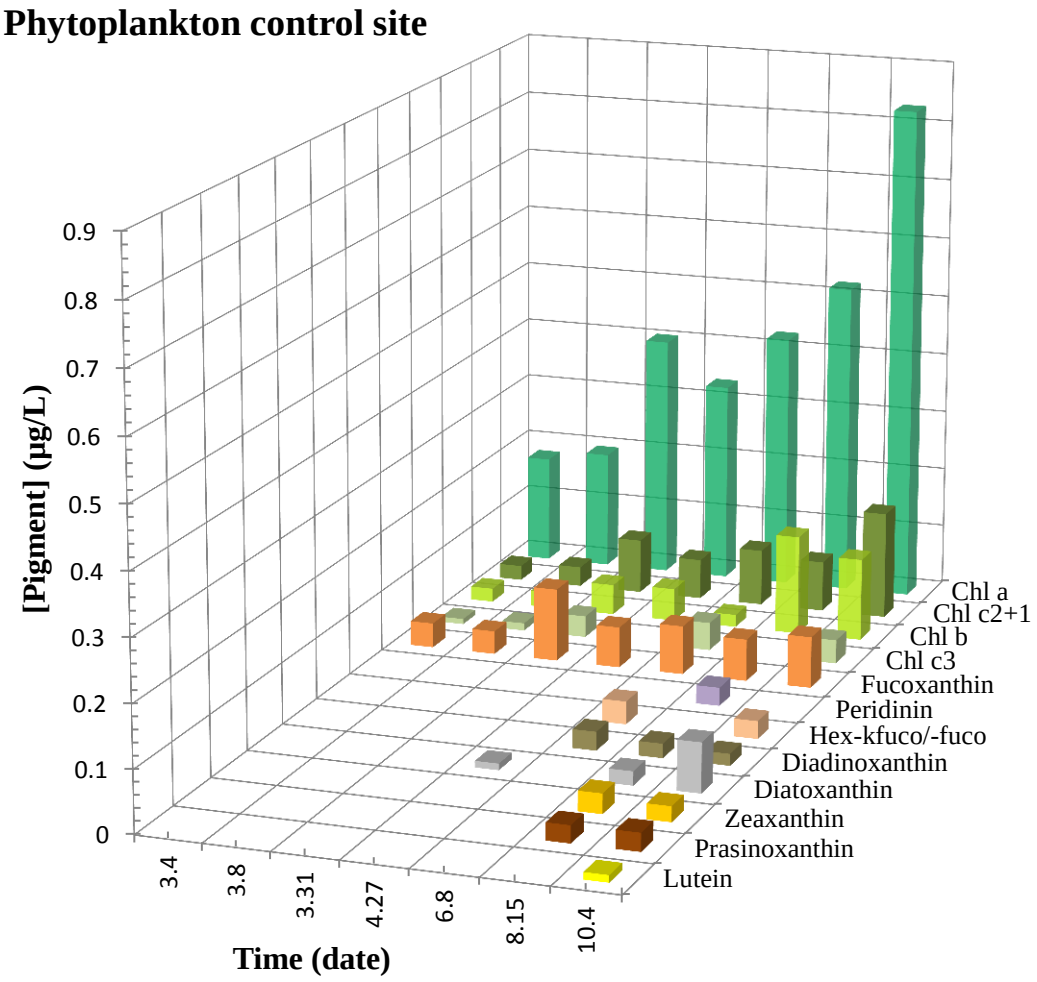

Figure 13: HPLC isolated pigments from phytoplankton extracts obtained from filtrated sea water that is identified and quantified and given as pigment concentration ([Pigment]) (µg L^{-1} filtrated sea water). Water samples collected at the control site (Lamøvågen), March 4rt, 8th and 31st, April 27th, June 8th, August 15th and October 4th 2016. Each column shows the average (n=3), and the pigments representing the columns are shown to the right. (Hex-kfuco/-fuco = 19'-Hexanoyloxy-4-ketofucoxanthin/19'-Hexanoyloxyfucoxanthin).

Relative abundance of phytoplankton identified with the light microscope (at 200X) was during the spring (sampled March 3rd, 8th and 31st) dominated by diatoms, and mainly by *Skeletonema costatum* during the two first samples and pennate diatoms March 31st. Late summer/autumn (August 15th and October 4th) was dominated by dinoflagellates within the Gymnodiniales and Peridiniales. Phytoplankton observed on 400X was smaller than 10 µm. Chryptophyta, and mainly *Teleaulax* sp. was consistent abundant in all samples. Haptophyta was present in all samples, dominated by *Chrysochromulina* sp. and *Prymnesium* sp. during spring and *Phaeocystis pouchetii* late spring and autumn. August 8th (Måsøval and control)

had a bloom of *Emiliania huxleyi* as the samples was full of loose coccoliths. Prasinophytes was only observed from April 27th and later. April 27th at Måsøval had a bloom of *Hetrocapsa rotundata* (dinoflagellate) that were not present at the control site.

3.2.2 Zooplankton

Zooplankton naturally occurring in water masses around the farm, which are wild Atlantic salmon's most important source of Astaxanthin. Measured *in vitro* pigment absorbance (OD (λ)) of zooplankton (Figure 15), collected from April to August, 700 m south of the fish farm. Absorbance spectra show a couple of weak shoulders at 416 and 445 nm, a clear maximum peak at 474-475 nm indicating presence of Astaxanthin, and a small peak at 666±1 nm indicating presence of Chlorophyll *a*. Astaxanthin, light pink line is put in the graph for a relative comparison. Figure 15 shows clearly that it is almost only Astaxanthin or Asta-like carotenoids present in pigment extracts from the zooplankton samples.

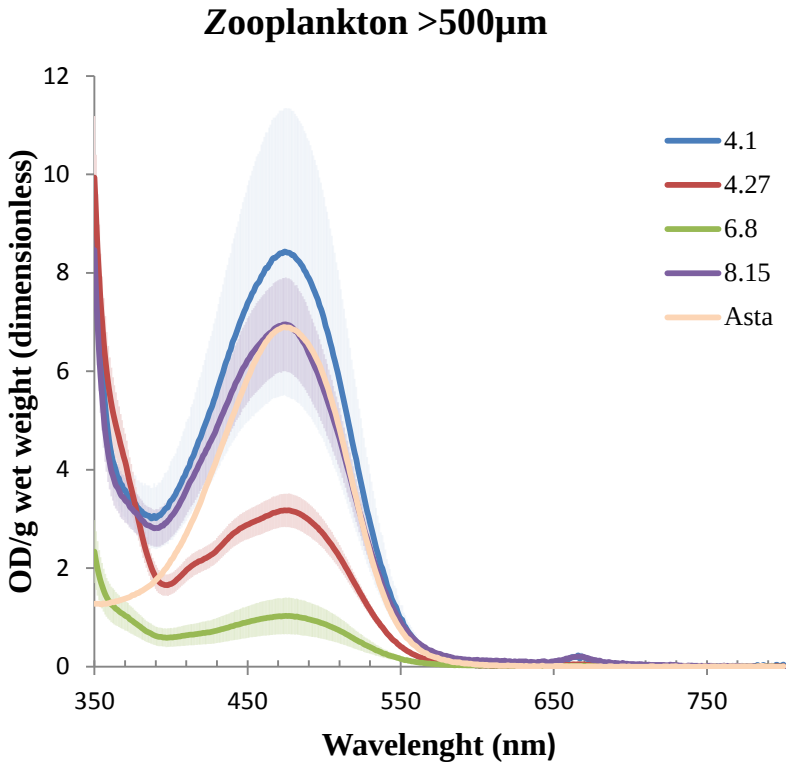

Figure 15: *In vitro* absorbance spectra (OD (λ)) zooplankton (bigger than 500 micrometer) containing Astaxanthin (µm) g^{-1} wet weight (n=3), collected 700 m south of the fish farm from April to October. Dates of collected samples are shown to the right of the graph with correspondingly color of the line, and ±SD is shown as shaded areas.

Relative abundance of different zooplankton caught in the net draw was identified in a stereo loupe. Samples from April 1st was dominated by Copepods, then Leptomedusae and eggs from Atlantic cod. The relative amount of biomass caught was not very big compared with the other months. April 27th was dominated primarily by Copepods, then Comb-jellies (Ctenophora) and Leptomedusae. During our samplings of zooplankton, Copepods had its peak at this date. June 8th was similar to April 27th. August 15th was dominated by Copepods, Krill (Euphausiacea) and Leptomedusae, but in small amounts. On October 5th it was also collected zooplankton, but with relative low concentrations, hence too little material for pigment measurements. Dominating this day was Leptomedusae and Ctenophora, and only small amount of Copepods was present this date.

3.2.3 Filter feeder: Blue mussel

Investigating filter feeders placed next to pen no. 8 at Måsøval salmon pen using *in vitro* absorbance (OD (λ)) of Blue mussels stomach collected at the fish farm (Figure 16A), and at the control site (Figure 16B), from March to October shows information on pigment content of obtained food of the Blue mussel. All absorbance signatures of Blue mussels obtained maximum peaks or shoulders at similar wavelengths, and they are as follows, a vague shoulder at 395 nm, peaks/shoulders between 414-420 nm, 444-451 nm with 449 nm as the most abundant one, and a shoulder at 474-476 nm. This shows the presence of carotenoids. Then mainly absorbance spectra from March and April have small peaks or shoulders at 538 nm, 564 nm and 606 nm. A distinct peak can be seen at 664 nm at four of the months, one month at 665 nm, and the last month one has its peak at 667 nm. This shows the presence of chlorophylls, including Chlorophyll *a* (indicated by absorbance at 664-667 nm). Chlorophyll *a* was low in August and October, but they contain in addition a shoulder at 676-677 nm, and 681-682 nm, which indicates degraded chlorophylls. Generally March, April and June for the fish farm, and March and April for the control site has the highest quantitative absorbance from pigments, while later during the year there is a clear less absorbance at all wavelengths. No clear differences are shown between fish farm samples and control samples, and no clear similarities are shown in the shapes of the spectra within the belonging month.

Sample 3.31 fish farm and 8.15 control are slightly underestimated due to mistakes done in the lab, but this is not affecting the main purpose of this project because the shape of the signatures would be the same.

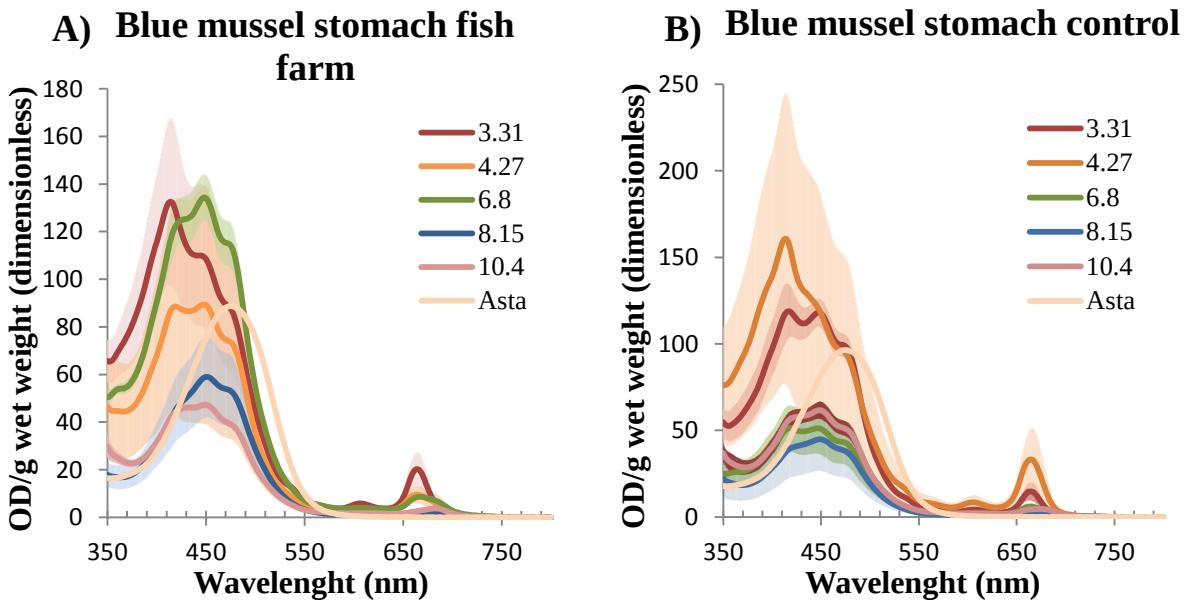

Figure 16: *In vitro* absorbance spectra (OD (λ)) of Blue mussel stomach containing phytoplankton pigments g^{-1} wet weight (n=3) collected at **A)** fish farm, and **B)** control site, from March to October. Dates of collected samples are shown to the right of the graphs with correspondingly color of the line, and ±SD is shown as shaded areas. Note different y-axis.

