Metabolomics as a Tool to Evaluate Salmonid Response to Alternative Feed Ingredients

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Abstract

Aquaculture has largely expanded in the last decades to satisfy the growing market demands for fish products. Fishmeal and fish oil, which are traditionally used in salmonid feeds, are becoming unsustainable. Development of aquafeeds based on alternative ingredients are needed to overcome the ecological challenges. Importantly, when using the new diets, fish growth performance, fish health and food quality need to be considered. In the thesis, different substitutes of fishmeal and fish oil were evaluated by using NMR and MS-based metabolomics.

The Baltic Sea is one of the most threatened water bodies and has environmental problems, such as contamination and eutrophication. The use of Baltic Sea-sourced nutrients after certain treatments in fish feeds would recycle the less valuable nutrients for human back into the food chain, and may promote an environmental-friendly aquaculture system.

In the thesis, we found that use of detoxified fishmeal and fish oil reduced adverse effects on fish health related to energy metabolism and hepatotoxicity, compared with the untreated diets. Moreover, the decontaminated fish materials containing high content of n-3 fatty acids were found to be valuable sources of fish feeds. Additionally, a new Baltic blend diet composed of Baltic Sea-sourced decontaminated fishmeal, blue mussel and baker's yeast was fed to Arctic char (*Salvelinus alpinus*) for 10 months. Based on the metabolomics results, the dietary content of betaine, trimethylamine-N-oxide and aromatic amino acids needs to be modified, in order to achieve a better growth performance. The hepatic metabolic heterogeneity of salmonids was also observed in the thesis.

Furthermore, the sphingolipids in salmonids skin were characterized for the first time. We found that reduction in dietary levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) changed the fatty acid composition in glycerol-phospholipids subclasses and sphingolipid composition in skin of Atlantic salmon (*Salmo salar*). These changes potentially disturb the barrier function of fish skin.

These findings provide new information on application of metabolomics in development of alternative aquafeeds.

Keywords: Baltic Sea, ceramide, DHA, EPA, fatty acids, fish metabolism, glycerol-phospholipids, *Mytilus edulis*, *Saccharomyces cerevisiae*, sphingolipids

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Dedication

To my parents

To learn without thinking is blindness; to think without learning is idleness.
学而不思则罔，思而不学则殆。
Confucius

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Cheng, K.***, Wagner, L., Pickova, J., & Moazzami, A.A. (2016). NMR-based metabolomics reveals compartmental metabolic heterogeneity in liver of Arctic char (*Salvelinus alpinus*). *Canadian Journal of Zoology*, 94(9), pp. 665-669.

II. **Cheng, K.***, Wagner, L., Moazzami, A.A., Gómez-Requeni, P., Schiller Vestergren, A., Brännäs, E., Pickova, J., & Trattner, S. (2016). Decontaminated fishmeal and fish oil from the Baltic Sea are promising feed sources for Arctic char (*Salvelinus alpinus* L.) – Studies of flesh lipid quality and metabolic profile. *European Journal of Lipid Science and Technology*, 118(6), pp. 862-873.

III. **Cheng, K.***, Müllner, E., Moazzami, A.A., Carlberg, H., Brännäs, E., & Pickova, J. Metabolomics approach to evaluate a Baltic Sea-sourced diet for cultured Arctic char (*Salvelinus alpinus* L.). (submitted)

IV. **Cheng, K.***, Mira, M.B., Du, L., Ruyter, B., Moazzami, A.A., Ehtesham, E., Venegas, C., & Pickova, J. Reducing dietary levels of EPA and DHA have major impacts on the composition of different skin membrane lipid classes of Atlantic salmon (*Salmo salar* L.). (manuscript)

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* Corresponding author.

The author contributed to the following publications during her PhD studies which were not included in the thesis:

- Carlberg, H., **Cheng, K**., Lundh, T., & Brännäs E.* (2015). Using self-selection to evaluate the acceptance of a new diet formulation by farmed fish. *Applied Animal Behaviour Science*, 171, pp. 226-232.

- Carlberg, H.*, Brännäs, E., Lundh, T., Pickova, J., **Cheng, K.**, Trattner, S., & Kiessling, A. (2014). Performance of Arctic char (*Salvelinus alpinus*) fed with Baltic Sea-sourced ingredients. *Reports of Aquabest project* 10/2014.

- Carlberg, H., Lundh, T., **Cheng, K.**, Pickova, J., Langton, M., Gutiérrez, J.L.V., Kiessling, A., & Brännäs, E.* In search for protein sources: evaluating an alternative to the traditional fish feed for Arctic char (*Salvelinus alpinus* L.). (Under reviewed by *Aquaculture*)

- **Cheng, K.***[#], Carlberg, H.[#], Brännäs, E., Lundh, T., Kiessling, A., & Pickova, J. Family effects on growth performance and flesh lipid quality of Arctic char (*Salvelinus alpinus* L.) fed an alternative feed derived from the Baltic Sea region. ([#] equal contribution; manuscript)

The contribution of Ken Cheng to the papers included in this thesis was as follows:

I. Participated in planning of the work together with the supervisors. Was mainly responsible for the analytical work, the evaluation of results and manuscript writing.

II. Performed most of the analytical work, including lipid and metabolomics analysis in muscle, and hepatic gene expression analysis. Was responsible for the evaluation of results and manuscript writing.

III. Participated in sample collection and planning of laboratory work together with the supervisors and co-authors. Was responsible for the experimental work, the evaluation of results and manuscript writing.

IV. Participated in method evaluation of sphingolipidomics analysis together with supervisors and co-authors. Was responsible for planning of the work, the sphingolipidomics analysis, the evaluation of results and manuscript writing.

Abbreviations

AA	arachidonic acid
ALA	α-linolenic acid
CC	commercial-type control diet
Cer	ceramide
CFM	crude fishmeal
CFO	crude fish oil
CV-ANOVA	cross-validation ANOVA
DFM	defatted fishmeal
DHA	docosahexaenoic acid
EFSA	European Food Safety Agency
EPA	eicosapentaenoic acid
EPA+DHA	1:1 mixture of EPA and DHA
ESI-QTOF	electrospray ionization-quadropole/time of flight
FA	fatty acids
FAS	fatty acid synthase
FDR	false discovery rate
GC-FID	gas chromatogram-flame ionization detector
GHR	growth hormone receptor
GlcCer	glucosyl-ceramide
GPL	glycerol-phospholipids
GSH	glutathione
HUFA	highly unsaturated fatty acids
IGF	insulin like growth factors
K-factor	Fulton's condition factor
LA	linoleic acid
LC-PUFA	long-chain polyunsaturated fatty acids
MS	mass spectrometry
MUFA	mono-unsaturated fatty acids
NC	negative control diet

NMR	nuclear magnetic resonance
OPLS-DA	orthogonal partial least squares-discriminant analysis
P_1–P_4	part 1−4
PC	phosphatidylcholine
PCA	principal components analysis
PE	phosphatidylethanolamine
PI	phosphatidylinositol
POP	persistent organic pollutants
PPARs	peroxisome proliferator-activated receptors
PS	phosphatidylserine
RT-PCR	reverse transcription-polymerase chain reaction
Sa	sphinganine
SAFA	saturated fatty acids
So	sphingosine
SPFO	semi-purified fish oil
Sph	sphingomyelin
SREBP-1	sterol-regulator element-binding protein-1
TAG	triacylglyceride
TCA	tricarboxylic acid
TLC	thin layer chromatography
TMAO	trimethylamine-N-oxide
TSP-d_4	sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuterio propionate
VIP	variable importance for the projection

1 Introduction

1.1 Aquaculture and aquafeeds

Fish are supplied by wild capture fisheries and aquaculture, and converted into human food or non-food uses, such as fishmeal and fish oil for feeds (Figure 1). World fish consumption per capita increased from an average of 9.9 kg in the 1960s to 20 kg in 2014, and the increasing trend is expected to continue (FAO, 2016). Since the fisheries production has been fairly constant during the last decades, aquaculture has had to adapt to enable this increased production. Global aquaculture production increased to about 74 million tonnes in 2014, which corresponded to almost half of the aquatic food consumed by human (FAO, 2016).

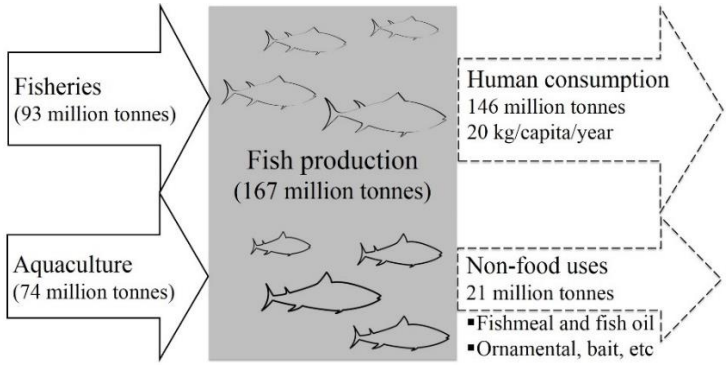

Figure 1. World fish production and utilization (FAO, 2016).

The proportion of fish used for direct human consumption has increased significantly in the last decades, from 67% in the 1960s to 87% in 2014. The remaining fish were destined for non-food uses, of which 76% was used to

produce fishmeal and fish oil (FAO, 2016). Fishmeal is a brown flour obtained by cooking, pressing, drying and milling whole fish and fish residues in fish processing, whereas fish oil is a brown or yellow liquid obtained in the process of pressing. Fishmeal usually contains 60−72% protein and 5−12% lipid, and fish oil is a good source of n-3 long-chain polyunsaturated fatty acids (LC-PUFA), especially eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) (Shepherd & Jackson, 2013). Fishmeal and fish oil are considered to be the most suitable ingredients for farmed-fish feeds based on their nutritional values.

Aquaculture is the largest consumer of fishmeal and fish oil (Figure 2), using about 68% of the total global fishmeal production and 74% of the total global fish oil production in 2012 (Tacon & Metian, 2015). With the growth of aquaculture production, the total consumption of fishmeal and fish oil in the aquaculture sector increased simultaneously (Naylor *et al.*, 2009; Tacon & Metian, 2008). Thus, the inclusion levels of fishmeal and fish oil in aquafeeds have decreased during the last decade (Shepherd & Jackson, 2013). For example, fishmeal inclusion rates in feeds for farmed Atlantic salmon (*Salmo salar*) decreased from >50% in 1995 to <30% in 2010 (Shepherd & Jackson, 2013). Much research has focused on reducing the dietary reliance on fishmeal and fish oil by using alternative ingredients in aquafeeds.

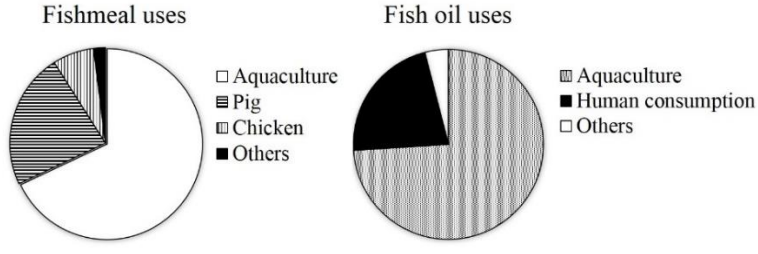

Figure 2. World utilization of fishmeal and fish oil by market in 2012 (adapted from Tacon & Metian, 2015).

