Novel ingredients for fish feed applications

An assessment of antinutrients and contaminants

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Nya foderingredienser till fisk: En överblick av antinutritionella substanser och potentiella föroreningar

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Abstract

The aquaculture industry is expanding, and so is the need for sustainable feed ingredients that can replace fish meal (FM) and soybean, thereby reducing the environmental pressure associated with fish feed production. Some emerging feed ingredients include mussel meal (MM) from the blue mussel (*Mytilus edulis*), black soldier fly meal (BSFM; *Hermetia illucens*), protein from the fungi *Paecilomyces variotii* (Mycoprotein), and leaf protein concentrates. To ensure the safety and suitability of these novel feed ingredients, they should be assessed for anti-nutritional factors (ANFs) and contaminants to mitigate health risks for both fish and human consumers. This thesis aims to ascertain the concentration of saponins, tannins, chitin, dioxins, metals, and PFAS in the aforementioned feed ingredients, as well as in the fish sludge and frass from the production of the black soldier fly larvae, and a FM, soybean meal (SBM), and soy protein concentrate (HP 310).

Three leaf protein concentrates (alfalfa protein concentrate, APC; white clover protein concentrate, WPC; ProteinMax biomass concentrate, PMC), SBM, and HP 310 were analysed for their saponin and tannin content. The chitin concentration in the BSFM and Mycoprotein was assessed. Dioxins were analysed in the MM, fish sludge, frass, and FM. All raw materials were examined for their metal and PFAS content.

Saponins and tannins were detected in all analysed feed ingredients, with the WPC exhibiting the highest saponin concentration and the PMC having the highest tannin concentration. The chitin concentration was greatest in the Mycomeal compared to the BSFM. Dioxins were identified at low levels in the MM, fish sludge and FM. The metal content varied among the raw materials, with WPC having an arsenic level exceeding the EU maximum limit established under Directive 2002/32/EC, rendering WPC unsuitable as a feed ingredient. PFAS was of no concern in these feed ingredients, as only low levels were detected in the MM, BSFM, and FM. In conclusion, all feed ingredients show promising results for inclusion in fish feed, with the exception of WPC, as ANFs and contaminants were present at low levels.

Keywords: Novel fish feed ingredients, anti-nutritional factors, contaminants, mussel meal, black soldier fly, leaf protein concentrates, *P. variotii*, aquaculture

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Abbreviations

Abbreviation	Description
ADF	Acid detergent fibre
ADIP	Protein linked to acid detergent fibre
ANF	Anti-nutritional factor
APC	Alfalfa protein concentrate
BSFL	Black soldier fly larvae
BSFM	Black soldier fly meal
CF	Crude fibre
CL	Crude lipids
CP	Crude protein
CT	Condensed tannins
DM	Dry matter
EU	European Union
F	Frass
FAO	Food and Agriculture Organisation of the United Nations
FCR	Feed conversion ratio
FM	Fish meal
FS	Fish sludge
GE	Gross energy
HT	Hydrolysable tannins
LPC	Leaf protein concentrate
MM	Mussel meal
MY	Mycomeal
PCDDs	Polychlorinated dibenzo-p-dioxins
PCDFs	Polychlorinated dibenzofurans
PFAS	Per- and polyfluoroalkyl substances
PMC	ProteinMax biomass concentrate
POP	Persistant organic pollutant
RAS	Recirculating aquaculture system
SBM	Soybean meal
SLU	Swedish University of Agricultural Sciences
SSL	Spent sulphite liquor
TEQ	Total dioxin equivalents
WHO	World Health Organisation
WPC	White clover protein concentrate

1. Introduction and background

Aquaculture is the fastest-growing animal food sector in the world, and about two-thirds of the species being produced are fed, meaning they are provided with feed formulated to meet their nutritional requirements (FAO 2024). The feed has historically relied on a high inclusion of fish meal (FM) and fish oil, but there has been a reduction in use over the years due to increasing prices alongside higher demand (FAO 2024). Soybeans (*Glycine max*) have been one source of protein used in fish feed to reduce the amount of FM, however, this comes with problems related to deforestation and long transport resulting in high greenhouse gas emissions (Bergman et al. 2024). Thus, these ingredients must be further replaced with more sustainable options (Terova et al. 2019; FAO 2024). Replacing these ingredients can also reduce the feed-food competition and make more food available directly to humans (Hooft et al. 2025). There is, therefore, a need for other nutritious feed sources, mainly to provide protein, to reduce the environmental pressure of fish feed. Some of the emerging feed ingredients with the potential to replace FM and soybean are mussel meal (MM) from the blue mussel (*Mytilus edulis*), black soldier fly meal (BSFM; *Hermetia illucens*), protein from the fungi *Paecilomyces variotii,* and leaf protein concentrates (LPCs) derived from alfalfa (*Medicago sativa*), white clover (*Trifolium repens*), perennial ryegrass (*Lolium perenne*) and tall fescue (*Lolium arundinacea*). These ingredients can be produced locally and thus reduce the environmental footprint of fish feed (Damborg et al. 2020; Azad et al. 2023; Bergman et al. 2024).

When introducing novel feedstuffs for use in the food production chain, it is crucial that they are safe and that they aren't going to cause any health problems in both the fish and us humans when we consume the final product (Glencross et al. 2020; Albrektsen et al. 2022; Mensah et al. 2024). Therefore, a thorough evaluation of novel feedstuffs regarding the presence of anti-nutritional factors (ANFs) and environmental contaminants should be done to ensure their safety (Krogdahl et al. 2022). However, the aspect of ANFs and contaminants is often overlooked in research due to the focus being on the protein content and digestibility of the feed ingredient and the growth performance of the fish, which might lead to bias when interpreting results. It is sometimes speculated that negative effects caused by novel feed ingredients are due to ANFs (Langeland et al. 2016; Cardinaletti et al. 2019; Coburn et al. 2021; Hooft et al. 2024; Navarrete 2025). But they are rarely quantified, which is needed to conclude their effects. Further, contamination from heavy metals and persistent organic pollutants (POPs), such as dioxins and per- and polyfluoroalkyl substances (PFAS), is of concern for both fish and human consumption, which motivates the need to analyse these (Glencross et al. 2020).

ANFs are naturally occurring compounds synthesised by plants as a chemical defence mechanism to protect themselves from bacteria, viruses, and fungi and from being eaten by animals (Krogdahl et al. 2022; Glencross et al. 2020). Depending on the dosage, ANFs can have both positive and negative effects on the fish. Some of the negative effects include decreased feed palatability, impaired nutrient absorption, disruption of dietary nutrient balance, inhibited growth, digestive issues, immune system alterations, changes in gut microbiota, thyroid dysfunction, pancreatic enlargement, reduced blood sugar levels, and potential damage to the liver and kidneys (Krogdahl et al. 2022). A combination of different ANFs causes soybean meal-induced enteritis in salmonids, and it is therefore important to evaluate the combined effects of different ANFs in a diet since plant-based feed ingredients usually contain more than one (Knudsen et al. 2008; Iwashita et al. 2009; Krogdahl et al. 2015). At low dosages, ANFs can have positive effects such as antioxidative effects, improved immune system, and prebiotic properties (Krogdahl et al. 2022). Consequently, understanding the effect and content of ANFs in feed ingredients makes it possible to optimise feed recipes for good growth and health in farmed fish.

Contaminants do not naturally exist in plants but can be a result of microbial growth, remaining pesticides, drug residues, or environmental pollutants, for example (Krogdahl et al. 2022). They can have toxic effects on living organisms, including both fish and humans, and even be lethal depending on the compound, dose, length of exposure, and age (Krogdahl et al. 2022). All feedstuffs can be contaminated, however, it is more common in marine-derived feed sources (Glencross et al. 2020). To ensure feed ingredients are safe to use, it is motivated to analyse them for the content of contaminants.

1.1 Characteristics and effects of anti-nutritional factors

Saponins are a diverse group of glycosides that consist of a hydrophilic sugar chain and a hydrophobic steroidal or triterpenoid aglycon, which makes them amphipathic (Krogdahl et al. 2022; NRC 2011). They are heat-stable but can be extracted with the use of alcohol (Krogdahl et al. 2022). Saponins are mainly produced by legumes but by other plants as well, a few lower marine animals, and a few bacteria (NRC 2011). Hence, they can be found in both alfalfa and white clover since both are legumes (Sakamoto et al. 1992; Chatzifotis et al. 2006). Unfortunately, this causes concerns when using these in LPCs due to the potential content of saponins.

Diverse positive biological effects have been associated with saponins when part of the fish diet. Anti-bacterial and immune-stimulating effects of saponins have been observed in Atlantic salmon (*Salmo salar*) (Cortés et al. 2023). Gu et al. (2015) added 2 g kg^{-1} pure soy-saponins to a FM-based diet for Atlantic salmon fry, which resulted in increased growth and liver function compared to the fish receiving a FM-based control diet. It was suggested by the authors that soy-saponins might increase nutrient absorption (Gu et al. 2015). In the same study, a mix of ANFs was added to one experimental diet, and those results suggest that soy-saponins alleviate the negative effects of other ANFs since no effects on growth were observed.

However, saponins are part of the cause of enteritis in Atlantic salmon by increasing the permeability of the distal intestine (Knudsen et al. 2008; Chikwati et al. 2012). Moreover, poor development of the distal intestine has been observed in rainbow trout (*Oncorhynchus mykiss*) when saponins were added to the diet at 3.8 g kg^{-1} (Iwashita et al. 2008, 2009). It has also been suggested that saponins negatively affect growth due to intestinal damage and consequently nutrient absorption (Collins et al. 2013). When extracted saponins were added to the diet of juvenile European sea bass (*Dicentrarchus labrax)* at 1 and 2 g kg^{-1}, Couto et al. (2015) observed that growth performance was the same as in the fish receiving a control diet without saponins. However, at 2 g kg^{-1} added saponins, the enzyme activity in the distal intestine was reduced, and signs of inflammation were observed (Couto et al. 2015). Longer periods of being fed saponins may cause more adverse effects, as discussed by Couto et al. (2015) and Iwashita et al. (2009). Chikwati et al. (2012) observed that the effect of added saponins to the diet of Atlantic salmon depended on the other feed ingredients used, which resulted in different total amounts of saponins. One additional concern regarding saponins is their bitter taste, which decreases the feed intake in fish (Collins et al. 2013; Glencross et al. 2020). It is therefore of importance to take the total amount of saponins into consideration when formulating fish diets.

Tannins are compounds found in both terrestrial plants, mainly legumes and grains, and marine plants (Krogdahl et al. 2022). The ones found on land can be divided into three groups: condensed tannins (CT), hydrolysable tannins (HT), and complex tannins, which are a mix of CT and HT (Krogdahl et al. 2022; Molino et al. 2023). Tannins protect the plant from being eaten, pathogens, and other environmental stressors (Brutti et al. 2024). They can also protect the plant from oxidative stress, solar radiation, and dryness (Molino et al. 2023). All tannins are heat stable and affect both animals and humans by decreasing protein digestibility, either by making it unavailable by binding to the protein or by inhibiting enzymes (Kaushik et al. 2018, Molino et al. 2023). Tannins can also decrease the feed palatability and, thereby, feed intake, and together with decreased protein digestibility, the growth rate of fish fed diets containing tannins decreases (Kaushik et al. 2018). At the same

time, it has also been shown that tannins can improve feed intake in rainbow trout and that they exhibit antioxidant properties (Collins et al. 2013; Krogdahl et al. 2022; Brutti et al. 2024). Several studies have evaluated tannins as nutritional additives with varying results, a summary can be seen in Table 1. These results imply that the effects of tannins on growth performance and health are dependent on the dose, type of tannin, and the fish being fed.

