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The role of fishmeal, alternative protein sources and consumer perception in the environmental performance of turbot farming in Europe

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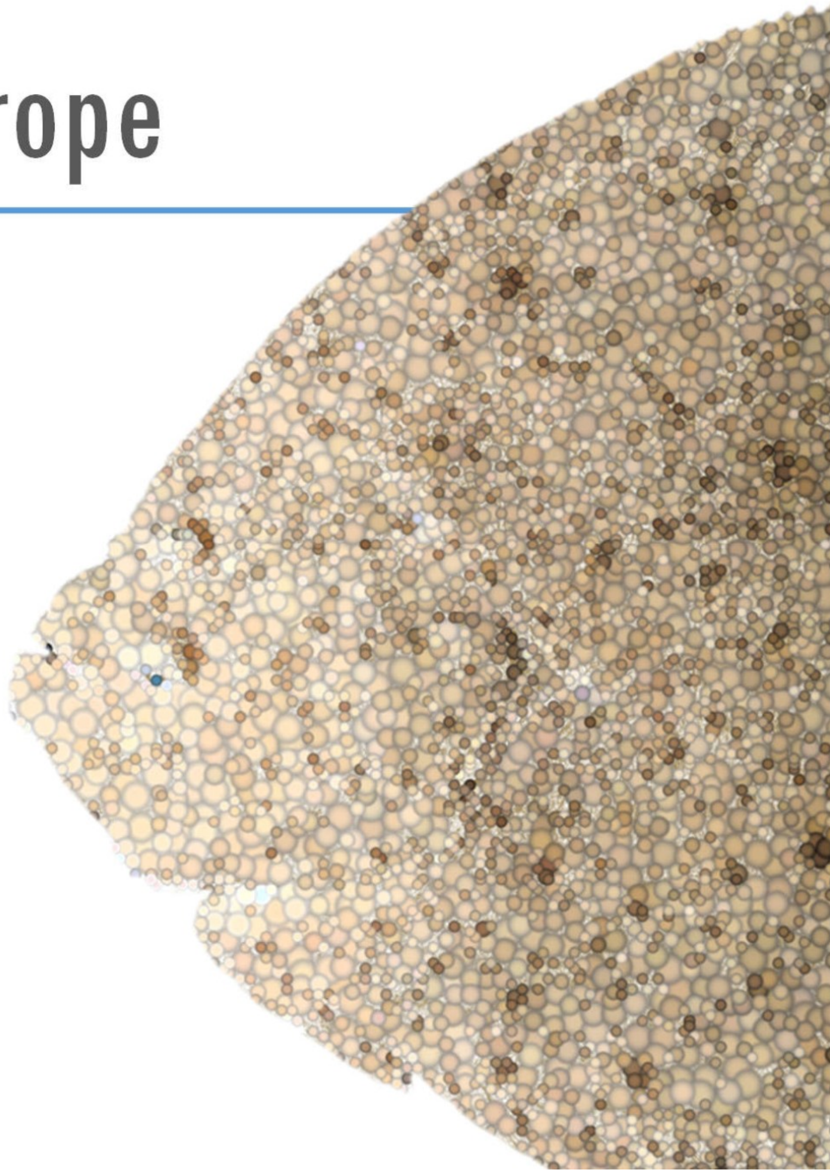

GAIN
Green Aquaculture Intensification

The role of fishmeal, alternative protein sources and consumer perception in the environmental performance of turbot farming in Europe

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Diese Arbeit ist inhaltlich identisch zu der beim Prüfungsamt eingereichten Version vom 21.12.2022. Jedoch wurden in der vorliegenden Arbeit Kopien der Fragebögen (Questionnaires) aus Manuskript IV dem Appendix hinzugefügt (Seiten 161-164) und auf Seite 9 darauf verwiesen.

Dedicated to my family

For our common future

Summary

Sustainable intensification of European aquaculture can be a solution to support future demand for high quality seafood, while ensuring environmental performance, animal welfare and social equality. Technological innovations in culture systems and feed production play a key role in the FAO's initiated *'Blue Transformation'* in aquaculture. In a transdisciplinary approach, this thesis contributes to this transformation by evaluating innovative feed formulations and identifying social factors that influence consumer perception of aquaculture. Feed formulations were developed in order to enhance environmental performance of turbot farming, by reducing the overall fishmeal content and replacing it with sustainable plant-based and animal-based protein sources. Addressing German consumers, a public survey was conducted focusing on sustainability, perception and attitude towards aquaculture.

In two feeding trials, this thesis investigated the effects of sustainable feed formulations on growth, feed utilization, nutrient digestibility and metabolism on turbot (*Scophthalmus maximus*) in the juvenile and grow-out phase. Plant-based and animal-based feed formulations are a suitable alternative for juvenile turbot yielding in similar growth as turbot fed with a commercial formulation. However, higher feed intake, observed when feeding the animal-based formulation, might be compensated by the lower feed cost in commercial applications. Comparing the metabolic profiles of juvenile turbot, revealed that replacing fishmeal with alternative protein sources leads to changes in energy allocation in liver and muscle tissue, while not affecting growth performance. Animal-based ingredients impaired the performance of turbot in the grow-out phase, whereas plant proteins in combination with insect meal and microbial biomass demonstrated great potential to reduce the fishmeal content by at least 30% the diets. As German consumers, perceive sustainability of aquaculture as low and voice that environmental concerns, animal welfare and the lack of vegetarian products restrain them from choosing aquaculture products.

Therefore, sustainable feed formulations can be a driving factor for increased acceptance of aquaculture in the public and facilitate a shift in consumption from wild-caught fish towards sustainable aquaculture products. Sustainable feed formulations with plant proteins, insects and microbial biomass can enhance the environmental performance of turbot farming in Europe. However, feeding turbot fishmeal-reduced diets might not match with consumer preferences for a presumably natural, high-quality product with health benefits. This together with a higher cost for sustainable aquaculture products could outweigh the consumer's environmental concerns that usually restrain consumption of aquaculture products.

Zusammenfassung

Um auch in Zukunft die Nachfrage nach hochwertigen Fisch und Meeresfrüchten zu decken, muss die Nachhaltigkeit der der europäischen Aquakulturproduktion verbessert werden. Ziel der von der FAO angestoßenen „*Blauen Transformation*" ist es, mittels technologischer Innovationen in Zuchtsystemen und Futterherstellung, die Auswirkungen auf die Umwelt zu reduzieren sowie das Tierwohl und soziale Gerechtigkeit zu gewährleisten.

Einem transdisziplinären Ansatz folgend trägt diese Arbeit zu diesem Wandel bei, indem sie nachhaltige Futterformulierungen für Steinbutt (*Scophthalmus maximus*) untersucht und soziale Faktoren identifiziert, welche die Wahrnehmung der Verbraucher bezüglich Aquakultur beeinflussen. Um die Umweltverträglichkeit der Steinbutt-Zucht zu verbessern, wurden Futterformulierungen entwickelt in denen der Gesamtgehalt an Fischmehl reduziert und durch nachhaltige pflanzliche und tierische Proteinquellen ersetzt wurde. Unter deutschen Verbrauchern wurde eine öffentliche Umfrage zum Verständnis von Nachhaltigkeit, sowie der Wahrnehmung und Einstellung zur Aquakultur durchgeführt.

Um die Auswirkungen von Futterformulierungen auf Wachstum, Futterverwertung, Nährstoffverdaulichkeit und Stoffwechsel von Steinbutt, Jungfisch- und Auswuchsphase zu untersuchen, wurden im Rahmen dieser Arbeit zwei Fütterungsversuche durchgeführt. Dabei zeigte sich, dass Futterformulierungen, die auf pflanzlichen und tierischen Rohstoffen basieren, für junge Steinbutte eine geeignete Alternative zu kommerziell genutzten Formulierungen sind. Bei gleichen Wachstumsergebnissen hatten Steinbutte, welche mit der kostengünstigen, auf tierischen Rohstoffen basierenden, Formulierung gefüttert wurden, eine geringere Futterverwertung. Ein Vergleich von Stoffwechselprofilen junger Steinbutte zeigte, dass der Ersatz von Fischmehl durch alternative Proteinquellen zu Veränderungen in der Energieverteilung im Leber- und Muskelgewebe führt, ohne das Wachstums zu beeinträchtigten. Tierische Rohstoffe beeinträchtigten Wachstum und Futterverwertung von Steinbutten in der Auswuchsphase, während pflanzliche Proteine, in Kombination mit Insektenmehl und mikrobieller Biomasse, zeigten, dass 30% oder mehr des Fischmehls in kommerziellen Futtermitteln ersetzt werden könnte. Die Umfrage zeigte, dass deutsche Verbraucher, die Nachhaltigkeit der Aquakultur als gering einschätzen und geben Umweltbedenken, Tierwohl und das fehlende Angebot von vegetarischen Produkten als Gründe an warum sie Aquakulturprodukte meiden.

Deshalb können nachhaltige Futterformulierungen ein treibender Faktor für eine höhere Akzeptanz der Aquakultur in the Öffentlichkeit sein und den Verbrauch von wild

gefangenem Fisch hin zu nachhaltigen Aquakulturprodukte verlagern. Wieviel Fischmehl im Futter von Steinbutt ersetzt werden kann hängt stark von der Auswahl der alternativen Proteinquellen ab. Nachhaltige Futterformulierungen mit pflanzlichen Proteinen, Insekten und mikrobieller Biomasse können stark dazu beitragen die Umweltverträglichkeit der Steinbutt-Zucht in Europa zu verbessern. Dennoch ist die Fütterung von Steinbutt mit fischmehlreduziertem Futter nicht im Sinne der Verbraucher, die sich ein möglichst naturbelassenes und qualitativ hochwertiges Produkt mit gesundheitsfördernden Nährstoffen wünschen. Dieser Wunsch zusammen mit dem höheren Preis für nachhaltige Aquakulturprodukte könnte dabei die Umweltbedenken der Verbraucher überwiegen.

Content

Abbreviations

AA	apparent availability
ADC	apparent digestibility coefficient
ALT	alternative ingredient
ANOVA	analysis of variance
BL	body length
BP	breaking point
BW	body weight
CE	circular economy
CF	condition factor
CTRL	control; commercial-like formulation
DFI	daily feed intake
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FC	formulation concept
FCR	feed conversion ratio
FI	feed intake
FM	fishmeal
FR	fishmeal replacement
GAIN	Green Aquaculture Intensification in Europe
HSI	hepato-somatic-index
MIX	feed formulation with balanced mixture of plant-based, animal-based and emerging feed ingredients
NMR	nuclear magnetic resonance
NoPAP/PLANT	feed formulation without (No) PAP/based on plant protein sources
PAP	processed animal protein
PC	principal component

Abbreviations

PCA	principal component analysis
PER	protein efficiency ratio
PLS-DA	partial least-squares discriminant analysis
PPC	plant protein concentrates
RAS	recirculating aquaculture system
SD	standard deviation
SDG	sustainable development goal
SGR	specific growth rate
TMAO	trimethylamine-N-oxide
VFI	voluntary feed intake
WG	weight gain

1 Introduction

"Development that meets the needs of the present generation without compromising the ability of future generations to meet their own needs".

This is how "Sustainable Development" was defined 35 years ago in the Brundtland Report, linking the biosphere with the society and economy it supports. In 2015, the United Nations member states adopted the "2030 Agenda for sustainable development" defining 17 sustainable development goals (see Figure 1) as an urgent call for action to improve food security and health while spurring economic growth, tackling climate change, and working to preserve the environment (UN, 2016). In this context, fish and other seafood play an important role in human nutrition (SDG2) and health (SDG 3) as source of high-quality protein, omega-3 fatty acids and essential minerals; and are associated among others with conservation and sustainable use of aquatic environments (SDG 14) and responsible production and consumption (SDG12) (FAO, 2022a).

Figure 1 "Wedding cake" model for the sustainable development goals, which is similar to the nested circle diagram, whereby the environmental dimension or system is the basis for the other two dimensions.

(Credit: Azote for Stockholm Resilience Centre, Stockholm University CC BY-ND 3.0.)

1.1 Sustainable intensification of aquaculture production in Europe - the role alternative protein sources in aquafeeds

The increasing population and wealth in Europe have led to an increase per capita in seafood consumption from 20.7 kg/year in 2005 (EUMOFA, 2019) to 24.2 kg/year in 2021, making the EU the third largest fish consumer (total consumption 11,149 tons) worldwide, after China and Indonesia (EUMOFA, 2022a). The decline in capture fisheries and a stagnating growth of aquaculture, resulted in an increasing dependence on imported seafood and a self-sufficiency rate of 38.9% in 2020 (EUMOFA, 2022a). To support the future demand for high quality seafood, the European aquaculture sector needs to be sustainably intensified, ensuring the reduction of environmental impacts, animal welfare and social equality as described in the FAO's (2022a) "Blue Transformation". In Europe, fish aquaculture is dominated by high-value and high-trophic/carnivorous marine fish species such as Atlantic salmon (*Salmo salar)*, Rainbow trout (*Oncorhynchus mykiss*), Gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) (EUMOFA, 2022a). Carnivorous species require high-protein diets (Turchini et al., 2019), which traditionally were based on wild fish, increasing the pressure on already overfished stocks, and competing with the use for human consumption (FAO, 2022a). In order to facilitate this transformation in the aquaculture sector, experts recommend technological innovations in culture systems and feed production as well as a shift in fish consumption towards low-trophic fish species such as carp and mullet (Klinger and Naylor, 2012, Waite et al., 2014).

To support the need for growth of aquaculture production an increase in feed volume will require sourcing of alternative ingredients to deliver protein, essential amino acids, and fatty acids as well as other micronutrients (Glencross et al., 2020, Naylor et al., 2021). In the past 20 years, the efforts of the science community and the aquaculture industry have significantly enhanced the efficiency in use of marine resources and considerably reduced the fishmeal content in the diets of marine fish from 50% in 1995 to 14% in 2017 (Naylor et al., 2009, Naylor et al., 2021). The challenge in formulating diets for carnivorous fish is to find alternative ingredients, which cover the species' nutritional requirements appropriately, reduce the environmental footprint and mitigate risks associated with the volatility in supply, quality, and price (Bostock, 2011, Glencross et al., 2020). Several alternative ingredients such as fish by-products (Whiteman and Gatlin, 2005, Bendiksen et al., 2011, Siddik et al., 2020, Naylor et al., 2021), plant proteins (Gatlin et al., 2007, Tacon et al., 2011, Hua et al., 2019, Naylor et al., 2021), and animal by-products (Campos et al., 2017, Karapanagiotidis et al., 2019, Woodgate et al., 2022) are already used in commercial diets. Other alternatives, such as insects, microbial biomasses or micro- and macroalgae, are emerging as ingredients for sustainable aquafeeds (Cottrell et al., 2020, San Martin et al., 2020). While from a nutritional point of view, wild-fish products are the “perfect” raw material as they contain high-quality protein with suitable amino acid and fatty acid profiles (Glencross et al., 2020); alternative ingredients have a different nutritional composition (proteins, lipids, carbohydrates, and ash), digestibility of nutrients, and availability of minerals and other micronutrients (Sugiura et al., 1998, Glencross, 2016, Hua et al., 2019). In carnivorous fish, this may affect growth and feed utilization, and lead to alterations in physiology, immune response and energy metabolism (Bonaldo et al., 2015, Aragao et al., 2020, Palma et al., 2020, Fernandes et al., 2021, Kaiser et al., 2022). Accordingly, physiological, economic, environmental, and societal needs have to be weighed against each other when formulating sustainable aquafeeds (Glencross et al., 2020, Naylor et al., 2021, FAO, 2022a).

1.2 The potential for sustainable intensification of turbot farming in Europe

Aquaculture of turbot (*Scophthalmus maximus*, Linnaeus, 1758; Figure 2) started 50 years ago in Europe and expanded the production to farming countries in the natural distribution range of turbot along the North Sea and Northeast Atlantic (FAO, 2022b).

Figure 2 Juvenile turbot (*Scophthalmus maximus*)

In Europe, turbot are currently cultured in land-based tanks (see Figure 3) connected to a flow-through or recirculating aquaculture systems (RAS), which allow wastewater treatment reducing eutrophication (Aubin et al., 2006). The commitment of the turbot farming industry to environmental management (ISP 14001 and EMASII system), the production of a high-quality product, and improved animal welfare demonstrates the efforts made by the industry to farm responsibly (FAO, 2022b). Despite being a niche species, 65% of the EU's turbot production comes from aquaculture with 11.757 tons and a value of 90 million euros (EUMOFA, 2022a, EUMOFA, 2022b) and almost all is consumed within its member states (EUMOFA, 2018).

Figure 3 Turbot farming facility in Cabo Vilán, Galicia, Spain
(Credit: P. Lameiro, CC BY-SA 3.0, via Wikimedia Commons)

Aquafeeds are identified as a driving factor to improve the environmental performance of turbot farming in Europe as the production of FM, soybean and wheat; the main protein sources in current feed formulations contributes to eutrophication and climate change (Aubin et al., 2006, Iribarren et al., 2012). Therefore, reduction of the FM content and the integration of sustainable plant and other alternative protein sources would improve the environmental performance of turbot farming. However, turbot is strictly carnivorous during its life cycle (FAO, 2022b) and has a high demand for protein (55%) (Oliva-Teles et al., 2022) and a low tolerance to FM replacement.

Depending on the alternative ingredients used, significant effects on growth and feed performance as well as organismic parameters were observed at replacement levels of more than 30-35% for juvenile turbot (5-100 g) (Bonaldo et al., 2011, Kroeckel et al., 2012, Bonaldo et al., 2015, Hermann et al., 2016, Bai et al., 2017, Chen et al., 2018, Bai et al., 2019). Unfortunately, only a few studies investigated the potential of FM reduction in diets for turbot in the grow-out phase (> 100 g) (Fuchs et al., 2015, Weiß and Buck, 2017), missing the opportunity to improve the environmental performance through aquafeeds.

1.3 How to "sell" sustainable intensified fish to the consumer?

Sustainable intensification measures will most likely increase the price for consumers due to higher costs for sustainable aquafeeds, technical innovation and use of renewable energy. Even though consumers often voice the preference of sustainable food (Black and Cherrier, 2010, Schoolman et al., 2014, Kapferer and Michaut-Denizeau, 2019) and the willingness to pay the higher costs (De Pelsmacker et al., 2005, Stubbe Solgaard and Yang, 2011), the reality is different. EU's seafood consumers are spending less than 5 euros per kilo of seafood (EUMOFA, 2022a), and as Portuguese consumers (Misund et al., 2020) are willing to pay more for sustainable seafood, consumers of high-income countries such as Norway (Misund et al., 2020), Belgium and Germany (Bronnmann and Hoffmann, 2018) are not willing to pay a higher price. In order to sell sustainably produced fish to consumers, the aquaculture sector needs to understand and mitigate social factors that restrain the purchase and consumption of aquaculture products.

2 Aims and outline of the thesis

This PhD thesis was rooted in the EU's Horizon2020 project "Green Aquaculture Intensification in Europe (GAIN)", which aimed to transform Europe's aquaculture sector through sustainable intensification of aquaculture. In a transdisciplinary approach, this thesis contributed to this transformation by (1) evaluating eco-efficient feed formulations, which reduce the diets' environmental impact by partially replacing fishmeal content with sustainable alternatives and (2) identifying social factors that influence the consumer's perception and acceptance of aquaculture.

2.1 Evaluation of eco-efficient feed formulations

The eco-efficient diets were formulated to reduce FM content and replace it with different sustainable feed ingredients following different concepts (see Figure 4). The concepts were based on cost-efficient ingredients such as processed animal proteins (PAPs) or consumer-oriented ingredients containing plant proteins (PLANT/NoPAP) without PAPs, evading consumers' food safety concerns related to the use of PAPs. The MIX concept should represent a balanced mixture of the other two concepts.