In vitro absorbance (OD (λ)) of Blue mussels mantel taken from the same individuals as used for sampling of stomach, collected at the fish farm (Figure 17A), and at the control site (Figure 17B), from March to October. The absorbance spectra gives information on pigments taken up and stored by the organisms either directly or transformed trough metabolism. Readings from the two graphs shows a clear similarity between these absorbance spectra with a shoulder at 372 nm and 422 nm. Two maximum absorbance peaks can be seen at 452±2 nm and 479 nm with a variation from 475 nm to 480 nm. This shows the presence of carotenoids. No absorbance of light at higher wavelengths which means no content of chlorophylls. The spectra show no clear similarities or patterns in quantity of absorbance between the months at the different locations, or throughout the year.

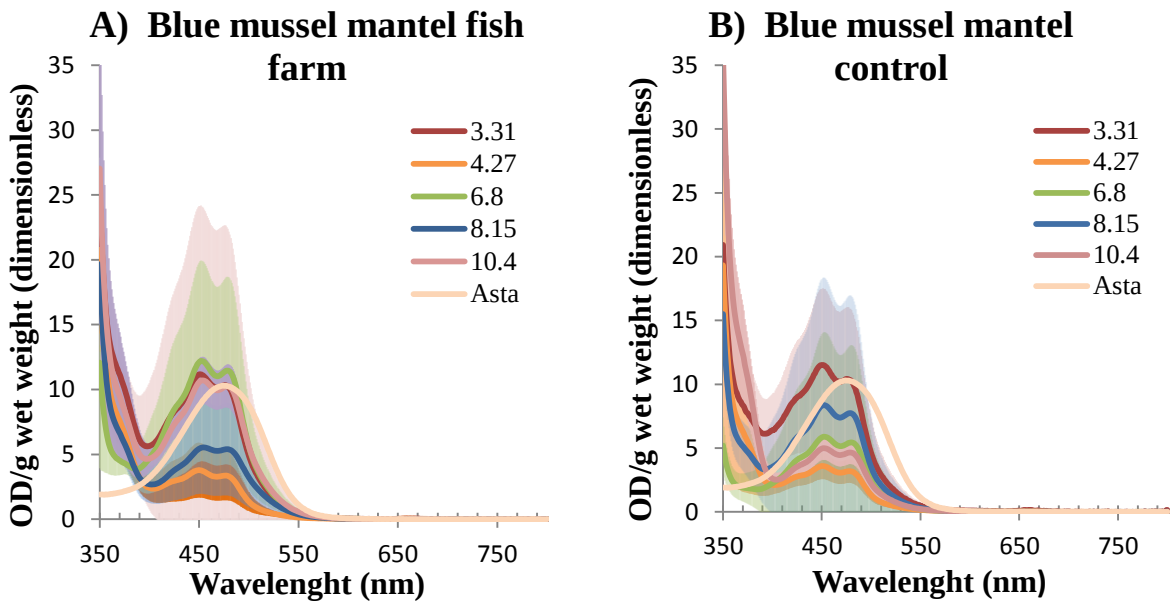

Figure 17: *In vitro* absorbance spectra (OD (λ)) of Blue mussel mantel containing carotenoids g^{-1} wet weight (n=3), collected at **A)** fish farm, and **B)** control site, from March to October. Dates of collected samples are shown to the right of the graphs with correspondingly color of the line, and ±SD is shown as shaded areas.

Coloration of Blue mussel mantel showed a big variation in concentration between replicates, as seen in ±SD in Figure 17. To show an example of the difference, pictures of mantel collected April 27th at the control site are shown in Figure 18. Three replicates from one sample day are shown to give a better picture of the big difference in pigmentation between individuals. All samples with replicates can be found in Appendix 4 for the fish farm and Appendix 5 for controls.

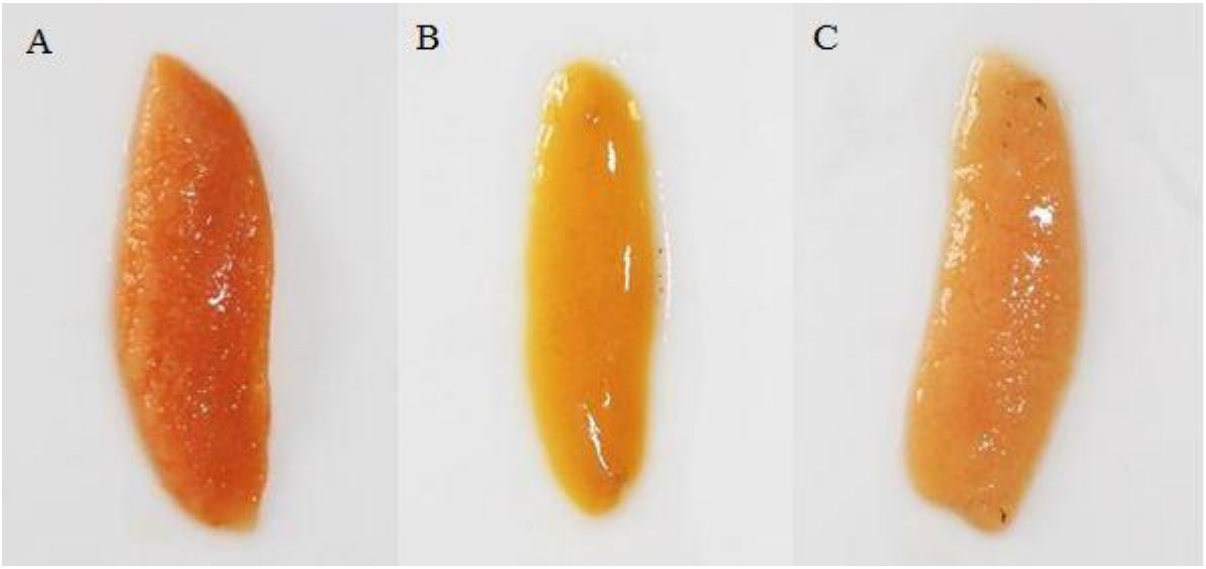

Figure 18: Coloration of Blue mussel mantle collected April 27th 2016 at the control site, indicating biological differences in pigmentation in three replicates. **A, B, C:** Replicate 1, 2 and 3, respectively (Photo: Kamilla Sporsheim).

3.2.4 Bottom feeder: Whelk

Investigating a benthic feeder in the surrounding ecosystem of the fish farm by looking at *in vitro* absorbance (OD (λ)) of Whelk foot, that provided with information on pigment composition stored in muscle tissue, either directly or transformed trough metabolism (Figure 19A). Correepondingly, the Whelk stomach gave information about pigment content in obtained food, found on the seabed (Figure 19B). Collected from March to May at Hammerbergskjæret (Ham), Kolskjæra (Kol) or Bukkholmen (Bukk). Absorbance spectra of Whelk foot (Figure 19A), shows a shoulder at 525±2 nm, a maximum peak at 446-450 nm, and a smaller peak at 476 nm. All spectra except Bukk 5.3 have in addition a shoulder at 534 nm. This shows that only carotenoids are present, and by looking at Figure 19A it can be seen that the concentration are very low, and tissue seen by eye, appear almost white.

Absorbance spectra of Whelk stomach (Figure 19B), shows a small shoulder at 375 nm, a maximum absorbance peak at 408-413 nm, shoulders/peaks at 446-450 nm and 474 nm. Then some of the samples have small peaks at 535, 606 and 554 nm (see Figure 19B). All of the spectra have a maximum peak at 664-665 nm. This shows the presence of carotenoids and chlorophylls.

General trends when comparing Figure 19A and B, is that Whelk foot contain carotenoids, and absence of chlorophylls (no absorbance higher than 570 nm), while Whelk stomach

contain substantial amounts of phytoplankton pigments (Chlorophyll *a* and carotenoids). In both figures Ham 3.1 show the lowest pigment concentration, and Kol 4.29 have the highest.

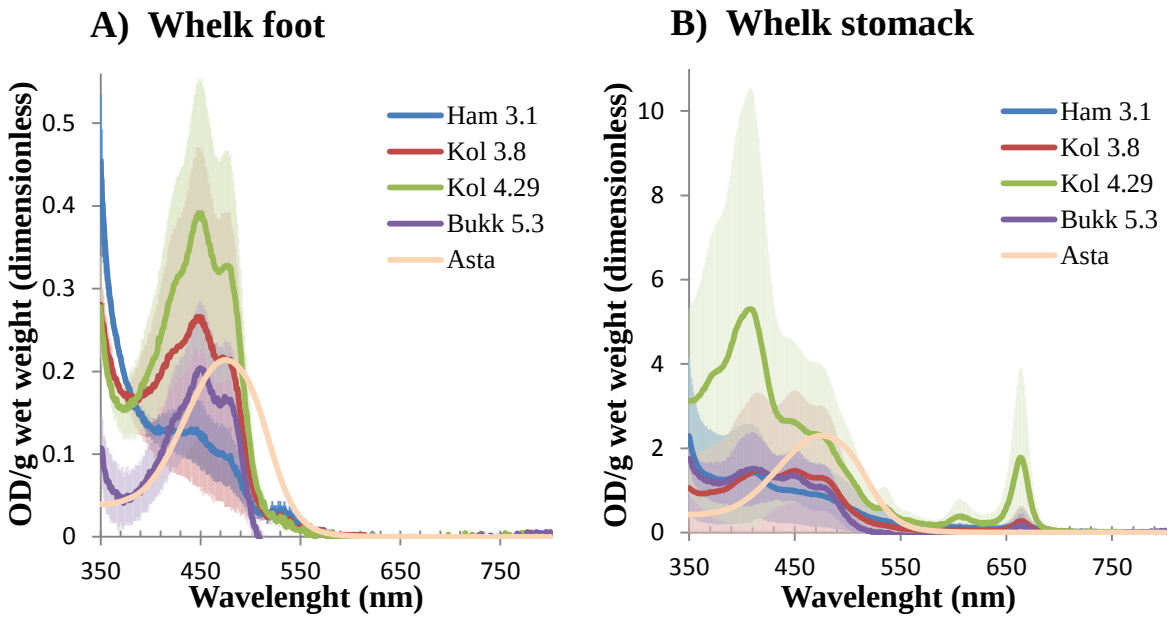

Figure 19: *In vitro* absorbance spectra (OD (λ)) of Whelk **A)** foot, containing carotenoids, and **B)** stomach, containing phytoplankton pigments g^{-1} wet weight (n=3) from March to May. Collected 400 – 600 m from pen raised salmon. Whelk caught at Hammerbergskjæret (blue line) located 2 km south of the fish farm and Kol 3.8 are considered as control samples for whelk. Dates of collected samples are shown to the right of the graphs with correspondingly color of the line, and ±SD is shown as shaded areas. Note different y-axis.