1.2 Alternative feed sources

Selections of alternative feed ingredients are usually based on fish growth performance, health and welfare, final product quality, consumer acceptance, human health benefits and ecosystem stress. Competitive price is also an important characteristic for the selection of aquafeed substitutes (Tacon & Metian, 2015; Naylor *et al.*, 2009; Thompson *et al.*, 2008).

Plant-derived proteins have been widely used as alternatives for fishmeal in fish feeds, such as soy protein concentrate and wheat gluten, which both possess

high protein content and favourable amino acid profile (Thompson *et al.*, 2008; Gatlin *et al.*, 2007; Kaushik *et al.*, 1995). Compared with fishmeal, vegetable-based alternatives contain more compounds that are indigestible and anti-nutritional factors, such as fibre, insoluble carbohydrate, phytate, saponins and phytoestrogens, which leads to low nutrient digestibility and low palatability (Naylor *et al.*, 2009). Further improvements in using plant-based protein sources in fish feeds are needed, for example, by developing new plant products with balanced essential nutrients, selective fish breeding and dietary manipulation by exogenous enzyme treatment (Naylor *et al.*, 2009).

Other important substitutes of fishmeal are animal-derived proteins, such as seafood by-products, terrestrial animal meat, feather meal, bone meal and blood meal. Animal by-products have a more complete amino acid profile and higher digestibility than plant proteins. However, there are constraints on using animal products in aquafeeds due to a large variation in product quality, consumer acceptance, risk of disease transmission and legislation restrictions in EU (Naylor *et al.*, 2009; Thompson *et al.*, 2008).

Single-cell organisms such as fungi and bacteria have been successfully used in aquafeeds as protein sources. For instance, zygomycete fungi (*Rhizopus oryzea*) is rich in protein and has similar amino acid profile as fishmeal (Vidakovic *et al.*, 2015; Olsen, 2011). Single-cell biomass can be produced on human waste sources, but the high cost and small-scale production currently constrain their use in aquafeeds (Naylor *et al.*, 2009).

Furthermore, n-3 LC-PUFA, especially EPA and DHA are essential fatty acids (FA) for fish growth and health (Ruyter *et al.*, 2000d; Ruyter *et al.*, 2000c). Usually, terrestrial plant-derived oils contain high concentrations of n-6 and n-9 FA, such as linoleic acid (LA; 18:2n-6) and oleic acid (18:1n-9), and terrestrial animal-derived oils high in saturated FA and cholesterol. However, both plant oil and animal oil almost lack EPA and DHA, and have a relatively high ratio of n-6/n-3 FA (Turchini *et al.*, 2009; Pickova & Mørkøre, 2007). Compared with fish oil, plant and animal oils have the advantages on price and sustainability. As a result, blending them with fish oil is a commonly used procedure in formulating fish diets.

Among other alternatives, genetically modified plant oils, marine sourced lipids, such as krill oil and microalgae oil which contain high content of EPA and DHA, were found to be promising substitutes for fish oil in some studies. So far these alternatives do not offer an economically and ecologically sustainable solution for fish farming (Olsen, 2011; Sissener *et al.*, 2011; Pickova & Mørkøre, 2007).

1.2.1 Decontaminated fishmeal and fish oil from the Baltic Sea

The Baltic Sea is a unique brackish, shallow and cold environment on the planet. Its only connection to the Atlantic Ocean is through the narrow Öresund strait, which limits water exchange between the two water bodies, and makes the Baltic Sea particularly low saline and sensitive to pollutants. Today, the Baltic Sea is one of the world's most threatened ecosystems, due to land-based human activity leading to industrial and municipal waste (Allsopp *et al.*, 2001). Contaminants, particularly organic pollutants can accumulate in fatty fish and harm fish and mammals health after consumption. Reportedly, consumption of some pollutants, such as persistent organic pollutants (POP) and heavy metals, leads to metabolic disorders and diseases in fish and mammals (Kokushi *et al.*, 2012; Ibrahim *et al.*, 2011; Ruzzin *et al.*, 2010). Therefore, it is recommended that direct consumption of fatty Baltic fish is limited (www.slv.se), even though the level of POP has decreased significantly in the recent decades.

Using the decontaminated fish raw materials from the Baltic Sea in fish feeds could be a strategy to increase the sustainability of aquaculture. It was previously reported that Atlantic salmon fed decontaminated fish oil containing less POP did not show apparent negative effects on growth performance, feed conversion ratio and fillet quality parameters (Lock *et al.*, 2011; Olli *et al.*, 2010). Fish after feeding with decontaminated fish raw materials contained low levels of POP in fish fillet (Sprague *et al.*, 2010). Thus, after a process of decontamination, pelagic fatty fish from the Baltic Sea could be valuable sources of ingredients for aquafeeds.

1.2.2 Blue mussel from the Baltic Sea

Another severe and widespread environmental threat to the Baltic Sea is eutrophication, which is the overload of nutrients in water bodies and can threaten water biodiversity and ecological balance. The main excess of nutrients causing eutrophication are nitrogen and phosphorus, mostly coming from agricultural run-off (Lindahl *et al.*, 2005).

Blue mussel (*Mytilus edulis*), a filter-feeding bivalve mollusc with excellent nutrient-binding capacity has been suggested for use to reduce nutrients in the eutrophic Baltic Sea (Lindahl, 2013). Due to the blue mussel's small size caused by low salinity and temperature, mussel growing in the Baltic Sea is less interesting for human consumption (Lindahl, 2013; Westerbom *et al.*, 2002). The non-food grade blue mussel has a high protein content and amino acid composition similar to fishmeal, particularly essential amino acids, such as methionine, cysteine and lysine for fish (Kikuchi & Sakaguchi, 1997; Berge & Austreng, 1989). It has been shown that use of blue mussel improved the

palatability of plant protein-based diets and growth of fish (Nagel *et al.*, 2014; Kikuchi & Furuta, 2009). De-shelled blue mussel was considered as a competitive alternative to fishmeal in pellet diets and offered good growth performance and nutrient digestibility (Langeland *et al.*, 2016; Vidakovic *et al.*, 2015).

1.2.3 Baker's yeast cultivated on non-food substrates

Baker's yeast (*Saccharomyces cerevisiae*) has been widely used in human food as fermenting agent since ancient times. It can be cultivated on hydrocarbons and their derivatives, inorganic nitrogen and even waste raw materials with a high reproductive rate (Kuhad *et al.*, 1997). However, due to the high levels of nucleic acids, microorganism products are not suitable for direct human consumption on large scale. The potential usage of microbial protein production in fish feeds has been discussed in several studies, since fish having high liver urate oxidase activity, can degrade nucleic acids without health impairments (Andersen *et al.*, 2006). Baker's yeast contains high values of nutrients, such as protein, with a similar amino acid profile to that of fishmeal except sulphur-containing amino acids, vitamin B, pigments and complex carbohydrate, such as β-glucans and mannan oligosaccharide. It was shown that supplementation of oligosaccharides derived from the cell walls of baker's yeast improved soybean-induced enteritis and diarrhoea-like condition in salmon (Rakers *et al.*, 2013). Based on growth performance and nutrients digestibility, intact baker's yeast was found to be a promising dietary protein source for Arctic char (*Salvelinus alpinus*) and carp fingerlings (*Cyprinus carpio*) when using up to 40% and 30% in their diets, respectively (Vidakovic *et al.*, 2015; Korkmaz & Cakirogullari, 2011).

1.2.4 Oil sources deficient in EPA and DHA

A reduction in levels of EPA and DHA in fish feed is unavoidable in the current aquaculture. It is important to know the minimum requirements of EPA and DHA for salmonids and the potential impacts of EPA and DHA deficiency on fish health. Poultry oil contains similar content of saturated FA to standard fish oil, but it is comparatively low in 18:2n-6 and 18:3n-3, and lacks EPA and DHA. The particular FA composition makes poultry oil very suitable for a study on EPA and DHA requirements. By replacing fishmeal with a combination of rapeseed oil and poultry oil, an oil with no EPA, no DHA and constant content of 18:3n-3 can be made.

There are many studies on the impacts of dietary EPA and DHA on fish growth, early development and FA composition in fish tissues (Thomassen *et al.*, 2016; Ruyter *et al.*, 2000a; Ruyter *et al.*, 2000b), but few studies considered the effects of reducing dietary EPA and DHA on fish skin health.

1.3 Metabolomics

Omics-based studies involving genomics, transcriptomics, proteomics and metabolomics explore from genotype to phenotype of organisms (Figure 3). The study of specific or globally occurring small molecule (<1500 Da) metabolites in an organism, tissue, or biological fluid, is termed metabolomics. Compared with the other omics approaches, metabolomics provides information on what is actually occurring on a metabolic and physiological level. Small molecules comprise a range of endogenous and exogenous chemicals, including carbohydrates, peptides, amino acids, nucleic acids, vitamins, minerals, organic acids, polyphenols, and alkaloids (Samuelsson & Larsson, 2008; Wishart, 2008; Nicholson *et al.*, 1999). Metabolomics usually aims to investigate the possible phenotypic changes caused by environmental variations or xenobiotic agents, for example, diseases, diets, and drugs (Goodacre, 2007). It has been widely used in many research areas, e.g. system biology, agronomy, human nutrition, clinical disease diagnostics, and pharmaceutical research (Alfaro & Young, 2016). During recent years, metabolomics analysis have also been applied in aquaculture, including hatchery production, animal nutrition and welfare, disease and quality control of seafood products (Alfaro & Young, 2016; Viant, 2007).

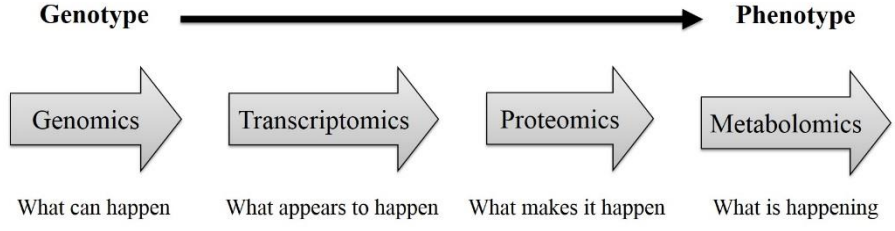

Figure 3. Diagram of omics-based studies (adapted from Alfaro & Young, 2016).

Liver is an important metabolomics centre where lipid and glycogen metabolism occurs, and has been widely used for metabolomics studies (Jang *et al.*, 2012; Kullgren *et al.*, 2010). Unlike the histological structure of mammalian liver, liver in salmonids have no any connective tissue septum or lobe architecture (Schär *et al.*, 1985). However, the content of glycogen and enzyme

activity were reported to be heterotopically distributed in liver of rainbow trout (*Oncorhynchus mykiss*). Thus, it has been suggested that metabolic profiles may vary in different sampling position, which would affect comparative metabolomics results.