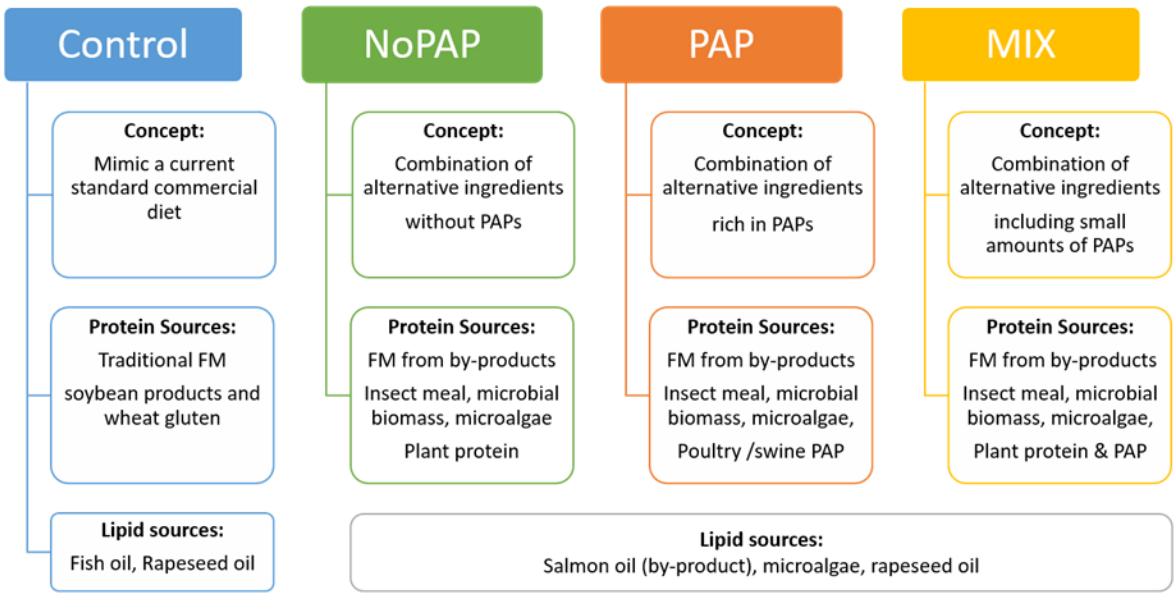

Figure 4 Feed formulation concepts and main ingredients used in the feeding trials with turbot in the juvenile phase (NoPAP/PLANT, PAP and MIX) and grow-out phase (NoPAP and PAP). FM: fishmeal; PAP: processed animal protein
(Adapted from Pereira et al., 2022)

Aims and outline of the thesis

Most feeding trials are conducted with younger and therefore faster growing turbot; hence, there is limited knowledge about the effects of dietary manipulation on larger and slower growing turbot. Yet, this knowledge is crucial for aquaculture species with a long production cycle as the greater feed input during the grow-out phase can magnify economic benefits as well as biological constraints for the farmer. Therefore, this thesis aims to evaluate the effects of partial fishmeal replacement by alternative protein sources on growth, feed utilization, nutrient digestibility and metabolism of turbot in the juvenile and grow-out phase.

Potential of emerging protein sources in diets for juvenile turbot (*Scophthalmus maximus*) in RAS

Based on established knowledge of the suitability of single feed ingredients in diets for turbot, the first feeding trial (Manuscript I) evaluates two novel feed formulations for sustainable turbot production, with moderate fishmeal replacement (20%) and using feed ingredients of terrestrial animal (PAP) and plant origin (PLANT). The diets were formulated to meet the nutritional demands of juvenile turbot. Growth and feed performance, apparent digestibility of nutrients, energy storage and apparent availability of minerals and trace elements are used to evaluate the effects of the dietary manipulations.

^{1}H-NMR-based metabolic profile in muscle and liver tissue of juvenile turbot (*Scophthalmus maximus*) fed with plant and animal protein sources

In order to verify the impaired nutritional status and reduced glycogen storage of juvenile turbot, ^{1}H-nuclear magnetic resonance (NMR) spectroscopy (Manuscript II) was used to examine if these alterations are rooted in metabolic processes related to the energy utilization in the muscle and in the liver. To determine the effects of the level of FM replacement, performance data of turbot fed with a diet with further reduced FM content (40% FM replacement) and a balanced mixture (MIX) of the feed ingredients used in the PLANT and PAP diets.

Effects of dietary plant and animal protein sources and replacement levels on growth and feed performance and nutritional status of market-sized turbot (*Scophthalmus maximus*) in RAS

Based on the results from the first feeding trial, the two formulation concepts were adapted to meet the nutritional requirements of turbot from the grow-out phase (Manuscript III). Being the longest phase in the production cycle, diets with a high level of FM replacement could reduce the feed costs making alternative formulation for marked-sized turbot with a good performance economically attractive. In a bi-directional approach, the third study investigated (1) the effects of two innovative feed formulation concepts based on sustainable feed ingredients without processed-animal protein (NoPAP) and with the inclusion of processed-animal protein (PAP) and (2) how much fishmeal could be replaced within these two formulation concepts (replacement levels 30% and 60%). Growth and feed performance, apparent digestibility of nutrients and minerals, and energy storage were used to evaluate the effects of the dietary manipulations.

2.2 Social limitations for the consumption of aquaculture products

Sustainable growth of the aquaculture sector will not be possible without consumers purchasing sustainably produced seafood. Social constraints such as misconceptions of the environmental performance and health benefits of aquaculture products could discourage consumers from purchasing sustainable fish.

The role of knowledge in the willingness to consume aquaculture products of different groups in Germany - factors that influence the consumer's perception and acceptance of aquaculture.

In order to mitigate social constraints in the acceptance of sustainable aquaculture products, the study (Manuscript IV) investigated, using a survey, how demographic factors influence the perception and acceptance of aquaculture with focus on sustainability of German consumers. The study was focused on younger age groups (25 years and younger and 26 to 39 years) by placing questionnaires (see Appendix Questionnaires) at a conference for young scientists and by a citizen science project with high school students.

3 Manuscripts

Manuscript I

Sustainable fish feeds: Potential of emerging protein sources in diets for juvenile turbot (*Scophthalmus maximus*) in RAS

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Abstract

In Europe, turbot aquaculture has a high potential for sustainable production but the low tolerance to fishmeal replacement in the diet represents a big issue. Therefore, this study investigated the effects of more sustainable feed formulations on growth and feed performance, as well as nutritional status of juvenile turbot in recirculating aquaculture systems. In a 16-week feeding trial with 20 g juvenile turbot, one control diet containing traditional fishmeal, fish oil and soy products and two experimental diets where 20% of the fishmeal was replaced with either processed animal proteins (PAP) and or with terrestrial plant proteins (PLANT) were tested. Irrespective of diets, growth performance was similar between groups, whereas the feed performance was significantly reduced in fish of the PAP group compared to the control. Comparing growth, feed utilization and biochemical parameters, the results indicate that the fish fed on PAP diet had the lowest performance. Fish fed the PLANT diet had similar feed utilization compared to the control, whereas parameters of the nutritional status, such as condition factor, hepato-somatic index and glycogen content showed reduced levels after 16 weeks. These effects in biochemical parameters are within the physiological range and therefore not the cause of negative performance. Since growth was unaffected, the lower feed performance of fish that were fed the PAP formulation might be balanced by the cost efficient formulation in comparison to the commercial and the PLANT formulations. Present study highlights the suitability of alternative food formulation for farmed fish.

Keywords: Insect meal, by-products, energy reserves, mineral, trace elements, circular economy

1 Introduction

Aquaculture has the potential to ensure a reliable supply of seafood for the globally increasing demands and sustainable growth. In order to conserve and sustainably use aquatic resources, the reduction of the environmental footprint of aquaculture practices has become a high priority for the scientific community, producers and consumers. One of the major concerns is the challenge to feed farmed fish with diets that are nutritious but at the same time economically and environmentally sustainable (Glencross et al., 2020). In the last decades, research efforts focused on the identification of major nutritional requirements for important farmed fish such as trout, salmon, sea bass, or seabream (FAO, 2020, Naylor et al., 2021). These efforts set the foundation for substitution of fish meal and fish oil originating from wild pelagic fish with other sources (Hardy and Barrows, 2003). This resulted in diet formulations with reduced fish content that improve growth and feed performance (Olsen and Hasan, 2012).

However, in many carnivorous farmed fish a total replacement of fish products in the diets is still not feasible. In order to reduce dependence on traditional fishmeal and fish oil, the use of fishery and aquaculture by-products are a good alternative for sustainable aquafeeds (Forster et al., 2005, Whiteman and Gatlin, 2005, Bendiksen et al., 2011, Hua et al., 2019). Processed raw materials such as hydrolysates are more energy efficient than fish meal from by-products and were shown to improve growth and feed performance in farmed fish (Siddik et al., 2020).

Terrestrial plant materials are commonly integrated into commercial fish diets (Gatlin et al., 2007, Tacon et al., 2011, Naylor et al., 2021, Abdel-Latif et al., 2022) enabling even fish-free diet formulations for carnivorous fish. In the case of soybean, however, these products are often associated with unsustainable production, long transportation and a high proportion of genetically modified strains. Furthermore, soybean meals and other vegetable ingredients introduce anti-nutrients, giving rise to a number of problems for the fish such as enteritis and nutrient uptake and bioavailability (Kaushik et al., 1995, Baeverfjord and Krogdahl, 1996, Storebakken et al., 1998). This can be offset - at least to a certain point- by refining the plant protein sources (Refstie et al., 2005, Naylor et al., 2009b, Glencross, 2016, Jia et al., 2022), which, however, introduces costs and results in a trade-off between fish welfare/health and feed cost. Other alternative protein and oil crops such as pea, rapeseed, and lupines proved to be suitable for fish feeds (Burel et al., 2000b, Øverland et al., 2009, Glencross et al., 2011, Zhang et al., 2012, Omnes et al., 2015). However, the availability at a competitive price and regular supply in sufficient quality is still a major issue that needs to be solved (Bähr et al., 2014, Glencross et al., 2020). Moreover, many consumers question whether plant materials are an acceptable and appropriate feed ingredient for carnivorous fish (Feucht and Zander, 2015).

Plant material as a basic commodity is used in a wide range of human consumption, feed for terrestrial livestock, biofuels and many other industrial applications. Therefore, the competition is high and aquaculture feed producers should avoid to totally rely on plant materials. Therefore, researchers and feed producers emphasize that a broader range of alternatives is needed to facilitate the predicted increase in fed aquaculture production (Matos et al., 2017, FAO, 2020). Since the European crisis of the mad cow disease in 1990, terrestrial animal proteins were mostly banned from farmed animal feed formulations. Therefore, research on PAPs in fish feeds is scarce until recently. However, recent studies show that PAPs are suitable alternatives to fishmeal in fish diets (Lu et al., 2015, Wang et al., 2015, Campos et al., 2017, Wu et al., 2018, Karapanagiotidis et al., 2019). However, in 2013 non-ruminant PAPs (processed animal proteins) were re-authorized in the EU under very specific regulations allowing correctly categorized PAP in aquafeeds. The availability in large amounts in the EU and elsewhere as a by-product from food production and its nutritional value qualifies PAPs as a sustainable feed ingredient for fish (Tacon et al., 2011).

Recently authorized as novel food and feed in the EU, insect derived products, such as protein and lipids are valuable ingredients for aquaculture feeds. Insects can valorize unused plant material, not suitable for human consumption and transform it into valuable nutrients (Newton et al., 2005, van Huis, 2013). They are also part of the natural diet of many freshwater and marine fish species (Henry et al., 2015). Meals derived from the black soldier fly (*Hermetia illucens*) or mealworm (*Tenebrio molitor*) were already successfully tested in fish diets for carnivorous fish species such as Atlantic salmon (*Salmo salar*) (Li et al., 2020), rainbow trout (*Oncorhynchus mykiss*) (Stadtlander et al., 2017, Jozefiak et al., 2019, Rema et al., 2019) and red seabream (*Pargus major*) (Ido et al., 2019).

Other feed ingredients, such as micro- and macroalgae and microbial meals are emerging as suitable protein and lipid sources for aquafeeds. Microbial biomass, which is produced as a by-product from food, beer and biogas production, can be a valuable ingredient in aquafeeds (Oliva-Teles and Goncalves, 2001, Aas et al., 2006, Bendiksen et al., 2011, Tacon et al., 2011, Olsen and Hasan, 2012, San Martin et al., 2020). In particular, microalgae are a valuable source with essential fatty acids in diets with a low level of fish oil. Additionally, algae and yeast can act as functional ingredients, increasing the health of farmed fish and crustaceans (Refstie et al., 2010, Vallejos-Vidal et al., 2016, Dineshbabu et al., 2019, Wan et al., 2019).

Novel feed formulations with a broad spectrum of ingredients can balance the ingredients' quality, cost and availability, but most importantly, they need to satisfy the nutritional requirements of the farmed species. Thereby the effects of integrating alternative feed ingredients on fish performance and nutritional status have to be validated. In comparison to fishmeal, alternative ingredients differ in nutritional composition, digestibility of nutrients and availability of minerals (Sugiura et al., 1998b, Glencross, 2016). This may affect growth, nutrient utilization and whole body composition of carnivorous fish and lead to an altered energy metabolism and energy allocation. Plant-based and carbohydrate-rich diets influenced the energy reserves, such as the hepatic content of glycogen and lipid in Atlantic salmon, rainbow trout (Krogdahl et al., 2004), Gilthead seabream (*Sparus aurata*) (Robaina et al., 1995), and turbot (*Scophthalmus maximus)* (Miao et al., 2016a). Furthermore, plant-based diets affected the mineral composition and availability in rainbow trout (Read et al., 2014, Antony Jesu Prabhu et al., 2018) and Atlantic salmon (Storebakken et al., 2000, Silva et al., 2019).

Turbot is an important species in EU aquaculture due to its high value and reputation and low competition with fisheries production (EUMOFA, 2018). It has a high potential for sustainable production due to the controlled farming cycle, production practices (RAS and flow-through systems) and its robustness, enabling high-density farming and domestication (FAO, 2005 (FAO, 2005, Aksungur et al., 2007, Li et al., 2013, Bischoff et al., 2018, EUMOFA, 2018). However, as a carnivore, turbot has a low tolerance to fishmeal reduction (Burel et al., 2000a, Burel et al., 2000b, Nagel et al., 2012, von Danwitz et al., 2016) and is a sensitive and thus suitable candidate for testing novel feed formulations. Therefore, the present study aims to evaluate the effects of two novel feed formulations for sustainable turbot production, with moderate fishmeal replacement and using feed ingredients of terrestrial animal and plant origin, on the growth and feed performance, apparent digestibility of nutrients, energy storage and apparent availability of minerals and trace elements.

2 Material & Methods

2.1 Experimental Diets

All experimental diets were formulated to be isonitrogenous (530 g kg^{-1}). Due to species' behavior and size, 3mm pellets with positive buoyancy (floating) were manufactured by extrusion at SPAROS LDA (Olhão, Portugal). All diets, including the control diet, were produced using the same facility and extrusion parameters to minimize technological differences. There were three treatments, including two novel formulations and one control diet, which was mimicking a typical current commercial formulation used for turbot. In the control diet, the main protein sources were fishmeal (500 g kg^{-1}), wheat gluten (110 g kg^{-1}) and soy protein concentrate (100 g kg^{-1}). In the two experimental diets, the commercial fishmeal was fully replaced with fish by-products (meal and hydrolysates) and the overall fish-derived content was reduced by 20% to 400 g kg^{-1}. The remaining protein was sourced with emerging ingredients such as insect meal, single cell meal and algae meal. Soy-derived ingredients were replaced by pea protein and pea starch. Furthermore, in all experimental diets DHA-rich algae and rapeseed oil replaced 60% of fish oil. The content of the respective experimental diets as well as the control diet is shown in Tables 1 and 2. Once the experimental feeds were produced, they were delivered from Portugal to the experimental facility at the Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research (AWI) in Bremerhaven (Germany). Before and during the trials the feed was stored at 4°C to ensure continuous quality of the diets throughout the feeding experiment.

2.2 Experimental setup

Juvenile turbot (*Scophthalmus maximus*) were purchased from France Turbot (L'Épine, France), transferred in specified transport containers overland to the recirculating aquaculture systems (RAS) of the Centre for Aquaculture Research (ZAF) at AWI, acclimated to the RAS for two weeks prior to starting the 16 week (112 days) experimental trial. A total of 750 turbots with a mean weight (± SD) of 20.2 ± 0.4 g and a mean total length of 10.1 ± 0.1 cm were randomly distributed into 15 tanks (50 fish per tank, 5 tanks per diet). The RAS consisted of 36 tanks, each with a bottom area of 1 m² and a volume of approx. 700 L. The condition of the process water was monitored constantly with a SC 1000 Multiparameter Universal Controller (Hach Lange GmbH, Germany) and the nutrient concentration was measured with the QuAAtro39 AutoAnalyzer (SEAL Analytical, Germany) twice a week (see Table 3).

Table 1 Formulation (%) of the experimental diets for juvenile turbot (*Scophthalmus maximus*).

Ingredients	Control	PAP	PLANT
Fishmeal[1]	50.00	0.00	0.00
Fishmeal (by-product)[2]	0.00	35.00	35.00
Fish hydrolysate (by-product)[x]	0.00	5.00	5.00
Insect meal (Hermetia illucens)[x]	0.00	5.00	5.00
Porcine hemoglobin[3]	0.00	2.50	0.00
Poultry meal[4]	0.00	10.20	0.00
Microbial protein meal (methanotrophic bacteria)[x]	0.00	2.50	2.50
Yeast protein meal (Saccharomyces cerevisiae)[x]	0.00	2.50	2.50
Microalgae meal (Arthrospira platensis)[1]	0.00	0.00	2.00
Microalgae meal (Chlorella vulgaris)[5]	0.00	0.00	0.50
Microalgae meal (Tetraselmis chuii)[5]	0.00	0.00	0.20
Soy protein concentrate[6]	10.0	0.00	0.00
Pea protein concentrate[7]	0.00	5.00	12.40
Wheat gluten[7]	11.00	10.00	11.50
Soybean meal[8]	4.00	0.00	0.00
Wheat meal[9]	8.00	0.00	0.00
Pea starch[10]	4.00	8.99	8.89
Fish oil[1]	11.60	4.64	4.64
DHA-Rich algae (Schizochytrium)[11]	0.00	1.08	1.08
Rapeseed oil[12]	0.00	3.44	4.64
Rapeseed lecithin[13]	0.00	0.80	0.80
Vitamin and mineral premix[14]	1.00	1.00	1.00
Vitamin C[15]	0.05	0.05	0.05
Vitamin E[15]	0.05	0.05	0.05
Betaine HCl[16]	0.00	0.50	0.50
Macroalgae mix[17]	0.00	0.50	0.50
Antioxidant[18]	0.18	0.18	0.18
Sodium propionate[19]	0.10	0.10	0.10
L-Tryptophan[20]	0.00	0.15	0.15
DL-Methionine[21]	0.00	0.30	0.30
L-Taurine[16]	0.00	0.50	0.50
Yttrium oxide[22]	0.02	0.02	0.02

Control: commercial-like formulation, PAP: processed animal protein, PLANT: plant based protein.