3.2.5 Grab sampling

Composition of benthic macrofauna gives an indication of the health state of the seabed, and to get sediment samples a Van Veen Grab was used the last sampling day of this project, October 5th for the benthic taxonomy study. Two replicates were collected at each site. At Måsøval the replicates, Grab I and Grab II was taken respectively 150 m and 100 m from the fish farm. At the control site the replicates was taken close to each other, at the same depth and at the same distance from nearby island (Hellskjæret). General observations when grab sampling at Måsøval was hard bottom substrates and small boulders, due to difficulties in closing the grab properly, or having an empty grab returning to surface. This resulted in different amount sediment collected in each replicate. When retrieving a grab containing

sediments it consisted of fine grained sand (<0.5 mm) and mud. Grab sampling at Hellskjæret was a lot easier, and bottom substrate was characterized with gravel, shell sand (ca. 0.5 cm) and sand (0.5 mm) with bigger grain size than Måsøval.

Overview of phylum/classes found at each site (replicates added together) and number of different families or genuses within them are shown in Figure 20. Polychaeta is the most abundant and diverse at both sites, and Bryozoa are most abundant and diverse for the control site alone. Anthozoa and other Mollusca are also only found at the control site. Nemertea, Echinoidea and Priapulida are only found at Måsøval.

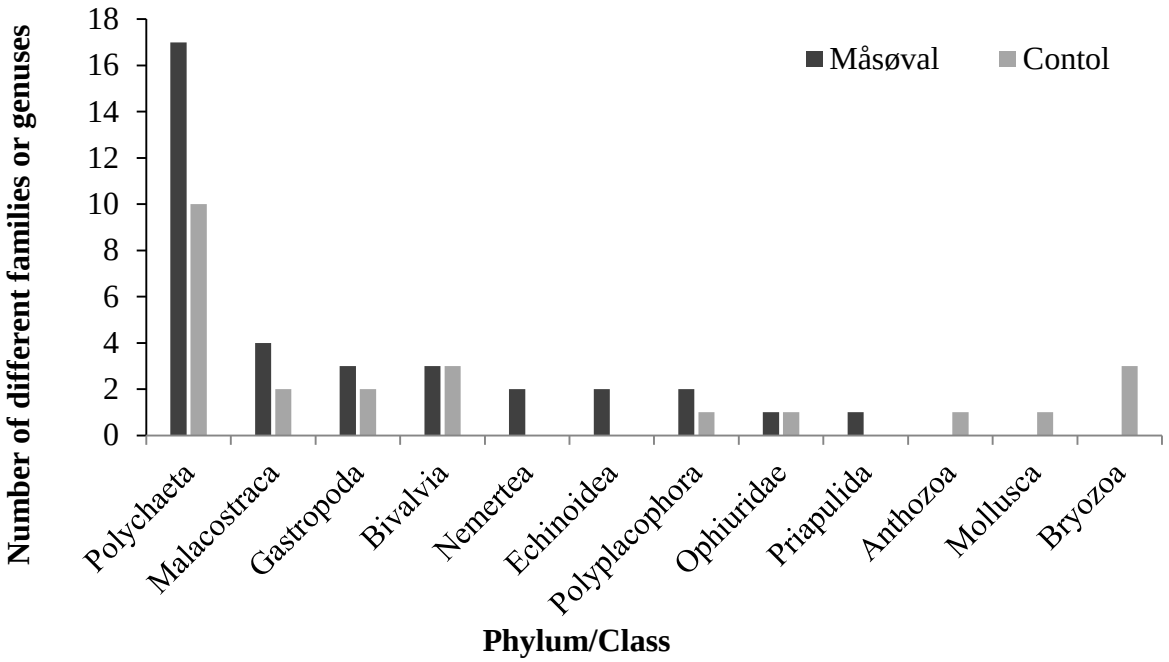

Figure 20: Overview of benthic organisms at Måsøval and controls (Hellskjæret), collected with a Van Veen Grab. Two replicates at each sites was determined taxonomically, and added together in the figure. The figure shows number of different families or genus's observed per phylum or class.

Organisms found in grab (Table 3), determined to lowest taxonomic level as possible, i.e. to phylum, subclass, family, genus or species. Counting of individuals of each species was not carried out in this project. Figure 21 shows two examples of organisms found at both Måsøval and control site. An equality test was performed to check whether if the two replicates collected at the same site can be compared with each other. The outcome of the test shows that equality between replicates at Måsøval was 12.5 % out of total 40 different species.

Equality between replicates at control site was 10.7 % out of total 28 species. Equality between Måsøval and control was 17.7 % out of total 51 species. For the equality calculations see Appendix 6. This is very low values for equality between replicates, which means a big variation between replicates and not suitable for comparison.

Table 3: List of all collected organisms from grab sampling, done at Måsøval (n=2) and Hellskjæret (n = 2). Classification is done to the lowest taxonomic level as possible to phylum, subclass, family, genus or species.

Måsøval:	**Control:**
Ampharetidae	*Arenicola marina*
Arctica islandica	*Bryozoa*
Buccinum sp.	*Capitellidae*
Cerebratelus marginatus	*Chaetopterus* sp.
Cirratulidae	*Circimphalus casina*
Echinus elegans	*Crisia* sp.
Flabelligeridae	*Gari* sp.
Galathea intermedia	*Glyceridae*
Galathea strigosa	Hexacorallia
Gibbula sp.	*Lacuna* sp.
Glyceridae	*Lucinoma borealis*
Hydroidus norvegica	*Membranipora membranacea*
Leptochiton asellus	*Munidopsis serricornis*
Lucinoma borealis	*Ophiotrichidae*
Macropipus sp.	*Orbiniidae*
Nassarius incrasatus	*Paguridae*
Nemertea	*Polyplacophora* sp.
Nephtys sp.	*Pomatoceros triqueter*
Ophellidae acuminata	*Sabellidae*
Ophiura albida	*Serpula vernicularis*
Orbinidae	*Spirorbis* sp.
Paguridae	*Terebellidae*
Pherusa sp.	*Thyasira* sp.
Phylldocidae	*Trivia arctica*
Polynidae	
Polyplacophora sp.	

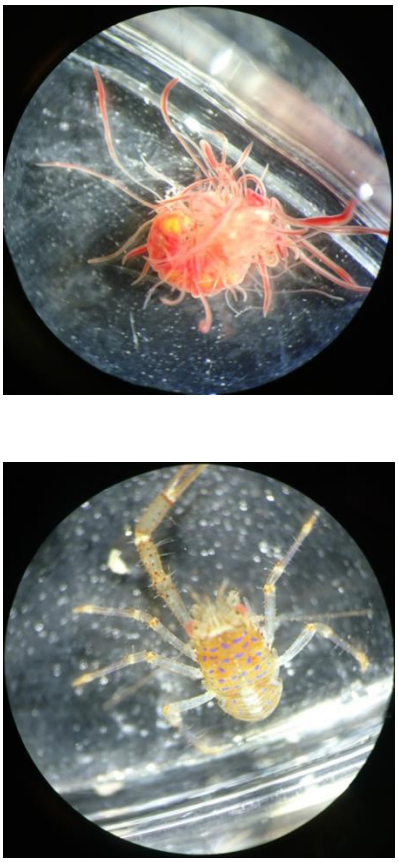

Figure 21: Upper photo: The Polychaeta *Terebellidae.* Lower: The Malacostraca *Paguridae*, both found at Måsøval and control site. (Photos: Gustav Nore)

Pomatoceros trequeter
Priapulus caudatus
Sabellida sp.
Scalibregma inflatum
Sphaerodoridae
Strongylocentrotus sp.
Syllidae
Terrebellidae
Thyasira sp.

Pigment content of collected sediments gives information of what is living in or on the seabed that is too small to identify with eye in the taxonomy study. *In vitro* absorbance (OD (λ)) of seabed sediments (Figure 22) collected October 5th, 150 m, 100 m and two 50 m from the fish farm, and two next to Hellskjæret as controls. All absorbance spectra shows similarities in shape with a shoulder at 375 nm and 398 nm, a maximum absorbance peak at 416-420 nm, 438-445 nm, and a shoulder at 470-473 nm. This indicates presence of carotenes and xantophylls. The absorbance spectra show also a small shoulder at 538 nm, a small peak at 608–613 nm, and a distinct peak at 666 nm, with one specter at 667 nm (Control II) . These indicate chlorophylls, including degraded.

The control samples have the least total absorbance, meaning the lowest concentration of pigments. Sample 50 m II have the highest concentration of pigments. Other than this there is not a clear pattern in concentration of pigments. All spectra show clear chlorophylls and carotenoids content, with high amount of carotenoids relative to chlorophylls.

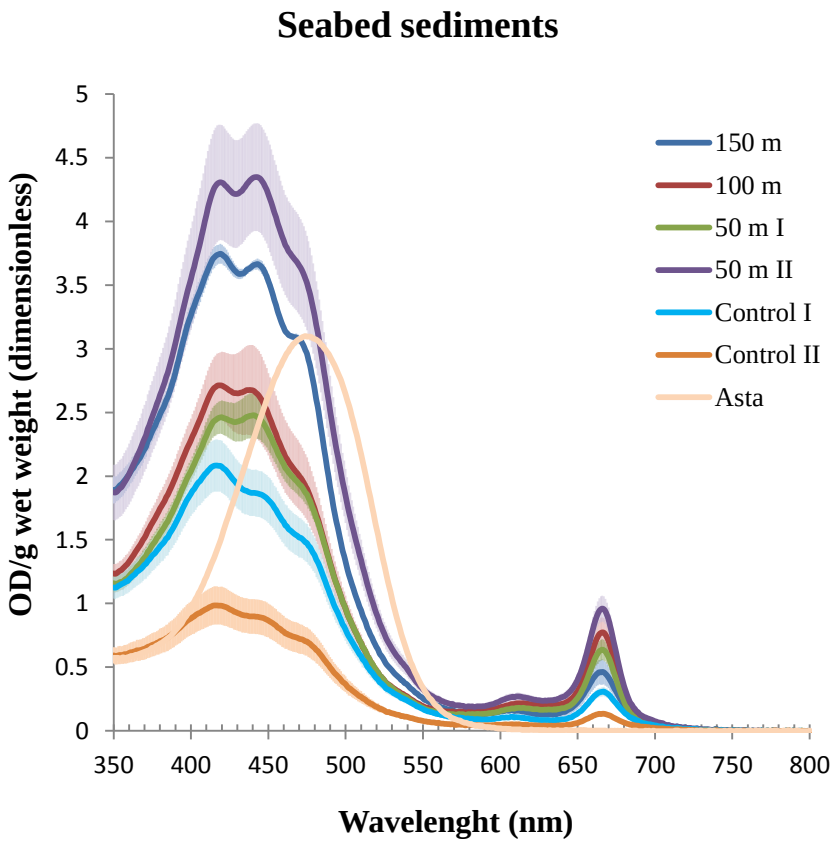

Figure 22: *In vitro* absorbance spectra (OD (λ)) of seabed sediments containing chlorophylls and carotenoids g^{-1} wet weight (n=3) collected October 5th 2016. Sediments were collected with the use of a Van Veen Grab at 150 m, 100 m and two at 50 m from the Måsøval pen. Two grab samples collected as controls (Control I + II). Samples with belonging color are shown to the right of the graph, and ±SD is shown as shaded areas.