1.3.1 Metabolomics analytical approaches

The widespread application of metabolomics in expanding research fields is attributed to the simultaneous development of advanced analytical approaches and multivariate statistical analysis. The most commonly used, high-throughput and high-resolution analytical platforms for metabolomics studies are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) coupled with chromatography. Both techniques are robust and reliable for measuring the metabolites in bio-samples, and are often used in combination because they have their own advantages (Young & Alfaro, 2016). For instance, NMR is a non-destructive, highly quantitative and reproducible technique, requiring minimal sample preparation. Nevertheless, NMR has relatively low sensitivity compared with MS-based measurements. The development of chromatographic methods coupled to MS detection has decreased the complexity of sample matrix, enhanced the sensitivity and selectivity of the analysis, and broadened the applicability of MS-based metabolomics approach. However, the process of sample purification and separation prior to the mass analyser makes MS-based analysis more time-consuming (Pan & Raftery, 2007).

Metabolomics dataset are usually very large and complex. Univariate methods cannot account for correlation between metabolites and it is difficult to detect group differences when only minor variations exist (Young & Alfaro, 2016). Furthermore, due to the high numbers of variables, univariate analysis needs to be corrected to account for multiple testing correction, for example, using false discovery rate (FDR) and Bonferroni correction (Noble, 2009). Multivariate analysis, such as principal components analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA), can reduce the complexity of datasets and clarify the relationships among metabolites and samples, and thus provide complementary information that helps data interpretation (Young & Alfaro, 2016; Trygg *et al.*, 2007).

1.3.2 Application of metabolomics in aquafeed evaluation

It is necessary to properly evaluate a new formulated fish feed which contains unusual ingredients before commercial usage, in order to assure fish health and welfare. Due to the complexity of biological responses, nutritional metabolomics

studies can get insights into metabolic changes in a holistic manner, and help us understand the complex interactions between nutrition, health and organism (Alfaro & Young, 2016; Goodacre, 2007). Currently, metabolomics-based approaches have been initially used in nutritional research in aquafeeds, such as to investigate the effects of food deprivation (Sheedy *et al.*, 2016; Kullgren *et al.*, 2010), nutrient supplementation (Wagner *et al.*, 2014; Cajka *et al.*, 2013), and dietary substitution of fishmeal and fish oil (Castro *et al.*, 2015; Jin *et al.*, 2015; Abro *et al.*, 2014; Schock *et al.*, 2012; Bankefors *et al.*, 2011) and dietary imbalance (Prathomya *et al.*, 2017; Maruhenda Egea *et al.*, 2015) on fish.

Metabolomics is usually helpful for generating some hypotheses on preliminary mechanism of metabolic changes by using biochemical pathways (Young & Alfaro, 2016). Usually, metabolites participate in several metabolic pathways which can influence their levels. Metabolomics in combination with studies on the expression of key genes involved in particular metabolic regulation and cellular signalling pathways may offer more ideas on responsible metabolic changes (Castro *et al.*, 2015; Jin *et al.*, 2015), such as fatty acid synthase (FAS), growth hormone receptor/insulin like growth factors (GHR/IGF) axis, peroxisome proliferator-activated receptors (PPARs) and sterol-regulator element-binding protein-1 (SREBP-1) (Chen *et al.*, 2013; Ruzzin *et al.*, 2010; Casals-Casas *et al.*, 2008; Castillo *et al.*, 2004).

1.4 Fish, a valuable source of LC-PUFA

Fish is important as human food providing high quality protein rich in essential amino acids, vitamins like A, D and B_{12}, and minerals including calcium, iodine, zinc, iron and selenium. In addition, fish is a valuable food source of LC-PUFA, particularly EPA and DHA for humans.

1.4.1 Importance of LC-PUFA on human health

LC-PUFA usually refers to the FA having ≥2 double bonds and ≥18 carbon chain length. They are not only essential nutrients for human, but also modulate and prevent certain diseases. EPA and DHA, which are also called n-3 highly unsaturated fatty acids (HUFA; ≥3 double bonds and ≥20 carbon chain length, Figure 4), are vital structural components of phospholipids, particularly in cellular membranes of brain and retina. Clinical intervention studies showed the beneficial effects of n-3 FA in improving cardiovascular disease, inflammation, and perhaps type 2 diabetes (Mozaffarian & Rimm, 2006; Connor, 2000). The n-6 FA, typically arachidonic acid (AA; 20:4n-6), are the precursors of

eicosanoids which function as signalling molecules and control body systems mainly in immunity and the nervous system.

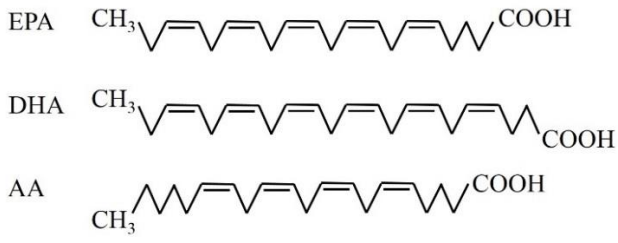

Figure 4. Chemical structure of n-3 highly unsaturated fatty acids, eicosapentaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (DHA; 22:6n-3) and arachidonic acid (AA; 20:4n-6).

A high intake of n-6 FA would shift the physiologic state to pro-thrombotic and pro-aggregatory status with increases in blood viscosity and vasoconstriction, and decreases in bleeding time (Simopoulos, 1999). Due to the increased dietary intake of n-6 FA, like animal meat, vegetable oils from corn, sunflower seeds, and soybean, humans consume higher proportions of n-6 FA, but lower n-3 FA. The ratio n-6/n-3 FA is nowadays close to 20-30:1 in western diets, instead of 1-2:1 in traditional diets. With the ratio increasing, the prevalence ratio of cardiovascular disease and type 2 diabetes is increasing (Simopoulos, 2002).

1.4.2 Recommendation on intake of LC-PUFA

Many authorities and organisations of nutrition and health have given dietary recommendations for the intake of EPA and DHA. The European Food Safety Agency (EFSA) has recommended daily intake of 250 mg/day EPA and DHA for adults (about 1−2 servings/week of oily fish) which appeared sufficient for primary prevention of cardiovascular diseases (EFSA Panel on Dietetic Products, Nutrition 2010). The American Heart Association recommends consumption of at least two servings of fish per week for the general population, taking 1 g/day of EPA and DHA for patients with documented coronary heart disease, and taking 2−4 g/day of EPA and DHA for patients needing triacylglyceride (TAG) lowering (Kris-Etherton *et al.*, 2002).

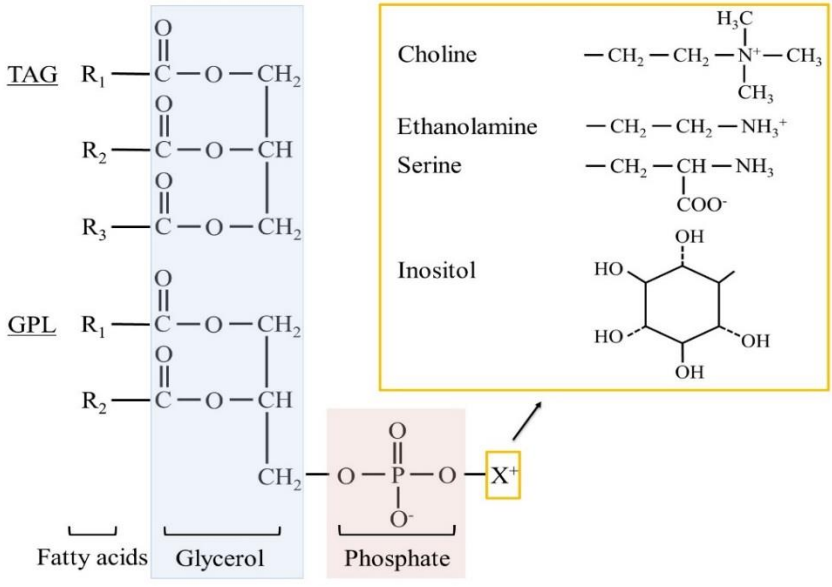

Figure 5. Chemical structure of neutral (triacylglycerol, TAG) and polar (glycerol-phospholipids, GPL) lipids.

1.5 Lipids and fatty acids in salmon

1.5.1 Lipids and sphingolipidomics in salmon

Lipids can be defined as compounds that are soluble in organic solvent and broadly are classified as neutral and polar lipids (Figure 5). Neutral lipids are completely soluble in non-polar solvents, including TAG, diacylglyceride and monoacylglyceride, wax esters, sterols, sterol esters and free FA, while polar lipids possess a wide range of solvent solubility based on their non-lipid head groups, such as glycerol-phospholipids (GPL), sphingolipids, sulpholipids and glycolipids (Sargent *et al.*, 2002).

TAG is the most abundant lipid class in fish lipids and play important roles, such as storage of energy, and maintaining reproduction, growth and health in fish. GPL including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) and sphingolipids such as sphingomyelin (Sph) are essential components of cell membranes to maintain the proper fluidity and functions of cell membrane, such as nutrients transportation, enzyme activity, and signal transduction (Bell & Koppe, 2010; Tocher *et al.*, 2008; Yang *et al.*, 2000).

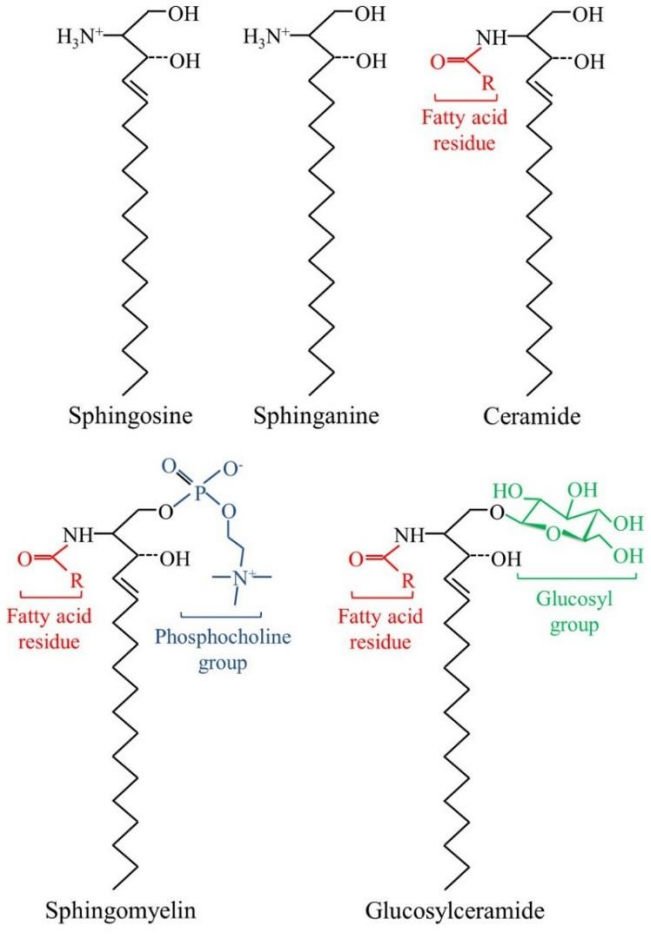

Figure 6. Chemical structure of sphingosine, sphinganine, ceramide, sphingomyeline and glucosylceramide.