Table 1. Summary of the effects of tannins on fish health and performance.

Reference	Tannin type	Dose, g kg^{-1} DM	Fish species	Positive effects	Negative effects
Yang et al. 2025	CT	0.6–3.75	Juvenile largemouth bass (Micropterus salmoides)	Improved growth and intestinal health	
		9.38			Reduced growth and intestinal health issues
Qiu et al. 2023	HT	>1	Chinese sea bass (Lateolabrax maculatus)		Reduced growth, lower trypsin & lipase activity
		2–4			Lower feed intake, intestine/liver damage
Qiu et al. 2024	CT / HT	2	Chinese sea bass	Increased antioxidant capacity & immune response	Reduced growth and enzyme activity, intestine/liver damage, lower feed intake (CT worse than HT)
Brutti et al. 2024	CT	0.15[1]	Nile tilapia (Oreochromis niloticus)	Highest growth performance & immune response	
		0.15–1[1]		Higher survival rate in bacterial challenge	
Yang et al. 2024	HT	1–2	Largemouth bass	Improved digestibility in soybean meal diets	
		4			Reduced growth due to lower feed intake
Omnes et al. 2017	TA	>10 20-30	European seabass juveniles		Reduced protein digestibility Reduced feed intake and growth

CT, condensed tannins; HT, hydrolysable tannins; TA, tannic acid. [1]As is

Chitin is a well-studied bioactive mucopolysaccharide polymer that is said to be the second most abundant carbohydrate in living organisms after cellulose and it is found in the exoskeletons of crustaceans and insects and the cell walls of fungi (Karlsen et al. 2017; Rimoldi et al. 2019, 2023; Kaushik et al. 2022). This fraction is also known as animal fibres and is generally seen as non-digestible by fish according to Rimoldi et al. (2019, 2023) and Yandi et al. (2023). However, chitinase, the enzyme that degrade chitin, can be found in several fish species with varying activity (Hasan et al. 2023; Mengkrog Holen et al. 2024; Pascon et al. 2025). Chitin can act as an energy sink if not utilized, which can reduce growth in fish (Karlsen et al. 2017). Supplementation of chitin in the diet for Atlantic salmon higher than 1 % has been shown to negatively affect growth (Karlsen et al. 2017). A negative effect might be due to lower digestibility, which could be due to the chitin binding to digestive enzymes (Eggink et al. 2022). Further, chitin might not just act as an antinutrient by decreasing the digestibility of the feed, it can also cause an overestimation of the crude protein since it contains nitrogen (Janssen et al. 2017).

In contrast, chitin can act as a potential prebiotic and improve the gut microbiota of fish (Terova et al. 2019). Rimoldi et al. (2023) found positive effects on the gut microbiota when chitin was included as a functional feed from two different sources in the diet of rainbow trout. However, the amount of chitin in each diet was not presented, and it is therefore not possible to conclude how much chitin is accepted by the fish.

1.2 Characteristics and effects of contaminants

The term 'dioxins' refers to two chlorinated core structures: polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), each of which has multiple structural congeners (Glencross et al. 2020). There are 75 PCDDs and 135 PCDFs, of which 7 and 10 are toxic, respectively (Van den Berg et al. 2006). The most toxic one is 2,3,7,8-tetrachlorodibenzodioxin (TCDD), and the toxicity of all the other variants is measured in relation to this one (Van den Berg et al. 2006). They are often a product of combustion and manufacturing processes (Mikolajczyk et al. 2020). When consumed by fish, dioxins can cause fin necrosis, haemorrhages, reduced growth, fertility issues and mortality (EFSA 2018). The impact dioxins have on humans includes immune and enzyme disorders, possible human carcinogens, birth defects, and even death at high exposure (Glencross et al. 2020). Since dioxins are lipophilic, they tend to accumulate in the fat of fish, which causes concerns for human consumption, especially regarding fat fish from the Baltic Sea (Glencross et al. 2020; Mikolajczyk et al. 2020; Commission Regulation (EU) 2023/915). Due to bioaccumulation, older fish have a higher content of dioxins in their fatty tissue (Hagebro 2004).

Heavy metals at certain doses also have toxic properties, and the ones of most concern in fish feed are arsenic (As), cadmium (Cd), lead (Pb), and mercury (Hg) (Krogdahl et al. 2022; Glencross et al. 2020). Fish can be exposed to heavy metals both through the water and the feed, with varying effects depending on the route of exposure (Luo et al. 2015; Naz et al. 2023). Both soil and oceans can be contaminated by heavy metals, and they can therefore find their way into several feed sources (NRC 2011; Krogdahl et al. 2022). The toxicity depends on the chemical form of the metal, which also affects its potential to accumulate in the muscle of the fish (Biancarosa et al. 2019; Glencross et al. 2020). Inorganic arsenic (iAs) is toxic, and the organic form is non-toxic. In contrast, the other way around is true for mercury and lead (Krogdahl et al. 2022). It is therefore important to analyse which form the metal is in when evaluating a feed ingredient. One of the concerns with heavy metals is that some of them accumulate in the fish that eat contaminated feed, which in turn can cause health risks for humans consuming the fish (Krogdahl et al. 2022; (Singh & Sharma 2024). It is possible to perform

decontamination processes to reduce the content of dioxins in feed ingredients, however, this is not possible to do for contamination by heavy metals (Glencross et al. 2020).

Cd binds to proteins in fish, but it doesn't accumulate in fish muscle, however, it has been shown to accumulate in the gonads of Nile tilapia (Luo et al. 2015; Glencross et al. 2020). It can cause fertility issues, depressed growth and immune system alterations in fish (Naz et al. 2023; Singh & Sharma 2024). Pb is one of the most toxic non-radioactive metals and causes damage to the nervous system when ingested (Glencross et al. 2020; Naz et al. 2023). Hg is also known for being very toxic, and it is the most well-studied metal due to its association with aquatic environments and its ability to accumulate in fish tissue (Adamse et al. 2017; Glencross et al. 2020). Methylmercury, the organic form of Hg, can induce oxidative stress and have a negative effect on the nervous system and reproduction of fish (Naz et al. 2023; Singh & Sharma 2024). Some As species is toxic even at low levels and can cause oxidative stress, epithelial lifting and developmental arrest (Singh & Sharma 2024).

PFAS are organic chemicals with several areas of use, including surface protectors, firefighting foam, hydraulic oil, metal-plating, and ski waxes (Johansson & Undeman 2020). They are very stable in the environment and can transform into different PFASs, they are therefore considered POPs (Johansson & Undeman 2020). The various substances can be hard to monitor since there are over 4000 known PFASs and there aren’t analysis methods for all of them (Johansson & Undeman 2020). Some are more known than others and have been banned for production, however, this has led to others being produced instead, and the phase-out of PFAS is thus difficult (Johansson & Undeman 2020). PFAS have been found to accumulate in rainbow trout when fed spiked feed, but to decrease when fed non-spiked feed (Goeritz et al. 2013; Vidal et al. 2019). No effect on fish health was observed in these studies (Goeritz et al. 2013; Vidal et al. 2019). However, several biological effects have been observed in zebrafish (*Danio rerio*) receiving PFAS in their diet, including development issues, oxidative stress, endocrine and metabolic disruption, as well as behavioural and gene expression alterations (Kreychman et al. 2024).

There are limits on the allowed content of dioxins, As, Cd, Pb and Hg in both feed for fish and human food (Directive 2002/32/EC; Commission Regulation (EU) 2023/915). The limits for animal feed regulate both different types of feed ingredients and complete feeds. The limit for dioxins is expressed as the sum of PCDD/F in World Health Organisation toxic equivalents (WHO-TEQ), which is calculated using the WHO-TEF (toxic equivalency factors) (Van den Berg et al. 2006). A compilation of relevant limits can be found in Table 2. However, there are

currently no limits for PFAS in feed and for other heavy metals. For fish intended for human consumption, there are limits on four of the PFAS (Commission Regulation (EU) 2023/915).

Table 2. Compilation of relevant maximum allowed contents of undesirable substances (Directive 2002/32/EC).

		Heavy metals[2]			
	Sum of dioxins[1]	**As**	**Cd**	**Pb**	**Hg**
Feed materials		2		10	0.1
Feed materials of animal origin	0.75		2		
Feed materials of plant origin	0.75		1		
Fish, other aquatic animals and products derived thereof	1.25	25[3]			0.5
Complete feed for fish	1.75	10[3]	1	5	0.2

[1]Maximum content in ng WHO-PCDD/F-TEQ kg^{-1} relative to a feed with a moisture content of 12 %. [2]Maximum content in mg kg^{-1} relative to a feed with a moisture content of 12 %. [3]The content of iAs must be lower than 2 mg kg^{-1}.

1.3 ANFs and contaminants found in novel feed ingredients

1.3.1 Potential of mussel meal in fish diets

Blue mussels have the potential to become an important ingredient in fish feed due to their favourable amino acid content and potential to be produced locally (Langeland et al. 2016; Azad et al. 2023). Mussel meal provides a good taste to the feed and show potential for inclusion to arctic charr (*Salvelinus alpinus*) (Carlberg et al. 2015; Vidakovic et al. 2016). Additionally, mussels are environmentally friendly since they counteract eutrophication by taking up excess nutrients from the environment in which they are produced (Kotta et al. 2020; Azad et al. 2023). However, since mussels are filter feeders, they can accumulate various contaminants such as dioxins, heavy metals, and PFAS, which is of concern when they are used in feed or directly consumed by humans (Parolini et al. 2020; Gomez-Delgado et al. 2023).

Gomez-Delgado et al. (2023) observed accumulation of As, Cd, Pb and Hg in blue mussels grown in Norway and that it varied depending on the season, size of the mussels, temperature, salinity, and phytoplankton blooms. They also found As to be of most concern since high levels were detected that sometimes exceeded the limits for feed and food (Directive 2002/32/EC; Commission Regulation (EU) 2023/915). It has also been shown that the concentration of As in blue mussels depend on where they were grown (Sloth & Julshamn 2008). Tibon et al. (2023) found the amount of arsenic to decrease in blue mussels even though they were fed

algae spiked with arsenic, however, they explained this to the content of arsenic during their experiment being lower than the natural levels the mussels were exposed to before the trial.

1.3.2 Potential of black soldier fly meal in fish diets

Several studies have been done to evaluate insect meals as substitutes for FM in fish diets. BSFM has a high nutritional value, which makes it a good replacement (Rimoldi et al. 2019; Fisher et al. 2020; Eggink et al. 2022). Insects are also a natural part of the diet of many fish species and, therefore, well accepted by the fish (Henry et al. 2015). Additionally, the black soldier fly larvae (BSFL) can be produced with waste in the growth substrate, which makes BSFM a circular and sustainable feed ingredient (Albrektsen et al. 2022). However, one obstacle to how much BSFM can be included in the fish diet is the content of chitin present in the exoskeleton of the BSFL (Eggink et al. 2022). Results from previous studies with BSFM in the experimental diets of fish vary between positive and negative effects, depending on the chitin content of the feed and the aim of the study. As presented in Table 3, the effect of chitin on growth is either the same as in the control diet or negative at higher levels of chitin, whereas the effect on the gut microbiota is always positive.