The samples measured by the spectrophotometer (Figure 22) were also measured by the HPLC technique (Figure 23). From readings of Figure 23, both the control samples have the least pigment concentration and pigment diversity. Sample 50 m II (Måsøval) have the highest pigment concentration, and decreasing with the order from 50 m II to 100 m and 150 m. All sediment samples had big constituents of degraded chlorophylls and carotenoids, and the many of them are not included in Figure 23, due to difficulties in determination and quantification. HPLC measurements show that absorbance at 470-473 nm contain Astaxanthin, this can indicate dead zooplankton sunk to the bottom, benthic zooplankton containing Astaxanthin, feces containing eaten zooplankton from wild pelagic fish or Astaxanthin coming from pen raised fish feed. In addition to a big variety of pigment groups that indicate different groups of phytoplankton are present (see introduction). Chlorophyll *a*

includes all photosynthetic algae, Chromophyta (including diatoms, Fucoxanthin and Chlorophyll c_1 + c_2), then followed by Chlorophyceae and Prasinophyceae II (Lutein, Chlorophyll *b*, β,β-Carotene and β,ε-Carotene) and Cyanophyceae (Zeaxanthin) to mention the most abundant pigments present.

Chlorophyll *a* was ranging between 0.27 and 7.00 µg g^{-1} for both sites, but with a relative low [Chlorophyll *a*] relative to total [pigments], compared with phytoplankton in water masses, this indicate more degraded Chlorophyll *a*, i.e. not as healthy cells as in the water surface.

Coefficient of variation in percentage (±CV %), the ratio of standard deviation (±SD) to the average are shown in Appendix 7. ±CV % is between 1.26 % and 173.21 %. The reason for the high values are due to absence of pigment in one or two of the replicates, which makes ±SD higher than the average pigment content of the samples. Samples with pigment content in all three replicates were not more than 56 %.

When comparing absorbance (OD (λ)) (Figure 22) with HPLC isolation (Figure 23) it can be seen that a both figures show carotenoids as a big constituent part relative to chlorophylls. The control samples are similar in concentrations, and in pigment diversity in terms of chlorophylls and carotenoids. Grab sample 50 m I, have low total absorbance (OD), but the second highest [Chl *a*]. This indicates low (relative) content of carotenoids. 150 m sample have the second highest total absorbance (OD), and low [Chl *a*], this indicates high (relative) concentration of carotenoids.

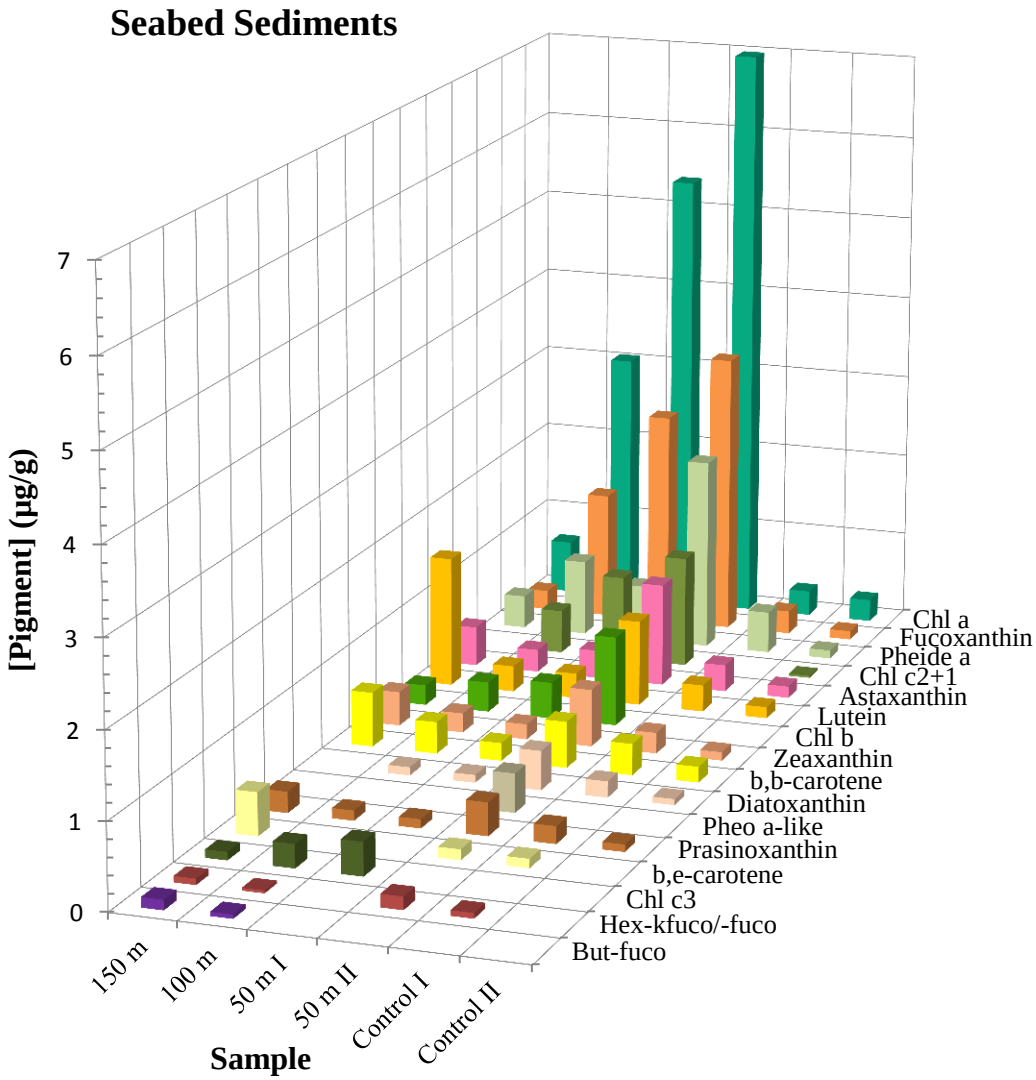

Figure 23: The main pigments from seabed sediment extracts, which is identified and quantified with HPLC. The samples are characterized with high pigment content, a major constituent is xanthophyll (including Astaxanthin), and carotenes are present. Less chlorophyll relative to total pigment content is present than phytoplankton in water masses. Samples were taken October 5th 2016, 150 m, 100 m, and two at 50 m from the fish farm. As controls, two samples were taken at Hellskjæret for comparison. Each column shows the average (n=3), and the pigments representing the columns are shown to the right. (Chl = Chlorophyll, Hex-kfuco/-fuco = 19'-Hexanoyloxy-4-ketofucoxanthin/19'-Hexanoyloxyfucoxanthin, But-fuco = 19'-Butanoyloxyfucoxanthin)

To verify the expected peak of Astaxanthin in HPLC isolation of pigments to be certain, Asta std. (Figure 24A) was compared to a sediment sample assumed containing Astaxanthin (Figure 24B) to compare the retention time of the peak in the chromatogram. The peak had the same retention time and Astaxanthin was easy to determine in the sediment samples.

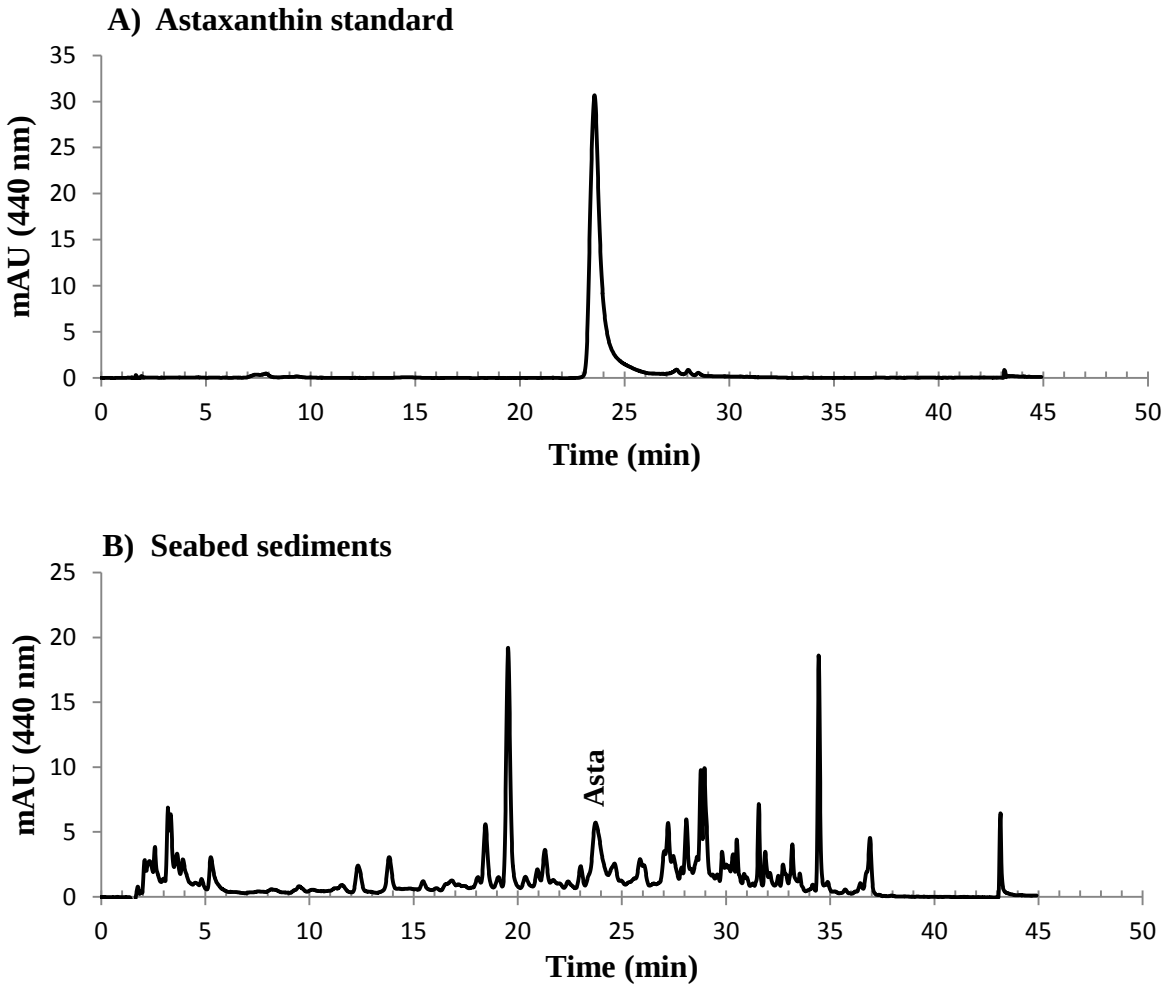

Figure 24: HPLC chromatograms that verify the presence of Astaxanthin in a **A)** Astaxanthin standard and **B)** Seabed sediments from sample 50 m II at Måsøval (one of the replicates), measured in milli Absorbance Unit (mAU) as a function of time in minutes.

3.2.6 *In situ* video survey

To obtain an overview of bottom condition and health status below the fish farm, *in situ* video survey with the use of a small Remotely Operated Vehicle (ROV). Two video survey lines were performed at Måsøval, below pen no. 8 at 17 m depth, and two at the control site (Hellskjæret) at 43 m depth for comparison in environmental conditions. The observed conditions at Måsøval showed the presence of settled organic waste from the above lying pen, covering some of the natural bottom substrates, which consist mostly of sand and small grained shell sand and few small boulders. Patches of white mats was abundant on the seabed. This indicates the presence of *Beggiatoa* sp., bacteria that live in sulfur- rich environments. In

the water column it was observed a lot of big (< 1 cm) free floating organic particles, with a high sinking rate and smaller particles preventing good visibility (Figure 25).