Sphingolipids play a determinant role in water retention and permeability-barrier function in skin of terrestrial vertebrates, such as Sph, glucosyl-ceramide (GlcCer) and ceramide (Cer) (Kendall & Nicolaou, 2013; Feingold, 2007). Cer is composed of a sphingosine (So) or sphinganine (Sa) and a fatty acid (Figure 6), and can be produced by hydrolysis of Sph and GlcCer by acidic sphingomyelinase and β-glucocerebrosidase, respectively, or *de novo* synthesis from L-serine and palmitoyl CoA (Figure 7). Reportedly, essential FA deficiency led to an interruption in sphingolipids metabolism, thereafter causing abnormal epidermis function of permeability barrier in mammals (Feingold & Elias, 2014; Pullmannová *et al.*, 2014). In contrast to human skin, fish epidermis lacks of a keratinised layer and hairs, but has a mucous layer and bone tissues-

related scales (Rakers *et al.*, 2013). To the best of our knowledge, there is only one article that has measured the total content of Sph and GlcCer in fish skin (Duan *et al.*, 2010). The composition and function of sphingolipids in salmon skin are still unknown.

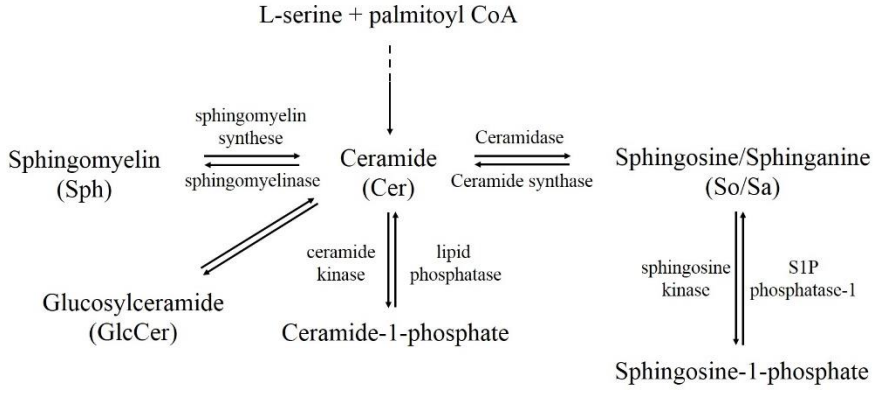

Figure 7. Main steps in the metabolic pathways involved in the sphingolipidomics (adapted from Kendall & Nicolaou, 2013).

1.5.2 Fatty acids and their metabolism in salmon

FA consist of a carbon chain with an aliphatic carboxylic acid at one end and a methyl group at the other (Figure 4). FA are designated on the basis of chain lengths, degree of unsaturation and the position of the double bonds. Based on the numbers of double bonds, FA are classified as saturated fatty acids (SAFA), mono-unsaturated fatty acids (MUFA) and PUFA. The FA in fish usually contain even numbers of carbon atoms in straight chains. The most important and abundant PUFA in fish are generally the n-3 series, such as α-linolenic acid (ALA; 18:3n-3), EPA and DHA, and n-6 series, such as LA and its metabolic product AA (Bell & Koppe, 2010).

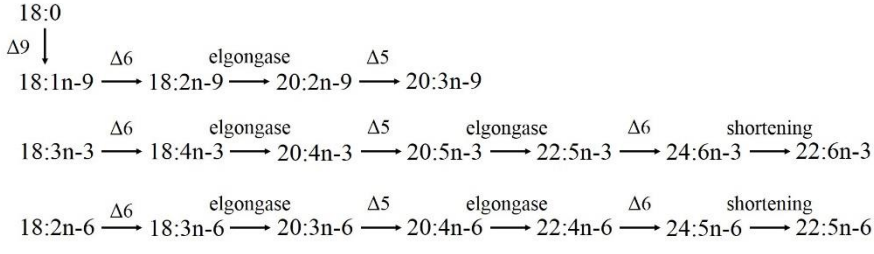

Figure 8. Elongation and desaturation (Δ5, Δ6 and Δ9) pathways of C18 FA to their long-chain polyunsaturated fatty acids (adapted from Bell & Koppe 2010).

The SAFA 16:0 and 18:0 can be synthesized *de novo* in fish by FA synthase, then MUFA 16:1n-9 and 18:1n-9 produced via Δ9-desaturase. However, fish lack desaturases to produce 18:2n-6 and 18:3n-3, which are thus regarded as essential FA and have to be obtained from food. Most fish, including freshwater and diadromous species, are able to convert 18:2n-6 and 18:3n-3 to HUFA, like 20:4n-6, 20:5n-3 and 22:6n-3 (Figure 8), but the endogenous synthesis of HUFA from PUFA is usually inefficient, particularly the production of 22:6n-3 (Bell & Koppe, 2010; Tocher, 2009; Sargent *et al.*, 2002).

2 Objectives

The overall aim of this thesis was to evaluate different alternative ingredients in feeds for Arctic char and Atlantic salmon, in order to reduce the reliance on fishmeal and fish oil traditionally used in aquaculture. The dietary effects on fish growth performance, lipid composition in tissues (white muscle, liver and skin), and metabolic profile in plasma and tissues (white muscle, liver and skin) were explored.

Specific objectives were to:

I. Investigate the intra-hepatic variation in metabolic profile of Arctic char by using ^{1}H NMR-based metabolomics.

II. Explore the effects of using decontaminated Baltic Sea-sourced fishmeal and fish oil in feed for Arctic char, by analysing lipid composition, metabolic responses in liver and muscle, and hepatic gene expression.

III. Evaluate a new fish feed containing mixture of Baltic Sea-sourced fishmeal, blue mussel and baker's yeast as protein sources for Arctic char by using ^{1}H NMR-based metabolomics.

IV. Study the impacts of reducing dietary EPA and DHA on FA profile in GPL subclasses and sphingolipidomics in skin of Atlantic salmon.

3 Materials and methods

A brief description of materials and methods used in the thesis are shown in this chapter. For more detailed description of each method, check the papers.

Table 1. *Summary of study design for Paper I-IV.*

Study	I	II	III	IV
Fish species	Arctic char	Arctic char	Arctic char	Atlantic salmon
Initial size *g* [a]	103.7±2.7	131.3±12.2	50.1±13.2	52.8±0.8
Final size *g* [b]	276.5±17.4	237.9±5.3	628.0±4.0	379.7±96.5
Trial duration	15 weeks	11 weeks	10 months	6 months
Treatment	Liver anatomical position: part 1−4	Diet [c]: Control DFM+SPFO DFM+CFO CFM+SPFO CFM+CFO	Diet [d]: Control Test	Diet [e]: NC 0.5, 1.0, 1.5, 2.0% EPA, DHA and EPA+DHA (1:1) CC
Tank number per treatment	3	1	3	2 or 3
Fish number per tank	12	10	495	70
Samples	liver	liver, muscle	liver, muscle, plasma	skin
Sample number per treatment	6	6 or 10	18 or 24	2 or 3 five-fish pooled samples
Analyses	Metabolomics	Metabolomics Gene expression Lipid content Fatty acid profile	Metabolomics	Sphingolipidomics Fatty acid profile in glycerol-phospholipid subclasses

[a] Mean ± SD.
[b] Mean ± SE.

[c] Control diet was purchased from Skretting, Norway; the Baltic Sea-sourced fish materials (CFM, crude fishmeal; CFO, crude fish oil; DFM, defatted fishmeal; SPFO, semi-purified fish oil) were from TripleNine, Denmark.

[d] Control diet was similar to the commercial feed for Arctic char; test diet was composed of Baltic Sea-sourced fishmeal, blue mussel and baker's yeast as protein sources. The diets were manufactured at Laukaa Aquaculture station, Finland.

[e] Negative control diet (NC) contains no EPA and no DHA; commercial-type control diet (CC) contains 2.2% eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) formulated by BioMar, Norway. Experimental diets were produced by Nofima, Norway. EPA+DHA, 1:1 mixture of EPA and DHA.

3.1 Experimental design

A summary of study design is presented in Table 1. Arctic char in Paper I−III were reared at Aquaculture Centre North in Kälarne, Sweden, while Atlantic salmon in Paper IV were reared at Nofima Institute in Sunndalsøra, Norway. Before handling, fish were anaesthetized using tricaine methamesulfonate. All the tissue and plasma samples were frozen immediately in liquid nitrogen and stored at −80°C until analysis.

The experiments followed the guidelines of the Animal Care and Welfare at the Swedish University of Agriculture Sciences (Paper I, II and III), EU Directive 2010/63/EU (Paper I) and Norwegian Ministry of Education and Research (Paper IV).

3.1.1 Paper I

Arctic char were distributed into three 700-L tanks (n=12 fish/tank) provided with a flow-through system (10 L/min, 5−17 °C). Fish were fed a fishmeal-based experimental diet close to a commercial fish feed for 15 weeks. Feeding was done *ad libitum* at a rate of 1% body weight per day using belt feeder.

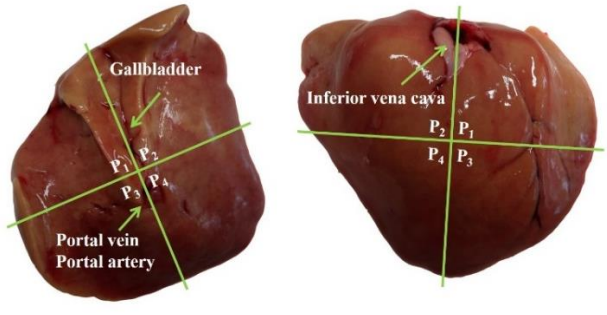

Figure 9. Schema for sampling of Arctic char liver in Paper I; P_1−P_4 part 1−4.

Fish were randomly selected from three tanks (n=2 fish/tank). Liver samples without gall bladder were cut into four parts along the direction of gall bladder

and at right angles to this, keeping the thicker and wider part of tissue upwards. According to the position, samples were named as part 1−4 (P_1−P_4; Figure 9).

3.1.2 Paper II

Arctic char were individually PIT tagged and randomly divided into five tanks (n=10 fish/tank) in a flow-through system at 10 °C. Fish were fed one commercial control or one of the four experimental diets formulated with Baltic Sea-sourced fish materials for 11 weeks. Defatted fishmeal (DFM) was produced by removing lipids from crude fishmeal (CFM) using organic solvent, while semi-purified fish oil (SPFO) was purified from crude fish oil (CFO) by activated carbon adsorption. The four experimental diets were DFM+SPFO, DFM+CFO, CFM+SPFO and CFM+CFO. The content of organic pollutants including polychlorinated biphenyls, polycyclic aromatic hydrocarbons, polybrominated diphenyl ethers were measured by an authority research centre.

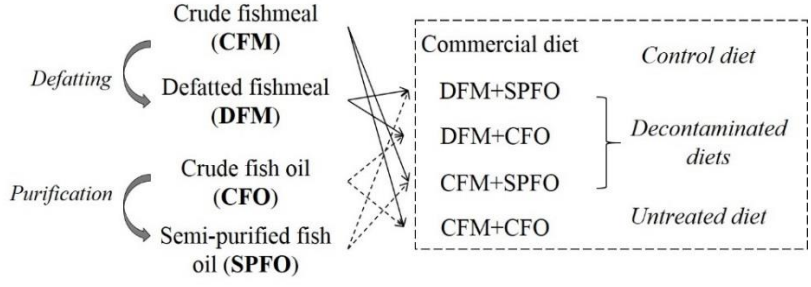

Figure 10. Schema of experimental design in Paper II.