[x] not disclosed; [1] Sopropêche, France; [2] Conserveros Reunidos S.A., Spain; [3] SONAC BV, The Netherlands; [4] SAVINOR UTS, Portugal; [5] Allmicroalgae, Portugal; [6] ADM, The Netherlands; [7] Roquette Frères, France; [8] CARGILL, Spain; [9] Casa Lanchinha, Portugal; [10] COSUCRA, Belgium; [11] Alltech, Ireland; [12] Henry Lamotte Oils GmbH, Germany; [13] Novastell, France; [14] DL-alpha tocopherol acetate, 255 mg; sodium menadione bisulphate, 10 mg; retinyl acetate, 26000 IU; DL-cholecalciferol, 2500 IU; thiamine, 2 mg; riboflavin, 9 mg; pyridoxine, 5 mg; cyanocobalamin, 0.5 mg; nicotinic acid, 25 mg; folic acid, 4 mg; L-ascorbic acid monophosphate, 80 mg; inositol, 17.5 mg; biotin, 0.2 mg; calcium panthotenate, 60 mg; choline chloride, 1960 mg. Minerals (g or mg·kg-1 diet): copper sulphate, 8.25 mg; ferric sulphate, 68 mg; potassium iodide, 0.7 mg; manganese oxide, 35 mg; organic selenium, 0.01 mg; zinc sulphate, 123 mg; calcium carbonate, 1.5 g; excipient wheat middlings; [15] DSM Nutritional Products, Switzerland; [16] ORFFA, The Netherlands; [17] Ocean Harvest, Ireland; [18] Kemin Europe NV, Belgium; [19] Disproquímica, Portugal; [20]Ajinomoto EUROLYSINE S.A.S, France; [21] EVONIK Nutrition & Care GmbH, Germany; [22] Sigma Aldrich, USA

Table 2 Chemical composition of the experimental diets as fed.

	Control	**PAP**	**PLANT**
Moisture (%)	4.1	7.3	6.7
Crude Protein (%)	52.9	52.8	52.8
Crude Lipid (%)	16.5	16.2	18.1
Ash (%)	7.1	10.5	9.9
Gross Energy (MJ kg^{-1})	23.1	20.8	21.2
Minerals and trace elements			
Calcium (Ca; g kg^{-1})	8.1	22.1	19.1
Potassium (K; g kg^{-1})	4.0	7.4	7.6
Magnesium (Mg; g kg^{-1})	1.8	1.7	1.9
Sodium (Na; g kg^{-1})	4.7	6.8	7.6
Phosphorus (P; g kg^{-1})	10.1	14.5	14.5
Ca/P ratio	0.8	1.5	1.3
Arsenic (As; mg kg^{-1})	7.1	6.1	5.8
Copper (Cu; mg kg^{-1})	29.4	18.6	19.1
Iron (Fe; mg kg^{-1})	278.9	347.3	319.6
Manganese (Mn; mg kg^{-1})	71.8	90.6	69.8
Zinc (Zn; mg kg^{-1})	206.9	174.5	186.3
Amino acids (%)			
Arginine (Arg)	3.55	3.24	3.49
Histidine (His)	1.24	1.18	1.17
Isoleucine (Ile)	2.07	1.95	2.16
Leucine (Leu)	3.26	3.26	3.27
Lysine (Lys)	3.13	3.33	3.34
Threonine (Thr)	2.09	1.97	2.02
Tryptophan (Trp)	0.23	0.28	0.28
Valine (Val)	2.09	2.35	2.31
Methionine (Met)	1.07	1.14	1.21
Cysteine (Cys)	0.26	2.34	0.28
Phenylalanine (Phe)	2.35	0.26	2.39
Tyrosine (Tyr)	1.99	1.97	2.01
Alanine (Ala)	2.25	2.58	2.43
Glycine (Gly)	2.42	2.52	2.16
Proline (Pro)	3.02	2.66	2.57
Serine (Ser)	2.28	2.11	2.13
Taurine (Tau)	0.84	0.84	0.80

Control: commercial-like formulation, PAP: processed animal protein, PLANT: plant based protein. Values are expressed as means from duplicates.

Table 3 Water parameters (n=112) and nutrient concentrations (n=32)

Temperature (°C)	pH	Conductivity (mS cm^{-1})	Oxygen (%)	Ammonium (mg L^{-1})	Nitrite (mg L^{-1})	Nitrate (mg L^{-1})
16.4 ± 0.2	7.6 ± 0.1	52.1 ± 1.3	103.5 ± 4.3	0.2 ± 0.1	0.5 ± 0.3	155.1 ± 37.0

Values are expressed as means ± SD.

The fish were fed twice a day (9 am and 2 pm) ad libitum. After the fish were fed in the afternoon (30 min later), the remaining pellets were netted (mesh size 1 mm) from the tanks, dried for 24 h at 50°C and weighed. To account for potential weight loss of the non-eaten pellets, duplicates of each experimental diet (2 g each) were incubated at 16°C and 100 cycles per minute in 100 mL water which was taken from the experimental recirculation system (30 ‰ salinity) (Obaldo et al., 2002). After 30 min the content was sieved (mesh size 1 mm), the collected pellets were dried for 24 h at 50°C and weighted. The weight loss was used to calculate the loss factor for later correction of the recovered non-eaten pellets (see formula (6)).

2.3 Measurements and sampling

Fish were weighed to 0.2 g precision and measured in length to 0.5 cm precision every 4 weeks. At the end of the 16 week trial 6 individuals from each of the 15 tanks were sampled, from which 3 fish were used for tissue sampling to determine the energy reserves and 3 fish per tank were sampled as whole fish for proximate and mineral analysis. Fish were anaesthetized with 500 mg L^{-1} tricaine methanesulfonate (MS-222; Sigma Aldrich, Germany). After recording weight (precision 0.01 g) and length (precision 0.5 cm), fish were sacrificed and tissues (liver and filet without skin) were rapidly sampled on ice. The liver of three fish per tank (n = 15 fish per diet) was weighted with 0.0001 g precision to determine the hepato-somatic index (HSI). Both tissues were frozen in liquid nitrogen and stored at -80°C until further analysis. For digestibility analysis, the faeces were sampled by stripping anaesthetized fish and pooled from one tank, centrifuged at 4°C and 3,000 x *g* for 5 min, and the pellets were frozen at -80°C until further analysis. To gain sufficient tissue mass, the whole fish bodies were pooled (from the 3 fish taken per tank), cut into small pieces and stored at -20°C until further analysis.

2.4 Chemical analysis of diets, whole body and faeces

The chemical analysis of the diets was conducted in duplicates (see Table 2) and of the whole body and faeces as pooled replicates per tank (n = 5 tanks per diet). The whole body samples were minced frozen using a meat grinder (MADO Primus, Germany), refrozen at -20°C and then freeze-dried for 48 h. The samples of the experimental diets and faeces were freeze-dried for 24 h. The experimental diets and whole body samples were further homogenized in a knife grinder (5000 rpm, 30 s, Grindomix GM 200; Retsch, Germany).

The moisture content, ash, crude protein, crude lipid and energy of the experimental diets, whole body fish and faeces was determined after AOAC (1980). Moisture content of the feeds was determined by drying the samples at 105°C for 24 h. The moisture content of the whole body and faeces was determined by freeze-drying. Total ash content was determined by combustion of the samples in a muffle oven at 550°C for 6 h. The total nitrogen in the feed and whole body samples was determined following the automated Kjeldahl Method. Due to small sample volume in the faeces samples, the total nitrogen was determined after the Dumas Method. For all samples the measured total nitrogen was converted to equivalent crude protein (%) by the numerical factor of 6.25. Crude lipid was determined by acid hydrolysis. Gross energy was measured in an adiabatic bomb calorimeter (Model 6100; Parr Instrument, Germany).

For the analysis of the mineral content, 0.2 g of freeze-dried and homogenized samples of the experimental diets, whole body and faeces was digested in 3 mL nitric acid HNO_3 (65%, trace grade) in a microwave oven (CEM MARS5, Germany) according to DIN EN 13805 (2014). After digestion, the samples were diluted with milli-q water to 50 mL. Calcium, potassium, magnesium, phosphorus, arsenic, copper, iron, manganese, yttrium and zinc concentrations were analyzed in an ICP-OES (iCAP7400; Fisher Scientific, Germany). As reference fish muscle (ERM – BB422, EU) was used.

2.5 Glycogen and crude lipid content of liver and muscle tissue

Following the procedure described by Keppler and Decker (1988) glycogen content was determined photometrically after enzymatic hydrolysis of glycogen to glucose. Briefly, filet and liver samples (3 individual fish per tank; 15 fish per diet in total) were grinded under liquid nitrogen and approx. 200 mg tissue was homogenized in 5x volume of ice-cold 0.6 M perchloric acid (PCA) (w:v). After one cycle of 20 s at 6000 rpm and 3°C using Precellys 24 (Bertin Technologies, France) samples were sonicated for 2 min at 0°C and 360 W (Branson Ultrasonics Sonifier 450; Fisher Scientific, Germany) and homogenates were immediately divided for the analysis of total and free glucose concentrations. Due to small volume the individual samples of liver and muscle were pooled (n = 5 tanks per diet) for the crude lipid content. Following the method of Folch et al. (1957) and Postel et al. (2000) the lipids in the muscle and liver tissue were extracted with 2:1 dichloromethane-methanol (v:v) and an aqueous solution of 0.88% KCl (w:v). Crude lipid content was determined gravimetrically to the nearest 0.001 g and calculated as the percentage of lipids of tissue wet weight.

2.6 Data analysis (Calculations and statistics)

The growth parameters were based on body weight (BW) and body length (BL) and calculated as follows.

(1) $Weight\ gain\ (WG, g) = BW_{final} - BW_{initial}$

(2) $Relative\ growth\ rate\ (RGR, \%\ d^{-1}) = 100 \times (e^{\frac{ln(BW_{final}) - \ln(BW_{initial})}{feeding\ days}} - 1)$

(3) $Condition\ factor\ (CF) = 100 \times \frac{\mathrm{BW}}{\mathrm{BL}^3}$

(4) $Hepato - somatic\ index\ (HSI) = 100 \times \frac{liver\ weight}{BW_{final}}$

The feed performance parameters, daily feed intake (DFI) and feed conversion ratio (FCR) were based on the feed intake (FI) in g of the offered amount of feed and the uneaten feed, which is corrected by the soluble loss factor. Total FI and WG for FCR were corrected for the lost biomass through mortalities and sampling.

(5) $Total\ Feed\ Intake\ (FI_{total}, g) = Feed_{offered} - (Feed_{uneaten} \times factor_{soluble\ loss})$

(6) $factor_{soluble\ loss} = 1 + [1 - (\frac{Feed_{final}}{Feed_{initial}})]$

(7) $Daily\ feed\ intake\ (DFI, \%\ BW\ d^{-1}) = 100 \times FI_{total} / \frac{BW_{final} + BW_{initial}}{2} \times Feeding\ days^{-1}$

(8) $Feed\ conversion\ ratio\ (FCR) = \frac{FI_{total}}{WG}$

(9) $Protein\ efficiency\ ratio\ (PER) = \frac{weight\ gain}{crude\ protein\ intake}$

The apparent digestibility (ADC) of the dietary nutrients and the apparent availability (AA) of minerals were based on the amount of the inert yttrium marker in the diet and faeces and the respective nutrient or element in faeces and diets.

$$(10) ADC\ dry\ matter\ (\%) = 100 - (100 \times \frac{yttrium_{diet}}{yttrium_{faeces}})$$

$$(11)\ ADC\ nutrient\ (\%) = 100 - (100 \times \left(\frac{yttrium_{diet}}{yttrium_{faeces}} \times \frac{nutrient_{faeces}}{nutrient_{diet}}\right)$$

$$(12)\ AA\ (\%) = 100 - (100 \times \left(\frac{yttrium_{diet}}{yttrium_{faeces}} \times \frac{element_{faeces}}{element_{diet}}\right)$$

The glycogen content was calculated based on the concentration of total glucose ($c_{total\ glucose}$) subtracted by the concentration of free glucose ($c_{free\ glucose}$).

$$(13) c_{total/free\ glucose} (\mu mol\ mg^{-1}) = (\Delta A \times V_{assay\ total}) / \frac{\varepsilon \times d \times V_{sample}}{c_{tissue}} \times DF$$

Whereas, ΔA = the change in absorption, Vassay total = the total measurement volume of the assay (ml), Ɛ = the coefficient of extinction at 339 nm (6.3 mL μmo^{-1} cm^{-1}), d = the thickness of layer for the cuvette (1 cm), Vsample = the sample volume (mL), DF = the dilution factor and c_{tissue} = the concentration of tissue wet weight in crude extract (mg mL^{-1}).

The glucose concentration was converted to glycogen content using the molecular weight of the glucosyl moiety in glycogen with Mr = 162 g mol^{-1}.

$$(14)\ Glycogen\ content\ (mg\ g^{-1}\ wet\ weight\ tissue) = \left(c_{total\ glucose} - c_{free\ glucose}\right) \times 162$$

2.7 Statistical analysis

Statistical analysis was conducted with Sigma Plot (12.5, Systat Software, Germany). One-way Analysis of Variance (ANOVA) was used to determine significant differences between the treatments. Whenever there were statistically significant differences an All Pairwise Multiple Comparison Procedure was performed using the Holm-Sidak method (overall significance level p = 0.050) to find the difference within the treatments. Values are given as means ± standard deviations.

3 Results

3.1 Growth and feed performance

The experimental feed formulations did not significantly affect the growth of the juvenile turbots and the survival during the experimental period was high with 0% mortalities (Table 4). After 16 weeks, the fish from the control group increased their weight 3.25 fold (65 g), whereas the fish fed with the experimental diets only increased their weight 3.10 fold (by an average of 62 g). The fish accepted all diets with a daily feed intake (DFI) of 0.99 ± 0.03% BW d^{-1} (n = 15 tanks). The feed conversion ratio (FCR) in the fish from the control group was significantly lower than in the fish from the PAP group, with no significant differences to the fish from the PLANT group. The protein efficiency ratio (PER) was significantly higher in the fish from the control group than in the PAP group with no significant differences to the fish from the PLANT group. The condition factor (CF) was significantly affected by the experimental diets (Table 4). The CFs of fish from PLANT group were significantly lower than of the fish in the control and the PAP group. However, the CF significantly increased in all treatments from the initial to the final (t-test; $p < 0.001$).

Table 4 Performance parameters of the juvenile turbot (*Scophthalmus maximus*) fed the experimental diets for 16 weeks (n = 5 tanks per diet).

	Control	PAP	PLANT	F	p
Initial body weight (g)	20.2 ± 0.3	20.1 ± 0.5	20.4 ± 0.4	0.402	0.677
Final body weight (g)	85.2 ± 9.7	82.9 ± 6.1	82.1 ± 9.5	0.183	0.835
Weight gain (g)	65.0 ± 9.5	62.8 ± 5.9	61.7 ± 9.2	0.203	0.819
Relative growth rate (% d^{-1})	1.29 ± 0.09	1.27 ± 0.06	1.25 ± 0.09	0.293	0.751
Daily feed intake (% BW d^{-1})	0.98 ± 0.03	1.01 ± 0.04	1.00 ± 0.03	0.985	0.402
Feed conversion ratio	0.87 ± 0.03^{b}	0.92 ± 0.03^{a}	0.90 ± 0.02^{ab}	5.059	0.026
Protein efficiency ratio	2.17 ± 0.07^{a}	2.06 ± 0.06^{b}	2.10 ± 0.05^{ab}	4.031	0.046
Initial condition factor	1.96 ± 0.02	1.94 ± 0.02	1.95 ± 0.03	0.767	0.486
Final condition factor	2.11 ± 0.01^{a}	2.10 ± 0.01^{a}	2.07 ± 0.02^{b}	7.750	0.007

Control: commercial-like formulation, PAP: processed animal protein, PLANT: plant based protein. Values are expressed as means ± SD, values with different letters within the same line are significantly different ($P < 0.050$), F and p values from One-way-ANOVA

3.2 Whole Body composition, apparent digestibility and energy reserves

The whole body composition with moisture content, crude protein, ash content and energy content did not differ significantly between fish fed the different diets (Table 5). Fish from the PAP group had a significantly lower crude lipid content than the fish from the control group with no significant differences to the fish from the PLANT group. The apparent digestibility coefficients (ADC) of dry matter and crude protein were significantly higher in the fish from the control group compared to the fish from the experimental groups, whereas the ADC of energy was not affected (see Table 5).

Table 5 Proximate whole body composition on wet weight basis and apparent digestibility coefficient of juvenile turbot (*Scophthalmus maximus*) fed the experimental diets for 16 weeks (n = 5 tanks per diet).

	Control	PAP	PLANT	F	P
Moisture (%)	74.1 ± 1.3	76.6 ± 0.9	74.4 ± 3.5	1.802	0.207
Crude Protein (%)	16.2 ± 1.1	15.2 ± 0.8	16.1 ± 2.6	0.491	0.624
Crude Lipid (%)	4.5 ± 0.4^{a}	3.5 ± 0.4^{b}	3.9 ± 0.5^{ab}	6.585	0.012
Ash (%)	3.7 ± 0.3	3.9 ± 0.1	4.3 ± 0.6	3.039	0.086
Gross Energy (MJ kg^{-1})	5.2 ± 0.6	4.6 ± 0.2	5.3 ± 0.8	2.130	0.162
Apparent digestibility coefficient					
Dry matter (%)	83.2 ± 1.1^{a}	77.1 ± 1.7^{b}	77.2 ± 1.9^{b}	23.103	<0.001
Crude protein (%)	92.0 ± 0.5^{a}	89.8 ± 0.7^{b}	89.7 ± 0.7^{b}	22.626	<0.001
Gross Energy (%)	85.2 ± 0.8	86.4 ± 2.2	86.3 ± 1.1	1.027	0.388

Control: commercial-like formulation, PAP: processed animal protein, PLANT: plant based protein. Values are expressed as means ± SD, values with different letters within the same line are significantly different ($P < 0.050$), F and p values from One-way-ANOVA

The hepato-somatic index (HSI) of fish fed the control diet was significantly higher than that of the fish from the experimental groups (Table 6). The hepatic glycogen was significantly higher in the fish fed the control than in the fish fed the PLANT diet, with no significant difference to the fish from the PAP group. The hepatic lipid content, glycogen, and lipid content in the muscle of turbot showed no significant differences between groups (see Table 6).

Table 6 Hepato-somatic index (n = 15 fish per diet), glycogen in wet tissue (n = 15 fish per diet) and lipid content in dry tissue (n = 5 tanks per diet) in the liver and muscle of juvenile turbot (*Scophthalmus maximus*) fed the experimental diets for 16 weeks.

	Control	PAP	PLANT	F	P
Hepato-somatic index	1.8 ± 0.3[a]	1.5 ± 0.3[b]	1.5 ± 0.3[b]	4.177	0.022
Liver glycogen (mg g^{-1})	63.7 ± 23.9[a]	48.0 ± 17.2[ab]	46.4 ± 16.0[b]	3.666	0.034
Liver lipid (mg g^{-1})	452.0 ± 56.5	486.6 ± 54.9	527.2 ± 64.8	2.044	0.172
Muscle glycogen (mg g^{-1})	1.7 ± 0.7	1.8 ± 0.7	2.1 ± 0.7	1.411	0.255
Muscle lipid (mg g^{-1})	64.3 ± 20.6	90.8 ± 37.4	77.0 ± 35.2	0.861	0.447

Control: commercial-like formulation, PAP: processed animal protein, PLANT: plant based protein. Values are expressed as means ± SD; values with different letters within the same line are significantly different ($P < 0.050$), F and p values from One-way-ANOVA

3.3 Mineral analysis of the diets, mineral balance and apparent availability

The mineral content of the whole body showed no significant differences in the fish from all diets, except for arsenic and copper concentration in the PLANT-feeding fish was significantly higher than in the control fish, with no significant differences to PAP (Table 7).