Figure 25: Bottom condition under pen no. 8 at Måsøval at 57 m depth. Upper image: shows high particle concentration in water masses, accumulated organic particles on the seabed and white mats of sulfide-oxidation bacteria *Beggiatoa* sp.. Lower image: A close up picture of *Beggiatoa* sp. and a burrowing Polycgaeta. Obtained from the *in situ* video survey (Pictures: Frame grabs pictures of video filmed with use of an ROV SeaBotix, NTNU AUR-Lab)

Seabed condition at the control site (Hellskjæret) looked healthy an unaffected from aquaculture industry (Figure 26). Bottom substrates consist of shell sand, gravel and many small boulders, which is a bit different habitat than at Måsøval. Water quality was good with limited amount of particles and good visibility.

Figure 26: Pictures that shows bottom condition at the control site (Hellskjæret), low particle concentration in water masses, low accumulation of organic particles on the seabed and organisms of brown macroalgae, red calcareous macroalgae and calcareous Polychaeta. (Pictures: Frame grabs pictures of video filmed with use of an ROV SeaBotix, NTNU AUR-Lab)

Organisms observed from the video survey on the two different sites where different (Table 4). Måsøval was dominated by different types of fish and organisms living in the sediments like Polychaeta. Starfish (Asteroidea), Sea urchin (Echinoidea) and Sea anemones (Actiniaria) was also observed. At the control site, remarkable fewer fishes were observed, but brown macroalgae (Phaeophycea) and green algae (Manostromataceae) were present in contrast to Måsøval.

Table 4: Observed families or order with *in situ* video survey at Måsøval (n=2) at 17 m depth, and at control site (Hellskjæret) (n=2) at 43 m depth.

Måsøval (9 families)	**Hellskjæret (8 families)**
Actiniaria	Astropectinidae
Arenicolidae	Cancridae
Arcticidae	Desmarestiaceae
Asteroidea	Leptomedusae
Cyclopteridae	Monostromataceae
Echinoidea	Phaeophyceae
Gadinae	Pleurochloridellaceae
Pleuronectidae	Serpulidae
Serpulidae	
Spirorbidae	

Ending the result section with a short summary of all the absorbance maximum peaks obtained from readings of the graphs with correspondingly key pigments verified with HPLC (Table 5). The table gives an overview of all collected tissue samples, and their absorbance maximum peaks (OD (λ_{max})) in nanometer wavelengths of light, together with some of the most important pigments in this project separated with HPLC that absorbs light at matching wavelengths. Astaxanthin are present in fish feed, Atlantic salmon muscle tissue and feces, Astaxanthin standard, zooplankton and in seabed sediments. Surface water samples confirmed with HPLC are not containing Astaxanthin. Blue mussel stomach and mantle, Whelk stomach and foot have a shoulder in the part of the absorbance spectra (474-480 nm) that can with

uncertainty show the presence of Astaxanthin, due to difficulties to distinguish from other pigments with similar absorbance maximum peaks.

Table 5: Overview of collected samples and the corresponding *in vitro* absorbance maximum peaks (OD (λ_{max})) measured with the spectrophotometer in nanometer (nm) of wavelengths, with their corresponding key pigments identified with the help of HPLC that absorbs light within these wavelengths of light. HPLC performed on Astaxanthin standard, Phytoplankton, and seabed sediments. Wavelengths marked red is confirmed Astaxanthin.

Sample	**λ_{max} (nm)**	**Pigment markers (HPLC)**
Pellet	420, 449, 471	Astaxanthin
Atlantic salmon, muscle tissue	409-416, 449, 471-476	Astaxanthin
Atlantic salmon, faeces	378, 400, 418, 446, 467-470	Astaxanthin
Astaxanthin standard	474	Astaxanthin
Phytoplankton	419, 434, 438, 475, 583, 615, 638, 665	Chl *a*, *b* and $c_{1\text{-}3}$, Fuco, Peri, Diadino, Diato, Pras, Zea, Hex-kfuco/-fuco, Lut
Zooplankton	370, 412, 445, 474-475, 666	Astaxanthin, Chl *a*
Blue mussel, stomach	395, 414-420, 444-451, 474-476, 538, 564, 606, 664, 676-677, 681-682	Carotenoids and Phytoplankton pigments
Blue mussel, mantle	372, 422, 452, 475-480	Carotenoids
Whelk, foot	425, 446-450, 476, 534	Carotenoids
Whelk, stomach	374, 408-413, 446-450, 474, 535, 606, 664-665	Carotenoids and Phytoplankton pigments
Sediment	375, 398, 416-420, 470-473, 538, 608-613, 666 (-667)	Asta, Chl *a*, *b* and $c_{1\text{-}3}$, Fuco, Pheide *a*, Lut, Zea, Diato, Pheo *a*-like, Pras, Hex-kfuco/-fuco, But-fuco, ββ-Car, βε-Car,

4. Discussion

Following sections will discuss the results and methods in this project, ending the discussion with challenges and future perspectives. The main focus is Astaxanthin and its potential use as a pigment marker for organic pollution from pen raised Atlantic salmon. To the author knowledge, this is the first study looking at Astaxanthin as a bio/pigment marker tracing the pathway of fish feed through different key organisms in the marine ecosystem. This can be further used to improve already existing monitoring procedures (MOM, Modelling-Ongrowing fish farms-Monitoring) in terms of organic loadings, by using the proposed method "Astaxanthin method" applied in this study to provide a quick, easy and applicable approach for enhanced information for monitoring and preferably reduce man-made pollution in marine ecosystems.

4.1 Farm raised Atlantic salmon

4.1.1 Feed pellet, the input of Ataxanthin and organic loading to a fish farm

Astaxanthin is the major constituent of pigmentation of Atlantic salmon feed pellets, in addition to other substances present and affecting the absorbance spectra compared with the Astaxanthin standard. The overall major constituents of fish feed are 24-30 % oil and 40-46 % protein. Mostly from plants, such as rapeseed oil, soy protein and legume protein, and only a small part is marine, providing the salmon with food either taken up, or released into surrounding ecosystem (Wang et al. 2013).

Underestimation of [Asta] is due to difficulties when soaking the compact feed pellets in water. Due to different size fractions of the pellets, the biggest (PW3) did not soak water as easy as the smallest (PW1) leading to less grams in total per wet weight. This is not affecting the shape of absorbance spectra which is the key information here.

4.1.2 Atlantic salmon

Atlantic salmon muscle tissue consists mainly of Astaxanthin and this is verified with a comparison with an Astaxanthin standard. The first muscle tissue samples appeared white by eye and increased steadily in coloration with time, Astaxanthin concentration as a function of time verifies this. This shows an increase food given with increased size of the fish. Farm

raised Atlantic salmon is fed with feed pellets added artificial Astaxanthin to obtain the redness (Breithaupt 2007; Foss et al. 1984), due to consumers preferences (Alfnes et al. 2006). Variations between individuals in pigment concentration (±SD), is due to specifically choosing replicates with different sizes, and the reason for a lower pigment than expected content for the last sample day are most likely due to smaller individuals sampled since pigment content are shown to increase with size.

Astaxanthin concentration in feces is directly correlated to feed pellets that are not used in the metabolism of the fish, and the results shows that at feeding start, the fish have not managed to utilize the food. Then, from April to July feces contained very high values of Astaxanthin, 30 μg g^{-1} in feces compared with 50 μg g^{-1} added in the feed pellet (July sample), which may indicate overfeeding or low efficiency of food utilization. In addition, personal observations when dissecting the fish, was that high amounts of undigested food in rectum strongly indicates overfeeding. This can further be used for a better feed management. Giving suitable amount of food is important for the industry's economy, and better for the surrounding ecosystem that receive the excess fish feed either as feces or uneaten feed pellets.

4.2 Surrounding ecosystem of the fish farm

4.2.1 Phytoplankton

Water surface did not contained detectable values of Astaxanthin. That is reasonable due to big particles i.e. high sinking rate. Beyond that water samples provide with good background information of what is present in the water surface. The most abundant phytoplankton groups present were diatoms, dinoflagellates, prymnesiophytes, chryptophytes and prasinophytes, with seasonal variations in dominance. A slightly higher total pigment concentration was present at the fish farm, this can possibly be due to natural variations or because of a higher content of dissolved inorganic nutrients released from the farm that can cause a higher Cholorophyll *a* concentration (indication of phytoplankton biomass) (Wang et al. 2014).

Absorbance (OD (λ)) of pigment extract, HPLC isolation and taxonomic identification with light microscopy provides combined good and reliable information about, the combined pigment composition present in an extract, chemotaxonomy and taxonomic identity and abundance. These methods together contribute to either quality assurance, or easier find sources or error.

Alloxanthin, a pigment marker for chryptophytes was not detected with HPLC, but results from the microscopy identification show the presence of chryptophytes in all samples. This can be due to either too low pigment concentrations or difficulties in separation between Alloxanthin- and Diatoxanthin peaks when analyzing the chromatograms.

Pigmentation of phytoplankton follows seasonal blooms, with an increase in pigmentation in spring and autumn. Phytoplankton community is dominated by diatoms during spring, and dominated by dinoflagellates autumn. This is as recorded before in the Trondheimsfjord (Sakshaug and Myklestad, 1973).

The microscopy counts and identification where done to verify pigment measurements but used with caution due to sources of error in taxonomic identification. It's not a trained personal skill, challenging work and time consuming. Improvements have been done through the process, and that can have an influence on the quality of especially the first samples examined.

4.2.2 Zooplankton

Zooplankton consisted mostly of copepods containing Astaxanthin or Astaxanthin-like pigments, the major Astaxanthin source for wild Atlantic salmon (Andersson et al. 2003). When comparing absorbance spectra of Atlantic salmon muscle tissue and zooplankton they are very similar, sharing the same dominant absorbance maximum peak for Astaxanthin (471-474 nm). Zooplankton obtains their pigment compositions from their food source, phytoplankton, and transform the pigments through metabolism, and further transferred to higher trophic levels as pelagic fish (Andersson et al. 2003).

4.2.3 Filter feeder: Blue mussel

Results shows that Blue mussel is mainly feeding on phytoplankton present in the water surface instead of organic loading from the fish farm (measured Astaxanthin), but total pigment content do not follow total pigment content measured for phytoplankton in this project, which may indicate that Blue mussel is feed selective to different types of phtoplankton. It is reported that more than 50 % of carotenoids present in Blue mussel are Alloxanthin (main pigment in chryptophytes), in addition to Zeaxanthin, lutein, diatoxanthin, antheraxanthin and β-Carotene to mention some. Some of them were also present in Blue

mussel feces. Fucoxanthin where absent (main pigment in diatoms) (Campbell 1970). This indicates that food obtained by Blue mussel is primarily phytoplankton, due to phytoplankton pigments, and Blue mussels prefer phytoplankton as a food source. Previous it is reported that Blue mussel feeding mostly on fish farm waste during winter months when phytoplankton concentrations are low, and can only utilize small fractions of fish farm waste (Handå et al. 2012). This is important information regarding IMTA (integrated multi-trophic aquaculture), whether if it's a good solution to use Blue mussel as a mitigation strategy to remove some of the excess organic waste from aquaculture industry.

Blue mussel mantel consists only of different types of carotenoids, and show big variations between individuals in pigment concentration (big ±SD). This can be due to specifically choosing replicates with different sizes, and/or individual differences.

4.2.4 Benthic feeder: Whelk

Whelk collected in close distance (minimum 400 m) to the fish farm did not show any increased value of Astaxanthin compared to control samples. The results indicates that Whelk is feeding on microphytobenthos due to the characteristic signatures of Chlorophyll *a* and its derivatives in stomach, and concentrations of pigments can be believed to follow concentrations of microphytoplankton due to a higher concentration in late April, and lower for May. Whelk foot consists only of carotenoids, due to no absorption of pigments higher than 570 nm, and in low concentrations due to rough absorbance spectra and almost white tissue.