At the end of the experimental trial, fish body weight and length were measured. White muscle and liver samples from 10 fish were dissected (Figure 10).

3.1.3 Paper III

Arctic char (n=2970) from Arctic superior strain in the Swedish breeding programme were individually PIT tagged. They were divided into six tanks (three tanks/diet; Figure 11) with 495 fish each. To acclimatise the environment, fish were fed a commercial diet (Skretting) for four months before the experimental trial.

The experimental feeding trial started by giving the 1:1 mixture of experimental and commercial feeds for one month, and fish were then fed the experimental diets for the remaining nine months. The water temperature ranged

from 1.2°C to 13.8°C. Fish thinning was separately conducted in May and September to ensure suitable biomass in tank and fish welfare.

In December, all fish in tanks were weighed and their length measured to calculate Fulton's condition factor (K-factor), [Body weight in g ÷ (Total body length in cm)^3 × 100]. Tissues (liver and white muscle) and plasma were sampled for metabolomics and lipid analyses.

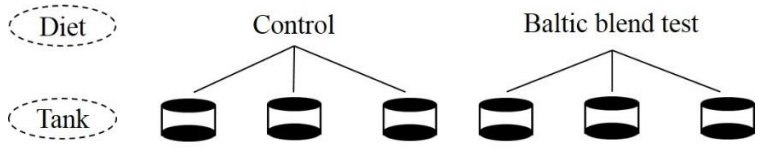

Figure 11. Schema of experimental design in Paper III

3.1.4 Paper IV

A commercial-type diet containing 2.2% EPA and DHA (1:1) was used as a control (CC), and a diet depleted in EPA and DHA was referred as negative control (NC). Different levels (0.5, 1.0, 1.5 and 2.0% of the feed dry weight) of EPA, DHA and 1:1 mixture of EPA and DHA (EPA+DHA) were formulated. Atlantic salmon were randomly divided into 33 tanks (n=70 fish/tank; 2 tanks/diet for the 0.5%, 1.0% and 1.5% EPA, DHA and EPA+DHA groups; 3 tanks/diet for the NC, CC and 2.0% EPA, DHA and EPA+DHA groups; Figure 12). All tanks were supplied with 15 L/min seawater at ambient temperature (6.3−13.8°C). Fish were fed a commercial diet (Skretting) prior to the experimental trial by using automatic disc feeders.

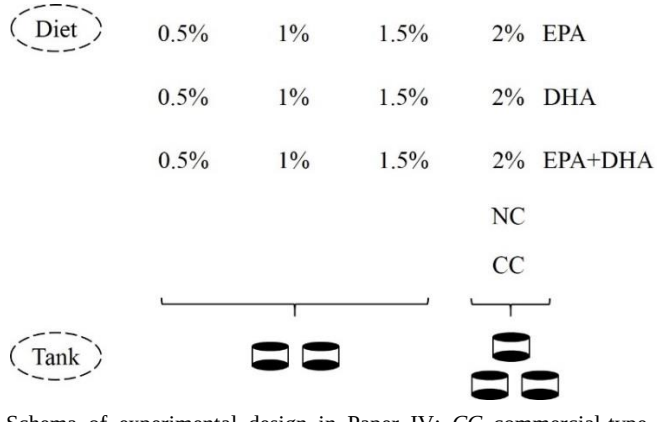

Figure 12. Schema of experimental design in Paper IV; *CC* commercial-type control, *DHA* docosahexaenoic acid, *EPA* eicosapentaenoic acid and *NC* negative control.

During the feeding trial, fish skin samples were taken twice, when average fish body weight reached 200 g and 400 g, respectively. Fish skin samples from five fish per tank were collected and pooled.

3.2 ^{1}H NMR-based metabolomics analysis

Metabolites in tissues (liver and white muscle; Paper I−III) and feeds (Paper III) were extracted using methanol:chloroform (2:1, *v/v*), as previously described (Wagner *et al.*, 2014; Moazzami *et al.*, 2011). The aqueous (polar) and chloroform (nonpolar) phases were collected separately. The aqueous phases were dried, mixed with 600 µL sodium phosphate buffer (0.25/0.135 mol/L, pH 7.0), and filtered in 3-kDa Nanosep centrifugal filters to remove proteins in samples. After addition of D_2O (50 µL) and sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuterio propionate (TSP-d_4, 30 µL, 0.3 mmol/L) to the filtrates, the aqueous samples were analysed in 5-mm NMR tubes by 600 MHz Bruker NMR spectrometer, using zgesgp pulse sequence. The chloroform phases in Paper II were dried and re-dissolved in $CDCl_3$ (600 µL, 99.96% D). ^{1}H NMR spectra were obtained using zg30 pulse sequence.

Plasma samples (60 µL) in Paper III were filtered in 3-kDa Nanosep centrifugal filters. After mixture with Millipore water (55 µL), sodium phosphate buffer (50 µL, 0.4 mol/L, pH 7.0), TSP-d_4 (10 µL, 5.8 mmol/L) and D_2O (15 µL), samples were analysed by 600 MHz NMR using zgesgp pulse sequence in 3 mm Bruker NMR tubes. NMR settings used for metabolomics analysis were summarized in Table 2.

Table 2. *NMR setting used for tissues and plasma metabolomics analyses in Paper I-III.*

Sample	Tissues (liver and muscle)		Plasma
	Polar	Nonpolar	
Temperature (°C)	25	20	25
Scans	128	128	512
Data points	65,536	65,536	65,536
Spectral width (Hz)	17,942	12,019	17,942
Acquisition time (s)	1.8	2.7	1.8
Relaxation delay (s)	4	3	4

All NMR spectral data were processed using Bruker TopSpin 3.1. Data in aqueous phase were Fourier-transformed after multiplication by a line broadening of 0.3 Hz and referenced to the standard peak TSP-d_4 at 0.0 ppm. Each spectral baseline and phase were corrected manually (Wagner *et al.*, 2014). The ^{1}H NMR signals were identified according to the ChenomX NMR Suite 6.1

library, the Human Metabolome Database (www.hmdb.ca) and previous literature (Moazzami *et al.*, 2012; Bankefors *et al.*, 2011; Moazzami *et al.*, 2011; Kullgren *et al.*, 2010; Samuelsson *et al.*, 2006). Concentrations of metabolites were calculated from spectra using ChenomX NMR Suite Profiler after accounting for overlapping signals, and expressed in µmol/g tissues or feed and µmol/L plasma. Spectra of chloroform phase in Paper II were integrated using Bruker software Amix 3.7.3, and the results were expressed as intensity, without unit.

3.3 Lipid analysis

Total lipids in tissues (white muscle and liver) and feeds in Paper II and III were extracted in hexane:isopropanol (3:2, *v/v*), as previously described (Mráz & Pickova, 2009). Total lipids in fish skin (Paper IV) were extracted in chloroform:methanol (2:1, *v/v*), according to Folch *et al.* (1957). Lipid content was determined by weighing the lipids after solvent evaporation.

Total lipids in liver samples of Paper II were separated into TAG and GPL by thin layer chromatography (TLC), with hexane:diethylether:acetic acid (85:15:2, *v/v/v*) as mobile phase and silica gel plates as stationary phase (Mráz & Pickova, 2009). Lipids were methylated to fatty acid methyl esters with boron trifluoride and analysed by gas chromatography-flame ionization detector (GC-FID) in a split mode, equipped with a fused silica capillary column (Trattner *et al.*, 2008).

The GPL faction in skin samples of Paper IV were isolated from the other lipid classes by TLC using a mixture of petroleum ether, diethyl ether and acetic acid (113:20:1, *v/v/v*) as mobile phase. The GPL bands were scraped off and soaked in a solvent of chloroform, methanol, acetic acid and water (50:39:1:10, *v/v/v/v*) to elute GPL from silica gel. GPL subclasses (PC, PE, PS and PI) were further separated by TLC with a mixture of chloroform, methanol, acetic acid and water (100:75:6:2, *v/v/v/v*) as mobile phases (Mason & Waller, 1964). GPL after scraping off were methylated with benzene, methanolic HCl and 2,2-dimethoxypropane (10:10:1, *v/v/v*) and analysed by GC-FID.

3.4 Gene expression analysis

Lysis reagent QIAzol was added in fish liver samples in Paper II before RNA isolation. After precipitation by chloroform, the total RNA in water phase were purified using RNeasy Mini kit. The concentration of purified RNA was normalised to 250 ng/µL, and then reverse transcription was performed by using a High Capacity RNA-to-cDNA Kit or a TATAA Grandscript cDNA Synthesis

Kit. The primers (IGF-I and IGF-II) were designed by using Primer-BLAST, and the other primers (GHR-I, PPARα, PPARβ1A, PPARγ, SREBP-1 and FAS) were designed based on available salmon sequences (Vestergren *et al.*, 2012; Skiba-Cassy *et al.*, 2009; Plagnes-Juan *et al.*, 2008; Gómez-Requeni *et al.*, 2005). Elongation factor 1AA was chosen as the reference gene (Olsvik *et al.*, 2005).

The gene expression was evaluated by using reverse transcription-polymerase chain reaction (RT-PCR). The relative expression was calculated as $\Delta C_T=2^{-\Delta\Delta CT}$, and reported as fold change (Livak & Schmittgen, 2001).

3.5 Sphingolipidomics analysis

Sphingolipids (Cer, GlcCer, Sa, So and Sph) in fish skin samples (Paper IV) were extracted and analysed by liquid chromatography-electrospray ionization-quadropole/time of flight mass spectrometer (LC- ESI-QTOF MS). Briefly, skin samples (containing sphingolipids internal standards) were extracted twice in chloroform:methanol (1:2, *v/v*), as previously described (Kelly *et al.*, 2011; Shaner *et al.*, 2009). Due to the high content of Sph in skin, the amounts of Sph were separately determined by analysing part of extracts using C12:0 Sph as internal standard. Rest extracts were used for other sphingolipids quantification. Samples were dried and re-dissolved in ethanol for analysis.

The separation of sphingolipids was achieved on an HILIC column, with buffer A (1% formic acid and 10 mM ammonium formate in water) and buffer B (0.1% formic acid in acetonitrile) as mobile phases. Spectra were acquired in positive ionization mode and a sodium formate solution (4 µL formic acid, 20 µL 1 M NaOH, 100 mL H_2O and 100 mL 2-propanol) was used as MS calibrant. Peak heights of the compounds with interests were calculated based on the assigned m/z and retention times using Mzmine. Concentrations were determined against the internal standards and expressed in nmol/g tissue.

3.6 Data analysis

Univariate data analysis was done by SAS. Data in percentage were firstly square-root-arcsine transformed before testing. Data distribution of normality and homoscedasticity were checked. After passing the tests, either before or after log-transformation, data were compared by using General Linear Model ("proc glm" in SAS) in Paper II and IV, and Mixed Model ("proc mixed" in SAS) in Paper I and III. Otherwise, Mann-Whitney test was applied as non-parametric test. A P-value<0.05 was considered statistically significant. Tukey's test was used as a post-hoc test (Paper I, II and IV).