Chitin can negatively affect the digestibility of dry matter, crude protein, and NFE, and the lower protein digestibility might be due to chitin binding to some enzymes (Belghit et al. 2018; Eggink et al. 2022). It has also been discussed that a lower digestibility of protein might be due to it being bound to chitin (Weththasinghe et al. 2021). Furthermore, the defatting process of some BSFM may cause lipids to bind with chitin, thus affecting their availability for utilisation (Kroeckel et al. 2012). Kroeckel et al. (2012) argued that chitin may reduce the feed intake and thereby growth performance.

However, Weththasinghe et al. (2022a) found a positive effect on growth performance with the inclusion of insect meal in the diet of Atlantic salmon, which they partially attributed to the inclusion of chitin. Additionally, positive effects on the gut microbiota and gut health in rainbow trout and Atlantic salmon with BSFM in the diet have been observed, possibly due to the chitin (Huyben et al. 2019; Rimoldi et al. 2019; Terova et al. 2019; Weththasinghe et al. 2022b). Previous research has also shown positive effects on the number of goblet cells (Yandi et al. 2023), while another study showed upregulation of stress and inflammation-related genes with high BSFM diets in rainbow trout (Cardinaletti et al. 2019). However, neither study provided information on chitin levels in the diets. There is also a need for longer studies to discover the long-term effects of BSFM, and more specifically, chitin.

Table 3. Summary of the content and effect of chitin from BSFM in feed on fish.

Reference	Inclusion of BSFM, g 100 g^{-1} DM	Chitin in feed, g 100 g^{-1} DM	Chitin in meal, g 100 g^{-1} DM	Fish species	Effect, gut	Effect, growth	Source of BSFM
Rimoldi et al. 2019	8.9–27	0.55–1.51	4.96	Rainbow trout	Positive		Partially defatted prepupae
Bruni et al. 2018	18.8–37.7	1.05–2.09	5.56[1]	Rainbow trout	Positive	Same as control	Partially defatted larvae
Eggink et al. 2022	24–24.4	1–4.7[1]	1.8–15.4	Rainbow trout		Negative	Partially defatted larvae. Sieved into three size fractions.
Huyben et al. 2019	28	1.9	6.77[1]	Rainbow trout	Positive		Larvae
	28	2.8	9.97[1]		Positive		Defatted larvae
	27.5	3	10.9[1]		Positive		Prepupae
Weththasinghe et al. 2021	7.3–14.6	0.6–1.2[1]	8	Atlantic salmon		Same as control	Larvae
	29.2	2.3[1]				Negative	
Weththasinghe et al. 2022a (Growth)	17.9	1.44[2]	7.05[2]	Atlantic salmon	Positive	Positive	Full fat larvae
	12.9	1.44[2]	9.65[2]		Positive	Same as control	Defatted larvae
Weththasinghe et al. 2022b (Gut)	22.2	0.53[2]	2.15[2]		Positive	Positive	De-chitinased larvae
	6.6	1.43[2]	19.8[2]		Positive	Same as control	Exoskeleton of larvae
Terova et al. 2019	8.9–27	0.5–1.51	4.96	Rainbow trout	Positive	Same as control	Partially defatted prepupae
Kroeckel et al. 2012	15.7–31.7	1.6–3.2	9.6	Juvenile turbot		Same as control	Defatted prepupae
	46.5–72.3	4.7–7.3				Negative	

[1]Values have been calculated by the author based on information from the articles. [2]As is, %.

Other uncertainties regarding the safety of using insect meal include the risk of contamination from heavy metals, dioxins, and PFAS, since BSFL easily retain or accumulate contaminants from the growth substrate if present (Schmitt et al. 2019; Fels-Klerx et al. 2020; Jensen et al. 2022; Liland et al. 2023; Belghit et al. 2024). If the level of contaminants is high, the BSFL may accumulate more than what is allowed in feed ingredients (Alagappan et al. 2022; Belghit et al. 2024; Kofroňová et al. 2024). It is therefore important to make sure the amount of contaminants in the substrate is as low as possible to ensure the safety of the BSFM (Belghit et al. 2024).

1.3.3 Potential of *P. variotii* in fish diets

The filamentous fungi *P. variotii* has been evaluated as a fish feed ingredient due to its high nutritional value and sustainability aspects (Albrektsen et al. 2022; Wikandari et al. 2022; Hooft et al. 2024; Gaudhaman et al. 2025). Diets including *P. variotii* have been shown to have a lower environmental impact compared to when soy is used (Bergman et al. 2024; Hooft et al. 2025). Moreover, *P. variotii* can be produced with waste from various sources in the growth substrate and is, therefore, a circular protein source that does not compete with human food

production (Albrektsen et al. 2022; Wikandari et al. 2022). Additionally, *P. variotii* not only provides protein to the diet of fish, it also has potential health benefits, making it a functional feed as well (Hooft et al. 2024; Mensah et al. 2024). The quality and nutritional composition of *P. variotii* depend on the substrate it has grown on, the fermentation conditions, and how it is prepared before being used in feed (Bergman et al. 2024; Hooft et al. 2024; Mensah et al. 2024).

Bergman et al. (2024) conducted a study on rainbow trout in which soy protein was partially or fully replaced by extruded or non-extruded *P. variotii* at a level of 15 or 20 % of the diet. It was shown that the fish getting the diets with extruded *P. variotii* had the same feed conversion ratio (FCR) as the fish getting the control diet. Similar results were reported in another study on rainbow trout in which *P. variotii* was included at 30 % (Gaudhaman et al. 2025). In a study on Atlantic salmon in which up to 20 % of the protein content was replaced by *P. variotii,* the results showed an improved FCR with increasing levels of *P. variotii* in the feed (Hooft et al. 2024). However, the digestibility decreased linearly with higher inclusion rates, and it was discussed that it might have to do with the content of chitin and other parts of the cell wall of the fungi (Hooft et al. 2024). Still, these results imply that this is a promising protein source in fish feed.

1.3.4 Potential of leaf protein concentrates in fish diets

Alfalfa and white clover are perennial crops mainly grown to provide protein-rich forage for ruminants (Coburn et al. 2021). These can both be grown locally, and offer several environmental benefits, resulting in a more sustainable feed source than soy, for example (Stødkilde et al. 2018; Damborg et al. 2020; Coburn et al. 2021). They also provide a high protein yield per hectare and have a favourable amino acid content (Chatzifotis et al. 2006; Stødkilde et al. 2018; Møller et al. 2021; Chen et al. 2025). In order to increase the use of alfalfa and white clover, they can be processed into LPCs that can be properly digested by monogastric animals (Møller et al. 2021). Depending on the processing method, a green or white protein concentrate can be produced, with the white having higher protein content and improved flavour due to removing chlorophyll (Møller et al. 2021). However, the additional production steps required for the white protein concentrate make it more costly.

Alfalfa and white clover contain ANFs, such as saponins and tannins, which limit the inclusion of LPCs derived from these legumes in feed for fish (Sakamoto et al. 1992; Chatzifotis et al. 2006; Carlsen & Fomsgaard 2008; Møller et al. 2021; Grela et al. 2023). A few growth trials have been done to evaluate alfalfa protein concentrates (APC) on fish performance (Chatzifotis et al. 2006; Coburn et al. 2021; Chen et al. 2025; Navarrete 2025). Despite the differences in the nutritional

composition of the concentrates used in these studies, the inclusion of APC exceeding 10 % resulted in reduced growth performance, partly due to the lower digestibility of the experimental feeds and a decreased feed intake.

Alfalfa and white clover might also contain contaminants, and this depends on the contamination levels of the soil (Lanier et al. 2016; Rezaeian et al. 2020; Li et al. 2024). Heavy metals have been detected in different APCs, indicating that contaminants can remain in the LPC after processing (EFSA 2009).

1.4 Aim

This thesis aims to fill knowledge gaps on antinutrients and contaminants in novel fish feed ingredients, including some of the protein sources that have recently been the focus of research, such as MM, BSFM, *P. variotii*, and LPCs derived from alfalfa, white clover, perennial ryegrass and tall fescue. These feed ingredients may contain various ANFs and contaminants. This thesis focuses on saponins, tannins, chitin, dioxins, heavy metals and PFAS. A method for the analysis of saponins will be refined and standardised. Specific questions to be answered are: 1) What levels of anti-nutritional factors exist in these chosen feed ingredients? 2) What levels of heavy metals and chosen contaminants exist in the selected feed ingredients? 3) Based on the results, what are the risk factors connected to the ANFs and contaminants through the value chain, focusing on their effect on fish and the potential transfer to fish fillets?

2. Materials and methods

2.1 Raw materials

The raw materials included were MM, BSFM, Mycomeal (*P. variotii*), APC, white clover protein concentrate (WPC), ProteinMax biomass concentrate (PMC), the frass from the BSFL and fish sludge used in the substrate of the BSFL. A FM, soybean meal (SBM), and soy protein concentrate were also included for comparison with the novel feed ingredients.

The MM was provided by Ecopelag, Värmdö, Sweden. Blue mussels were farmed in the Baltic Sea. Processing included de-shelling with a pressure cooker at 130 °C, drying of the meat at 75 °C using infrared drying technology and milling.

BSFL and their residue, frass, were provided by the Department of Energy and Technology, SLU, Uppsala, Sweden. The BSFL were fed poultry feed until five days of age, thereafter, the BSFL were grown on a substrate of cabbage, brewer spent grains and fish sludge, comprising 15, 60 and 25 % of the substrate. They were harvested and euthanised by blanching after a total of 15 days. The fish sludge was provided by Smögenlax Aquaculture AB, Kungshamn, Sweden, from a land-based recirculating aquaculture system (RAS) rearing Atlantic salmon. Preparation of the BSFL, frass and fish sludge before analysis included freeze-drying and grinding.

Mycomeal was provided by Cirkulär AB, Lund, Sweden. It was produced from the filamentous fungi *P. variotii* in a continuous fermentation process at Cirkulär AB's plant. It utilised spent sulphite liquor (SSL) as the main substrate, which was provided by Sylvamo-Nymölla paper mill, Nymölla, Sweden. The processing of the Mycomeal included separation of the *P. variotii* from the fermented sulphite liquor, filtration, washing and incubation in a drying oven at < 70 °C until a moisture content of < 10 % had been reached. Lastly, a grain milling machine was used to produce a powder.

The APC, WPC and PMC were provided by the Department of Biological and Chemical Engineering at Aarhus University, Aarhus, Denmark. The APC was produced from alfalfa biomass, the WPC from 100 % white clover biomass, and the PMC from a biomass mixture of white clover, perennial ryegrass, and tall fescue, with the seeds comprising 20, 55 and 25 % of the mixture, respectively. The production steps for the LPCs have been summarised in Figure 1.