Sampling of Whelk was done on at Kolskjæra, Bukkholmen and Hammerbergskjæra (Figure 1). Between Bukkholmen and Koskjæra there is another fish farm located 600 m upstream from of Kolskjæra with feeding start March 30th, which makes the samplings of Whelk at this site also possible affected by the farm raised salmon industry. When using Astaxanthin as a biomarker it would be easier to compare samplings of Whelk if they were collected at the same site. The good side with having Whelk collected at different locations is to obtain a bigger picture of the area around the farm and possible influence from other fish farms in the area.

4.2.5 Benthic macro fauna taxonomy from grab sampling

Polychaetes were the most abundant and diverse class of organisms found at location fish farm and control site, with respectively 17 and 10 different species. A clear trend of more species found at the fish farm is present with a total of 35 different species compared with 24 at the control site. Nemertea, Echinoidea and Parapulida are only found at the fish farm. Bryozoa, Anthozoa and Mollusca (other than already mentioned e.g. Polychaeta) are only found at the control site. According to literature, is it expected to find more detritus feeders below a fish farm, due to more organic loading on the seabed, as long as organic pollution is within tolerant amount of what the benthic organisms can tolerate. Consequently the biodiversity will shift towards species that is tolerant to high amounts of organic matter and often oxygen depletion instead of a natural species composition (Kutti et al. 2007; Holte et al. 2004). It is difficult to determine whether there are any significant differences. The equality test shows that comparing replicates and different sampling locations should be doing with carefulness due to a greater variation between replicates collected at the same site, than between the locations.

Polychaetes have been used for a long time as indicator species for organic pollution, due to some are opportunistic to heavy or moderate polluted benthic environment. Absence of species is also of great informative value. Dominant species at heavy organic loadings are among the most used *Capitella capitata,* the genus *Ophryotrocha* and polydorid spionids, and *Heteromastus filiformis* at moderate loadings (Kutti et al. 2008; Rygg 2002). None of these are observed in our study. Groups of organisms such as genuses or family as indicators species cannot be used unless stated in literature, consequently a detailed taxonomic identification is needed. Generalization of species should be done with carefulness, e.g. *Glycera alba,* that is observed at the fish farm and control site, have earlier been used both to indicate polluted areas, and healthy areas (Pearson et al. 1983; Rygg 2002). Hence, it is reasonable to think that the benthic environment below the fish farm is not heavily polluted, but a more extensive and detailed study is needed to be able to generalize indicator species for organic pollution or a healthy ecosystem.

One interesting finding at the fish farm is the Penis worm *Priapulus caudatus*, which are tolerant to hydrogen sulfide and anoxia (Schreiber et al. 1996). It is important to take into consideration that often it is not the degree of organic matter that settles, but the degree of

oxygen depletion on the seabed that determine the benthic community, and this varies greatly with current conditions of the site. Different bottom substrates geographical and seasonal variations, sediment grain size and water depth when sampling can also be reasons for different organism biodiversity (Dean 2008; Holte et al. 2004; Kutti et al. 2007).

4.2.6 Pigment composition of seabed sediments

Astaxanthin is present on the seabed, at concentrations <1.2 µg g^{-1} sediment, this can be due to micro-meio zoobenthos containing Astaxanthin, dead zooplankton containing Astaxanthin sunk to the bottom, fish feces containing eaten zooplankton or Astaxanthin coming from pen raised fish feed. The fact that the control site is located far away from a fish farm and containing Astaxanthin makes it reasonable to think that Astaxanthin on the seabed originates from natural sources. Without HPLC measurements it would be difficult to determine Astaxanthin present at these concentrations (<1.2 µg g^{-1}) from the absorbance spectra.

Then in general pigment content of the seabed shows a huge variety and in relative high concentrations. Chlorophylls and carotenoids are both present in great amounts, and due to marker pigments present, microphytobenthos do possibly consist of Chromophyta, Chlorophyceae, Prasinophyceae and Cyanophyceae (Cyanobacteria). Diatoms and Cyanobacteria are reported to be among the main constituent of microphytobenthos (Brotas and Plante-Cuny 2003; Barranguet et al. 1997).

Måsøval have higher pigment content (max [Chl *a*] 7 µg g^{-1}) than at the control site (max [Chl *a*] 0.3 µg g^{-1}), this can be due to additional organic and inorganic loading coming from the fish farm, smaller grain size of sediments and/or less water depth crating greater light availability, that may can be more favorable conditions for microphytobenthos. On the other side, the control site has greater water depth where more inorganic and organic matter has the chance to settle, and less particles in water column that favor the chance of light to reach seabed.

When comparing pigments found on seabed with pigment content in water surface, the seabed has up to 7 times as high pigment concentration, and significant higher amounts of degraded chlorophylls and carotenoids.

When identifying pigment peaks from HPLC chromatograms and compare with known pigment standards there is a source of error, due to complex sediment samples containing

many small peaks not possible to determine e.g. degraded pigments (see Figure 24B). The identification of pigments where done as good as possible and with necessary help. The chosen pigment where consistent through all samples, so if mistake has occurred it will affect all samples sharing the same retention time.

4.2.7 *In situ* video survey

The fish farm has a higher content of settled organic waste covering some of the natural substances shown as brown mud covering rocks and shell sand, together with high particle content with high sinking rate in water column. As opposed to the control site with no visual accumulation of fish farm soured organic waste, due to absence of brown mud covering rocks and seabed, and good water quality shown as good visibility. However, the concentrations of organic waste from fish feed and feces covering the seabed below the fish farm were lower than expected. The presence of abundant amounts of pelagic and benthic fishes, and other benthic organisms shows that the excess food source of fish farm waste provides with a feast. It appear that benthic organisms are able to remove settled organic matter before it accumulates to great amounts, which is very good, and shows a healthy management of the fish farm.

Benthic communities observed with use of video show a clear difference between the fish farm and control site, with frequent occurrence of detritus feeders at the fish farm and no observations of brown and green macroalgae, and opposite for the control site. *Beggiatoa* sp. was abundant on the seabed at the fish farm, shown as patches of white mats on top of seabed substrates. These usually occur under low levels of oxygen, as these bacteria use sulfate instead of oxygen for anaerobic respiration, and the product are hydrogen sulphide (H_2S). Production of H_2S has shown to be positively correlated with sedimentation (Dahlbäck and Gunnarsson 1981). Very high levels of hydrogen sulphide over time on the seabed are a risk for the health of Atlantic salmon (Braaten 1983). The importance of this compared with low organic accumulation is hard to know whether of these tells the best story regarding benthic health state, since they provides with opposite information.

Comparing the video survey at Måsøval with the control site (Hellskjære) should do with carefulness due to different bottom substrates and water depth. However, the video survey was a quick and easy method to get a good overview of the benthic health state below the farm, which is clear affected, but did not appear heavily polluted.

4.2.8 Organic loading

Allowed area of influence is as much as 3000 m in diameter from one aquaculture farm (KILDE). Along the Norwegian coastline the fish farms are lying close to each other causing some affected areas to overlap against each other. This is important to take into consideration when putting up new farms, that they are not placed too close to each other making emissions affecting the natural ecosystems bigger than expected due to overlaps. Since salmon production in Norway are expected to increase by fivefold before 2050 (Olafsen et al 2012).

Guidelines for using MOM analyses today are carrying out as if a fish farm has bad environmental condition, production will continue until the farm has crossed the border of heavily polluted (NS 9410:16). My opinion here is that more should be done underway during production, and bad conditions should not be acceptable.

4.3 Challenges

Proper extraction of pigments in tissue samples was in some samples difficult, resulting in pigments still present in the tissue after filtration of the extract. In qualitative studies, this is not a problem when looking at the shapes of the absorbance (OD) spectra, but in quantitative studies this may pose a problem since pigment content per gram tissue will be underestimated.

Absorbance specter of phytoplankton was rough and a challenge to obtain maximum absorbance peaks in nm of wavelengths. That is due to low pigment concentration in the samples, and improvements can be done by filtrate more seawater on each filter. Higher pigment concentrations will also increase quality of HPLC separation and quantification. Absorbance specter of Whelk was also of low quality, but that is due to low pigment content in the tissue from nature. To get more concentrated extracts from Whelk more tissue and less organic solvent could be used, but then the risk of not be able to extract the tissue properly are present.

The studied salmon pen had a lice-skirt through the whole sampling period, reaching 10 m down the water column. This is affecting the local water current around the pen, and makes the organic waste sink straight downward first 10 m instead of following the natural more vertical water current. This might affect the uptake possibilities of feces and food spill of Blue mussels. The lice-skirt can also cause less dilution of organic wastes and more accumulation

to a smaller area at the seabed, since water current are strongest in surface waters and decreases down the water column, making feces and food spill sink directly downwards the first 10 m, instead of a more normal vertical transport following the natural water current.

Samplings of Whelk at Kolskjæra was originally intended to serve as a control, but it turned out that another fish farm was located in close distance, with a later feeding start. This is why only one of these samples is considered as controls. Sampling of Whelk was not easy to perform in close distance from the fish farm (minimum 400 m away). This makes it hard to distinguish between control individuals and possible affected individuals, and due to the long distance, only very high values of Astaxanthin released from the farm would reach that far.

When grab sampling hard bottom substrates, and small rocks preventing the grab to close properly resulted in different amount sediment collected in each replicate especially at the fish farm. This also shows that comparing the replicates should be avoided, and hence this is the reason for compiling the replicates in the result section. Hard bottom substrate may indicate relatively high water current, which is good for the organic waste to spread and dilute to bigger areas, keeping a healthier ecosystem surrounding the fish farm.

Topographical and hydrographical conditions should be better studied before the project started, to get a more strategically plan of the sampling locations and regime. The location used for grab sampling was a ridge in the bottom topography (Figure 3), which makes the location more exposure for water current, and less possibilities for organic matter to settle because particles tend to settle at the bottom of the slope or in holes on the seabed (NS 9410:2016). Grab sampling at the aquaculture site should therefore be taken directly downstream as done, but in addition samples should also be performed more to the west, at greater depth and at the bottom of the slope, with greater possibilities to find soft bottom substrates and more accumulated organic waste from the farm, and consequently a bigger chance of finding higher amounts of Astaxanthin on the seabed.

4.4 Future perspective

My recommendations for further surveys are to look more into if Astaxanthin can be used as a pigment/biomarker for organic pollution from fish farms, by being more specific relative to using organisms as biomarkers. Use benthic organisms eating organic wastes on the seabed, either species already found there, like Polychaeta or Sea cucumber, or by putting out

organisms in cages on the seabed. Organisms suited for using Astaxanthin as a biomarker are organisms eating detritus, due to more likely they are eating released food spill and feces. Consequently there is a chance that they also can be used in the future as benthic "cleaner" organisms. Whether the goal are to improve IMTA (integrated multi-trophic aquaculture) or mitigate for fish farm sourced organic wastes, the biggest problem lies on the seabed i.e. a great potential for IMTA. It will be important to develop threshold values for acceptable Astaxanthin content on the seabed or in organisms, as a measure of total organic loading released from a fish farm.

More use of combination strategies to develop better MOM-B and -C trend analysis, as *in situ* video survey and other new technology. For example other ROV mounted sensors, like Underwater Hyperspectral Imaging for a quick estimate of white sulfur mats, or organic waste coverage in combination with traditional incorporated methods like grab sampling. To get a quicker, easier and better insight in environmental health state of surrounding ecosystem to fish farms that provides with cheaper information of greater quality, and this thesis can be used in development of smarter ways of doing environmental monitoring.

More focus on mitigation solutions, like e.g. IMTA to remove redundant organic matter released from the fish farms. Polluted areas are not good, even if they are within the border of heavily polluted. Work for an enhanced knowledge-based science to develop good monitoring procedures and find good mitigation solutions for fish farm sourced organic loadings.