The SIMCA-P was used for multivariate data analysis, with all variables pareto-scaled. PCA models were applied to over view the dataset and search for outliers (Paper I−IV), and OPLS-DA models were performed to classify groups (Paper I−III). The significance of the OPLS-DA models was checked by using cross-validation ANOVA (CV-ANOVA; $P<0.05$) and overall cross-validation R^2. OPLS-DA loading plots and variable importance for the projection (VIP) plots were used to identify the discriminative metabolites (Paper I−III).

To take the false discovery into account in multiple testing, FDR controlled at 0.05 by using the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995) in Paper I and Bonferroni correction ($\alpha_\beta=0.05/n$, n is the metabolites numbers) in Paper I−III were applied (Noble, 2009).

4 Results

The important results in each paper are briefly described in this chapter. For more detailed and comprehensive results, such as tables and figures, check the papers.

4.1 Paper I

Separation between the four anatomic parts (P_1−P_4; Figure 9) of Arctic char liver was seen in the score plots of both PCA and OPLS-DA, which indicated the differences in metabolic profile between the polar portions of liver extracts, although the OPLS-DA model was not significant according to the value of Q^2Y and the *P* values of CV-ANOVA.

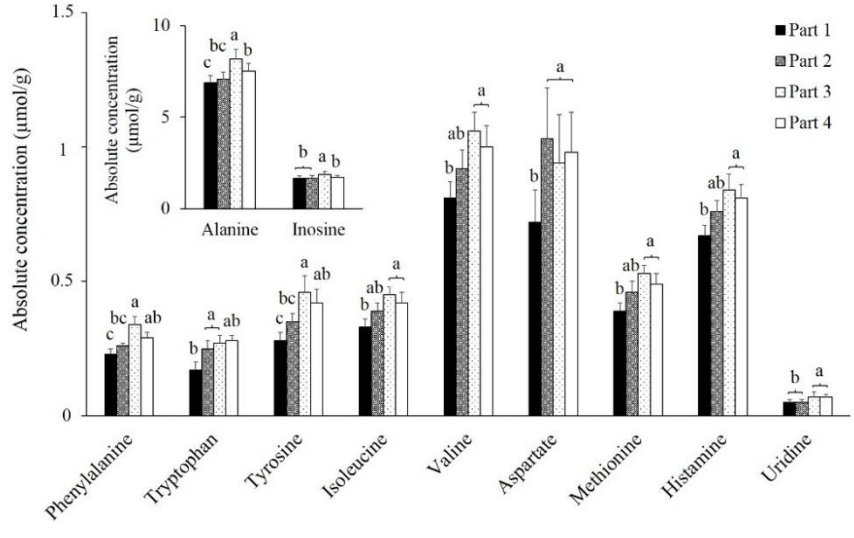

Figure 13. Absolute concentrations (μmol/g) of the discriminative metabolites in polar portions of fish liver extracts (mean±SE; n=6). a−c denote significant differences between the four liver parts.

Univariate results after FDR correction showed that 11 metabolites differed between fish liver parts: alanine, aspartate, histamine, inosine, isoleucine, methionine, phenylalanine, tryptophan, tyrosine, uridine and valine. Generally, the concentrations of these metabolites were lower in P_1 and higher in P_3 (Figure 13).

4.2 Paper II

Fish final body weight and lipid content in white muscle and liver did not differ between the groups. Compared with the commercial control, fish fed the Baltic Sea-sourced decontaminated and untreated feeds had higher content of 20:4n-3 and ∑SAFA, and lower content of 18:2n-6 and ∑n-6 in white muscle. FA composition in liver did not differ between groups.

Table 3. *Summary of the metabolic changes in aqueous samples of Arctic char fed the CFM+CFO feed (Paper II) and the Baltic blend test diet (Paper III), compared with the other dietary groups in the studies.*

	CFM+CFO in Paper II		Baltic blend test in Paper III		
	Liver	Muscle	Plasma	Liver	Muscle
−	GSH	formate glucose taurine	phenylalanine tyrosine TMAO		anseine TMAO
+	cholate choline glycine isoleucine leucine methionine phenylalanine tyrosine valine	pyruvate ADP	betaine N,N-dimethylglycine Myo-inositol	sn-glycerol-3-phosphocholine AMP	Alanine Glycine ADP aminoisobutyrate betaine

−/+ indicates concentrations of these metabolites were lower/higher in the CFM+CFO or the Baltic blend test dietary group.

Metabolomics studies in aqueous liver extracts showed that fish fed CFM+CFO had lower content of glutathione (GSH), but higher content of cholate, choline, glycine, isoleucine, leucine, methionine, phenylalanine, tyrosine and valine than the other groups (Table 3). Moreover, compared with the experimental groups, the control group had stronger signals corresponding to all FA except EPA and DHA, all FA except ∑n-3 and unsaturated FA.

Fish fed CFM+CFO contained less formate, glucose and taurine, and more pyruvate and ADP in aqueous white muscle than those fed CFM+SPFO (Table 3). Lipid profile in chloroform extracts of white muscle did not differ significantly between fish fed CFM+CFO and those fed CFM+SPFO.

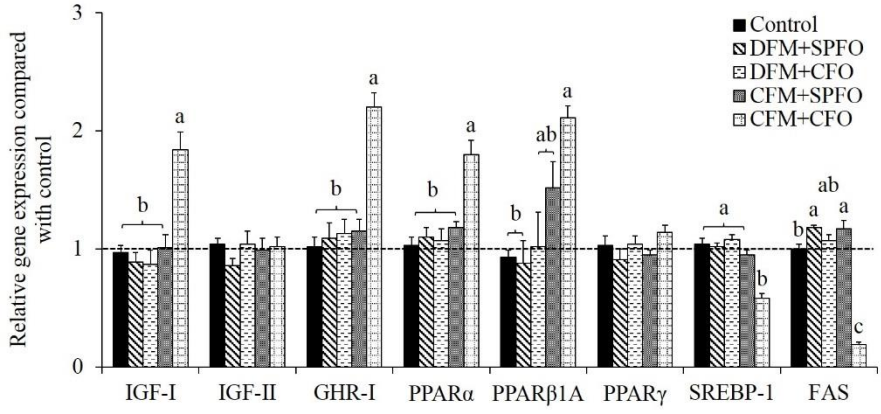

Figure 14. Relative gene expression in liver of fish fed a commercial standard diet and four experimental diets formulated with Baltic Sea-sourced fish diets, shown as fold change (mean±SE; n=6). a−c denote significant differences between the diets.

Hepatic mRNA levels of IGF-I, GHR-I, PPARα and PPARβ1A showed significantly up-regulation in fish fed CFM+CFO. However, the expression of SREBP-1 and FAS were down-regulated in fish fed CFM+CFO, compared with other dietary groups. Hepatic expression of IGF-II and PPARγ did not differ between treatments (Figure 14).

4.3 Paper III

Fish fed the test diet had a significantly lower K-factor than control, and tended to have less lipids in fillet (not statistically significant).

Metabolomics analysis on fish plasma, liver and white muscle showed dietary effects on fish metabolic profile. Compared with the control group, fish fed the test diet had higher levels of alanine in muscle, glycine in muscle, sn-glycerol-3-phosphocholine in liver, ADP in muscle, AMP in liver, 3-aminoisobutyrate in muscle, betaine in plasma and muscle, N,N-dimethylglycine in plasma and myo-inositol in plasma, and lower levels of phenylalanine in plasma, tyrosine in plasma, anserine in muscle and trimethylamine-N-oxide (TMAO) in plasma and muscle (Table 3).

Furthermore, the Baltic blend diet contained more betaine, and less myo-inositol and TMAO than the control diet.

4.4 Paper IV

Diets low in EPA and DHA changed FA composition in skin GPL subclasses. Generally, when dietary proportions of EPA and/or DHA increased from 0.5% to 2.0%, the FA composition in samples was close to the CC and far away from the NC, with the proportion of 22:6n-3 increasing and that of 20:4n-6 decreasing. Dietary impacts were stronger in PC and weaker in PS and PI. Differences were more pronounced at 400 g than 200 g.

Table 4. *The characteristics of fatty acid (FA) composition in skin glucosyl-phospholipid subclasses of Atlantic salmon fed the negative control (NC) diet and their changes with increasing levels of dietary EPA/DHA at 400 g.*

FA	NC				With increasing levels of EPA/DHA			
	PC	PE	PS	PI	PC	PE	PS	PI
16:0		Lower				↑		
20:2n-6	Lower							
20:5n-3	Lower				↑	↑		
22:6n-3	Lower	Lower	Lower	Lower	↑	↑		
16:1n-9							↓	
18:1n-9	Higher							
18:0					↓			
18:2n-6	Higher				↓			
18:3n-6	Higher				↓			
20:3n-6	Higher	Higher	Higher		↓	↓	↓	↓
20:4n-6	Higher	Higher	Higher		↓	↓		
22:5n-6			Higher					

↑/↓ indicates the proportion of FA in fish skin increased/decreased, with increasing levels of dietary EPA and/or DHA.

In the PC fraction at 400 g, fish fed the NC contained higher proportions of 18:1n-9, 18:2n-6, 18:3n-6, 20:3n-6 and 20:4n-6, and lower proportions of 20:2n-6, 20:5n-3 and 22:6n-3 in skin. With increasing levels of dietary EPA and/or DHA, the relative amounts of 20:5n-3 and 22:6n-3 increased in the DHA and EPA+DHA groups, and those of 18:0 in EPA+DHA group, 18:2n-6, 18:3n-6, 20:3n-6, and 20:4n-6 decreased. In the PE fraction at 400 g, the proportions of 20:3n-6 and 20:4n-6 were higher and those of 16:0 and 22:6n-3 lower in the NC. With the increasing levels of EPA and/or DHA in diets, the relative content of 16:0 in the EPA group, 20:5n-3 in the EPA and EPA+DHA groups and 22:6n-3

increased, and that of 20:3n-6 in the EPA and DHA groups and 20:4n-6 decreased. In the PS fraction at 400 g, fish fed the NC had more 20:3n-6, 20:4n-6, and 22:5n-6, and less 22:6n-3. With increasing levels of dietary EPA and/or DHA, the proportion of 16:1n-9 in the EPA+DHA groups and 20:3n-6 in the EPA and DHA groups decreased. In the PI fraction at 400 g, fish fed the NC contained lower proportion of 22:6n-3. With increasing levels of EPA and/or DHA in diets, the proportion of 20:3n-6 decreased (Table 4).