For all analyzed minerals, the apparent availability (AA) was highest in the control diet compared to the experimental diets, except for potassium and sodium, where the AA in the control was lowest (Table 7). No significant differences were found in the availability of calcium, arsenic and zinc. The potassium and sodium availability in the control was significantly lower than in the experimental diets. In contrast, the availability of magnesium, copper and iron was in the control significantly higher than in the experimental diets. The phosphorus availability was in the control diets significantly higher than in the PLANT diet, with no significant differences to the PAP diet. The manganese availability was in the PLANT diet significantly lower than in the control and PAP diet.

Table 7 Analyzed concentrations on wet weight basis in the whole body and apparent availability (AA) of minerals and trace elements in turbot (*Scophthalmus maximus*) fed the experimental diets for 16 weeks (n = 5 tanks per diet).

	Control	PAP	PLANT	F	P
Calcium (Ca; g kg^{-1})	10.41 ± 2.16	10.02 ± 1.91	12.40 ± 2.25	1.832	0.202
Potassium (K; g kg^{-1})	2.79 ± 0.35	3.02 ± 0.22	3.13 ± 0.48	1.061	0.376
Magnesium (Mg; g kg^{-1})	0.33 ± 0.05	0.35 ± 0.02	0.39 ± 0.05	3.138	0.080
Sodium (Na; g kg^{-1})	1.52 ± 0.21	1.63 ± 0.09	1.77 ± 0.20	2.305	0.142
Phosphorus (P; g kg^{-1})	6.34 ± 1.22	6.48 ± 0.89	7.70 ± 1.21	2.254	0.147
Ca/P ratio	1.64 ± 0.04	1.54 ± 0.08	1.61 ± 0.07	2.735	0.105
Arsenic (As; g kg^{-1})	0.72 ± 0.09[b]	0.83 ± 0.10[ab]	0.91 ± 0.08[a]	5.642	0.019
Copper (Cu; mg kg^{-1})	0.48 ± 0.06[b]	0.53 ± 0.02[ab]	0.60 ± 0.08[a]	4.288	0.021
Iron (Fe; mg kg^{-1})	4.74 ± 0.7	7.37 ± 2.52	6.67 ± 1.12	3.430	0.066
Manganese (Mn; mg kg^{-1})	13.25 ± 3.70	11.25 ± 1.59	15.27 ± 2.64	2.611	0.114
Zinc (Zn; mg kg^{-1})	11.22 ± 1.22	11.35 ± 0.61	13.23 ± 1.85	3.608	0.059
Apparent availability					
Calcium (Ca; %)	58.7 ± 14.9	45.6 ± 16.5	37.7 ± 25.8	1.451	0.273
Potassium (K; %)	88.1 ± 1.2[b]	93.4 ± 1.1[a]	94.0 ± 0.9[a]	46.210	<0.001
Magnesium (Mg; %)	-61.7 ± 22.6[a]	-151.5 ± 37.6[b]	-142.1 ± 37.4[b]	10.992	0.002
Sodium (Na; %)	-30.6 ± 37.5[b]	13.1 ± 19.2[a]	30.9 ± 5.5[a]	8.323	0.005
Phosphorus (P; %)	88.1 ± 1.9[a]	78.7 ± 7.2[ab]	77.8 ± 7.1[b]	4.578	0.033
Arsenic (As; %)	91.8 ± 2.0	90.4 ± 2.9	90.1 ± 2.1	0.751	0.493
Copper (Cu; %)	62.4 ± 5.4[a]	36.0 ± 2.8[b]	41.9 ± 3.5[b]	58.949	<0.001
Iron (Fe; %)	46.9 ± 4.3[a]	17.4 ± 3.6[b]	17.7 ± 7.1[b]	52.347	<0.001
Manganese (Mn; %)	72.5 ± 11.5[a]	52.8 ± 7.3[a]	32.4 ± 23.5[b]	8.156	0.006
Zinc (Zn; %)	46.0 ± 6.6	27.3 ± 15.9	26.2 ± 21.3	2.484	0.125

Control: commercial-like formulation, PAP: processed animal protein, PLANT: plant based protein. Values are expressed as means ± SD, values with different letters within the same line are significantly different ($P < 0.05$), F and p values from One-way-ANOVA

4 Discussion

In the present study, the more sustainable feed formulations, in which fish-derived ingredients were reduced by 20%, resulted in juvenile turbot with similar growth comparing to the control (commercial-type feed) group. This is congruent with literature where decreased growth and feed performance were observed when more than 30-35% of fishmeal was replaced by processed animal protein (Dong et al., 2016), insect meal (Kroeckel et al., 2012) and plant protein (Burel et al., 2000a, Fournier et al., 2004, Bonaldo et al., 2015, Hermann et al., 2016, von Danwitz et al., 2016, Bian et al., 2017). Nevertheless and not unexpected, compared to controls, fish that were fed the slightly leaner experimental diets were less capable of building up energy reserves as the HSI and the slightly lower liver glycogen show, possibly augmented by a slightly lower apparent digestibility of dietary protein (2%) and energy (3%) of the control diet compared to the experimental diets. The fish in the PAP group had the poorest feed conversion, whereas other parameters showed no clear picture. In any case, the results of present study indicate that successful substitution of traditional fishmeal and fish oil can be achieved in turbot. It can further be speculated that an equal fraction of digestible energy contents between the different diets would have led to an even better similarity in fish performance between the diets of the GAIN project alternative formulations as it was shown with similar formulation in Gilthead seabream (Aragao et al., 2020). Even though the crude lipid content in the PLANT diet was 2% higher than in the control and PAP diet, a possible effect of this on the turbot can be negligible. The crude protein level (52%) used in present study is sufficiently high for turbot to minimize possible effects of the differing crude lipid level as previously observed in juvenile turbot (Sevgili et al., 2014).

4.1 Growth and Feed Performance

The relative growth rate (RGR) was similar between all groups, on average 1.27 ± 0.08% d^{-1} (n = 15 tanks) indicating that the different diets did not affect turbots' energy allocation with respect to growth performance. In the present study, the RGR was higher by 0.01 percentage points compared to the specific growth rate (SGR), which is widely used in literature (i.e. Burel et al., 1996, Arnason et al., 2009, Bonaldo et al., 2011, Nagel et al., 2012). However, it is incorrect in concept to express the SGR as a percentage increase in daily weight, and therefore, the RGR is used instead (Hardy and Barrows, 2003). The presented RGR results were lower than those of similar sized turbot based on the majority of available literature data (Burel et al., 1996, Imsland et al., 1996, Arnason et al., 2009, Fuchs et al., 2015, Nagel et al., 2017), but moderate to high compared to turbot in commercial RAS (Baer et al., 2011).

The feed conversion ratio (FCR) and protein efficiency ratio (PER) was significantly better in the control than in the PAP group. However, the differences between the FCRs in all diets were small with a mean value of 0.90 ± 0.03 (n = 15) and the daily feed intake (DFI) was approximately 0.99% BW d^{-1} resulting in a RGR in the expected range (Burel et al., 1996). Furthermore, the turbot strain used in the present study might have a lower growth rate per se, as turbot exhibit counter gradient variation (Imsland et al., 2000). Strains from lower latitudes, such as France, show generally lower growth and feed efficiency compared to populations from higher latitudes, such as Norway and Iceland (Imsland et al., 2001).

In present study, the differences in FCR might be overestimated due to the higher moisture content in the experimental diets compared to the control diet. The same pattern as for the FCR was observed in the protein efficiency ratio, with the highest value for the control, followed by PLANT and PAP. In line with literature, in the present study the apparent digestibility coefficient (ADC) of protein in turbot decreased (> 90% in the control group with 450 g kg^{-1} fishmeal) when the fishmeal inclusion level is reduced (Regost et al., 1999, Bonaldo et al., 2011, Liu et al., 2014b, Bai et al., 2019, Li et al., 2019b). When combining all feed performance indicators, the fish from the control group had the best performance followed by the PLANT group and the PAP group where fish showed the lowest performance.

Even though the condition factor (CF) of turbot from the PLANT group showed statistically a difference to the control and PAP group (2.07 vs. 2.11 and 2.10, respectively), the physiological relevance is minor and does not indicate poorer nutritional status. CFs above 2 are indicate an overall good nutritional status of the fish, as presented CFs are similar to values of in previous studies (Fuchs et al., 2015, von Danwitz et al., 2016, Nagel et al., 2017, Weiß and Buck, 2017, Wanka et al., 2019). In previous studies, a reduced CF was observed in turbot fed with plant-based diets (Bonaldo et al., 2015) and insect meal based diets (Kroeckel et al., 2012) at a substitution/replacement level of more than 55%. In line with present study, reduced CF in fish fed with different PAPs was not observed in previous studies for European sea bass (Campos et al., 2017), Gilthead seabream (Karapanagiotidis et al., 2019) and rainbow trout (Lu et al., 2015). However, this might be biased by a lack of studies on this feed ingredient.

4.2 Nutritional and energy status

In this study, fish from the control group had a significantly higher hepato-somatic index (HSI) than the fish from the two experimental groups (1.8 vs. 1.5 and 1.5, respectively). The HSI of the control group is good for juvenile turbot (Dietz et al., 2012, Bonaldo et al., 2015, Nagel et al., 2017). The hepatic glycogen of the control, PAP and PLANT fed turbot (63.7 mg g^{-1}, 48.0 mg g^{-1} vs. 46.4 mg g^{-1}, respectively) followed a similar pattern indicating a positive correlation between glycogen as energy reserve in a good nutritional status and the HSI (Liu et al., 2014a, Guerreiro et al., 2015a, Zeng et al., 2015, Miao et al., 2016a). Hepatic glycogen serves in many fish species as an energy reserve and high glycogen deposition leads to increased liver weight in many fish species (Hemre et al., 2002). The liver lipid content was not affected by the diet, which is in line to a study by Guerreiro et al. (2015b) on European seabass that were fed with plant protein compared to fish protein. The effects of the diet on the hepatic lipid content might be minor since turbot does not store excess dietary lipid in the liver or muscle (Regost et al., 2001, Leknes et al., 2012, Liu et al., 2014a). The muscle glycogen and lipid content were not affected by the diets, whereas the muscle glycogen (1.9 mg g^{-1}, calculated for all animals, irrespective of diet group) was on the lower range of 1 – 12 mg g^{-1} compared to previous studies (Soengas et al., 1995, Pichavant et al., 2002, Miao et al., 2016a).

Considering the lower HSI and hepatic glycogen of the PAP and PLANT fed turbot, we can conclude that the experimental diets used in present study did alter the nutritional status of turbot to a certain degree without negatively affecting the growth. The decreased apparent digestibility of the experimental diets might have caused a reduced surplus on energy resulting in slightly smaller liver masses and thus HSI in PAP and PLANT fed turbot. Interestingly, it has been shown that the reduction and replacement of fishmeal with alternative feed ingredients could lead to contradicting results. Decreasing fishmeal content may lead to a decreased HSI (Kroeckel et al., 2012, von Danwitz et al., 2016, Gu et al., 2017, Bai et al., 2019, Wanka et al., 2019), unchanged HSI (Bonaldo et al., 2015, Fuchs et al., 2015, Wang et al., 2016, Weiß and Buck, 2017) or even increased HSI (Fournier et al., 2004, Dietz et al., 2012, Nagel et al., 2017). This aspect might be worth investigating in more detail to unravel the observed variation in HSI, hepatic glycogen and lipid dependent on alternative feed ingredients.

4.3 Mineral balance, utilization and availability

The concentrations of calcium, potassium, sodium, phosphorus, iron and manganese were lower in the control diet than in the experimental diets. The type of fishmeal used can explain the elevated ash, calcium and phosphorus content in the experimental diets. Fishmeal from fish by-products has a higher ash content containing much calcium and phosphorus due to a higher content of bones compared to traditional fishmeal (Olsen and Hasan, 2012). These differences, however did not significantly affect the concentration of minerals and trace element in the whole body of turbot, which are similar to those of other species (see meta-analysis by Antony Jesu Prabhu et al., 2016). However, the manganese concentration was twice as high as the maximum described for different fish species (Antony Jesu Prabhu et al., 2016) and for turbot in RAS (van Bussel et al., 2014). This might be due to accumulation effects in the whole body, which was already described in turbot (Ma et al., 2015) and Atlantic salmon parr (Lorentzen et al., 1996).

Even though there were no diet-dependent effects on the concentrations of calcium, arsenic and zinc, the apparent availability magnesium, copper and iron were significantly higher in the fish from the control group than in the fish fed with the experimental diets. Furthermore, the apparent availability of phosphorus and manganese was significantly reduced in fish fed the plant-based diet compared to the fish fed the control. Potassium and sodium had a significantly reduced apparent availability in the fish fed the control compared to the fish fed the experimental diets. Substances such as phytate in plant-based feed ingredients are known to bind minerals and, thus, reduce the availability of phosphorus, iron and zinc in fish (Kumar et al., 2012). The inclusion of rapeseed in diets lead to a reduced availability of phosphorus, manganese, iron and zinc, but increased copper availability in turbot (von Danwitz et al., 2016). The potassium and sodium availability was in general high, and was significantly higher in fish fed the experimental diets than in the control group (93% and 94% in PAP and PLANT vs. 88% in control). In contrast, the potassium availability in rainbow trout was higher in the fishmeal-based diet than in the plant-based diet (Antony Jesu Prabhu et al., 2015, Antony Jesu Prabhu et al., 2018).

Since the mineral concentrations in the whole body are similar in the fish from all experimental groups, it can be concluded that the mineral and trace element demand was sufficiently covered and that the elevated mineral concentration in the experimental diets was balanced by elevated excretion rates.

4.4 Prediction of feed and production costs

The present results of growth performance indicate that alternative feed formulations can be used in commercial aquaculture for juvenile turbot. Since feed costs are the largest cost factor in the production, small differences in the FCR can balance feed costs and could make more cost efficient formulations attractive. The animal-based formulation (PAP) presented in this study, has a lower cost with a commercial margin than the commercial-like control formulation, whereas the plant protein formulation (PLANT) is more expensive (see Table 8). Taking this study's FCRs into consideration, the feed costs to produce one ton of turbot is still lower with the PAP formulation than the control and the PLANT formulation. Feeding juvenile turbot with the PAP formulation could lead to a cost reduction of 10% compared to the control, whereas feeding the PLANT formulation would increase the costs by 12%.

Table 8 Estimated feed costs for typical turbot (*Scophthalmus maximus*) farms with the alternative feed formulations.

	Control	PAP	PLANT
Full cost with commercial margin (€ ton^{-1})	2373	2027	2569
Feed cost to produce fish (€ ton^{-1})	2064	1865	2312
Change over Control (%)		-10	12

Control: commercial-like formulation, PAP: processed animal protein, PLANT: plant based protein.

5 Conclusion

The present study highlighted that fish by-products are a suitable replacement for commercial fishmeal and that protein sources derived from terrestrial plants or animals can replace 20% of the overall fish-derived ingredients without compromising growth performance and body composition of juvenile turbot. These findings are a promising start for further research to find the optimal replacement of marine ingredients, in order to ensure acceptable feed utilization and deviations from nutritional status. Overall, the alternative diet formulations may produce leaner fish, which have the potential for muscle growth rather than adiposity and the slightly lowered apparent digestibility of protein suggest that waste production within a commercial aquaculture system would not be much higher than with feeding the control diet. Furthermore, the feed formulation based on processed animal protein (PAP) seems to be an economical feasible alternative for juvenile turbot since the lower feed related production costs balance the slightly poorer feed conversion. Further studies on turbot in the grow-out phase will investigate how a higher fishmeal replacement will affect the performance.

Besides the effects of the alternative feed formulations on fish performance, the economic and environmental benefits of the diets, the consumers' acceptance of the diet formulations need to be considered. Alternative feed ingredients, sourced through circular economy processes, could be more environmentally sustainable (Maiolo et al., 2020) but may also increase production costs. Hereby particularly, insect and algae production could be included in an integrated multi trophic aquaculture (IMTA) system, which reduces the environmental impact by recycling of nutrients (Barrington et al., 2009, Milhazes-Cunha and Otero, 2017). Many consumers are concerned that feed ingredients, such as by-products from terrestrial animals, may not be safe (Glencross et al., 2020). Furthermore, they express the concern that the feed formulations with high levels of plant ingredients might not be species appropriate and impair the animal welfare of cultured fish (Feucht and Zander, 2015). Therefore, in addition to the marketing of more sustainable aquaculture products in Europe such socio-economic aspects need to be considered when developing, new and innovative fish diets for commercial important fish species.

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Ethics approval

The experiments were performed under the guidelines of the local authority 'Food surveillance, animal welfare and veterinary service (LMTVet)' of the state of Bremen with the permission to carry out animal experiments (500-427-103-1/2019-1-19).

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Manuscript II

[1]H-NMR-based metabolic profiling in muscle and liver tissue of juvenile turbot (*Scophthalmus maximus*) fed with plant and animal protein sources

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Abstract

Circular-economy driven feed ingredients and emerging protein sources such as insects and microbial meals pose the potential to develop balanced feed formulations for high-trophic fish. Even though growth and feed performance are often unaffected at low inclusion levels, the metabolic effects are unknown. This study examined the metabolic response of juvenile turbot (*Scophthalmus maximus*) to diets with graded fishmeal replacement with plant, animal and emerging protein sources (PLANT, PAP, and MIX) in comparison to a commercial-like diet (CTRL). ^{1}H-nuclear magnetic resonance (NMR) spectroscopy was used to assess metabolic profiles of muscle and liver tissue after feeding the fish the experimental diets for 16 weeks. The comparative approach revealed a decrease in metabolites that are associated with energy allocation in both tissues of fish fed with fishmeal-reduced diets compared to the commercial-like diet (CTRL). Since growth and feeding performance were unaffected, the observed metabolic response suggests that the balanced feed formulations, especially at lower fishmeal replacement level, have the potential for industry application.

Keywords: by-product, compound, glycogen, glucose, TMAO, betaine

1 Introduction

The reduction of the environmental footprint of feeds has become a high priority in aquaculture research and industry (Glencross et al., 2020, Naylor et al., 2021). Fishmeal (FM), formerly the main ingredient in artificial diets for fish, is no sustainable choice due to its origin in limited stocks. Furthermore, the increasing demand for aquaculture diets is putting more pressure on this valuable marine resource (Hua et al., 2019). Even though the overall FM content has been drastically reduced during the last decades (FAO, 2022c), it has mostly been replaced by soybean-based protein sources. Soybean products however, are not a sustainable choice either, as its production is associated with tremendous effects on vulnerable ecosystems and people. Recent events and developments such as the COVID-19 pandemic and the Russia-Ukraine conflict that severely interrupted world trade highlighted the importance for locally sourced goods (Sarà et al., 2022). This, along with the challenge of formulating a feed for high-trophic fish that is both nutritious and economically and environmentally sustainable, requires an eco-efficient solution. The use of circular-economy (CE) driven feed ingredients based on other protein sources available in Europe, such as rapeseed, pea, microbial meals and land-animal proteins can reduce the conflict of use with human nutrition, diversify supply, and provide a viable alternative to FM and soybeans (Hua et al., 2019). All of the above-mentioned alternative feed ingredients have been assessed in various feeding trials with high-trophic fish focusing on key performance indicators such as growth, feed conversion, nutrient retention, digestibility and somatic indices (Fronte et al., 2019, Karapanagiotidis et al., 2019, Kaiser et al., 2021a). They are suitable to replace FM or alternatively soybean to a certain inclusion level without compromising these key performance indicators. However, effects in organs, tissues, transcriptome and metabolome can be detected even before the performance indicators are affected (Glencross et al., 2004, Øverland et al., 2009, Batista et al., 2016, Casu et al., 2017). Among the 'omics' approaches, metabolomic studies are attracting increasing interest in aquaculture research to gain a deeper understanding of how feed ingredients affect performance indicators (Alfaro and Young, 2018). In high-trophic fish species different metabolic pathways for glucose, amino and fatty acids are affected by full plant-based diets (Casu et al., 2017) or diets based on alternative ingredients such as land-based proteins (Schock et al., 2012, Roques et al., 2020a), single-cell proteins (Abro et al., 2014) or fish protein hydrolysates (Wei et al., 2017a). Thereby, liver and muscle are good target tissues for diet-dependent changes in metabolic profiles (Roques et al., 2020b). The liver plays a key role in the energy storage, deposited as glycogen and lipid, and in the digestion and muscle tissue, is a main part in a fish's body, where energy is used for locomotion and growth. However, many metabolomics studies did not regard the potential of balanced feed formulations to counteract the negative effects on the metabolome as observed with graded-level single ingredients diets (Schock et al., 2012, Wei et al., 2017a, Casu et al., 2019, Roques et al., 2020a).