5. Conclusions

Astaxanthin concentration in Atlantic salmon muscle tissue are shown to increase with the size of the fish, with performed feeding program, and overfeeding are indicated by high values of Astaxanthin in feces. As Astaxanthin is an expensive feed ingredient, control and monitoring of this is of economic value of the fish farm. In addition, this bridges economy with ecology.

Water surface 31.5 m from the pen did not contain Astaxanthin, this shows a high sinking rate of food spill and feces. This is also shown by high particle concentration further down the water column seen with the use of video operated with ROV.

Astaxanthin or Astaxanthin-like pigments dominate in zooplankton, and its wild Atlantic salmon most important source of food, it shows the natural origin of the red color of muscle tissue.

No accumulation of high concentrations of Astastaxanthin in organisms used as bio-indicators close the fish farm was observed, because no abnormal high values of Astaxanthin observed in Blue mussel feed uptake or mantle, Whelk feed uptake or foot. It should further be discussed if Blue mussel is a suited organism in terms of removing organic wastes from fish farms (IMTA), as shown in this study it is food selective choosing phytoplankton over other organic waste, especially during summer months (our sampling period). To use organisms for removing organic waste in the sea surface it is important to take into consideration that the uptake of nutrients from the farm may be reduced when the pen wear a sealed lice-skirt reaching 10 m down from the surface. Whelk as bio-indicator collected 400-600 m away from the farm showed to be too far away to detect increased values of Astaxanthin from a pen with biomass of 201 419 kg fish, halfway through a production cycle.

Quick dilution of organic wastes to larger areas is present due to good water current at the examined site Måsøval. Shown as hard bottom substrates, and good quality of the seabed sediments when grab sampling minimum 50 m from the pen. Organisms that indicate heavily polluted benthic environment are absent, this shows that this site is not heavily polluted, but a more extensive study is needed to draw any conclusions at this topic. The grab sampling should also be done closer to the pen. Astaxanthin was detected in seabed sediments, but in low concentrations, and this shows that high accumulated amounts of Astaxanthin can possibly be revealed with an easy and quick method as spectrophotometer. Threshold values

for Astaxanthin concentration are needed to be developed before this can be a part of a monitoring program.

This farm site looks healthy because benthic macro and micro fauna seems to able to remove settled organic wastes before it accumulates too much, but on the other side it is very clear that the seabed below the pen is affected from organic waste, in terms of mats of white sulfur bacteria (*Beggiatoa sp.*), and visible presence of organic waste. The video survey was a quick and easy method to get a good overview of the benthic health state below the farm. The biggest problem regarding released organic waste is on the seabed, and better mitigation solution should be performed to reduce this.

Overall, the excess organic contribution to this ecosystem does not provide with noticeable detectable or accumulated amounts of Astaxanthin. This support that the studied fish farm has a healthy management during the first seven months of a total fourteen month production cycle, and a healthy ecosystem is an indication of a healthy Atlantic salmon and good economy for the industry. But, with an array of fish pens along the coast, the added flux of organic waste into the ecosystem may affect the whole ecosystem.

6. References

6.1 Litterature

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6.2 Standards

NS-EN ISO 19493 (2007) Water quality – Guidance on marine biological surveys of hard-substrate communities. (ISO 19493)

NS 9410 (2016) Environmental monitoring of benthic impact from marine fish farms.

6.3 Web pages

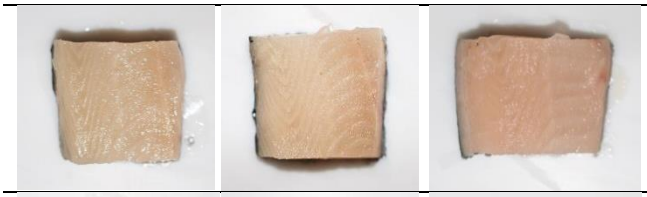
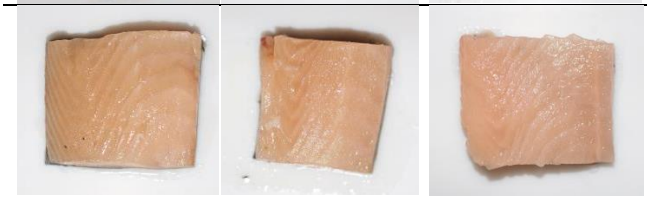
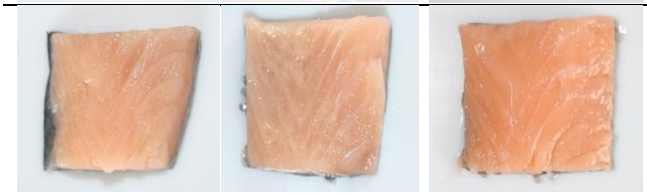
www.norgeskart.no

7. Appendces

Appendix 1. Whole individuals of *Salmo salar* replicates from each sample day with measured lenghts. n=3, replicate 1 to the left, no. 2 in the middle and no. 3 to the right.

			Date: 16.03.03 Lenght: 1: 29 cm 2: 36 cm 3: 29 cm
			16.04.12 1: 32cm 2: 29 cm 3: 30 cm
			16.05.03 1: 28 cm 2: 30 cm 3: 30 cm
			16.06.06 1: 39 cm 2: 33 cm 3: 39 cm
			16.07.04 1: 39 cm 2: 33 cm 3: 34 cm
			16.08.15 1: 42 cm 2: 43 cm 3: 44 cm
			16.09.19 1: 48.5 cm 2: 52 cm 3: 49 cm
			16.10.03 1: 50 cm 2: 62 cm 3: 54 cm

Appendix 2. Monthly pigmentation of *S. salar* muscle tissue, with measured weight. The same individuals as in appendix 1 (n=3), no. 1 to the left, no. 2 in the middle and no. 3 to the right. All pictures taken with the a opaque white plastic plate as reference in the back.

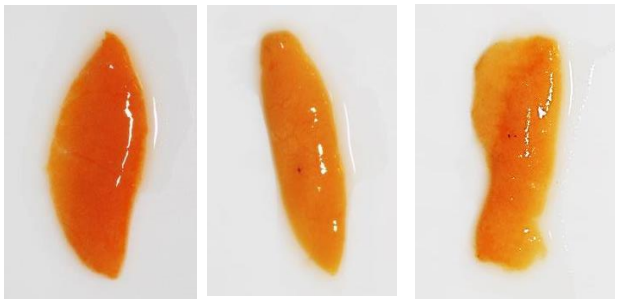
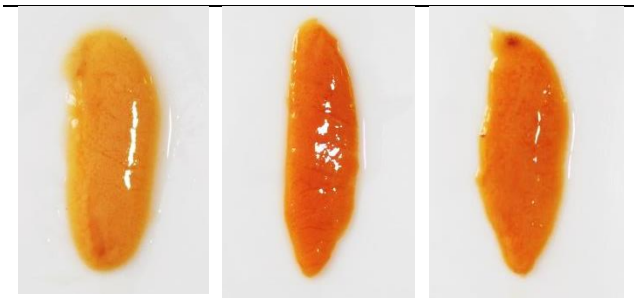
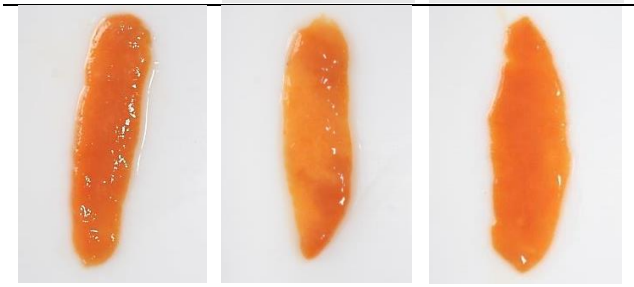
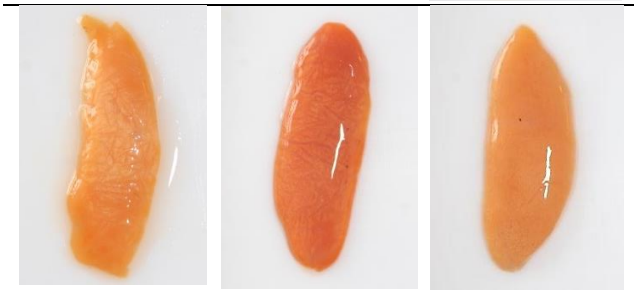
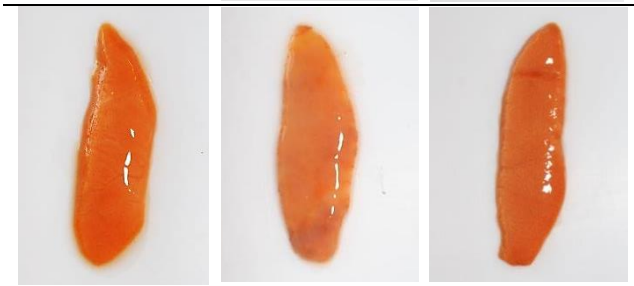
	Date: 16.03.03 Weight: 1: - 2: 0.39 kg 3: 0.27 kg
	16.04.12 1: 0.34 kg 2: 0.26 kg 3: 0.30 kg
	16.05.03 1: 0.25 kg 2: 0.29 kg 3: 0.31 kg
	16.06.06 1: 1.03 kg 2: 0.35 kg 3: 0.57 kg
	16.07.04 1: 0.60 kg 2: 0.36 kg 3: 0.37 kg
	16.08.15 1: 1.08 kg 2: 1.13 kg 3: 1.16 kg
	16.09.19 1: 1.61 kg 2: 2.19 kg 3: 1.83 kg
	16.10.03 1: 1.30 kg 2: 2.91 kg 3: 1.96 kg

Appendix 3: Phytoplankton HPLC measurements of quantitative pigment content as average (n=3), with standard deviation (± SD) and coefficient of variation in percentage (± CV%) for each pigment found at each sample site. To the left water samples from the fish farm. To the right water samples from control site.

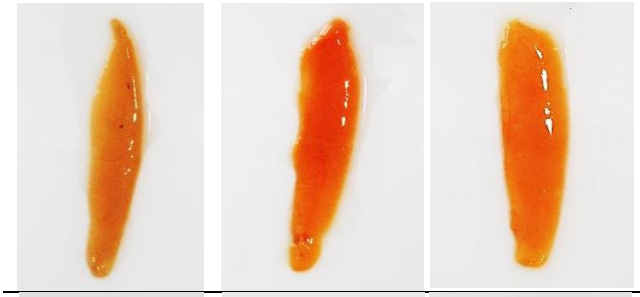
Pigment fish farm	3.4			3.8			3.31			4.27			6.8			8.15			10.4		
	Avg	SD	CV %	Avg	SD	CV %	Avg	SD	CV %	Avg	SD	CV %	Avg	SD	CV %	Avg	SD	CV %	Avg	SD	CV %
Chl c3	0.01	0.00	5.78	0.01	0.00	13.13	0.04	0.00	5.49	0.01	0.01	105.57	0.05	0.01	24.09	0.15	0.03	20.97	0.03	0.00	6.74
Chl c2+1	0.02	0.00	2.86	0.03	0.00	2.60	0.16	0.00	1.16	0.10	0.03	32.84	0.13	0.01	5.47	0.24	0.02	6.24	0.10	0.01	9.52
Peridinin										0.08	0.01	6.82				0.11	0.00	2.60			
Fucoxanthin	0.03	0.00	5.24	0.04	0.00	7.12	0.23	0.01	2.90	0.03	0.00	15.99	0.12	0.01	7.99	0.11	0.01	6.61	0.05	0.01	14.26
Prasinoxanthin																0.01	0.01	173.21	0.02	0.00	13.71
Hex-kfuco (Hex-fuco)													0.04	0.00	6.99	0.07	0.00	6.61	0.03	0.00	11.81
Diadinoxanthin							0.02	0.00	5.34				0.04	0.01	16.40	0.05	0.00	3.40			
Zeaxanthin																0.02	0.02	86.60	0.01	0.00	25.44
Diatoxanthin										0.03	0.00	14.18	0.03	0.00	15.64	0.04	0.00	4.63	0.04	0.01	15.89
Lutein																					
Chl b	0.01	0.00	6.77	0.02	0.00	4.14	0.04	0.00	8.26	0.10	0.00	3.34	0.07	0.00	1.77	0.20	0.06	33.07	0.07	0.01	15.81
Chl a	0.14	0.01	6.92	0.16	0.01	6.00	0.62	0.02	3.94	0.44	0.03	7.46	0.56	0.02	3.27	0.94	0.05	5.37	0.44	0.07	16.08