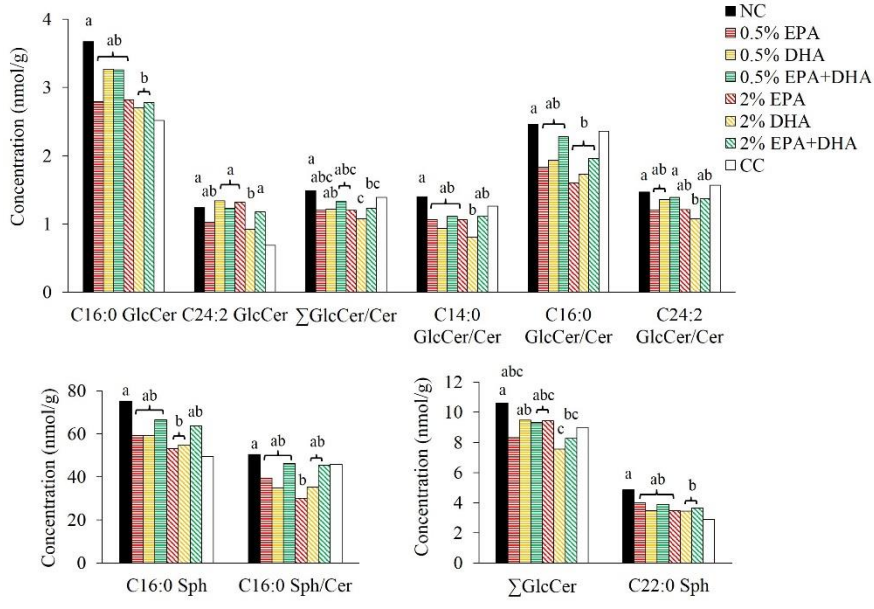

Figure 15. The absolute concentration (nmol/g) of important sphingolipids in skin of Atlantic salmon fed different experimental diets at 400 g (n=2 for 0.5% EPA and/or DHA groups, n=3 for other groups). a−c denote significant differences between diets (Tukey's test, $P<0.05$). Each statistical replicate originated from a five-fish pooled sample.

The effects of dietary EPA and DHA deficiency on sphingolipidomics (Cer, So, Sa, GlcCer, Sph, Sph/Cer and GlcCer/Cer) in fish skin were also studied (Figure 15). To sum up, fish in the experimental groups had higher concentrations of sphingolipids than those in CC, except C14:0 Cer, C14:0 GlcCer, C14:0 Sph and C20:2 Sph. Compared with the NC, there were decreased concentrations of C16:0 Sph, C16:0 Sph/Cer and C16:0 GlcCer/Cer, and increased concentrations of C24:2 Sph in 2.0% EPA; there were decreased concentrations of C14:0 GlcCer, C16:0 GlcCer, C24:2 GlcCer, ∑GlcCer, C16:0 Sph, C22:0 Sph, C14:0 GlcCer/Cer, C16:0 GlcCer/Cer, C24:2 GlcCer/Cer and ∑GlcCer/Cer in 2.0% DHA; and there were decreased concentrations of C14:0 GlcCer, C16:0 GlcCer, ∑GlcCer, C22:0 Sph and C16:0 GlcCer/Cer in 2.0%

EPA+DHA. Additionally, with increasing levels of dietary EPA and/or DHA, concentrations of the metabolites (C16:0 GlcCer, C24:2 GlcCer, ∑GlcCer, C16:0 Sph and C22:0 Sph) decreased gradually and were close to those in the CC (Figure 15). Moreover, most of sphingolipids in fish skin were more concentrated at 400 g than 200 g.

5 Discussion

In this thesis, the metabolic responses to different alternative fish feeds in liver, white muscle, plasma and skin were investigated by using ^{1}H NMR-based metabolomics and MS-based sphingolipidomics, to assess the potential impacts of diet on fish health and to understand the underlying mechanisms of differences in growth. Moreover, the effects of alternative diets on fish growth, lipid quality in fish fillet and hepatic gene expression were evaluated.

5.1 The hepatic heterogeneity of Arctic char

Heterogeneity of the metabolic profile in Arctic char liver were shown in Paper I. Most of the discriminative metabolites (alanine, aspartate, histamine, inosine, isoleucine, methionine, phenylalanine, tryptophan, tyrosine, uridine and valine) play a key role in fish physiology, such as the Cahill (glucose-alanine) cycle, amino acid catabolism and gluconeogenesis in liver.

Generally, P_1 and P_2 contain fewer discriminative metabolites than P_3 and P_4. Differences in metabolic profile of fish liver are probably due to the zonation of hepatic metabolism along the blood flow, which leads to gradients in the concentration of metabolites across the liver (Kline *et al.*, 2011; Yang *et al.*, 2008; Jungermann & Katz, 1989; Schär *et al.*, 1985). Based on our lab observation (Figure 9), P_1 and P_2 are close to the inferior vena cava where blood is carried away from the liver (efferent zone), and P_3 and P_4 are close to the hepatic artery and portal vein, where blood is carried towards the liver (afferent zone).

Furthermore, the inter-hepatic variation in metabolic profile may also be attributed to the heterogenetic distribution of parenchyma and non-parenchyma, such as sinusoids, microvascular and melano-macrophages (Agius & Roberts, 2003; Jungermann & Katz, 1989). For example, melano-macrophage centres which are concentrated in lipofuscin, melanin, ceroid and hemosiderin, and also

store cell-derived phospholipid and iron, are usually located in fish hepatic parenchyma around the portal regions (Agius & Roberts, 2003; Dutta & Datta-Mushi, 1996).

5.2 Metabolic responses to alternative feeds

5.2.1 Decontaminated fish materials from the Baltic Sea

In Paper II, fish fed the untreated Baltic Sea-sourced diet CFM+CFO contained higher concentrations of alanine, β-alanine, glucose, glycine, isoleucine, leucine, methionine, phenylalanine, tyrosine and valine in aqueous phase of liver extracts. It suggested that the untreated diet-induced disturbances on protein biosynthesis and catabolism, and energy metabolism pathways leading to the tricarboxylic acid (TCA) cycle (García-Sevillano *et al.*, 2014; Kokushi *et al.*, 2012; Bonga, 1997). Furthermore, compared with CFM+SPFO, CFM+CFO decreased the level of glucose and increased four-fold the level of pyruvate in white muscle, which indicated a metabolic shift in glycolysis from glucose to pyruvate (Figure 16). Reportedly, the reduced activity of oxidative enzymes and increased glycolytic capacities contributed to insulin resistance in humans with non-insulin dependent diabetes mellitus (Simoneau & Kelley, 1997). Stressors altered glycogenolysis in fish (Bonga, 1997) and insulin signalling pathway in mammals (Sargis *et al.*, 2012; Ibrahim *et al.*, 2011; Ruzzin *et al.*, 2010). Thus, the changes in amino acids and glucose in fish liver and white muscle implied the presence of stressors in the CFM+CFO diet, and suggested that CFM+CFO may induce changes in insulin signalling pathways in fish, similar to the insulin resistance observed in mammals.

IGF-I function similarly to insulin in metabolomics regulation, promoting glucose uptake and glycogen synthesis. In fish, IGF-I is even more effective than insulin (Caruso & Sheridan, 2011; Castillo *et al.*, 2004). Furthermore, it was reported that treatment with growth hormone which is functioned with GHR lead to an increased level of glucose in fish plasma, similar to the effects in mammals (Sangiao-Alvarellos *et al.*, 2005). Therefore, the up-regulated expression of IGF-I and GHR, together with the changes in metabolic profile, indicate the regulation of glucose metabolism was affected in CFM+CFO.

The increased level of choline in liver of CFM+CFO may be associated with degradation of PC and phosphocholine in cell membrane induced by contaminants in the diet (García-Sevillano *et al.*, 2014). On the other hand, the reduced expression of SREBP-1 and FAS in liver of CFM+CFO implied inhibited synthesis of PC from choline (Glunde *et al.*, 2011; Ridgway & Lagace,

2003). We found no significant differences in the content of phosphocholine or PL in liver so it is unclear whether the increased choline in CFM+CFO was due to promoted degradation or inhibited synthesis of PC. Zebrafish (*Danio rerio*) with alcoholic fatty liver had an increased concentration of choline in liver, compared with healthy-liver fish (Jang *et al.*, 2012), and yellow catfish (*Pelteobagrus fulvidraco*) exposed to waterborne copper had a decreased hepatic levels of SREBP-1 and FAS mRNA (Chen *et al.*, 2013). Thus, we speculated that the untreated diet CFM+CFO modified choline metabolism in liver and possibly induced liver damage.

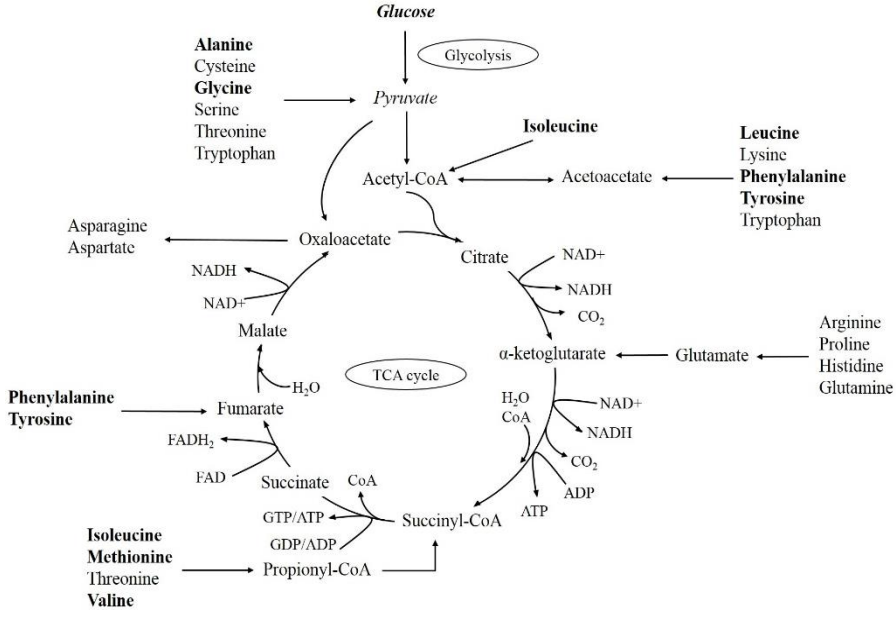

Figure 16. The CFM+CFO diet-induced changes in metabolic pathways, connecting amino acid catabolism to glycolysis and energy metabolism. The metabolites in **bold** were changed in liver of CFM+CFO, compared with other groups, and those in *italic* were changed in white muscle of CFM+CFO, compared with CFM+SPFO.

Cholate is an important component of bile acids and plays a role in regulation of glucose and lipid metabolism. Bile acid feedback repression allows the liver to control the synthesis of bile acid, and thus maintain a constant pool of bile acids (Li & Chiang, 2011). Thus, the increased concentration of cholate in CFM+CFO implies that the feedback regulation of bile acid were disturbed (Li & Chiang, 2011).

GSH is an antioxidant compound which can protect cells from free radicals and is associated with elimination of xenobiotics (García-Sevillano *et al.*, 2014; Valavanidis *et al.*, 2006). Taurine has protective function in heavy metal

detoxification in fish (Timbrell *et al.*, 1995). Thus, reduced concentrations of GSH and taurine in fish from CFM+CFO may be involved in detoxification, indicating an adverse health effects of the untreated diet to fish.

Based on our results, toxic effects were only observed when both sources of toxins (CFM and CFO) were included in the diets. According to the EU regulation for animal feed, levels of contamination such as polychlorinated biphenyls in the diets were relatively low. Thus, we speculated that when both CFM and CFO were used, there were enough identified or unidentified pollutants present, or synergistic effects of different compounds in diets.