Using ^{1}H-nuclear magnetic resonance (NMR) spectroscopy, this study assessed diet-dependent changes in metabolites of muscle and liver tissue of juvenile turbot (*Scophthalmus maximus*) after the fish were fed with eco-efficient feed formulations including plant and animal protein sources as well as emerging protein sources and graded FM replacement levels. Parts of the data for the growth and feed performance, as well as the glycogen content in the liver and muscle tissue of the fish have been previously published in Hoerterer et al. (2022b) and are presented in this study for better integration and discussion of the results from the metabolic profiling,

2 Material and Methods

2.1 Experimental design

The feeding experiment was carried out in the Centre for Aquaculture Research (ZAF) at the Alfred Wegener Institute HGF for Polar and Marine research in Bremerhaven, Germany. The juvenile turbot (*Scophthalmus maximus*) were purchased from France Turbot (L'Épine, France), and acclimated to the recirculating aquaculture system (RAS) for two weeks prior to starting the 16 weeks experimental trial. The RAS consisted of 36 tanks with a bottom area of one m² and a volume of 700 l and the water processing consisted of a drum filter, ozone treatment, protein skimmer, a nitrifying and a denitrifying biofilter. The physical parameters of the process water were monitored constantly (temperature 16.4 ± 0.2°C), pH (7.6 ± 0.1, conductivity 52.1 ± 1.3 mS cm^{-1}, and oxygen saturation 103.5 ± 4.3%; SC 1000 Multiparameter Universal Controller, Hach Lange GmbH, Germany). The concentrations for N-ammonium (0.2 ± 0.1 mg L^{-1}), N-nitrite (0.5 ± 0.3 mg L^{-1}), and N-nitrate (155.1 ± 37.0 mg L^{-1}) were measured with the twice a week (QuAAtro39 AutoAnalyzer, SEAL Analytical, Germany).

All experimental diets were formulated to be isonitrogenous (530 g kg^{-1}), isolipidic (160 g kg^{-1}) and isoenergetic (21 MJ kg^{-1}) and extruded as 3 mm pellets in a floating mode at SPAROS LDA (Olhão, Portugal). Three eco-efficient feed formulations with graded fishmeal (FM) content were tested against a control diet (CTRL), mimicking a typical current commercial formulation used for turbot. The CTRL diet contained conventional levels of FM (500 g kg^{-1}), wheat gluten (110 g kg^{-1}) and soy protein concentrate (100 g kg^{-1}) as the main protein sources. In the eco-efficient feed formulations, the commercial FM was fully replaced with FM and fish protein hydrolysates from by-products. The soybean ingredients as well as the remaining protein fraction was supplemented from emerging ingredient sources such as insect meal, microbial meal, and pea protein. In two diets, plant protein and microalgae (PLANT) and processed animal protein (PAP), respectively, replaced 20% of the FM. In the third diet, a mixture of processed animal protein, plant protein, cell meals, insect meal and microalgae (MIX) replaced 40% of FM. Furthermore, in all eco-efficient feed formulations DHA-rich algae and rapeseed oil replaced 60% of fish oil. The content of the respective experimental diets is shown in Table 1 and the proximate composition of the diets in Table 2.

For the experiment, 1000 turbot were weighed (initial body mean weight of 20.4 ± 0.6 g) and measured in total body length (initial body length 10.1 ± 0.1 cm) and randomly distributed to 20 tanks (50 individuals per tank, five tanks per diet). The fish were hand fed twice a day (9 am and 2 pm) ad libitum for a period of 16 weeks. The effects of the two feed formulations PLANT and PAP on growth and feed performance as well as the nutritional status was reported in a previous publication (Hoerterer et al., 2022b). The growth and feed performance as well as nutritional status of the fish from the MIX group were determined in the same way. In short, at the end final body weight (BW) and total body length (BL) were recorded from all remaining fish, to calculate the specific growth rate (SGR) and condition factor (CF) as follows:

(1) $SGR = 100 \times ln(final\,BW) - ln(initial\,BW) / growth\ days$

(2) $CF = 100 \times final\,BW / final\,BL^3$

The feed conversion ratio (FCR) was calculated from the total feed intake (FI) per fish during growth period divided by the weight gain as follows:

(3) $FCR = FI / final\,BW - initial\,BW$

Table 1 Formulation and proximate composition of the experimental diets for juvenile turbot (*Scophthalmus maximus*) on wet weight basis.

	CTRL*	PLANT*	PAP*	MIX
Level of fishmeal replacement	**0%**	**20%**	**20%**	**40%**
Ingredients (g kg^{-1})				
Fishmeal[1]	500			
Fishmeal (by-product)[2]		350	350	250
Fish hydrolysate (by-product)[x]		50	50	50
Insect meal (*Hermetia illucens*)[x]		50	50	75
Porcine hemoglobin[3]			25	
Poultry meal[4]			102	75
Microbial protein meal (*Methanotrophic bacteria*)[x]		25	25	50
Yeast protein meal (*Saccharomyces cerevisiae*)[x]		25	25	50
Microalgae meal [1,5]		27		38
Soy protein concentrate[6]	100			
Pea protein concentrate[7]		124	50	80
Wheat gluten[7]	110	115	100	100
Soybean meal[8]	40			
Wheat meal[9]	80			
Pea starch[10]	40	88.9	89.9	89.9
Fish oil[1]	116	46.4	46.4	46.4
DHA-Rich algae (*Schizochytrium*)[11]		10.8	10.8	18.8
Rapeseed oil[12]		46.4	34.4	34.4
Rapeseed lecithin[13]		8	8	8
Vitamin and mineral premix[14]	10	10	10	10
Vitamin C[15]	0.5	0.5	0.5	0.5
Vitamin E[15]	0.5	0.5	0.5	0.5
Betaine HCl[16]		5	5	5
Macroalgae mix[17]		5	5	5
Antioxidant[18]	1.8	1.8	1.8	1.8
Sodium propionate[19]	1	1	1	1
L-Tryptophan[20]		1.5	1.5	1.5
DL-Methionine[21]		3	3	3
L-Taurine[16]		5	5	6
Yttrium oxide[22]	0.2	0.2	0.2	0.2

CTRL: commercial-like formulation, PLANT: plant protein, PAP: processed animal protein, MIX: mixture of processed animal and plant protein. * Formulation and proximate composition of the CTRL, PLANT and PAP diets have been previously published in Hoerterer et al. (2022).

[x] not disclosed; [1] Sopropêche, France; [2] Conserveros Reunidos S.A., Spain; [3] SONAC BV, The Netherlands; [4] SAVINOR UTS, Portugal; [5] Allmicroalgae, Portugal; [6] ADM, The Netherlands; [7] Roquette Frères, France; [8] CARGILL, Spain; [9] Casa Lanchinha, Portugal; [10] COSUCRA, Belgium; [11] Alltech, Ireland; [12] Henry Lamotte Oils GmbH, Germany; [13] Novastell, France; [14] DL-alpha tocopherol acetate, 255 mg; sodium menadione bisulphate, 10 mg; retinyl acetate, 26000 IU; DL-cholecalciferol, 2500 IU; thiamine, 2 mg; riboflavin, 9 mg; pyridoxine, 5 mg; cyanocobalamin, 0.5 mg; nicotinic acid, 25 mg; folic acid, 4 mg; L-ascorbic acid monophosphate, 80 mg; inositol, 17.5 mg; biotin, 0.2 mg; calcium panthotenate, 60 mg; choline chloride, 1960 mg. Minerals (g or mg·kg-1 diet): copper sulphate, 8.25 mg; ferric sulphate, 68 mg; potassium iodide, 0.7 mg; manganese oxide, 35 mg; organic selenium, 0.01 mg; zinc sulphate, 123 mg; calcium carbonate, 1.5 g; excipient wheat middlings; [15] DSM Nutritional Products, Switzerland; [16] ORFFA, The Netherlands; [17] Ocean Harvest, Ireland; [18] Kemin Europe NV, Belgium; [19] Disproquímica, Portugal; [20] Ajinomoto EUROLYSINE S.A.S, France; [21] EVONIK Nutrition & Care GmbH, Germany; [22] Sigma Aldrich, USA

Table 2 Formulation and proximate composition of the experimental diets for juvenile turbot (*Scophthalmus maximus*) on wet weight basis.

	CTRL*	PLANT*	PAP*	MIX
Level of fishmeal replacement	**0%**	**20%**	**20%**	**40%**
Moisture (%)	4.1	6.7	7.3	7.5
Crude Protein (%)	52.9	52.8	52.8	52.6
Crude Lipid (%)	16.5	18.1	16.2	13.9
Ash (%)	7.1	9.9	10.5	9.4
Energy (MJ kg^{-1})	23.1	21.2	20.8	20.9

CTRL: commercial-like formulation, PLANT: plant protein, PAP: processed animal protein, MIX: mixture of processed animal and plant protein. * Underlined data from the CTRL, PAP and PLANT groups were previously published in Hoerterer et al. (2022)

2.2 Tissue collection and sample preparation

At the end of the experiment, three fish per tank (15 fish per diet) were sacrificed for tissue sampling. The fish were anaesthetized with 500 mg L^{-1} tricaine methanesulfonate (MS-222; Sigma Aldrich, Germany). After recording body weight (precision 0.01 g) and body length (precision 0.5 cm), fish were killed by separation of the gill artery and the liver and muscle tissue were rapidly sampled on ice, the liver was weighted (precision 0.001 g). The tissues were shock frozen in liquid nitrogen and stored at −80°C until further analysis. The hepato-somatic index (HSI) is the liver weight divided by the body weight of the sampled fish. The experimental diets were sampled from freshly opened bags and stored at −80°C until further analysis.

The sample preparation was adapted and performed according to Lannig et al. (2010). In short, feed, muscle and liver samples were grinded under liquid nitrogen and approx. 200-250 mg tissue was homogenized in 5x volume of ice-cold 0.6 M perchloric acid (w:v). After one cycle of 20 s at 6000 rpm and 3°C, using Precellys 24 (Bertin Technologies, France) samples were sonicated for 2 min at 0°C and 360 W (Branson Sonifier 450). Tissue homogenates were instantly divided for analysis of glycogen content and metabolite profile. Glycogen content was determined following the procedure described by Keppler and Decker (1988), photometrically after enzymatic hydrolysis of glycogen to glucose. Detailed steps and calculations, as well as the glycogen content in liver and muscle tissue of the fish from the CTRL, PLANT and PAP groups are described in Hoerterer et al. (2022b).

2.3 Untargeted ^{1}H-NMR based metabolic profiling

Homogenates of the experimental diets, muscle and liver tissues were centrifuged for 2 min at 0°C and 16,000g and supernatants were neutralized with ice cold KOH and PCA to pH 7.0-7.5. To remove precipitated potassium perchlorate samples were centrifuged again for 2 min at 0°C and 16,000g. The entire supernatant was transferred, shock-frozen in liquid nitrogen and stored an -80°C for later analysis. For ^{1}H-NMR spectroscopy analyses, samples were defrosted and dried in a rotational vacuum concentrator (RVC 2–18 HCl, Christ GmbH, Germany) at room temperature overnight. Afterwards samples were re-suspended 1:1 (w:v) in deuterated water (D2O) containing 0.05% of trymethylsilyl proprionate (TSP) (45010, Sigma Aldrich, USA) to a final concentration of 1 g mL^{-1} of the original frozen sample weight. TSP was used as a chemical shift and quantification standard. The re-suspended samples were centrifuged for 10 min at room temperature and 16,000g and for each sample 45 µL of the supernatant were transferred into NMR needles (1.7 mm capillary tube, FisherScientific, Country). One-dimensional ^{1}H-NMR spectra for feed and tissues extracts were acquired using a vertical 9.4 T wide bore magnet with Avance III HD (Bruker- GmbH, Germany) at 400.13 MHz with a 1.7 mm diameter triple tuned (^{1}H-^{13}C-^{15}N) probe (Georgoulis et al., 2022). The samples were measured using a Call-Purcell-Meiboom-Gill (Bruker protocol cpmgpr1d, TOPSPIN 3.5, Bruker GmbH, Germany) with water suppression at room temperature using the following parameters: Acquisition time (AQ) 4.01 s, sweep width (SW) 8802 Hz (22 ppm), delay (D1) 4 s, dummy scan (DS) 4, number of scans (ns) 128. Each spectrum was processed and analyzed with Chenomx NMR Suite 8.4 software (Chenomx Inc., Canada). Before analyzing, the spectra were corrected for phase, shim and baseline, and calibrated to TSP signal (at 0.0 ppm). The specific metabolites of the processed spectra were assigned using the internal database of Chenomx and literature data available for aquaculture fish (Casu et al., 2017, Wei et al., 2017b, Jarak et al., 2018, Roques et al., 2020a). The Chenomx software provided the concentration of the assigned metabolites, based on the concentration of the internal standard TSP (Schmidt et al., 2017). In total, signals of 25 compounds were annotated in the ^{1}H-NMR spectra of the experimental diets, 28 in liver and 32 in muscle extracts (see Supplementary materials Table S1).

2.4 Statistical analysis

The fish performance parameters for growth and feed utilization parameters as well as the condition factor were calculated as means of all fish per tank with five tanks per treatment. The sampled fish per treatment were considered as individual data points for the organ indices and the glycogen and glucose contents in the muscle and liver (N = 15). Metabolite concentrations in the liver and muscle tissue extracts of fish fed the experimental diets (CTRL, PLANT, PAP, and MIX) were analyzed using univariate and multivariate statistical analysis. The metabolite concentrations were transformed by applying a generalized log-transformation to stabilize the variance across the detected metabolite concentrations (Purohit et al., 2004). Unsupervised principle component analysis (PCA) and supervised partial least-squares discriminant analysis (PLS-DA) were applied using the Metaboanalyst web application (Metaboanalyst 5.0; Chong et al., 2019).

Fish performance parameters, individual nutritional parameters and the metabolites identified by the PLS-DA were analyzed using SigmaPlot (SigmaPlot 12.5, Systat Software Inc.). One-way ANOVA with post hoc Holm-Sidak method for all pairwise multiple comparison procedures was performed to detect and validate differences between the experimental groups (CTRL, PLANT, PAP and MIX). For differences between the different levels of FM replacement the groups were defined as CTRL = 0%, PLANT and PAP = 20% and MIX = 40%. The overall significance level was $P < 0.050$. Values are given as means ± standard deviation (SD).

3 Results

This study evaluated how three different feed formulations (PLANT, PAP and MIX) affected the metabolic profile of juvenile turbot fed for 16 weeks. To link the response in the metabolic profile to the growth and feed performance as well as the nutritional status of the fish, the performance of the MIX group (new data set) is presented in comparison to performance of CTRL, PLANT and PAP groups (data set previously published in Hoerterer et al., 2022)

3.1 Growth and feed performance and nutritional status of fish fed the different diets.

The growth and feed performance of the fish from the MIX group was not significantly affected by the diet and did not significantly differ from the fish of other experimental groups (see Table 3 and Hoerterer et al., 2022). Even though not significantly different, after 16 weeks, fish from the CTRL group had overall the best growth and feed conversion followed by the fish from the PLANT and PAP groups, and the fish from the MIX group having the lowest performance. The condition factors (CF) of fish from the MIX group were significantly lower compared to CFs of fish from the CTRL and the PAP group (One-Way ANOVA, P = 0.005), with no significant difference to CF of fish from the PLANT group. Additionally, at the level of FM replacement, fish from the MIX group (40%) had a significantly lower CF compared to the fish from the PLANT and PAP (20%) groups (One-way ANOVA, P = 0.025).

Table 3 Performance parameters of the juvenile turbot (*Scophthalmus maximus*) fed with different diets for 16 weeks.

	CTRL*	PLANT*	PAP*	MIX	P-value
Level of fishmeal replacement	**0%**	**20%**	**20%**	**40%**	
Initial body weight (g)	20.2 ± 0.3	20.4 ± 0.4	20.1 ± 0.5	20.3 ± 0.4	0.852
Final body weight (g)	85.2 ± 9.7	82.1 ± 9.5	82.9 ± 6.1	81.9 ± 7.3	0.914
SGR	1.28 ± 0.09	1.24 ± 0.09	1.26 ± 0.06	1.25 ± 0.07	0.852
FCR	0.87 ± 0.03	0.90 ± 0.02	0.92 ± 0.03	0.90 ± 0.04	0.109
CF	2.11 ± 0.01^{a}	2.07 ± 0.02^{ab}	2.10 ± 0.01^{a}	2.04 ± 0.04^{bc}	0.005

CTRL: commercial-like formulation, PLANT: plant protein, PAP: processed animal protein, MIX: mixture of processed animal and plant protein. SGR: specific growth rate, FCR: feed conversion ratio, CF: condition factor; *Underlined data from the CTRL, PAP and PLANT groups were previously published in Hoerterer et al. (2022). Values are shown as means ± SD (n = 5 tanks per diet), different letters (a, b, c) indicate significant differences between treatment groups detected one-way ANOVA and by Holm-Sidak method (P < 0.050).

The sampled fish (n = 15 per dietary treatment) had similar final body weight (see Table 4). Hepato-somatic indices (HSI) were significantly lower in fish from the MIX group (1.4 ± 0.2) than in fish from the CTRL group, with no significant differences to the PLANT and PAP groups (Hoerterer et al., 2022; One-Way ANOVA, P = 0.006).

The glycogen and glucose levels in the muscle of fish from the MIX group (1.6 ± 0.7 mg g^{-1}, 0.12 ± 0.04 mg g^{-1}, respectively) were not significantly different to the fish from the CTRL, PAP and PLANT groups (Hoerterer et al., 2022; Table 4). In contrast, the hepatic glycogen content in fish from the MIX group (41.6 ± 22.5 mg g^{-1}) was significantly lower than the fish from the CTRL group with no significant differences to the fish from the PAP and

PLANT groups (Hoerterer et al., 2022; One-way ANONA, P = 0.025). Even though not significant, the hepatic free glucose was highest in the fish from the MIX group, leading to a significantly two-fold higher hepatic glucose/glycogen ratio compared to the fish from the CTRL, PAP, and PLANT groups (One-way ANOVA, P = 0.009). Additionally, the level of FM replacement had significant effect on the HSI and hepatic glycogen, with significantly higher values in the fish from the CTRL group compared to the fish from the PLANT and PAP (20% FM replacement) and MIX (40% FM replacement) groups (One-Way ANOVA, post hoc Holm-Sidak method, P = 0.006, P = 0.002, respectively).