Pigment control site	3.4			3.8			3.31			4.27			6.8			8.15			10.4		
	Avg	SD	CV %	Avg	SD	CV %	Avg	SD	CV %	Avg	SD	CV %	Avg	SD	CV %	Avg	SD	CV %	Avg	SD	CV %
Chl c3	0.01	0.00	15.33	0.01	0.00	14.83	0.04	0.00	2.61				0.05	0.00	5.97				0.04	0.00	6.23
Chl c2+1	0.02	0.00	7.60	0.03	0.00	11.54	0.09	0.01	6.20	0.07	0.01	8.33	0.09	0.00	3.05	0.08	0.01	7.09	0.18	0.01	7.90
Peridinin																0.03	0.03	86.72			
Fucoxanthin	0.04	0.00	5.14	0.04	0.00	8.47	0.12	0.01	4.37	0.07	0.01	10.95	0.08	0.00	3.21	0.07	0.01	12.00	0.08	0.00	4.12
Prasinoxanthin																0.03	0.00	3.11	0.03	0.00	3.25
Hex-kfuco (Hex-fuco)													0.04	0.00	0.84				0.03	0.00	7.75
Diadinoxanthin													0.03	0.00	0.53	0.02	0.00	4.49	0.02	0.00	5.57
Zeaxanthin																0.03	0.03	86.89	0.02	0.00	4.39
Diatoxanthin										0.01	0.01	86.64				0.02	0.00	12.14	0.08	0.00	4.74
Lutein																			0.01	0.00	15.83
Chl b	0.02	0.00	7.44	0.03	0.00	9.47	0.05	0.00	4.32	0.05	0.00	4.68	0.02	0.03	141.42	0.16	0.01	4.02	0.14	0.01	8.86
Chl a	0.18	0.01	5.70	0.20	0.02	10.95	0.40	0.03	6.63	0.33	0.06	17.41	0.43	0.00	0.70	0.52	0.09	16.63	0.83	0.07	8.59

Appendix 4: Monthly pigmentation of blue mussel mantel collected at the fish farm, from March to October. Date collected, and lenght of whole mussels marked to the right (n=3). Replicate no. 1 to the left, no. 2 in the middle and no. 3 to the right. All pictures taken with the same opaque whte plastc plate as refernce in the back.

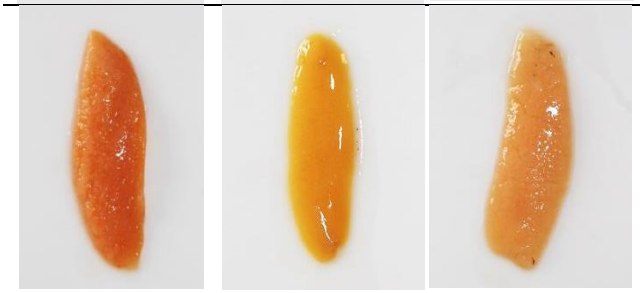
	Date: 16.03.31 Lenght: 1: 6.3 cm 2: 6.6 cm 3: 7.5 cm
	16.04.27 1: 6.4 cm 2: 7.0 cm 3: 6.9 cm
	16.06.08 1: 7.2 cm 2: 7.1 cm 3: 7.2 cm
	16.08.15 1: 6.5 cm 2: 7.0 cm 3: 7.5 cm
	16.10.04 1: 6.5 cm 2: 6.2 cm 3: 8.4 cm

Appendix 5: Monthly pigmentation of blue mussel mantel collected at the control site, from March to October. Date collected, and lenght of whole mussels marked to the right (n=3). Replicate no. 1 to the left, no. 2 in the middle and no. 3 to the right. All pictures taken with the same opaque white plastc plate as reference in the back.



Date:
16.03.31

Lenght:
1: 6.5 cm
2: 6.5 cm
3: 5.8 cm

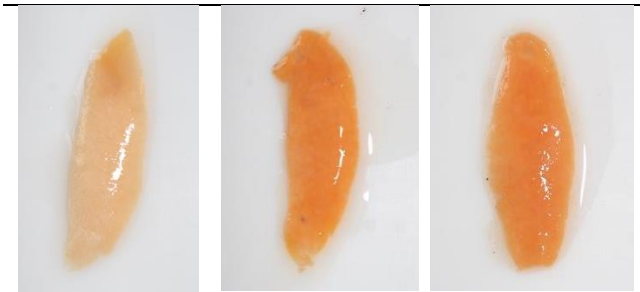


16.04.27

1: 7.6 cm
2: 6.5 cm
3: 7.7 cm

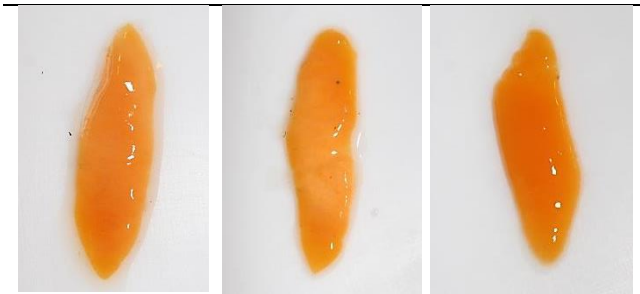
16.06.08

1: 7.1 cm
2: 7.0 cm
3: 7.6 cm



16.08.15

1: 6.4 cm
2: 6.7 cm
3: 6.0 cm



16.10.04

1: 6.2 cm
2: 6.5 cm
3: 5.6 cm

Appendix 6: Benthic fauna species list from grab sampling at Måsøval (n=2) and Hellskjæret (n=2) found at each location and replicate. Equality calculations are shown on the left side of the table.

		Måsøval		Hellskjæret		Equality		
Organisms	**Number**	**Grab I, 150m**	**Grab II, 100m**	**I**	**II**	**Måsøval**	**Hellskjæret**	**Måsøval+Hellskjæret**
Ampharetidae	1		1					
Arctica islandica	1	1						
Arenicola marina	1			1				
Buccinum sp.	1	1	1			1		
Bryozoa	1				1			
Capitellidae	1				1			
Cerebratulus marginatus	1	1						
Chaetopterus sp.	1				1			
Circimphalus casina	1			1				
Cirratulidae	1	1	1			1		
Crisia sp.	1			1				
Echinus elegans	1	1						
Flabelligeridae	1	1						
Galathea intermedia	1	1						
Galathea strigosa	1	1						
Gari sp.	1				1			
Gibbula sp.	1	1						
Glyceridae	1	1			1			1
Hexacorallia	1				1			
Hydroidus norvegica	1	1						
Lacuna sp.	1			1				
Leptochiton asellus	1	1						
Lucinoma borealis	1	1		1	1		1	1
Macropipus sp.	1	1						
Membranipora membranacea	1			1				
Munidopsis serricornis	1			1				
Nassarius incrassatus	1	1						
Nemertea	1		1					
Nephtys sp.	1	1						
Opheliidae acuminata	1	1						
Ophiotrichidae	1			1				
Ophiura albida	1	1						
Ophiuroidea	1				1			
Orbiniidae	1	1	1	1		1		1
Paguridae	1	1	1	1		1		1
Pherusa sp.	1		1					
Phylldocidae	1		1					
Polynoidae	1	1						
Polyplacophora sp.	1		1	1				1
Pomatoceros triqueter	1	1			1			1
Priapulus caudatus	1	1						
Sabellidae	1	1	1	1		1		1
Scalibregma inflatum	1	1						
Serpula vermicularis	1			1	1		1	
Sphaerodoridae	1	1						
Spirorbis sp.	1			1	1		1	
Strongylocentrotus sp.	1	1						
Syllidae	1	1						
Terrebellidae	1	1			1			1
Thyasira sp.	1	1			1			1
Trivia arctica	1			1				
Total #	51	30	10	15	13	5	3	9
Maximum number equality						40	28	51
% equality						12.50	10.71	17.65

Appendix 7: Sediment HPLC measurements of quantitative pigment content as average (n=3), with standard deviation (± SD) and coefficient of variation in percentage (± CV%) for each pigment found at each sample site.

Pigment	150 m			100 m			50 m I			50 m II			Control I			Control II		
	Avg	SD	CV %	Avg	SD	CV %	Avg	SD	CV %	Avg	SD	CV %	Avg	SD	CV %	Avg	SD	CV %
Chl c3				0.10	0.08	87.07	0.28	0.05	16.67	0.39	0.09	22.83						
Chl c2+1	0.05	0.04	86.74	0.52	0.15	28.40	1.01	0.39	38.57	1.33	0.22	16.86				0.03	0.04	173.21
Pheophorbid a	0.40	0.11	27.37	0.91	0.13	14.04	0.67	0.03	5.04	2.30	0.13	5.70	0.50	0.11	21.12	0.10	0.17	173.21
19'Butanoyloxyfucoxanthin	0.12	0.10	88.68	0.05	0.08	173.21												
Fucoxanthin	0.23	0.10	42.87	1.53	0.52	34.18	2.59	1.09	42.03	3.38	0.64	18.92	0.28	0.03	11.17	0.10	0.06	55.94
Prasinoxanthin	0.25	0.04	14.41	0.12	0.01	9.52	0.11	0.00	1.26	0.39	0.03	7.03	0.21	0.01	3.85	0.09	0.02	27.41
Astaxanthin	0.47	0.06	11.83	0.27	0.03	10.06	0.34	0.11	33.81	1.23	0.17	13.46	0.32	0.01	4.28	0.14	0.03	18.42
Hex-kfuco (Hex-fuco)	0.07	0.07	96.85	0.03	0.03	86.75				0.15	0.03	21.14	0.06	0.05	86.84			
Zeaxanthin	0.40	0.02	5.95	0.23	0.00	1.50	0.18	0.03	17.85	0.68	0.09	13.50	0.25	0.01	3.27	0.10	0.01	11.73
Diatoxanthin				0.10	0.02	20.83	0.09	0.01	16.22	0.46	0.07	16.11	0.19	0.02	11.15	0.07	0.00	5.50
Lutein	1.55	0.07	4.69	0.31	0.01	4.66	0.30	0.04	13.46	1.02	0.19	18.70	0.32	0.03	8.01	0.13	0.02	14.90
Chl b	0.24	0.41	173.21	0.36	0.12	34.99	0.43	0.14	32.17	1.06	0.47	44.23						
Chl a	0.63	0.34	52.88	3.05	0.84	27.48	5.36	2.29	42.66	6.98	1.26	18.11	0.30	0.02	7.31	0.27	0.10	35.86
Pheophytin a-like										0.46	0.16	34.65						
β,ε-Carotene	0.51	0.08	15.33							0.12	0.11	87.35	0.11	0.00	2.18			
β,β-Carotene	0.65	0.11	16.85	0.38	0.09	23.42	0.21	0.05	22.23	0.55	0.05	8.59	0.37	0.03	8.52	0.19	0.04	21.53