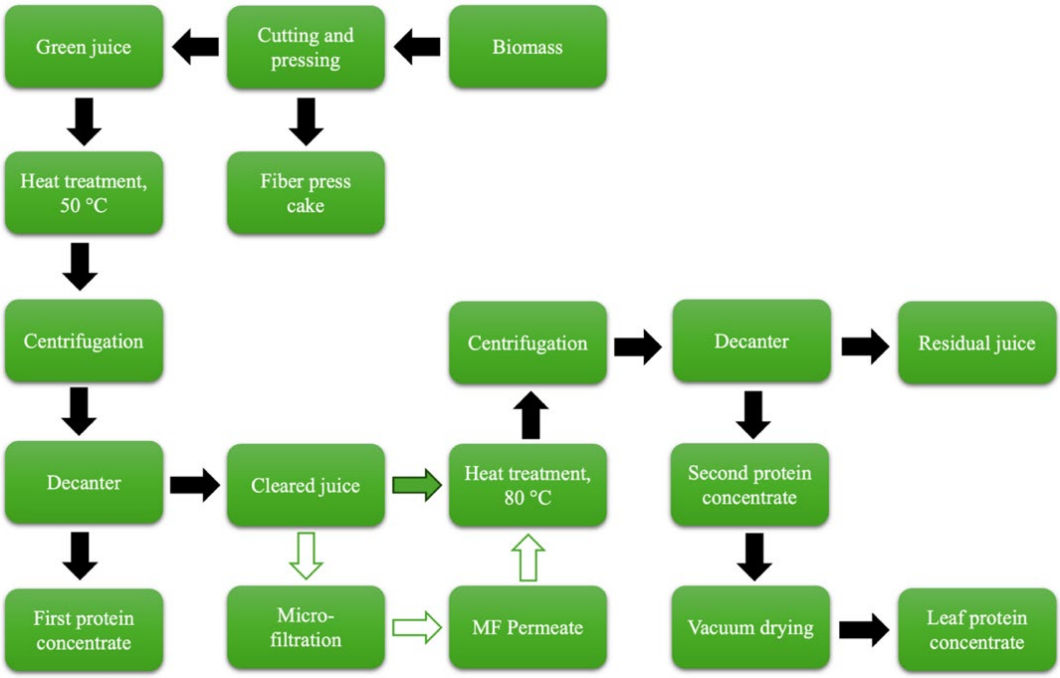

Figure 1. The production steps of the leaf protein concentrates. The green arrow indicates the production of the white clover protein concentrate and the ProteinMax biomass concentrate, and the white arrows indicate the additional steps for the production of the alfalfa protein concentrate.

FM was produced by Pelagia™, Bergen, Norway, soybean meal was purchased from Granngården, Uppsala, Sweden, and the soy protein concentrate HP 310 was provided by Hamlet Protein™, Horsens, Denmark.

2.2 Analysis of nutritional composition

The nutritional composition and the amino acid profile of the raw materials can be found in Tables 4 and 5. The dry matter (DM) was determined by drying all samples in an oven at 103 °C for 16 hours and weighing them after being cooled in a desiccator. Ash was determined by drying the samples at 550 °C for 3 hours and then weighing after being cooled in a desiccator. Crude protein (CP) was determined by analysing the total nitrogen (N) according to the Kjeldahl method using a 2520 Digestor, Kjeltec 8400 Analyser unit and an 8460-sampler unit (FOSS Analytical A/S, Hilleröd, Denmark). N was multiplied by 6.25 to receive the content of CP, except for the BSFM, for which 4.76 was used to account for the presence of non-protein N (NMKL, 1976; Janssen et al. 2017). Analysis of crude lipids (CL) was performed according to the Official Journal of the European Union (2009) using a Hydrotec 8000 and a Soxtec 8000 Extraction Unit (FOSS Analytical A/S, Hilleröd, Denmark). Crude fibre (CF) was analysed according to Jennische and Larsson (1990). For analysis of gross energy (GE), an isoperibol bomb calorimeter (Parr 6300, Parr Instrument Co., Moline, IL, USA) was used. Amino acids were analysed by Eurofins Biopharma Product Testing Sweden AB in

Uppsala, Sweden, using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS). The analysis of DM, ash, CP, CL, CF, and GE was conducted at the Department of Applied Animal Science and Welfare, SLU, Uppsala, Sweden.

Table 4. Nutritional composition and amino acid profile of the studied feed ingredients (expressed as g 100 g^{-1} DM if nothing else is stated).

Ingredient	**MM**	**BSFM**	**MY**	**APC**	**WPC**	**PMC**	**FM**	**SBM[1]**	**HP310**
Dry matter (%)	96.2	98.0	94.3	95.0	94.6	94.6	90.0	88	92.1
Ash	9.4	9.9	12.7	7.8	26.1	4.8	17.2	6.8	7.8
Crude protein	72.6	36.9	50.5	81.8	44.7	62.4	76.2	53.4	61.3
Crude fat	8.9	24.3	6.4	0.1	3.5	6.3	8.7	4.3	1.4
Crude fibre	0.2	6.3	7.9	0.1	0.9	0.0	0.0	6.3	4.3
Gross energy (MJ kg^{-1} DM)	22.8	26.7	21.1	22.2	19.2	25.0	20.6	20.2	20.3
Sum of EAA	31.3	18.9	19.4	40.5	21.7	31.4	31.8	23.4	26.2
Essential amino acids									
Arginine	4.6	2.3	2.9	5.8	2.8	3.9	4.5	3.9	4.2
Histidine	1.5	1.4	1.0	2.4	1.1	1.5	1.5	1.5	1.6
Isoleucine	3.0	1.9	2.0	3.7	2.2	3.3	3.0	2.5	2.8
Leucin	4.8	3.2	3.3	7.3	4.2	6.0	5.4	4.1	4.7
Lysine	5.6	3.0	3.3	5.2	2.8	4.0	5.9	3.3	3.7
Methionine	1.9	0.7	0.7	1.6	0.9	1.3	2.1	0.7	0.8
Phenylalanine	3.0	1.8	1.9	5.0	2.7	3.8	2.9	2.8	3.2
Threonine	3.6	2.0	2.1	4.5	2.2	3.4	3.1	2.1	2.3
Valine	3.4	2.7	2.3	5.1	2.7	4.1	3.4	2.5	2.9
Non-essential amino acids									
Alanine	3.4	3.1	2.7	5.0	2.7	4.3	4.8	2.3	2.7
Aspartic acid	7.6	4.4	4.8	8.7	4.5	6.8	7.2	6.1	7.4
Cysteine +Cysteine	1.2	0.5	0.4	0.7	0.3	0.4	0.7	0.8	1.0
Glutamic acid	8.6	5.8	5.3	9.3	4.8	7.2	10.1	9.3	11.2
Glycine	4.2	2.5	2.2	4.5	2.5	3.6	5.0	2.3	2.6
Hydroxyproline	0.2	<0.2[2]	<0.2[2]	<0.2[2]	<0.2[2]	<0.2[2]	0.6		<0.2[2]
Ornithine	<0.01[2]	<0.01[2]	0.2	<0.01[2]	<0.01[2]	<0.01[2]	<0.01[2]		<0.01[2]
Proline	3.1	2.9	2.0	3.8	2.2	3.1	3.2		3.3
Serine	3.5	2.1	2.1	3.0	2.0	3.1	3.2	2.7	3.1

MM, mussel meal; BSFM, black soldier fly meal; MY, Mycomeal; APC, alfalfa protein concentrate; WPC, white clover protein concentrate; PMC, ProteinMax biomass concentrate; FM, fish meal; SBM, soybean meal. [1]Data was collected from Granngården (n.d.) and IAFFD (2025), where possible. [2]As is, g 100 g^{-1}

Table 5. Nutritional composition of the frass and fish sludge (expressed as g 100 g^{-1} DM if nothing else is stated).

	Dry matter (%)	Ash	Crude protein	Crude lipids
Frass	20.8	8.5	19.6	
Fish sludge	11.3	15.7	35.0	22.5

2.3 Analysis of anti-nutritional factors

Analyses of ANFs were done at the Department of Applied Animal Science and Welfare, SLU, Uppsala, Sweden. An overview of what analyses were done on which materials is shown in Table 6.

Table 6. Overview of ANF and contaminant analyses done on the respective materials.

	ANFs		Contaminants			
Sample	**Saponins**	**Tannins**	**Chitin**	**PFAS**	**Metals**	**Dioxins**
APC	X	X		X[1]	X	
WPC	X	X		X	X	
PMC	X	X		X	X	
Soybean meal	X	X		X	X	
HP 310	X	X		X	X	
Mycomeal			X	X	X	
Mussel meal				X	X	X
BSFM			X	X	X	X[1]
Frass				X	X	X
Fish meal				X	X	X
Fish sludge				X	X	X

ANFs, anti-nutritional factors; PFAS, Per- and polyfluoroalkyl substances; APC, alfalfa protein concentrate; WPC, white clover protein concentrate; PMC, ProteinMax biomass concentrate; BSFM, black soldier fly meal. [1]Analysis not recieved

2.3.1 Extraction and quantification of saponins

The method for the analysis of saponins is based on multiple established methods (Cheok et al. 2014; Le Bot et al. 2022; Mora-Ocación et al. 2022). The extraction of saponins was done by weighing 1 g of sample into 50 ml Falcon tubes. Next, 20 ml of ethanol: water 1:1 (v/v) was added to each tube. One blank was prepared with no sample. The tubes were mixed thoroughly for approximately 20 seconds using a vortex machine before being put in an ultrasonic bath for 15 minutes at 60 °C. After half of the time had passed, the tubes were shaken manually, and at the end of the extraction, all tubes were vortexed again. The extracts were left to cool to room temperature before being filtered through 2 µm filter paper (Munktell, Ahlstrom, Helsinki, Finland) into evaporation flasks to remove solid residues. Using a rotary evaporator (Büchi Labortechnik, Switzerland), the samples were

evaporated until approximately 3 ml was left. Next, 10 ml of distilled water was added, and the extracts were transferred into 15 ml Falcon tubes.

For the spectrophotometric analysis, a series of standards with oleanolic acid (Thermo Scientific, CAS No. 508-02-1) was prepared to create a calibration curve. First, a stock solution with a concentration of 5 mg ml^{-1} was made. An ultrasonic bath at 60 °C was used for a few minutes to allow the oleanolic acid to dissolve properly. This was later used to create 6 standard solutions with concentrations of oleanolic acid ranging between 100 and 3000 µg ml^{-1}. Vanillin (Sigma-Aldrich, CAS No. 121-33-5) was used to prepare a vanillin in ethanol solution with a concentration of 5 mg ml^{-1}.

The vanillin-sulfuric acid reaction was done by transferring 0.25 ml of each sample extract, blank and standards, 0.25 ml vanillin solution and 2.5 ml sulfuric acid (72 %) to glass tubes. Parafilm was placed on all tubes to prevent spills during vortexing. All samples were placed in a water bath for 15 minutes at 60 °C to allow the colour reaction to occur. After the samples had cooled to room temperature, absorbance was measured at 544 nm using a spectrophotometer (UV-1800 Shimadzu, Japan). A calibration curve was made by plotting the absorbance against the concentration of the standards (Appendix 2, Figure 3) and the concentration of saponins in the feed ingredient samples was calculated using the equations below.

$$Concentration\ in\ extract, \mu g/ml\ (C_1) = \frac{Absorbance - 0.169}{0.0004}$$

$$Concentration\ in\ the\ sample, \mu g/mg\ (C_2) = \frac{C_1 * total\ volume\ of\ extract, ml}{sample\ weight,\ mg}$$

$$Percentage\ of\ dry\ matter, \%\ of\ DM = \frac{C_2}{10 * DM}$$

2.3.2 Extraction and quantification of total phenolics and tannins

The extraction and quantification of total phenolics and tannins were done according to the method by FAO/IAEA (2000) with a few modifications. The chemicals used were Folin-Ciocalteu reagent 2 N (Sigma-Aldrich, CAS No. 12111-13-6), sodium carbonate (Merck, CAS No. 497-19-8), tannic acid (Sigma-Aldrich, CAS No. 1401-55-4), and polyvinylpolypyrrolidone (PVPP) (Sigma-Aldrich, CAS No. 9003-39-8). The sodium carbonate solution was prepared with 20 g of pure sodium carbonate instead of 40 g of sodium carbonate (x10 H_2O) used in the method. An ultrasonic bath was used for a few minutes to allow the sodium carbonate to dissolve properly. The tannic acid solutions used to make the calibration curve were prepared in duplicates. Absorbance was measured at 725 nm

with no reference, and it was later plotted against the tannic acid concentration to make a calibration curve (Appendix 2, Figure 4).