Table 4 Final body weight, organ indices and glycogen and glucose levels in wet tissue of muscle and liver of juvenile turbot (*Scophthalmus maximus*) fed with different diets for 16 weeks.

	CTRL*	PLANT*	PAP*	MIX	P-value
Level of fishmeal replacement	**0%**	**20%**	**20%**	**40%**	
Final body weight (g)	89.7 ± 27.2	91.5 ± 33.3	92.1 ± 28.8	87.9 ± 34.3	0.983
Hepato-somatic index (HSI)	1.8 ± 0.3^{a}	1.5 ± 0.3^{b}	1.5 ± 0.3^{b}	1.4 ± 0.2^{b}	0.006
Muscle glycogen (mg g^{-1})	1.7 ± 0.7	2.1 ± 0.7	1.8 ± 0.7	1.6 ± 0.7	0.298
Muscle glucose (mg g^{-1})	0.14 ± 0.05	0.12 ± 0.04	0.14 ± 0.04	0.12 ± 0.04	0.244
Muscle glucose/glycogen	0.12 ± 0.10	0.06 ± 0.03	0.09 ± 0.04	0.08 ± 0.04	0.114
Liver glycogen (mg g^{-1})	63.7 ± 23.9^{a}	46.4 ± 16.0^{ab}	48.0 ± 17.2^{ab}	41.6 ± 22.5^{b}	0.025
Liver glucose (x10^{-3} mg g^{-1})	2.9 ± 1.1	2.8 ± 0.6	2.6 ± 0.6	3.2 ± 1.1	0.284
Liver glucose/glycogen (x10^{-5})	5.7 ± 4.7^{b}	6.5 ± 1.9^{b}	6.0 ± 2.1^{b}	11.1 ± 7.8^{a}	0.009

CTRL: commercial-like formulation, PLANT: plant protein, PAP: processed animal protein MIX: mixture of processed animal and plant protein. *Underlined data from the CTRL, PAP and PLANT groups were previously published in Hoerterer et al. (2022). Values are shown as means ± SD (n = 15 fish per diet), different letters (a, b, c) indicate significant differences between treatment groups detected one-way ANOVA and by Holm-Sidak method (P < 0.050).

3.2 Patterns of compounds in feed

In the control and the three experimental diets 31 compounds were detected (see Table 5). Most compound concentrations did not differ between the diets but NMR spectroscopy nicely highlighted the supplementation of methionine and taurine showing approx. three- to ten-fold higher concentrations in the PLANT, PAP, and MIX diets compared to the CTRL diet. Even though betaine (in the form of betaine HCl) was supplemented to the diets, the betaine concentrations did not differ much between the diets. Creatine phosphate, lactate, N,N-dimethylglycine and O-phosphocholine had at least two fold higher concentrations in the experimental diets than in the CTRL diets. Only sarcosine concentrations were apparently two times higher in the CTRL diets compared to the experimental diets.

Table 5 Concentrations (mM) of compounds found in the aqueous extracts from the different diets.

	CTRL	PLANT	PAP	MIX
Level of fishmeal replacement	**0%**	**20%**	**20%**	**40%**
Acetate	23.9	27.9	24.4	22.0
Alanine	7.2	6.9	7.3	8.2
Betaine [suppl.]	20.1	20.4	20.9	24.2
Carnitine	1.3	1.3	1.3	2.3
Choline	10.5	11.8	11.2	11.1
Creatine	6.7	5.1	5.3	5.0
Creatine phosphate	1.2	3.8	5.7	2.7
Creatinine	8.7	8.0	8.1	7.4
Dimethylamine	10.0	18.1	17.1	11.4
Fumarate	0.1	0.1	0.0	0.1
Glucose-6-phosphate	4.4	4.6	3.9	3.3
Glutamate	6.6	8.3	7.0	9.2
Glycine	5.4	5.4	5.2	5.4
Isoleucine	1.3	3.3	1.3	1.8
Lactate	15.6	23.4	24.7	24.6
Leucine	6.5	6.6	7.1	6.8
Malonate	1.9	2.4	1.8	2.1
Methionine [suppl.]	1.4	15.1	12.7	14.9
N,N-Dimethylglycine	1.2	3.2	3.2	1.8
O-Phosphocholine	1.2	2.8	3.1	2.5
Sarcosine	4.1	2.1	2.0	1.7
Succinate	2.1	2.2	2.0	2.6
Taurine [suppl.]	11.8	38.4	35.1	30.5
Threonine	1.9	2.0	1.8	2.1
Valine	2.6	2.3	2.5	3.0

Control: commercial-like formulation, PAP: processed animal protein, PLANT: plant protein, MIX: mixture of processed animal and plant protein. Values are shown as means ± SD (n = 2); [suppl.] compounds were supplemented to the PLANT, PAP and MIX diets

3.3 ^{1}H-NMR based metabolic profile of the muscle and liver tissue

Univariate and multivariate statistical analysis were used to detect differences in the tissue metabolite concentrations between fish fed the different diets and the level of FM replacement (CTRL = 0%, PLANT and PAP = 20% and MIX = 40%). The composition of assigned metabolites in the muscle tissue did not differ between the experimental groups, except for varying compound concentrations. The principal component analysis (PCA) showed a heterogeneous group with no detected outliers. For the different dietary formulation groups the first principle component (PC1) explains 27.2% of the total variance (see Supplementary materials Figure S1).

The supervised partial least squares discriminant analysis (PLS-DA) score plot (Figure 1A) highlights the difference between the metabolic compounds found in the muscle samples. The fish from the CTRL group are distinctive from the fish from the other groups, whereas the fish from the MIX group over span the plots of the fish from the PLANT and PAP groups, showing that mixing the alternative feed ingredients could balance deficiencies in the plan-based and animal-based ingredients. The score plots PLS-DA of the liver tissue shows no separation between the dietary groups (Figure 1B).

3.5 Diet-dependent differences in metabolic profile in the muscle and liver tissue

The muscle extracts of fish from the CTRL group had a significantly higher concentration of betaine (One-way ANOVA; $P < 0.001$) than muscle tissue of fish from the other groups (PLANT, PAP and MIX; Figure 2A) with no significant differences related to the level of FM replacement (One-way-ANOVA, $P >= 0.050$). In contrast, the concentration of trimethylamine N-oxide (TMAO) in muscle tissue significantly decreased with the increasing level of FM replacement, with fish from the CTRL group having the highest, fish from the PLANT and PAP groups (20% FM replacement) intermediate and fish from the MIX group the lowest concentrations (One-way ANOVA; $P < 0.001$, Figure 2B). In liver tissue, betaine concentration was significantly lower in fish from the PAP group compared to the fish from the CTRL (One-way ANOVA, $P = 0.026$, Figure 2C). When analysed by the FM replacement, the betaine concentrations were significantly highest in fish fed the CTRL (1.1 ± 0.6 mM) compared to fish fed the MIX diet (40%; 0.66 ± 0.64) and fish fed with the PLANT and PAP (20%; 0.6 ± 0.5 mM) (One-way ANOVA, $P = 0.010$).

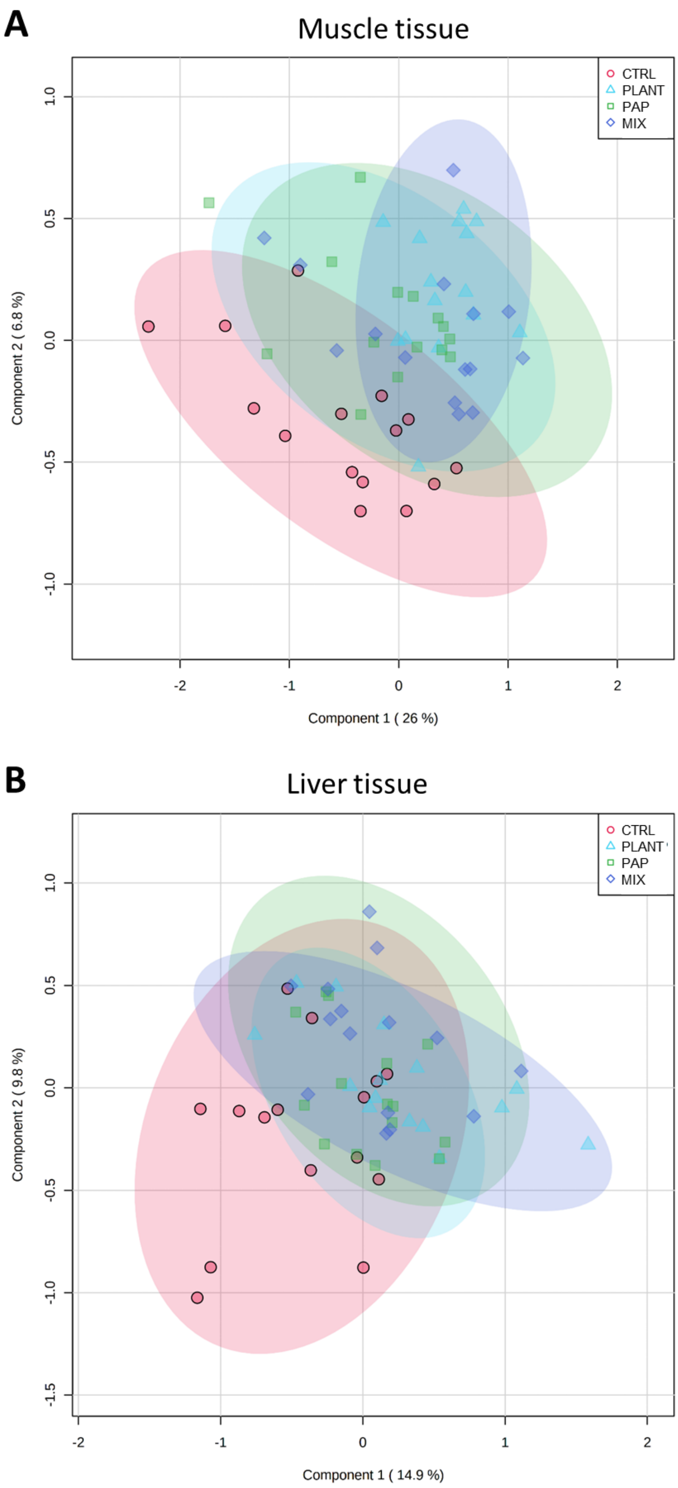


Figure 1 Score plots of the PLS-DA model for the concentrations of assigned metabolites determined in muscle (A) and liver (B) tissue of juvenile turbot (*Scophthalmus maximus*) fed with four experimental diets for 16 weeks. Ellipses correspond to a confidence interval of 95% for each group. CTRL: commercial-like formulation, PLANT: plant protein, PAP: processed animal protein, MIX: mixture of processed animal and plant protein.

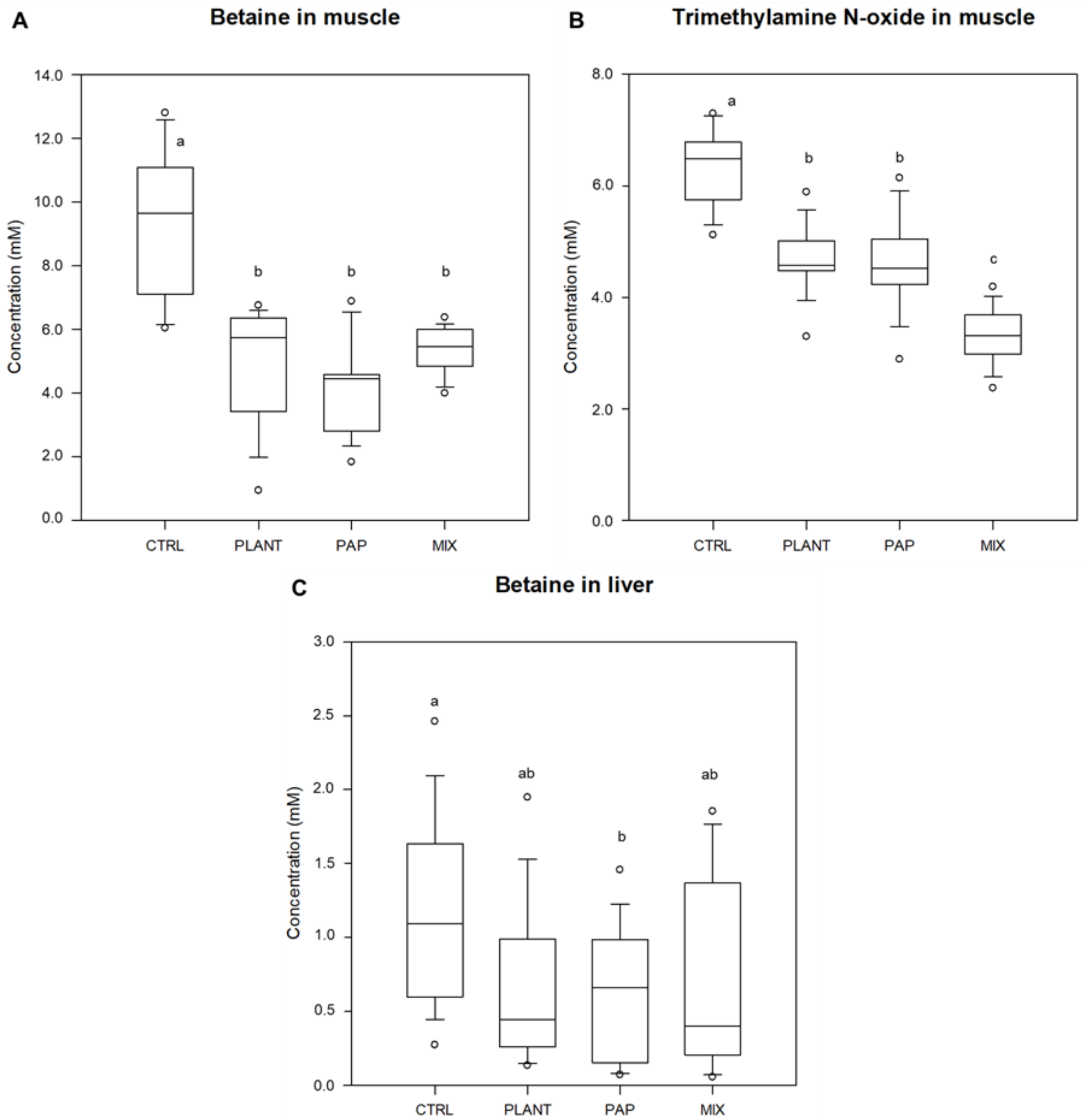


Figure 2 Concentrations of betaine (A) and trimethylamine N-oxide (B) in muscle and betaine (C) liver tissue of juvenile turbot (*Scophthalmus maximus*) fed with four experimental diets for 16 weeks (n = 15 fish per diet). Different letters (a, b, c) indicate significant differences between treatment groups detected by one-way ANOVA and Holm-Sidak method (P < 0.050). CTRL: commercial-like formulation, PLANT: plant protein, PAP: processed animal protein, MIX: mixture of processed animal and plant protein.

4 Discussion

In need of alternatives to traditional feed ingredients such as FM and soybean, feeds with ingredients from the circular economy offer the opportunity for sustainable development of European aquaculture. Turbot has a low tolerance to FM (FM) replacement, resulting in reduced growth and health when fed diets containing even low amounts of FM. The present study evaluated the metabolic response in liver and muscle tissue of juvenile turbot to three eco-efficient feed formulations at two levels of FM replacement.

4.1 Growth and feed performance

The feeding trial revealed that the three eco-efficient feed formulations with two FM replacement levels did not negatively affect growth and feed performance of juvenile turbot (see this study and Hoerterer et al., 2022). Only the condition factor showed a small decrease in fish fed the MIX formulation replacing 40% of the overall FM content with alternative feed ingredients. However, the observed decrease of 3.3% compared to CTRL fish is small and therefore the physiological relevance is minor. The present results suggest that fish-derived ingredients from by-products in combination with plant- and terrestrial animal-based ingredients allows a complete replacement of traditional feed ingredients and a reduction of 40% FM without compromising growth and feed performance. This is a progress compared to literature, where, decreased growth and feed performance were observed when more than 30-35% of FM was replaced with single ingredients such as land-based protein sources (Burel et al., 2000a, Kroeckel et al., 2012, Bonaldo et al., 2015, Fuchs et al., 2015, Dong et al., 2016, Hermann et al., 2016, Bian et al., 2017)

4.2 Metabolic response in the muscle

In the muscle tissue of the juvenile turbot, the formulations and the level of FM replacement negatively affected betaine and trimethylamine N-oxide (TMAO) concentrations. Betaine and TMAO are acting as osmolytes and are linked to the choline and methionine cycle. Osmolytes are often the main metabolite groups detected in the aqueous extracts of tissues from marine fish (Rebelein et al., 2018). In this study, betaine concentrations in the muscle tissue were not correlated to the level of FM replacement but were clearly reduced in the fish feed with the eco-efficient feed formulations. Betaine is known to be decreased in the muscle tissue of fasted rainbow trout (Kullgren et al., 2010) and red rum fed with soybean-based diets (Casu et al., 2017) and therefore might be a marker for energy deficiency. The supplementation of betaine HCl (5 g kg^{-1}) in the three eco-efficient feed formulations to act as a feed attractant seems not to have had any influence on the betaine concentrations in the muscle. Both betaine and choline, precursor of betaine, have similar levels in all experimental diets giving the same baseline to all groups. TMAO is also significantly lower in the muscle of the turbot fed with the eco-efficient feed formulations. In contrast to betaine, TMAO is correlated to the level of FM replacement. Similar to this study, TMAO was decreased in muscle tissue of red drum fed with soybean based diets (Casu et al., 2017). In contrast, TMAO was increased in the muscle of European Seabass fed with diets containing raw starch (Jarak et al., 2018). The role of betaine and especially TMAO as a marker for dietary manipulation needs to be further investigated since the response can strongly differ. Melis et al. (2017) suggested that TMAO might be a molecular marker for increased metabolic activity, in their study due to thermal stress. Therefore, the decreased betaine and TMAO concentrations in the muscle tissue of turbot could indicate reduced metabolic activity due to energy deficiency caused by the diets.

4.3 Dietary effects on energy storage, glucose metabolism and metabolic profile in liver

In contrast to unaffected growth and feed performance, the diet with a balanced mixture of processed animal protein, plant protein, cell meals, insect meal and microalgae (MIX) had a negative effect on the hepatic nutritional status of fish seen by decreased HSI and glycogen levels, most likely being correlation to the reduced FM content in the diet. These observations are in line with the findings for the other feed formulation groups (PLANT, PAP) reported in Hoerterer et al. (2022b). Both HSI and hepatic glycogen content are positively correlated in turbot (Liu et al., 2014a, Guerreiro et al., 2015a, Zeng et al., 2015, Miao et al., 2016b) as hepatic glycogen level serves as an energy storage in most fish species (Hemre et al., 2002). Compared to marine-based diets, plant-based diets seem to modulate glycolysis and gluconeogenesis in fish liver (Roques et al., 2020b). In this study, hepatic glucose content was not diet-dependent; however, the higher ratio of glucose to glycogen suggests that the glycogenolysis/glyconeogenesis was affected in the liver of the fish from the MIX group. A higher glucose/glycogen ratio could indicate that glucose was mobilized due to energy deficiency (Roques et al., 2020b). Decreased hepatic glycogen together with increased hepatic glucose content was observed in turbot when fed with plant-based diets (Wei et al., 2017a). Energy deficiency could be caused by decreased digestibility and availability of dietary nutrients such as protein (Hoerterer et al., 2022b) and presumably lipids in the eco-efficient feed formulations. This might have led to the mobilization of glucose as a source of energy (Kullgren et al., 2010) resulting in a decreased storage capacity of hepatic glycogen content accompanied by significantly lowered HSI in turbots fed the MIX diet compared to CTRL fish.