5.2.2 Baltic blend diet

The test feed in Paper III composed of the Baltic Sea-sourced blended ingredients as protein contained higher level of betaine than the fishmeal-based standard diet, which is probably due to the inclusion of blue mussel in the diet (Nagel *et al.*, 2014). Higher concentrations of betaine in fish plasma and tissues of the test group should mainly originate from the diet (Lever & Slow, 2010). In connection to this, we observed that the test group had an increased level of N,N-dimethylglycine derived from the methylation pathway of homocysteine to methionine (Lever & Slow, 2010) and higher levels of the subsequent metabolites (sarcosine, glycine and serine) in the single carbon metabolic pathway (Figure 17). Similarly, the lower level of TMAO in the test diet was consistent with the lower level of TMAO in plasma and muscle of fish fed the test diet (Seibel & Walsh, 2002; Charest *et al.*, 1988; Agustsson & Strøm, 1981).

Betaine and TMAO were considered as important osmolytes in fish, and also implicated in lipogenesis and lipid storage (Lever & Slow, 2010; Seibel & Walsh, 2002). The content of betaine in plasma was negatively related to body obesity markers in mammals (Lever & Slow, 2010; Konstantinova *et al.*, 2008; Eklund *et al.*, 2005), whereas the content of TMAO in muscle was positively correlated to body lipid level (Seibel & Walsh, 2002). Thus, the higher level of betaine and lower level of TMAO in fish from the test group were consistent with the lower body mass, lower value of K-factor and a trend of decreasing flesh lipid content in fish fed the test feed, which were observed in the same feeding trial.

Choline can be metabolized into trimethylamine by gut microbiota, and trimethylamine is then absorbed and metabolized to TMAO in liver (Prathomya *et al.*, 2017; An *et al.*, 2013). Recently, it was found that replacement of fishmeal with microbial altered the gut microbiota composition in Arctic char (Nyman *et al.*, 2017). To determine whether the higher level of TMAO in the control group is related to the activity of gut microbiota, further studies are needed.

In the present study, we found fish from the test group contained higher concentrations of ADP and AMP in muscle and liver, which is probably due to the dietary inclusion of baker's yeast, having large amounts of nucleotides (Lee 2015). In line with this, fish from the test group contained more 3-aminoisobutyrate in muscle, which is a common end-product formed from thymine in nucleic acid metabolism.

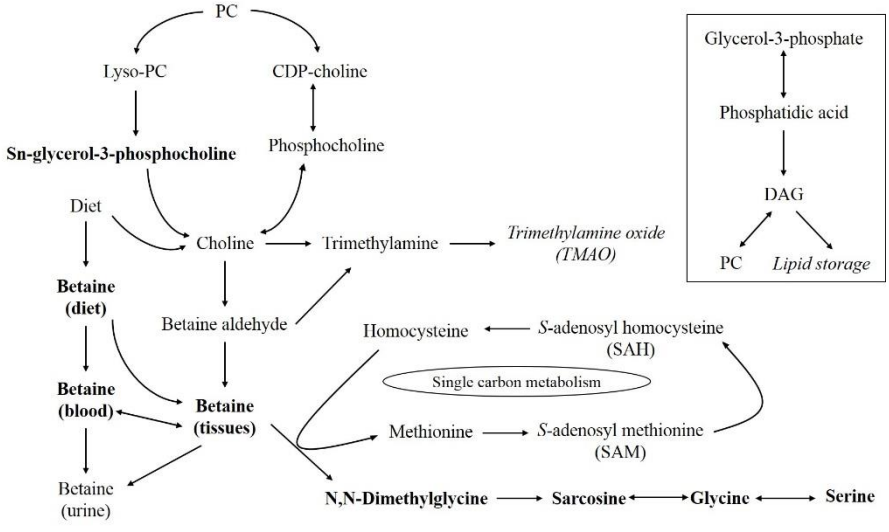

Figure 17. The Baltic blend test diet-induced changes in metabolic pathways of choline, betaine and single carbon metabolism. Concentrations of metabolites in **bold** were increased in fish fed the test diet, those in *italic* were decreased.

Furthermore, the higher level of myo-inositol in the control diet might be another reason for heavier final body weight in the control group, because myo-inositol plays an important role in stimulating growth and improving digestive capacity (Jiang *et al.*, 2009; Holub, 1986). Nevertheless, fish fed the test diet had a higher level of myo-inositol in plasma. This implied that the endogenous myo-inositol metabolism in fish was interrupted by the test diet. Myo-inositol, the most abundant inositol, can be synthesized from glucose *in vivo* (Holub, 1986). In Paper III, we found that the concentration of glucose in liver and the final body weight were lower in fish fed the test diet. These findings agree with others, in that piglets with lower birth weight had higher concentration of myo-inositol and lower level of glucose in plasma (Nissen *et al.*, 2010).

Tyrosine is an important substrate for the synthesis of neurotransmitters and thyroid hormones, which can regulate fish appetite and growth, and can be synthesized from phenylalanine (Li *et al.*, 2009). The lower levels of tyrosine and phenylalanine in plasma of the test group implied that the test feed had

insufficient nutritional components required for fish growth, compared with the control feed.

5.2.3 EPA and DHA deficiency

It has been reported that stresses can result in an activation of sphingomyelinases and ceramide synthases in sphingolipidomics pathway, and later an accumulation of Cer and GlcCer (Chalfant & Del Poeta, 2011; Nagai *et al.*, 2011). In Paper IV, it was observed that EPA/DHA levels in experimental diets decreased, concentrations of GlcCer and Sph tended to increase in fish skin. The up-regulated levels of GlcCer and Cer may offer cellular protection (Chalfant & Del Poeta, 2011; Nagai *et al.*, 2011) and GlcCer may improve the skin barrier function of mammals (Yeom *et al.*, 2012; Tsuji *et al.*, 2006). Although we did observe significant changes in Cer content, increased ratios of C16:0 Sph/Cer and GlcCer/Cer in the EPA/DHA deficient groups did emphasis that deficiency of EPA and DHA lead to interrupted sphingolipidomics, and possibly interrupted barrier function of fish skin (Pullmannová *et al.*, 2014). Similar protective effects of EPA and docosapentaenoic acid have been found in aged rats, by modifying the generation of Cer and sphingosine-1-phosphate in hippocampus (Kelly *et al.*, 2011).

Moreover, DHA was found to be more effective than EPA on modifying the sphingolipid composition in skin, because most improvements were observed in the 2% DHA and 2% EPA+DHA groups. Several previous works have also shown that DHA was more efficient than EPA in increasing fish growth and survival rates (Kanazawa, 1997; Watanabe, 1993).

5.3 Dietary effects on fish growth and lipid profile

We found no differences in final body weight or lipid content in white muscle or liver between the contaminated and decontaminated dietary groups in Paper II. For the FA profile in white muscle and liver, main differences were shown between the control and experimental diets. The control feed contained more plant-sourced oil, whereas experimental diets were composed of fishmeal and fish oil from the Baltic Sea. Thus, compared with experimental groups, fish fed the commercial diet had more n-6 FA and less n-3 FA in flesh. Baltic Sea-sourced fish materials that are decontaminated would be a valuable source of n-3 FA for fish feed.

In Paper III, lower lipid content in fish white muscle and lower value of K-factor in the Baltic blend test group were consistent with changes in metabolic

profile (see 5.2.2). This finding agrees with previous finding that fish fed the test diet had lower body mass than control (Carlberg, 2016).

The FA composition in GPL subclasses (PC, PE, PS and PI) of Atlantic salmon skin were also affected by the dietary EPA and DHA in Paper IV. When levels of dietary EPA and DHA decreased, the levels of 20:5n-3 and 22:6n-3 were markedly reduced in PC and PE. Simultaneously, to compensate for the loss of these FA, levels of 20:3n-6 and 20:4n-6 in GPL subclasses increased. Moreover, there was more 18:2n-6 in the NC, due to the higher inclusion of poultry oil and rapeseed oil in the NC diet. The increased accumulation of n-6 PUFA in the EPA/DHA deficient groups indicated an increased desaturation and elongation of 18:2n-6 to longer-chain n-6 PUFA. Our results agree with findings in liver and blood of Atlantic salmon of others (Ruyter *et al.*, 2000d; Ruyter *et al.*, 2000c).

The impacts of dietary essential FA on FA composition in skin were more notable in the PC and PE fractions, compared with PS and PI. This suggests that PS and PI are more conservative and resistant to FA changes, or possibly caused by transferring the FA from other lipids, such as TAG (Ruyter *et al.*, 2000d).

A previous study showed that dietary supplementation with fish oil lead to an incorporation of EPA/DHA into epidermal GPL and increased epidermal levels of PUFA-derived hydroxyl FA in guinea pig, which reduced the chronic inflammatory skin disorders (Miller *et al.*, 1991). Thus, together with increased concentrations of GlcCer and Sph in the NC group, the decreased EPA/DHA incorporated into skin GPL in the NC suggests that dietary EPA and DHA could enhance the protective function of fish skin, even though the significant differences in epidermal histology (mucous cell density and epidermal thickness) were not observed in the same study, due to the limited sample number.

6 Main findings and conclusions

This thesis evaluated Baltic Sea-sourced raw materials as feed for salmonids by using ^{1}H NMR-based metabolomics, and assessed the impacts of reducing dietary EPA and DHA on fish skin polar lipid composition by using GC-FID and MS based sphingolipidomics.

The main findings and conclusions obtained in the thesis are:

- The different metabolic profiles were observed in the four liver parts of Arctic char, which suggests the metabolic heterogeneity should be considered in future metabolomics studies on fish liver, particularly comparative studies.

- The process of decontamination reduced the negative impacts on fish metabolism and gene expression associated with energy metabolism and hepatic toxicology, compared with the untreated diet from the Baltic Sea.

- After decontamination, Baltic Sea-sourced fish materials with high levels of n-3 LC-PUFA would be valuable ingredients as fish feeds.

- The formulation of Baltic blend diet, which is composed of Baltic Sea-sourced fishmeal, blue mussel and baker's yeast as protein sources, needs to be modified to achieve better growth performance of Arctic char. For instance, the content of betaine should be reduced and that of TMAO and aromatic amino acids should be increase.

- Different types of Cer, GlcCer, Sph, So and Sa in skin of salmonids were characterised for the first time.

- Reduction of dietary EPA and DHA modified the FA composition in GPL subclasses and sphingolipidomics in skin of Atlantic salmon, which might affect fish skin health.

- NMR and MS-based metabolomics approaches were useful tools to assess the potential impacts of dietary compounds on fish health, and can be effectively used to develop new aquafeeds.

7 Perspectives

This thesis presents novel information on using metabolomics in the development of aquafeeds. Based on the findings presented, the following investigations are of interest to support sustainable aquaculture and assure fish health and product quality:

- Re-evaluate the impacts of Baltic blend test diet on fish growth performance after dietary modification, based on our metabolic results.

- Identify more metabolites as markers of fish malnutrition and potential diseases such as gut health, and use them to evaluate novel aquafeeds.

- Correlate metabolic profile in fillet with fish product quality, such as sensory quality, and use the metabolites as markers for fish selective breeding.

- Investigate continuously the functional mechanism of GPL and sphingolipids in maintaining osmotic homeostasis of fish skin.

- Inspect the sphingolipidomics in other fish tissues, except skin and their physiological functions.

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