To extract phenolics, 200 mg of each sample was weighed directly into 15 ml Falcon tubes. To analyse total phenolics, 0.02 ml of each extract was transferred to glass tubes, and distilled water was added to bring the total volume to 0.5 ml. For the analysis of simple phenolics, 0.06 ml of the second supernatant and 0.44 ml of distilled water were transferred into glass tubes.

2.3.3 Quantification of chitin

The quantification of chitin was done according to Marono et al. (2015), by which the content of chitin is estimated by subtracting the amount of protein linked to acid detergent fibre (ADIP) from the ash-free acid detergent fibre (ADF). For the analysis of ADF and ADIP, 1 g of each sample was weighed into P2 glass filter crucibles, 100 ml of AD solution was added, and all samples were gently boiled for one hour. They were thereafter filtered and washed with hot water until all foam had disappeared, followed by two washes with acetone. The samples were then dried overnight at 103 °C, cooled in a desiccator and weighed. For the determination of ash-free ADF, the dried samples were incinerated at 500 °C for 90 minutes, cooled in a desiccator and weighed. The weight of the ash was then subtracted from the weight of the sample after the extraction and drying to receive the ash-free ADF content. For the determination of ADIP, the extracted and dried sample was transferred into a Kjeldahl tube, and the CP was determined as previously described.

2.4 Analysis of contaminants

Dioxins were analysed based on Method 1613B (U.S. EPA 1994a) and EN 16190 (European Standards 2019) using High-Resolution Gas Chromatography-Mass Spectrometry (HR-GC/MS) by ALS Czech Republic, Pardubice, Czech Republic. The maximum possible (Upperbound) WHO-TEQ for the sum of dioxins was calculated according to Van den Berg et al. (2006) and Commission Regulation (EU) 2017/644 using the measured value or the numerical value of the limit of detection for each congener. For the analysis of metals, the samples were initially digested in nitric acid with the assistance of a microwave, following SS-EN 13805:2014 (SIS 2014) and then analysed using Inductively Coupled Plasma Sector Field Mass Spectrometry (ICP-SF/MS) according to SS-EN ISO 17294-2:2023 (SIS 2023) and US EPA Method 200.8:1994 (U.S. EPA 1994b) by ALS Scandinavia AB, Luleå, Sweden. PFAS were analysed by ALS Italy, Zoppola, Italy, using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) or Gas

Chromatography Tandem Mass Spectrometry (GC-MS/MS), depending on the target compound. The contaminants analysed are listed in Table 7.

Table 7. Contaminants included in the analysis.

Dioxins	**Metals**	**PFAS**
2,3,7,8-tetraCDD	Arsenic	Perfluoro-n-pentanoic acid (PFPeA)
1,2,3,7,8-pentaCDD	Cadmium	Perfluoro-n-hexanoic acid (PFHxA)
1,2,3,4,7,8-hexaCDD	Chromium	Perfluoro-n-heptanoic acid (PFHpA)
1,2,3,6,7,8-hexaCDD	Cobalt	Perfluoro-n-octanoic acid (PFOA)
1,2,3,7,8,9-hexaCDD	Copper	Perfluoro-n-nonanoic acid (PFNA)
1,2,3,4,6,7,8-heptaCDD	Lead	Perfluoro-n-decanoic acid (PFDA)
Octachlorodibenzodioxin	Manganese	Perfluoro-n-undecanoic acid (PFUnDA)
2,3,7,8-tetraCDF	Mercury	Perfluoro-n-dodecanoic acid (PFDoDA)
1,2,3,7,8-pentaCDF	Nickel	Perfluoro-n-tridecanoic acid (PFTrDA)
2,3,4,7,8-pentaCDF	Zinc	Perfluoro-n-tetradecanoic acid (PFTeDA)
1,2,3,4,7,8-hexaCDF		Perfluorobutanesulfonic acid (PFBS)
1,2,3,6,7,8-hexaCDF		Perfluoroheptanesulfonic acid (PFHpS)
1,2,3,7,8,9-hexaCDF		Perfluorohexanesulfonic acid (PFHxS)
2,3,4,6,7,8-hexaCDF		Perfluorooctanesulfonic acid (PFOS)
1,2,3,4,6,7,8-heptaCDF		Perfluorodecanesulfonic acid (PFDS)
1,2,3,4,7,8,9-heptaCDF		Perfluoropentanesulfonic acid (PFPeS)
Octachlorodibenzofuran		Perfluorononane sulfonic acid (PFNS)
		Perfluorododecanesulphonic acid (PFDoS)

PFAS, Per- and polyfluoroalkyl substances

2.5 Data management and statistical analysis

All collected data were stored in Microsoft Excel for Mac, version 16.94 (Microsoft Corporation, Redmond, WA, USA). Data for saponin, chitin, tannins, and total phenolics were analysed using GraphPad Prism v10. Given that the results were obtained in duplicates, only descriptive statistics were performed, including calculations of mean and standard deviation to summarisse the data.

3. Results

3.1 Anti-nutritional factors

3.1.1 Saponins, total phenolics and tannins

Saponins and phenolics were found in all analysed plant-derived feed ingredients. The content of saponins varied between 1.26 and 6.79 g 100 g^{-1} DM, with WPC having the highest and APC the lowest content (Figure 2a). The highest content of total phenolics and tannins was found in the PMC (0.86 and 0.29 g 100 g^{-1} DM, respectively) and the lowest in HP 310 (0.66 and 0.10 g 100 g^{-1} DM, respectively) (Figure 2b).

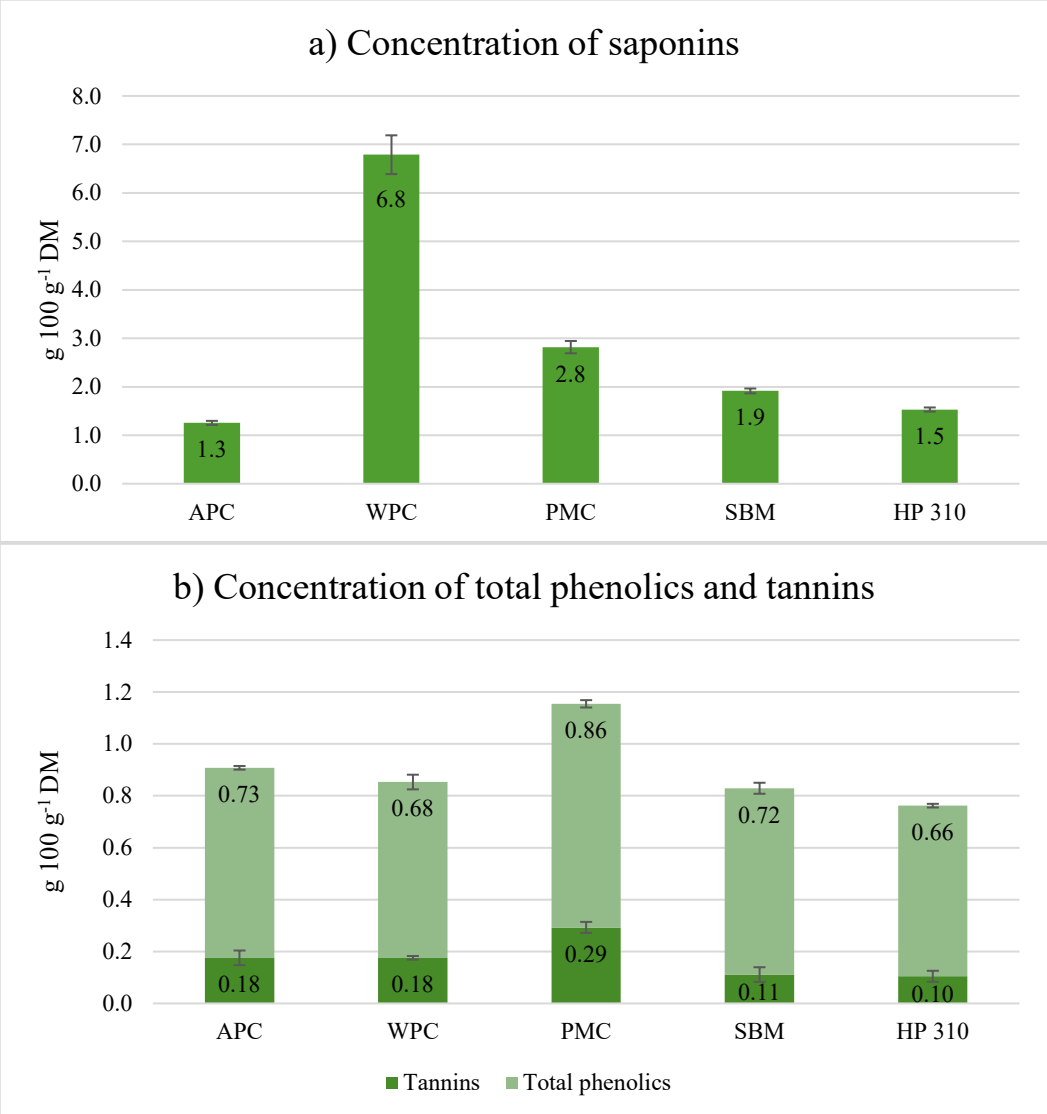

Figure 2. The content of a) saponins and b) total phenolics and tannins in the leaf protein concentrates, soybean meal and soy protein concentrate (mean ± SD, n=2).

3.1.2 Chitin

The highest chitin content was found to be 12.9±0.3 g 100 g^{-1} DM in the Mycomeal, and 4.3±0.15 g 100 g^{-1} DM in the BSFM (n=2).

3.2 Contaminants

3.2.1 Dioxins

Dioxins were not detected in the frass, however, three congeners were found in the MM, FM and fish sludge (Table 8). The MM showed the highest total concentration, followed by the fish sludge and fish meal (4.44, 2.79 and 2.36 ng kg^{-1} DM, respectively). When comparing the calculated maximum possible WHO-TEQ for the raw materials with the EU maximum allowed content for the sum of dioxins according to Directive 2002/32/EC, no raw material exceeded the limit (Figure 3).

Table 8. Dioxin content in the selected raw materials (expressed as ng kg^{-1} DM).