^{1}H-NMR-based metabolic profiling revealed reduced betaine concentrations in the liver of juvenile turbot fed with the PAP diet. As reviewed by Roques et al. (2022b) the choline cycle can be affected in fish fed diets with plant-based ingredients leading to altered choline, betaine, N, N-dimethylglycine and dimethylamine and O-phosphocholine concentrations. The choline cycle is linked to the lipid metabolism and alterations are an indicator for large differences in the lipid composition (Roques et al., 2020b) and unbalanced supply of other methyl donors such as methionine (Maruhenda Egea et al., 2015). The decrease of betaine could be linked to the significantly lower whole body lipid of the turbot fed with the PAP diet (Hoerterer et al., 2022). However, the difference is small and the results showed that the alternative formulations with various sources for lipids (CE-salmon oil, rapeseed and microalgae) and the supplemented methionine (3 g kg^{-1}) balanced possible deficiencies in the single ingredients used. Furthermore, there were no effects detected on metabolic compounds related to amino acid catabolism such as leucine and valine (Wagner et al., 2014, Wei et al., 2017b, Roques et al., 2020a), suggesting that the eco-efficient feed formulations are suitable to balance the amino acid profiles of the single ingredients with the usual amino acids supplemented to the diet.

4.4 Metabolites as markers of alteration of metabolism induced by eco-efficient feed formulations

In this study, the diet-dependent effects on the ^{1}H-NMR-based metabolic profile in the muscle and liver were small; however, differences between the diets were detected through univariate and multivariate analysis. The muscle tissue seems to be a better indicator for the effects of eco-efficient feed formulations on the metabolic profile than liver tissue in turbot. In PLS-DA, the CTRL group was separated from the eco-efficient feed formulations. Furthermore, it revealed that the MIX diet, being a mixture of the other two feed formulations, is located in between the PLANT and PAP circles. In addition, the level of FM replacement was also a driving pattern in the separation of the groups, highlighting the linear correlation between the FM replacement the performance of aquaculture. This study's experimental diets were designed to meet the species demand for all essential nutrients and since the growth and feed performance was not affected, we expected only small differences in the ^{1}H-NMR-based metabolic profile. The level of FM replacement or else inclusion level of the alternative feed ingredients were compared to other experimental study low. In their model, Hua and Bureau (2009) described depressed growth only at high dietary inclusion levels of plant-protein in fish diets, whereas low level showed no effect. Higher levels of FM replacement might lead to a clearer picture on the effects of the eco-efficient feed formulations on the metabolic profile of turbot as it was shown for inclusion levels of insect meal in rainbow trout (Roques et al., 2020a), soybean meal in red drum (Casu et al., 2019) or FM replacement in cobia (Schock et al., 2012).

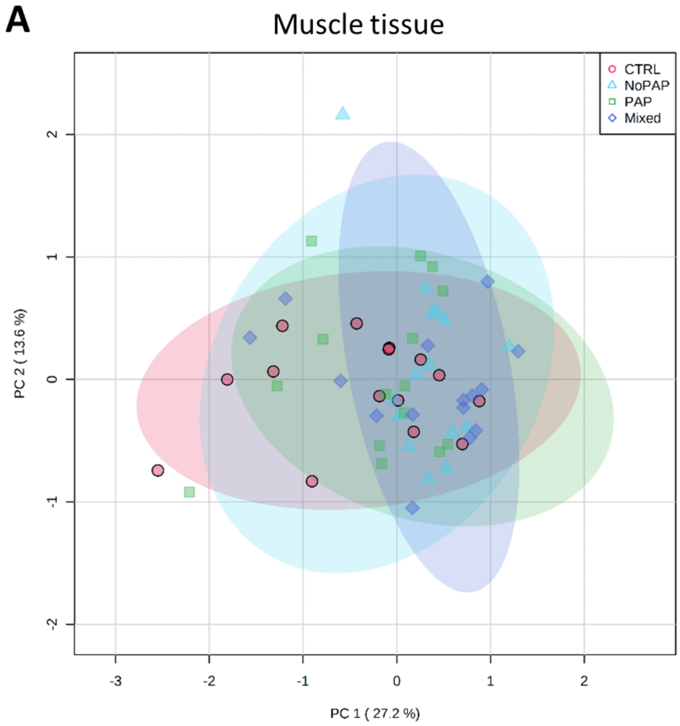
5 Conclusion

This study's results highlight that ^{1}H-NMR-based metabolic profiles are a suitable tool to detect early alterations in the metabolism of juvenile turbot fed with eco-efficient feed formulations. Research on alternative fish feed ingredients usually focuses on one single ingredient replacing the main protein source, mostly FM. Most studies showed that there is a point of inflection for the level of FM replaced, when growth and feed performance indicators are significantly affected. Often, physiological indicators, such as HSI, immunological parameters etc., show a significant change before this point. This is also the case in this study. These effects play an important role, when the feed formulations are applied to commercial aquaculture. With additional environmental stressors such as changing temperatures, water quality and pathogens, these effects might result in reduced growth and feed performance. Therefore, further investigation on the diet-dependent effects on the metabolome of other life stages of turbot is important. The grow-out phase from 100 g onwards is with approx. 18 months the longest and alterations in the physiology might have a magnified effect on the growth and feed performance leading to the economic success of the aquaculture farm.

6 Supplementary Materials

Table S1: Assigned compounds in the ^{1}H-NMR-spectrum muscle (M) and liver (L) tissue of juvenile turbot (*Scophthalmus maximus*) fed with the different diets (F).

Class	Compound	^{1}H chemical shift (ppm)	Extract
Organic acids	Acetate	1.9	F, M, L
	Adenine	8.2	M
	ADP	8.5	M
Amino acids, dipeptides	Alanine	1.5	F, M, L
	AMP	8.6	M
Amino acids, dipeptides	Anserine	6.8; 3.8	M
Amino acids, dipeptides	Arginine		M
Amino acids, dipeptides	Aspartate	2.8	M
	ATP	8.2; 4.4	M, L
Amines and N-containing compounds	Betaine	3.9; 3.3	F, M, L
Amino acids, dipeptides	Carnitine	3.2	F, M, L
Amines and N-containing compounds	Choline	3.2	F, M, L
Amines and N-containing compounds	Creatine	3.9; 3.0	F, M, L
Amines and N-containing compounds	Creatine phosphate	3.9; 3.0	F, M, L
Amines and N-containing compounds	Creatinine	4.0; 3.0	F, L
Amines and N-containing compounds	Dimethylamine	2.7	F, L
	Dimethyl sulfone	3.2	L
Organic acids	Formate	8.4	M, L
Organic acids	Fumarate	6.5	F, M, L
Sugars	Glucose-6-phosphate	5.2	F, M, L
Amino acids, dipeptides	Glutamate	2.3	F, M, L
Amino acids, dipeptides	Glycine	3.5	F, M, L
Amino acids, dipeptides	Isoleucine	1.0	F, M, L
Organic acids	Lactate	4.1; 1.3	F, M, L
Amino acids, dipeptides	Leucine	0.9	F, M, L
Organic acids	Malonate	3.1	F, M, L
Amino acids, dipeptides	Methionine	2.1	F, M, L
Amines and N-containing compounds	N,N-Dimethylglycine	2.9	F, M, L
Amines and N-containing compounds	O-Phosphocholine	3.2	F, L
Amino acids, dipeptides	Proline	2.0	M
Amino acids, dipeptides	Sarcosine	3.6; 2.7	F, M, L
Organic acids	Succinate	2.4	F, M, L
Amines and N-containing compounds	Taurine	3.4; 3.2	F, M, L
Amino acids, dipeptides	Threonine	1.3	F, M, L
Amines and N-containing compounds	Trimethylamine N-oxide	3.3	M
Amino acids, dipeptides	Valine	1.0	F, M, L



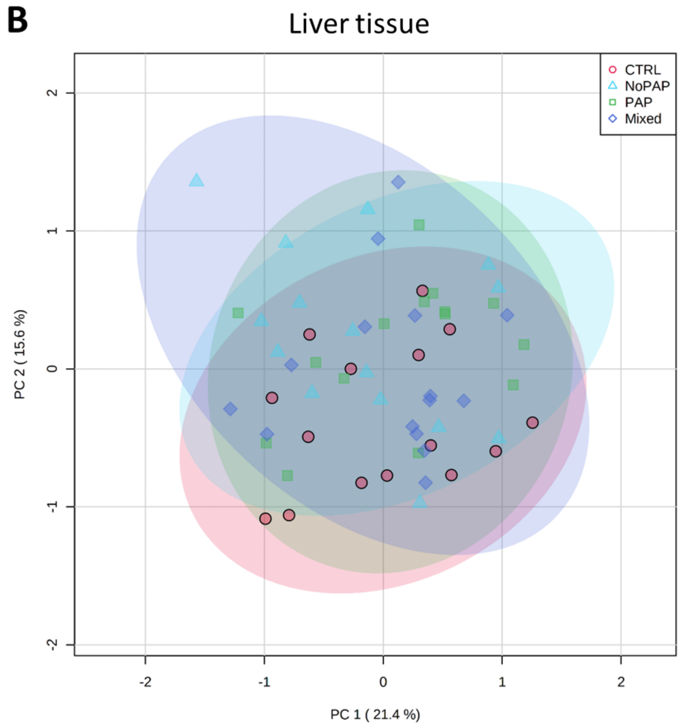


Figure S1 Principal component analysis (PCA) of the concentrations of assigned metabolites determined in muscle (A) and liver (B) of juvenile turbot (*Scophthalmus maximus*) fed with four experimental diets for 16 weeks. CTRL: commercial-like formulation, PLANT: plant protein, PAP: processed animal protein, MIX: mixture of processed animal and plant protein.

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"All authors have read and agreed to the published version of the manuscript."

Institutional ReviewBoard Statement

The experiments were conducted under the guidelines of and the animal study protocol was approved by the local authority 'Food surveillance, animal welfare and veterinary service (LMTVet)' of the state of Bremen (500–427-103–1/2019–1-19).

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Manuscript III

Effects of dietary plant and animal protein sources and replacement levels on growth and feed performance and nutritional status of market-sized turbot (*Scophthalmus maximus*) in RAS

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Abstract

One part of aquaculture sustainability is reducing the environmental footprint of aquaculture feeds. For European aquaculture, this means finding feed ingredients that are produced within the economic community, and that are not in conflict with human consumption. This is especially challenging when formulating diets for carnivorous fish such as turbot with low tolerance to fishmeal replacement that are both nutritious and economically and environmentally sustainable. Therefore, we investigated the effects of two novel and innovative feed formulation concepts on growth and feed performance and the nutritional status of market-sized turbot in a recirculating aquaculture system. In a 16-week feeding trial, 440 turbot (300 ± 9 g) were fed twice a day with a control diet (CTRL), based on a commercial formulation, and four experimental diets. The experimental diets were designed to investigate the effects of two formulations concepts based on sustainable terrestrial plant proteins (NoPAP) or processed animal proteins (PAP) and of 30% and 60% fishmeal replacement with emerging feed ingredients (fisheries by-products, insect meal and fermentation biomass). Turbot from the CTRL group had a similar growth and feed performance than fish fed the NoPAP30 formulation, with a significant decline of performance in the fish fed both PAP formulations and the NoPAP60. Comparing the two formulation concepts with each other the voluntary feed intake and protein efficiency ratio on tank basis as well as the individual weight gain and relative growth rate was significantly higher in the fish from the NoPAP groups than PAP groups. Furthermore, the apparent digestibility of nutrients and minerals was significantly reduced in the fish fed with the diets with 30% and 60% fishmeal replacement level compared to the fish from the CTRL group. In conclusion, the performance of the fish fed the NoPAP30 formulation concept highlights the potential of the used combination of sustainable ingredients, such as fisheries by-products, insect meal, microbial biomass and plant protein for turbot. Furthermore, this study shows that turbot has a higher tolerance to the incorporation of plant and insect protein than of processed animal protein.

Keywords: insect meal, grow-out phase, by-products, digestibility, mineral, consumer, sustainable, circular economy (CE)

1 Introduction

Anthropogenic activities, such as exploiting natural resources, building infrastructures and producing food have altered and shaped our planet and its ecosystems leading us into the new Anthropocene epoch (Lewis and Maslin, 2015). Within the Anthropocene, aquaculture may pose a risk as it can have a range of environmental impacts and its reliance on terrestrial agricultural products, fished marine protein and by competing with human food consumption for fish feeds (Troell et al., 2014, Keys et al., 2019). To conserve and sustainably use the valuable ecosystems and resources, the reduction of the environmental footprint of aquaculture feeds has become a high priority for the scientific community, producers and consumers (Glencross et al., 2020, Naylor et al., 2021). Furthermore, global crises have highlighted the importance of locally sourced raw materials and the reduction of the dependence on imports in many production sectors (Folke et al., 2021, Sarà et al., 2022).

For European aquaculture, this means to utilize sustainable feed ingredients that are produced within the economic community, such as plant protein from lupines and peas, and that are used for human consumption, such as insect meal and by-products from food processing of marine and terrestrial animals. Research efforts focused on the identification of major nutritional requirements for important farmed fish such as trout, salmon, sea bass or seabream (FAO, 2020, Naylor et al., 2021), resulting in diet formulations with reduced fish content that improve growth and feed performance (Olsen and Hasan, 2012, Hua et al., 2019). However, in many carnivorous farmed fish, a total replacement of fish products in the diets is still not feasible. The needed fish protein can be sourced from side-streams of the aquaculture production sector (Vazquez et al., 2020, Malcorps et al., 2021), which are a good alternative for sustainable aquafeeds (Forster et al., 2005, Whiteman and Gatlin, 2005, Bendiksen et al., 2011, Hua et al., 2019). Processed raw materials such as fish protein hydrolysates are more energy efficient than fishmeal from by-products and were shown to improve growth and feed performance in farmed fish (Siddik et al., 2020). Other alternative feed ingredient sources are processed animal proteins from poultry and pork by-products of the human food industry. The availability in large amounts in the EU and elsewhere as a byproduct from food production and its nutritional value qualifies processed animal proteins (PAPs) as a sustainable feed ingredient for fish (Tacon et al., 2011, Lu et al., 2015, Wang et al., 2015, Campos et al., 2017, Wu et al., 2018, Karapanagiotidis et al., 2019). Insect meal derived from the black soldier fly (*Hermetia illucens*) or mealworm (*Tenebrio molitor*) is also a promising feed ingredient for carnivorous fish such as Atlantic salmon (*Salmo salar*) (Li et al., 2020), rainbow trout (*Oncorhynchus mykiss*) (Stadtlander et al., 2017, Jozefiak et al., 2019, Rema et al., 2019). Insect larvae can valorize unused plant material, not suitable for human consumption, and transform it into valuable nutrients (Newton et al., 2005, van Huis, 2013). Microbial biomass, which is produced as a by-product from food, beer and biogas production, can be a valuable ingredient in aquafeeds (Oliva-Teles and Goncalves, 2001, Aas et al., 2006, Bendiksen et al., 2011, Tacon et al., 2011, Olsen and Hasan, 2012, San Martin et al., 2020). Life-cycle assessment revealed that insect meal and poultry by-product meal are the most sustainable alternatives for partial fishmeal replacement (Maiolo et al., 2020).

The European fish consumers prefer high value carnivorous species such as salmon, trout, sea bass, and turbot (Petereit et al., 2022b). Our approach within the GAIN2020 project (EU H2020 grant no. 773330) was to develop alternative feed formulation concepts for a range of European aquaculture species such as salmon, trout (Maiolo et al., 2021), sea bream (Naya-Català et al., 2021), sea bass (Petereit et al., 2022a) and turbot (Hoerterer et al., 2022b) based on various proteins sourced through circular economy tapping by-products and side-streams from food production sectors.

The life cycle of a fish in aquaculture includes many different stages with different needs for nutrition. Focusing on the life stages that are fed with compound diets, the protein and energy requirements of carnivorous fish generally decrease with increasing size (Kousoulaki et al., 2015). Most feeding studies concentrate on the juvenile stage of fish, since they respond quicker to nutritional changes due to their fast growth, making studies more cost efficient since statistically relevant growth differences appear in a shorter time span (Charles Bai et al., 2022). However, it is also important to investigate the effects of alternative feed ingredients and formulation on fish in the grow-out phase. The grow-out phase is for many larger marketed fish such as salmon and turbot, the longest phase accounting of 1-2 years (FAO, 2022b, FAO, 2022a). Turbot is usually transferred at 4-6 month of age and with a body weight of approximately 100 g into land-based flow-through or recirculation aquaculture systems (RAS) to start the grow-out phase (EUMOFA, 2022a, FAO, 2022b). Feeding costs account for 50%-60% of the overall production costs in carnivorous fish species making feed quality an important issue for a successful farming operation in European aquaculture production (Davis and Hardy, 2022). Here, alternative feed ingredients, which are often cheaper, offer the potential of reducing feed costs. However, compared to traditional feed ingredients such as fishmeal and soybean products, they differ often in quality, nutritional composition, digestibility of nutrients and availability of minerals (Glencross, 2016, Hua et al., 2019). This may affect growth, nutrient utilization and whole body composition (Bonaldo et al., 2015, Aragao et al., 2020) of carnivorous fish and lead to an altered energy metabolism and energy allocation (Palma et al., 2020).

In this study, we used a variety of key performance indicators for growth, feed utilization and nutritional status to find an alternative formulation for marked-sized turbot to the currently used commercial formulation, which has a similar performance and is economically attractive. In a bi-directional approach, we investigated (1) the effects of two novel and innovative feed formulation concepts based on sustainable feed ingredients such as plant protein, krill meal, insect meal and microbial biomass without (NoPAP) and with the inclusion of processed animal protein (PAP) and (2) how much fishmeal could be replaced within these two formulation concepts (replacement levels 30% and 60%).

2 Material and Methods

2.1 Experimental diets

Based on the nutrient requirements of marked sized turbot, five experimental diets were formulated to be isonitrogenous (crude protein. ~520 g/kg as fed), isolipidic (crude lipid: ~160 g/kg as fed) and isoenergetic (gross energy: 20 MJ/kg as fed) and manufactured as 6 mm floating pellets by extrusion at SPAROS LDA (Olhão, Portugal). The control diet (CTRL) was formulated to mimic a current standard commercial diet used for turbot with 40% fishmeal combined with 42% plant ingredients. One aspect of the feeding trial was to replace 30% and 60% of the fishmeal content in the novel formulation concepts. Additionally, the remaining fish-derived protein sources were fully replaced by fishmeal and fish protein hydrolysate (4% FHP) from fisheries by-products. The difference in fishmeal content was replaced by a combination of krill meal, insect meal and fermentation biomass (21% and 31% respectively). One formulation concept contained 30% plant protein concentrates (PPC) (NoPAP) and the second formulation concept was based on 12% PPCs and on 21% processed animal proteins (PAP). Furthermore, 50% of traditional fish oil in the control diet was replaced by salmon oil from by-products and algae oil. Moreover, in the novel formulation concepts the selenium and iodine from the vitamin and mineral premix was replaced by enriched macro and microalgae to increase the bioavailability of selenium and iodine for turbot. The formulation and chemical composition of the experimental diets is shown in Table 1 and Table 2. Before and during the trials the feed was stored at 4°C to ensure continuous quality of the diets throughout the feeding experiment.