	MM	**FM**	**FS**	**F**
2,3,7,8-tetraCDD	<0.0035	<0.0025	<0.0017	<0.018
1,2,3,7,8-pentaCDD	<0.0067	<0.005	<0.0037	<0.038
1,2,3,4,7,8-hexaCDD	<0.018	<0.013	<0.01	<0.15
1,2,3,6,7,8-hexaCDD	<0.011	<0.0095	<0.0092	<0.1
1,2,3,7,8,9-hexaCDD	<0.012	<0.011	<0.011	<0.12
1,2,3,4,6,7,8-heptaCDD	<0.016	<0.012	<0.011	<0.17
Octachlorodibenzodioxin	<0.029	<0.02	<0.019	<0.2
2,3,7,8-tetraCDF	1.98	1.11	0.24	<0.016
1,2,3,7,8-pentaCDF	0.9	1.09	2.38	<0.13
2,3,4,7,8-pentaCDF	1.56	0.16	0.17	<0.079
1,2,3,4,7,8-hexaCDF	<0.0081	<0.0059	<0.0045	<0.079
1,2,3,6,7,8-hexaCDF	<0.0064	<0.0048	<0.004	<0.034
1,2,3,7,8,9-hexaCDF	<0.0087	<0.0068	<0.0054	<0.073
2,3,4,6,7,8-hexaCDF	<0.0089	<0.0068	<0.0049	<0.063
1,2,3,4,6,7,8-heptaCDF	<0.01	<0.007	<0.0067	<0.093
1,2,3,4,7,8,9-heptaCDF	<0.024	<0.018	<0.019	<0.2
Octachlorodibenzofuran	<0.022	<0.015	<0.014	<0.15

MM, mussel meal; FM, fish meal; FS, fish sludge; F, frass

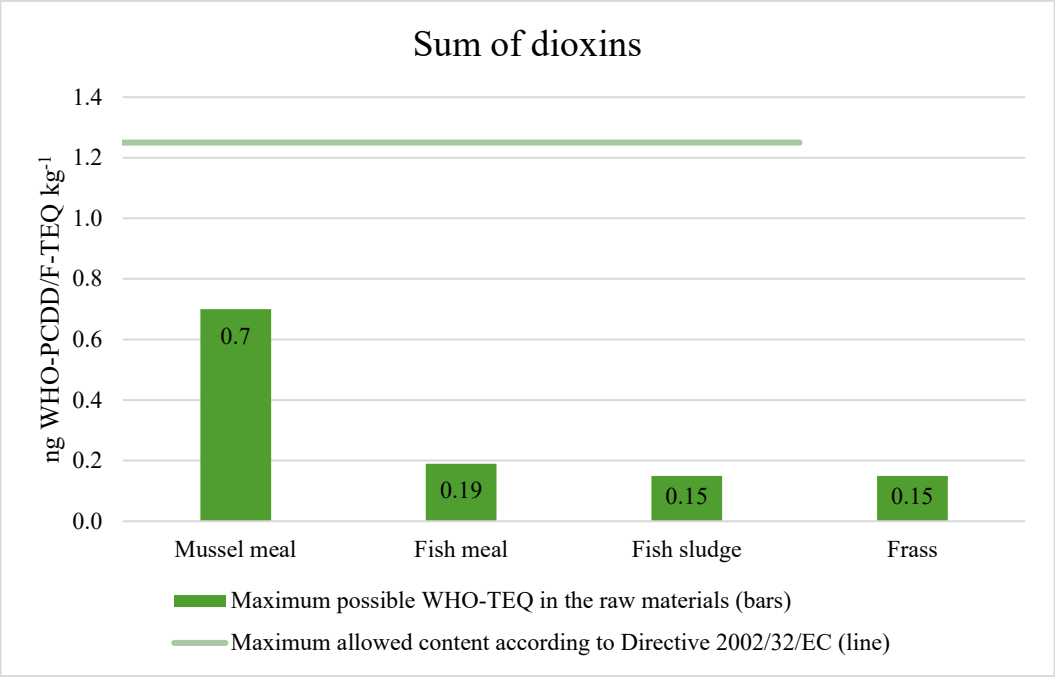

Figure 3. A comparison between the maximum possible WHO-TEQ in the raw materials and the allowed maximum content according to Directive 2002/32/EC relative to a feed ingredient with 12 % moisture.

3.2.2 Metals

Most of the raw materials had similar contents of metals, however, some raw materials differed from the others. The WPC is distinguished from other raw materials by its higher content of Pb, Cr, Co, Mn, and Ni (Table 9), which is reflected in the total amount of metals (Figure 4). Additionally, the content of As in the WPC exceeded the allowed maximum content (Directive 2002/32/EC). The content of Zn was highest in the fish sludge, which makes this raw material stand out as well, however, this is not to be used in fish feed. The MM was notable for its cadmium content, while the PMC was distinguished by its copper content compared to the other raw materials.

Table 9. Content of metals in the selected raw materials (expressed as mg kg^{-1} DM).

	As	Cd	Cr	Co	Cu	Pb	Mn	Hg	Ni	Zn
MM	5.33	1.14	0.81	0.56	11.54	0.70	114.4	0.05	2.53	110.2
BSFM	0.15	0.25	0.82	0.09	14.59	0.09	157.1	<0.01[2]	0.49	239.8
MY	0.31	0.08	1.74	0.12	9.93	0.43	305.4	<0.01[2]	1.09	94.80
APC	<0.08[2]	0.87	7.13	0.13	22.95	0.60	302.2	0.01	3.35	117.9
WPC	3.60[1]	0.20	34.04	2.32	21.35	9.08	572.9	0.04	10.78	74.42
PMC	0.44	0.10	4.14	0.26	45.56	1.32	143.8	0.05	1.26	37.32
FM	8.02	0.29	0.17	0.04	2.81	<0.04[2]	4.2	0.13	0.19	65.11
F	0.24	0.03	1.93	0.18	12.91	0.07	43.25	<0.01[2]	1.28	144.5
FS	1.04	0.29	1.19	0.29	12.54	0.09	136.8	0.02	0.82	370.9
SBM	<0.08[2]	0.06	0.57	0.19	18.30	0.08	39.32	<0.01[2]	8.16	50.45
HP 310	<0.08[2]	0.02	0.13	0.04	15.53	<0.04[2]	35.40	<0.01[2]	0.60	65.36

As, arsenic; Cd, cadmium; Cr, chromium; Co, cobolt; Cu, copper; Pb, lead; Mn, manganese; Hg, mercury, Ni, nickel; Zn, zinc; MM, mussel meal; BSFM, black soldier fly meal; MY, Mycomeal; WPC, white clover protein concentrate; PMC, ProteinMax biomass concentrate; FM, fish meal; F, frass; FS, fish sludge; SBM, soybean meal. [1]The value exceeds the EU limit (Directive 2002/32/EC). [2]As is, mg kg^{-1}.

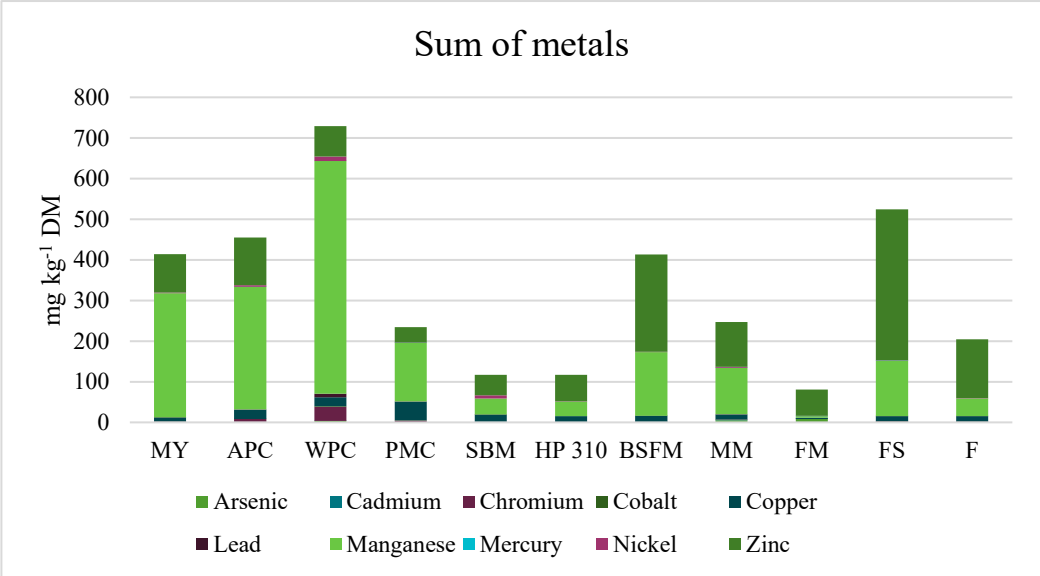

Figure 4. The sum of metals in each raw material.

3.2.3 PFAS

Most of the analysed raw materials did not contain any PFAS, however, three compounds were detected in the MM, BSFM and FM, with FM exhibiting the highest content (Table 10). PFOS was detected in all three feed ingredients at concentrations of 0.61, 0.49 and 0.44 µg kg^{-1} DM, respectively, for the MM, BSFM and FM. PFNA was detected in the BSFM and FM at concentrations of 0.11 and 0.12 µg kg^{-1} DM, respectively, and PFUnDA was detected in the FM (0.29 µg kg^{-1}

DM). All other PFAS were below the limit of detection of 0.05 µg kg^{-1} and are therefore not included in the table below.

Table 10. Concentration of the detected PFAS in the MM, BSFM and FM (µg kg^{-1} DM).

PFAS	MM	BSFM	FM
PFOS	0.61	0.49	0.44
PFNA	<RL	0.11	0.12
PFUnDA	<RL	<RL	0.29

PFAS, Per- and polyfluoroalkyl substances; MM, mussel meal; BSFM, black soldier fly meal; FM, fish meal; PFOS, Perfluorooctanesulfonic acid; PFNA, Perfluoro-n-nonanoic acid; PFUnDA, Perfluoro-n-undecanoic acid; RL, Reporting limit

4. Discussion

The results acquired in this thesis add to the potential of using MM, BSFM, Mycoprotein and LPCs in future fish feed. ANFs and contaminants are present at varying degrees, making some feed ingredients better than others for use in fish feed. Both dioxins and PFAS were detected at low levels or not at all, whereas the level of metals differed more across the raw materials. The levels of the toxic metals were low, but with some exceptions, especially regarding the WPC.

4.1 Anti-nutritional factors

4.1.1 Saponins and tannins

The results show that all plant-based protein concentrates contained both saponins and tannins (Figure 2). The APC contained a lower concentration of saponins compared to the SBM and HP 310, whereas the WPC and PMC had a higher saponin concentration. The tannin concentration was higher in all the LPCs compared to the SBM and HP 310. Previous research on APC has shown the saponin content to vary between 0.08 and 2.9 g 100 g^{-1}, which depends on the variety of alfalfa, stage of maturation at harvest, and the processing method (Livingston et al. 1979; Tava et al. 1999; EFSA 2009; Grela et al. 2023). The result acquired in this thesis falls in the middle of this range. The tannin content of another APC was previously found to be 0.15 g 100 g^{-1}, which is lower than the obtained result for the APC analysed in this thesis (Grela et al. 2023). No previous analyses on the content of saponins and tannins in the WPC and PMC have been conducted, and it is therefore difficult to conclude if these results fall within a reasonable range.

Given that all legumes contain saponins, it is not surprising that the WPC contains more than the PMC, since the content of white clover is higher in the WPC compared to the PMC. It is, however, surprising that the PMC contains more tannins than the WPC, since those are also more commonly found in legumes. Perhaps there are differences in the maturation of the white clover before harvest or different varieties, since that can result in different concentrations of tannins (Burggraaf et al. 2003). More research is needed to determine the reason.

The APC and HP 310 analysed in this thesis were the same as those used in the study by Navarrete (2025). It was found in that study that the feed intake of rainbow trout decreased significantly with the inclusion of APC at 20 % in the diet, which equals a saponin and tannin content of 0.55 and 0.056 g^{-1} DM in the diet, respectively. The control diet had a saponin and tannin concentration of 0.3 and 0.02 g^{-1} DM, respectively, coming from the soy protein concentrate. Chen et al.

(2025) did not observe any decrease in feed intake in rainbow trout with the inclusion of an APC up to 20 %, which replaced the fishmeal. However, the inclusion of soy was lower in their diets than in those prepared by Navarrete (2025), resulting in a lower total concentration of saponins and tannins. The inclusion of APC did, however, result in a decreased feed digestibility (Chen et al. 2025). The feed ingredients used by Chen et al. (2025) were also different from those used by Navarrete (2025) and might contain lower amounts of ANFs. Since the WPC and PMC contained higher concentrations of saponins and tannins, respectively, than the APC and soy, it can be expected that lower concentrations of these in the diet will be accepted by fish. However, Omnes et al. (2017) concluded that no more than 1 % of tannins should be included in the diet of European seabass, and since the analysed feed sources in this study contain up to 0.29 g 100 g^{-1}, there shouldn't be any concerns with including these in feed to carnivorous fish species. However, more research is needed on the effect of tannins on the salmonid species relevant for Swedish aquaculture production. Additionally, Chikwati et al. (2012) suggested that it is important to consider the total concentration of ANFs in a fish diet, thus, the inclusion of LPCs might result in better fish performance if it replaces the soy instead of the FM. In conclusion, the accepted inclusion of LPCs in a fish diet depends on the other ingredients used and, thereby, the total concentration of ANFs.

As previously mentioned, SBM is known to induce enteritis in salmonids, and saponins together with other ANFs are said to be the cause (Krogdahl et al. 2015). Knudsen et al. (2008) observed severe enteritis in Atlantic salmon fed a diet containing 25 % SBM and a saponin content of 0.18 g 100 g^{-1} on a dry matter basis. This saponin content is lower than what was estimated for the control diet used in the study by Navarrete (2025), and other research on the content of saponins and tannins in SBM and soy protein concentrates has shown lower concentrations than what was acquired in this thesis (Knudsen et al. 2006, 2008; Collins et al. 2013; Pokharel et al. 2023). It can therefore be challenging to determine if and to what extent the feed ingredients in this study can be incorporated into a diet to prevent enteritis or other negative effects. However, the methods used to analyse the saponin content differ between studies, which might render any comparison unfair with the current project. Moreover, the processing of soy protein concentrates is expected to reduce the levels of ANFs, however, according to the obtained results, both the SBM and HP 310 exhibited similar levels of saponins and tannins. It is thus important to have a standardised method for analysis to compare results.

4.1.2 Chitin

The chitin content in the BSFM presented in different studies varies between 1.8 and 15.4 g 100 g^{-1} on a dry matter basis, and this depends on the life stage of the BSFL at harvest as well as the preparation method of the BSFM (Table 3). The

acquired results for chitin in BSFM in the current study were 4.3 g 100 g^{-1}, which falls within the range presented by other studies. However, the measured concentration might be underestimated due to limitations of the method used for analysis. As discussed by Marono et al. (2015), the ADIP is overestimated due to some of the nitrogen being part of the chitin. According to Finke (2007), as little as 10 % of the nitrogen in the ADF can come from amino acids, depending on insect species, meaning that up to 90 % can come from chitin. To receive a more correct value of chitin, the amino acids linked to the ADF would have to be analysed and subtracted from the ash-free ADF (Finke 2007). However, this would be more costly. Considering the higher price and challenging timeframe, it was decided not to proceed with this option for the current project.

The acquired results show Mycomeal to contain 12.9 g 100 g^{-1} chitin, and similar results have been reported by the producer. However, it can be estimated based on previous research that *P. variotii* contain up to 4.5 g 100 g^{-1} chitin (Domenech et al. 1994; Hooft et al. 2024). According to Domenech et al. (1994), the cell wall of *P. variotii* contains 42.7 to 47.3 % β-glucan-chitin, of which 19.6 to 30 % is chitin, depending on the specific strain, and according to Hooft et al. (2024), the cell wall makes up about 26-32 %. Perhaps the differences in strain cause these differences in chitin content.

Based on previous studies, it appears that a chitin content below 2 g 100 g^{-1} DM of the diet does not negatively affect growth performance in rainbow trout or Atlantic salmon (Bruni et al. 2018; Terova et al. 2019; Weththasinghe et al. 2021, 2022a). This implies that the BSFM analysed in this thesis can be included at up to 46.5% on a dry matter basis in a diet for Atlantic salmon and rainbow trout without negatively impacting fish performance, if only the chitin content is considered. Hence, if the same limit of acceptable chitin in fish feed applies to the Mycomeal, then 15.5 % on a dry matter basis can be included in a diet without negative effects on fish performance. However, studies have shown inclusion of up to 20 and 30 % on an as-is basis of *P. variotii* to be accepted by rainbow trout (Bergman et al. 2024; Gaudhaman et al. 2025). Their product, though, differed from the Mycomeal analysed in this thesis due to differences in production, which limits a direct comparison. Additionally, it is suggested by Mensah et al. (2024) that ß-glucans, which are a part of the cell wall of *P. variotii,* have immunostimulatory properties, and positive health effects have been observed in Atlantic salmon fed a diet including *P. variotii*, possibly due to the ß-glucans (Hooft et al. 2024). It is, therefore, not possible to conclude how much Mycoprotein can be included in salmonid diets based solely on the content of chitin. Hence, these findings highlight the importance of considering the combined effects of chitin and ß-glucans, as well as other bioactive compounds, when evaluating a novel feed ingredient and whole fish feed recipes.

4.2 Contaminants

4.2.1 Dioxins

All of the analysed raw materials showed low or non-detectable levels of dioxins that were below the maximum limit for the sum of dioxins according to Directive 2002/32/EC, and the feed materials can, therefore, be used in fish feed without restrictions. The MM contained the highest concentration of dioxins compared to the other analysed materials, which might have to do with the mussels' origin from the Baltic Sea, since that is the most contaminated sea on Earth (HELCOM 2023). Measures have been taken to reduce contamination, however, once hazardous substances enter the Baltic, they are difficult to remove due to their persistence, and they can therefore end up in marine organisms (HELCOM 2023). Dioxins have historically been of most concern due to high emissions, however, emissions have been reduced since the 1990s, which is reflected in the levels of dioxins in fish captured in the Baltic Sea (Mikolajczyk et al. 2021).

It has been shown that dioxins accumulate over time in Atlantic salmon reared to market size and that it doesn't reach a steady state, meaning the longer the production cycle is, the more dioxins will be accumulated (Berntssen et al. 2010). The dioxin levels in the feeds used in the study by Berntssen et al. (2010) were below the limit for maximum content allowed in feed (Directive 2002/32/EC), and the fish fillets had concentrations of dioxins below the maximum limit for food. This suggests that the dioxin level found in the MM analysed in this study shouldn't be of concern for use in fish feed, even though it is higher than what was obtained for the FM.

4.2.2 Metals

All feed ingredients, except for the WPC, have levels of metals that are allowed in fish feed and can therefore be used without restriction (Directive 2002/32/EC). Unfortunately, the WPC contain As at a higher concentration than what is allowed in a feed ingredient of vegetable origin and can therefore not be used in feed. When examining the content of heavy metals in different feed and feed ingredients, marine-derived sources are often of more concern than others (Adamse et al. 2017). This statement can be confirmed by the received results, as the content of As, Cd, and Hg is highest in either the MM or FM, or both, compared to the other feed ingredients (Table 9). Fortunately, the levels do not exceed the limit for what is allowed in these feed ingredients since these are marine-derived (Directive 2002/02/EC). The results are also similar to what was observed by Hannisdal et al. (2025), who found that FM has the highest content of As, Cd and Hg compared to other fish feed ingredients. On the contrary, Pb, Ch, Co and Mn are much higher in

the WPC compared to any other feed ingredient analysed in this thesis, although Pb is still within the allowable limit set in Directive 2002/32/EC. One explanation for the high content of certain metals in the WPC could be contamination of the soil the white clover has grown on (Ghiani et al. 2014; Lanier et al. 2016). It is also possible that the contamination happened during the production of the WPC, but since the WPC and PMC went through a similar production process, this explanation is uncertain.

Since the content of As in the mussel meal is higher than 2.3 mg kg^{-1} DM, it should be analysed for iAs to make sure the limit isn't exceeded as stated in Directive 2002/32/EC. However, this is probably not necessary since iAs has been shown to only make up about 1.7-4 % of total As in different fish feeds containing either FM or MM, according to Silva et al. (2023), and less than 1.2 % of total As in fish feed according to Sloth et al. (2005). Even though these studies analysed whole fish feed and not MM specifically, it's still possible to conclude that the content of iAs won't be too high in the MM. The FM used for comparison in this study also had an elevated As content, but it has been approved for use in fish feed, and the content of iAs is therefore of no concern. Furthermore, it was also shown by Silva et al. (2023) that their analysed fish feed with either FM or MM had the same amount of total As, but the feed with MM had a higher level of iAs, showing the differences between the feed sources.

The BSFM analysed in this study shows accumulation of Cd, Pb, Cu, Mn and Zn, as these levels are higher in the BSFM compared to the frass. This results in a bioaccumulation factor (BAF) higher than one and thus a clear result of accumulation. This could be of concern regarding Cd and Pb since these are unwanted substances in the BSFM, but no toxic metal exceeds the maximum limit in the BSFM, rendering it safe for use in fish feed (Directive 2002/32/EC). The highest difference between the concentration in the BSFM and frass was found for Cd, which is in line with previous research showing that Cd is of most concern due to the high risk of accumulation (Alagappan et al. 2022; Belghit et al. 2024; Charlton et al. 2015; Schmitt et al. 2019; Fels-Klerx et al. 2020). The substrate given to BSFL needs to be monitored since high levels of contaminants can result in accumulation higher than what is allowed in feed ingredients (Belghit et al. 2024). However, the content of Cd and Pb in the frass and BSFM analysed in this study was low, which indicates that the content in the substrate used for rearing the BSFL was low too. It has been found that sludge is not an optimal growth substrate for BSFL due to reduced growth and content of undesirable substances that can accumulate (Schmitt et al. 2019; Belghit et al. 2024). However, the sludge used in the production of the BSFM analysed in this thesis had low levels of heavy metals and should therefore not be concerning for use in insect rearing in that respect.

As has been found to accumulate in fish fillets, however, no iAs has been found to accumulate in either Atlantic salmon or rainbow trout (Biancarosa et al. 2019; Granby et al. 2020; Silva et al. 2023). It was suggested by Silva et al. (2023) that iAs is either transformed or eliminated after uptake, since no retention was observed in the fillet or liver of Atlantic salmon. Additionally, it was found by Biancarosa et al. (2019) that a lower amount of total As was transferred to Atlantic salmon fillets when the fish were fed a diet containing BSFM, compared to a FM-based diet, indicating that different As species are accumulated differently in the fish (Biancarosa et al. 2019). Similar results were observed in a study on rainbow trout, where FM was found to be the source of accumulated As rather than the kelp incorporated into the experimental diets, which provided a higher amount of As (Granby et al. 2020). It seems like FM causes more concern for the accumulation of heavy metals in fish fillets than other feed ingredients, however, this should be further studied on novel feed ingredients. Biancarosa et al. (2019) and Granby et al. (2020) found no Cd or Pb in fish fillets from Atlantic salmon and rainbow trout, which isn't surprising since those have a low capacity for accumulation in fish due to poor uptake from the intestines (Ciardullo et al. 2008; Biancarosa et al. 2019; Glencross 2020). Hg is of more concern due to higher accumulation, however, as long as the content in the feed is below the maximum limit, it most likely won't exceed the maximum limit for fish muscle (Ciardullo et al. 2008). Overall, as long as the feed has a content of heavy metals below the maximum limit, the fish fillets will not be of concern for human consumption.

4.2.3 PFAS

The analysis of PFAS provided positive results, as the majority of the raw materials contained none. However, PFOS, PFNA and PFUnDA were found in the MM, BSFM and FM. For fish intended for human consumption, there is an allowed limit of 2 and 0.5 µg kg^{-1} for PFOS and PFNA, respectively, relative to a feed with 12 % moisture (Regulation (EU) 2023/915), and it has been found that both PFOS and PFNA can accumulate in rainbow trout, but with a BAF less than one (Goeritz et al. 2013; Vidal et al. 2019). The feeds used in the studies by Goeritz et al. (2013) and Vidal et al. (2019) were spiked with PFOS and PFNA, thus, the content was much higher than what was detected in the raw materials analysed in this thesis. Since the BAF is less than one, there shouldn't be a risk with using the analysed feed ingredients in which PFAS was detected. However, further studies are needed to investigate the accumulation of PFAS throughout the entire growth period of farmed fish and determine if these levels pose a concern. Additionally, it has been shown that the accumulation of PFAS in fish muscle is lower compared to other organs, and that removing skin from the fillet reduces the content of PFAS, making fish less of a concern for human consumption (Goeritz et al. 2013). Thus, regarding PFAS, all feed ingredients can be considered safe to use in fish feed.

4.3 Prospects for the novel feed ingredients

The level of contaminants in feed ingredients is highly batch-specific and varies based on the growth conditions. For example, other batches of the WPC may contain lower concentrations of As that do not exceed the maximum limit, making it allowed to be used in feed, while batches of the other feed ingredients might contain more than what is allowed according to Directive 2002/32/EC. Regarding insects and *P. variotii*, the presence of contaminants depends on the quality of the growth substrate, whereas the quality of the LPCs is influenced by soil contamination, and MM by the contamination of what the mussels consume. Despite these variations, the high protein content of all the novel feed ingredients assessed in this thesis shows promising potential for inclusion in aquafeeds. While the WPC have a lower protein content compared to the SBM and HP 310, it remains high. On the contrary, the PMC has a protein content similar to that of HP 310, and the APC exceeds both, making them more suitable for inclusion in aquafeeds compared to the WPC. The MM have a similar nutritional composition to the FM, suggesting it would be a good replacement. Although the BSFM and Mycomeal have a lower protein content than the FM and SBM, they are still valuable replacements to ensure the future sustainability of the aquafeed production. Additionally, to further increase the use of the WPC and PMC, research should focus on ways to decrease the concentration of ANFs. Since the concentration of saponins is lower in the APC compared to the WPC and PMC, it would be interesting to investigate whether that has to do with the production method, rather than the biomass used.

According to Hagebro (2004), it should be safer for humans to consume farmed fish since the feed can be controlled for unwanted substances and thus the potential transfer to fish fillets. Although the feed is the biggest source of contamination of fish fillets, it also depends on environmental factors within the rearing system of the fish (Luo et al. 2015; Lundebye et al. 2017). It was reported by Lundebye et al. (2017) that fillets from Norwegian wild-caught Atlantic salmon had significantly higher concentrations of dioxins, As and Hg compared to farmed Atlantic salmon, although still below the EU maximum limit (Directive 2002/32/EC). Monitoring of Norwegian fish feed and farmed fish shows levels of metals, fortunately, the levels for As, Cd, Hg and Pb never exceeded the EU maximum limit for fish feed and fillets (Bernhard et al. 2022; Sele et al. 2022). Additionally, there are levels of dioxins in fish fillets from farmed Atlantic salmon and rainbow trout, but these levels are also below the EU maximum limit (Bernhard et al. 2022). Analysis of fish caught in the Baltic Sea shows high content of dioxins, making them unsuitable for consumption (Mikolajczyk et al. 2021). This highlights the safety of consuming farmed fish.

5. Conclusion

In conclusion, the studied novel feed ingredients all show potential for inclusion in fish feed with a few exceptions. ANFs are present in the analysed feed ingredients at varying degrees. The content of saponins was highest in the WPC, and the concentration of tannins was highest in the PMC, which might make these potentially unsuitable for inclusion in fish feed. However, it is important to consider the total amount of ANFs in a fish diet, thus, the suitability depends on what they replace. The concentrations of dioxins and PFAS were low and of no concern in any of the tested feed ingredients. Regarding heavy metals, As was too high in the WPC, making this feed ingredient not suitable for use in fish feed, whereas the MM, Mycomeal, BSFM, APC and PMC had levels of heavy metals lower than the allowed EU maximum content and are therefore safe to use in fish feed. There will be some transfer of contaminants to fish fillets, however, there won't be any concerns for human consumption.

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Popular science summary

Safety and suitability of novel feed ingredients for use in aquafeeds

The aquaculture industry is growing, and so is the need for sustainable feed ingredients that can replace traditional components, such as fish meal and soybean, thereby reducing the environmental pressure associated with fish feed production. To ensure the safety of these novel feed ingredients, they should be evaluated for safety to ensure they do not pose health risks to either the fish or human consumers.

Some emerging feed ingredients with potential for use in aquafeed include mussel meal from the blue mussel (*Mytilus edulis*), black soldier fly meal (BSFM; *Hermetia illucens*), protein from the fungi *Paecilomyces variotii* (Mycoprotein), and leaf protein concentrates (LPCs) derived from alfalfa (*Medicago sativa*), white clover (*Trifolium repens*), perennial ryegrass (*Lolium perenne*), and tall fescue (*Lolium arundinacea*).

Two important categories of substances that should be assessed in feed ingredients are anti-nutritional factors (ANFs) and environmental contaminants. ANFs are naturally occurring compounds that can exert both positive and negative effects on fish health and performance. While some ANFs may benefit fish in small amounts, others can reduce feed palatability or interfere with digestion, depending on the type and dose. By identifying the concentration of these compounds, it is possible to estimate the inclusion rate of a feed ingredient in a fish diet. Contaminants, such as heavy metals or persistent chemicals, can be toxic and are regulated by the European Union under Directive 2002/32/EC.

To evaluate the safety and suitability of these new feed ingredients for use in fish feed, the concentrations of a few ANFs and contaminants were analysed in the current work. ANFs were detected in the LPCs at varying degrees, making them more or less suitable for inclusion in fish feed. However, it is important to consider the total amount of ANFs in fish feed, thus, what these LPCs replace is also important. One type of ANF was assessed in the BSFM and Mycomeal. However, based on the level that was found, it is still difficult to conclude the suitability of the feed ingredients.

The analysed contaminants were within safe levels in all feed ingredients except one, making them safe for use in fish feed. Some transfer of these contaminants into fish fillets can be expected, however, at low levels, and the fish consuming these feed ingredients are thus safe for human consumption.

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Appendix 1. Calibration curves.

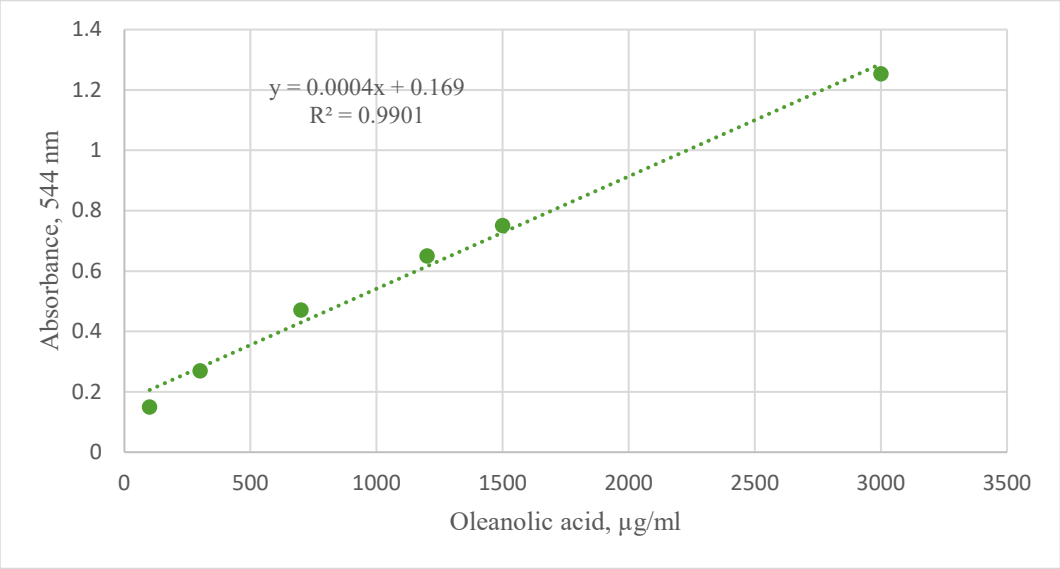

Figure 1. Calibration curve for quantification of saponins with correlation coefficient (R2) and linear equation.

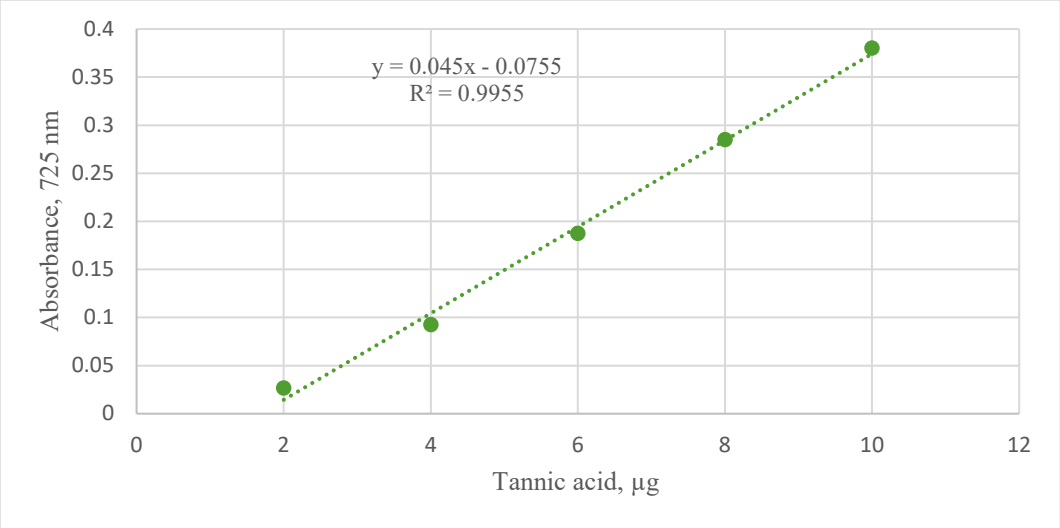

Figure 2. Calibration curve for quantification of total phenolics with correlation coefficient (R2) and linear equation.

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