Table 1 Formulation of the five experimental diets (Manufacturer of feed ingredients) for market-sized turbot

	Experimental diets				
	CTRL	NoPAP30	PAP30	NoPAP60	PAP60
Ingredients (%)					
Fishmeal LT70[1]	40.00	0.00	0.00	0.00	0.00
Fishmeal 60 (by-products)[2]	0.00	28.00	28.00	16.00	16.00
Fish protein hydrolysate[1]	4.00	0.00	0.00	0.00	0.00
Fish protein hydrolysate (by-products)[3]	0.00	4.00	4.00	4.00	4.00
Krill meal Qrill AQUA[4]	0.00	3.50	3.50	3.50	3.50
Porcine blood meal[5]	0.00	0.00	6.00	0.00	6.00
Poultry meal[6]	0.00	0.00	15.00	0.00	15.00
Insect meal[7]	0.00	8.75	8.75	13.70	13.70
Fermentation biomass[8]	0.00	8.75	8.75	13.70	13.70
Soy protein concentrate[9]	10.00	7.50	0.00	7.50	0.00
Wheat gluten[10]	14.20	10.65	0.00	10.65	0.00
Wheat meal[11]	17.83	11.65	12.17	12.18	12.65
Fish oil[1]	7.00	3.50	3.50	3.50	3.50
Salmon oil[12]	0.00	3.80	3.80	5.60	5.50
Algae oil[13]	0.00	1.00	1.00	1.20	1.20
Rapeseed oil[14]	4.50	2.70	1.40	1.20	0.00
Vitamin & Mineral Premix[15]	1.00	0.00	0.00	0.00	0.00
Vitamin & Mineral[15] *without Se & I	0.00	1.00	1.00	1.00	1.00
Vitamin E50[16]	0.10	0.10	0.10	0.10	0.10
Betaine HCl[17]	0.05	0.05	0.05	0.05	0.05
Macroalgae[18]	0.00	2.00	2.00	2.00	2.00
Macroalgae Se-rich[18]	0.00	0.05	0.05	0.05	0.05
Microalgae Se-rich[19]	0.00	0.30	0.30	0.30	0.30
Antioxidant[20]	0.20	0.20	0.20	0.20	0.20
Sodium propionate[21]	0.10	0.10	0.10	0.10	0.10
Monoammonium phosphate[22]	1.00	1.50	0.30	2.30	1.20
L-Lysine HCl 99%[23]	0.00	0.80	0.00	0.95	0.10
L-Taurine[17]	0.00	0.08	0.01	0.20	0.13
Yttrium oxide[24]	0.02	0.02	0.02	0.02	0.02

CTRL: commercial-like formulation, formulation concepts (FC): NoPAP: plant-based protein, PAP: processed animal protein, level of fishmeal replacement (FR): 30/60

[1] Sopropêche SA, France; [2] Conserveros Reunidos SA, Spain; [3] Blue whiting (Micromesistius poutassou) CP 49.9%, CF 1.0%, GAIN - Instituto de Investigaciones Marinas - CSIC, Spain; [4] AKER Biomarine AS, Norway; [5] SONAC BV, The Netherlands; [6] SAVINOR UTS, Portugal; [7] Black soldier fly (Hermetia illucens) CP 57.8 %, CF 8.5 % (Supplier not disclosed); [8] Methylococcus capsulatus CP 68.2%, CF 9.8% (supplier not disclosed); [9] ADM WILD BV, The Netherlands; [10] Roquette Frères, France; [11] Casa Lanchinha Lda, Portugal; [12] CF 98.3%, 4.6% EPA; 5.2% DHA, Sopropêche SA, France; [13] Alltech, Ireland; [14] Henry Lamotte Oils GmbH, Germany; [15] Vitamins (IU or mg kg^{-1} diet): DL-alpha tocopherol acetate, 255 mg; sodium menadione bisulphate, 10 mg; retinyl acetate, 26000 IU; DL-cholecalciferol, 2500 IU; thiamine, 2 mg; riboflavin, 9 mg; pyridoxine, 5 mg; cyanocobalamin, 0.5 mg; nicotinic acid, 25 mg; folic acid, 4 mg; L-ascorbic acid monophosphate, 80 mg; inositol, 17.5 mg; biotin, 0.2 mg; calcium panthotenate, 60 mg; choline chloride, 1960 mg. Minerals (g or mg kg^{-1} diet): copper sulphate, 8.25 mg; ferric sulphate, 68 mg; manganese oxide, 35 mg; zinc sulphate, 123 mg; calcium carbonate, 1.5 g; potassium iodide*, 0.7 mg; organic selenium*, 0.01 mg; excipient wheat middlings; [16] DSM Nutritional Products Ltd, Switzerland; [17] ORFFA Additives BV, The Netherlands; [18] GAIN - Salten Havbrukspark AS, Norway; [19] GAIN - Wageningen University & Research, The Netherlands; [20] Kemin Europe NV, Belgium; [21] Disproquímica, Portugal; [22] Aliphos BV, The Netherlands; [23] Indukern SA, Portugal; [24] Sigma Aldrich, USA

Table 2 Chemical composition of the five experimental diets for market-sized turbot as fed

	Experimental diets				
	CTRL	NoPAP30	PAP30	NoPAP60	PAP60
Proximate composition					
Moisture (%)	5	5.5	5.7	7.1	6
Crude Protein (%)	52.9	51.6	51.2	50.5	51.7
Crude Lipid (%)	17.2	15.9	15.9	16.2	16.2
Ash (%)	8.8	9.3	9.9	7.5	8.5
Energy (MJ/kg)	19.9	19.7	19.6	19.1	19.7
Mineral composition					
Calcium (Ca, g/kg)	15.1	20.9	23.2	13.5	18
Copper (Cu, mg/kg)	12.8	18.7	18.1	18.5	18.1
Iron (Fe, mg/kg)	120.9	192.7	281.1	254.6	364.8
Potassium (K, g/kg)	8.4	6	6.5	5.3	6.2
Manganese (Mn, mg/kg)	19.5	28.3	20.3	22.3	23.4
Sodium (Na, g/kg)	9	6.6	6.3	5.7	5.4
Phosphorus (P, g/kg)	13.9	15.7	15.7	14.4	15
Selenium (Se, mg/kg)	2.2	3	3.3	3	2.3
Zinc (Zn, mg/kg)	61.2	72.7	74.9	64.5	72

CTRL: commercial-like formulation, formulation concepts (FC): NoPAP: plant-based protein, PAP: processed animal protein, level of fishmeal replacement (FR): 30/60

2.2 Experimental setup

Turbot (*Scophthalmus maximus*) were purchased from France Turbot (L'Épine, France), transferred in specified transport containers overland to the Centre for Aquaculture Research (ZAF) at the Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research (AWI, Bremerhaven, Germany). The fish were acclimated to the recirculating aquaculture system (RAS) for two weeks prior to starting the 16 week (112 days) experimental trial. A total of 440 turbots with a mean weight (± SD) of 300.0 ± 8.5 g and total length of 25.3 ± 0.1 cm were randomly distributed into 20 tanks. The five experimental treatments were randomly distributed between the 20 tanks with four replicate tanks per treatment to counteract any possible tank effects. For more precise growth parameters 330 fish (300.0 ± 47.0 g and 25.3 ± 1.0 cm) were individually tagged with pit tags (7x1.35 mm, ISO 11784, Loligo Systems AS, Denmark). The RAS consisted of 36 tanks each containing 700 L with a bottom area of 1 m² each. The temperature (17.5 ± 0.1°C), pH (7.6 ± 0.1), conductivity (53.4 ± 1.8 mS/cm), and oxygen saturation (97.3 ± 5.3%) of the process water was monitored constantly (N = 112 days, SC 1000 Multiparameter Universal Controller, Hach Lange GmbH, Germany). The ammonium (0.12 ± 0.05 mg/L), nitrite (0.4 ± 0.3 mg/L), and nitrate (193.6 ± 34.5 mg/L) concentration was measured with an automated analyzer (QuAAtro39 AutoAnalyzer, SEAL Analytical, Germany) twice a week (N = 28). The fish were fed in the morning (9 am) a weight adapted portion (1% of estimated tank biomass) and in the afternoon (2 pm) ad libitum. In the afternoon, 30 min after the feeding the remaining pellets were netted (mesh size 1 mm) from the tanks and counted.

2.3 Measurements and sampling

Fish were weighed to 0.2 g precision and measured in length to 0.5 cm precision and tags were read to track individual growth every four weeks. At the end of the 16-week trial, five fish from each of the 20 tanks were sampled for tissue sampling to determine the energy reserves and three fish per tank were sampled as whole fish for proximate and mineral analysis of the carcass. Fish were anesthetized with 500 mg/L tricaine methane sulphonate (MS-222, Sigma Aldrich, Germany) prior to exsanguination. After taking the weight

(precision 0.01 g) and length (precision 0.5 cm) fish were killed and tissues (liver and filet without skin) were rapidly sampled on ice. The liver of 20 fish per treatment was weighed with 0.0001 g precision to determine the hepato-somatic index (HSI). Both tissues were shock frozen in liquid nitrogen and stored at -80°C until further analysis. For digestibility analysis, the feces were sampled by stripping anesthetized fish and pooled from all fish in one tank, centrifuged at 4°C and 3,000g for 5 min, and the pellets were frozen at -80°C until further analysis. To gain sufficient tissue weight the fish carcasses were pooled (from the three fish taken per tank), and cut into small pieces and stored at -20°C until further analysis.

For analysis of the marketable products of turbot three fish per tank (n = 12 per diet and N = 60 in total) were killed with a blow to the head and separation of the gill artery. The fish were weighed to 0.1 g precision and measured in length to 0.5 cm precision, visceral and filet removed and weighted to 0.1 g precision to determine the percentage of marketable gutted whole fish and filet yield.

2.4 Chemical analysis of diets, whole body and feces

The chemical analysis of the diets was conducted in duplicates (n=10), while carcass and fecal samples were pooled on tank level with four replicate tanks per experimental diet. The carcass samples were minced frozen using a commercial meat grinder homogeneously and refrozen The samples of the experimental diets homogenized in a knife grinder (5000 rpm, 30 s, Grindomix GM 200, Retsch, Germany).

The dry matter, ash, crude protein and crude lipid of the experimental diets and fish carcass were determined after AOAC (1980). Dry matter content of the diets and carcass was determined by drying the samples at 105°C for 24 h. Total ash content was determined by combustion of the samples in a muffle oven at 550°C for 6 h. The total nitrogen in the feed and carcass was determined following the automated Kjeldahl Method. Due to small sample volume in the feces samples, the total nitrogen in the freeze-dried feces was determined after the Dumas Method. For all samples, the measured total nitrogen was converted to equivalent crude protein (%) by the numerical factor of 6.25. Crude lipid was determined by acid hydrolysis. Gross energy was measured in an adiabatic bomb calorimeter (Model 6100; Parr Instrument, Germany).

For the analysis of the mineral content, 0.2 g of freeze-dried and homogenized samples of the experimental diets, carcass and feces were digested in 3 ml nitric acid HNO_3 (65%, trace grade) in a microwave oven (CEM MARS5, Kamp-Lintfort, Germany) according to DIN EN 13805 (2014). After digestion, the samples were diluted with milli-q water to 50 mL. Calcium, potassium, magnesium, phosphorus, arsenic, copper, iron, manganese, selenium, yttrium and zinc concentrations were analyzed in an ICP-OES (iCAP7400, Fisher Scientific, Schwerte, Germany). As reference fish muscle (ERM – BB422, EU) was used.

2.5 Glycogen of liver and muscle tissue

Following the procedure described by Keppler and Decker (1988) glycogen content was determined photometrically after enzymatic hydrolysis of glycogen to glucose. Filet and liver samples of 5 individual fish per tank (20 fish per treatment) were grinded under liquid nitrogen and approx. 200 mg tissue was homogenized in 5x volume of ice-cold 0.6 M perchloric acid (PCA) (w:v). After one cycle of 20 s at 6000 rpm and 3°C using Precellys 24 (Bertin Technologies, France) samples were sonicated for 2 min at 0°C and 360 W (Branson Sonifier 450) and homogenates were immediately divided for the analysis of total and free glucose concentrations.

2.6 Data analysis

The growth parameters were based on initial body weight (BW, g), final BW and final body length (BL, cm) and calculated as follows.

(1) $Weight\ gain\ (WG, g) = final\ BW - initial\ BW$

(2) $Specific\ growth\ rate\ (SGR) = 100 \times \frac{\ln(final\ BW) - \ln(initial\ BW)}{feeding\ days}$

(3) $Condition\ factor\ (CF) = 100 \times \frac{final\ BW}{final\ BL^3}$

(4) $Hepato - somatic\ index\ (HSI) = 100 \times \frac{liver\ weight\ (g)}{final\ BW}$

The feed performance parameters, Voluntary feed intake (VFI) and feed conversion ratio (FCR) were based on the total feed intake (FI) in g of the offered amount of feed and the uneaten feed.

(5) $Total\ Feed\ Intake\ (FI, g) = Feed_{offered} - Feed_{uneaten}$

(6) $Voluntary\ feed\ intake\ (VFI, \%\ average\ body\ weight\ (ABW)/d) = 100 \times FI / \left(\frac{initial\ BW + final\ BW}{2} / feeding\ days\right)$

(7) $Feed\ conversion\ ratio\ (FCR) = \frac{FI}{WG}$

(8) $Protein\ efficiency\ ratio\ (PER) = \frac{WG}{crude\ protein\ intake\ (g)}$

(9) $Whole\ fish\ gutted\ (\%) = 100 \times \frac{BW - viscera\ weight\ (g)}{BW}$

(10) $Filet\ yield\ (\%) = 100 \times \frac{filet\ weight\ (g)}{BW}$

The apparent digestibility (ADC) of the dietary nutrients and minerals were based on the amount of the inert yttrium marker in the diet and feces and the respective nutrient or element in feces and diets.

(11) $ADC\ dry\ matter\ (\%) = 100 - \left(100 \times \frac{yttrium_{diet}}{yttrium_{feces}}\right)$

(12) $ADC\ protein\ (\%) = 100 - \left(100 \times \frac{yttrium_{diet}}{yttrium_{feces}} \times \frac{protein_{feces}}{protein_{diet}}\right)$

(13) $ADC\ mineral\ (\%) = 100 - \left(100 \times \frac{yttrium_{diet}}{yttrium_{feces}} \times \frac{mineral_{feces}}{mineral_{diet}}\right)$

The glycogen content was calculated based on the concentration of total glucose subtracted by the concentration of free glucose. The difference between Absorptions ΔA (A2 – A1), the total measurement volume of the assay $V_{assay\ total}$ (mL), the coefficient of extinction at 339 nm (Ɛ = 6.3 mL/µmol cm), the thickness of layer d = 1 cm for the cuvette, the sample volume V_{sample} (mL), the dilution factor (DF) and the concentration of tissue wet weight in crude extract c_{tissue} (mg/mL).

(14) $Total\ glucose/free\ glucose\ concentration\ (c_{total\ glucose/free\ glucose}, \mu mol/mg) = \frac{\Delta A \times V_{assay\ total}}{Ɛ \times d \times V_{sample}} / c_{tissue} \times DF$

The glucose concentration was converted to glycogen content using the molecular weight of the glucosyl moiety in glycogen with Mr = 162 g/mol.

(15) $$Glycogen\ content\ (mg/g\ wet\ weight\ tissue) = (c_{total\ glucose} - c_{free\ glucose}) \times 162$$

Statistical analysis was conducted with Sigma Plot (12.5, Systat Software, Germany). One-way Analysis of Variance (ANOVA) was used to determine significant differences between the five treatments. Two-way ANOVA was used to determine significant differences and interaction between the novel formulation concepts (NoPAP vs. PAP; FC) and level of fishmeal replacement (30% vs. 60%; FR). Whenever there were statistically significant differences an All Pairwise Multiple Comparison Procedure was performed using the Holm-Sidak method (overall significance level P = 0.05) to find the difference within the treatments. Values are given as means ± standard deviations (SD).

3 Results

We present results relevant to the commercial culture on a tank basis (Table 3). The fish from the CTRL group had a significantly higher biomass gain (One-way ANOVA; P = 0.041) and specific growth rate (SGR) (One-way ANOVA; P = 0.010) than in the fish from the NoPAP60, PAP30 and PAP60 groups, with no significant difference to the fish from NoPAP30 group. The feed conversion ratio (FCR) was significantly (One-way ANOVA; P = 0.008) increased in the fish from the NoPAP60, PAP 30 and PAP60 groups compared to the fish from the CTRL groups), with no difference to the fish from the NoPAP30 group. The protein efficiency ratio (PER) was highest in the fish from the CTRL group and significantly decreased (One-way ANOVA, P = 0.001) in the fish from the NoPAP60, PAP30 and PAP60 groups, with no significant difference to the fish from the NoPAP30 group. The voluntary feed intake (VFI) with an average value of showed no significant differences between all experimental groups, with a slightly higher value in the fish from the PAP60 group. Fish fed with the PAP formulation concept (FC) had a significantly increased VFI (Two-way ANOVA 'FC'; P = 0.028) and decreased PER compared (Two-way ANOVA 'FC'; P = 0.017) to fish fed the NoPAP formulations, with no effect by replacement level (FR) and no interaction between formulation concept and replacement level (Two-way ANOVA 'FC x FR').

3.2 Individual growth performance

Due to the higher number of total replicates (N = 330), the analysis of the growth of the individually tagged fish revealed more comprehensive effects of the dietary formulations (Table 4). The fish from the CTRL group had a significantly (One-way ANOVA; $P = 0.001$) higher final BW than the fish from the PAP30, NoPAP60 and PAP60 groups, with no significant differences to the NoPAP30 group. The condition factor (CF) significantly increased (N = 330 fish; t-test; $p < 0.001$) from initially 1.8 ± 0.2 to finally 2.0 ± 0.2 in all groups. However, only the CF from the fish in the PAP30 group was significantly higher than in the fish from the PAP60 group (One-way ANOVA; $P = 0.012$). The weight gain (WG) and specific growth rate (SGR) in the fish from the CTRL group was significantly (One-way ANOVA; $P < 0.001$) higher than in the fish from the NoPAP60 PAP30 and PAP60 groups, with no significant differences to the NoPAP30 group. Furthermore, the fish from the NoPAP30 group had a significantly higher WG and RGR than the fish from the PAP60 group, with no significant differences to the PAP30 and NoPAP60 group.

Considering the replacement levels, the CF, WG and SGR of the fish from 30% groups were significantly higher than in the 60% groups (Two-way ANOVA ‘FR’, $P = 0.001$, $P = 0.050$, $P = 0.033$, respectively). Furthermore, the WG and SGR of the fish fed with the NoPAP formulation were both significantly higher compared to the fish from the PAP groups (Two-way ANOVA ‘FC’, $P = 0.038$, $P = 0.048$). However, no interaction was found between the formulation concept and replacement level (Two-way ANOVA ‘FC x FR’).

Table 3 Performance parameters of turbot fed five experimental diets (n=4)

	Experimental diets					Two-Way ANOVA		P-value
	CTRL	NoPAP30	PAP30	NoPAP60	PAP60	FC	FR	FC x FR
Initial BW (g)	303.2 ± 10.4	301.5 ± 3.7	298.4 ± 13.9	298.9 ± 7.9	299.7 ± 7.3	0.796	0.888	0.664
Final BW (g)	511.2 ± 38.0	485.0 ± 21.6	458.2 ± 31.6*	458.8 ± 34.9*	446.2 ± 11.4*	0.163	0.175	0.601
Biomass gain (g)	4057.9 ± 695.0	3458.0 ± 136.5	2956.9 ± 581.4*	3051.1 ± 679.1*	2989.5 ± 205.4**	0.248	0.435	0.362
SGR (%/day)	0.47 ± 0.05	0.44 ± 0.05	0.37 ± 0.08*	0.38 ± 0.10*	0.36 ± 0.03**	0.091	0.082	0.664
VFI (% ABW/day)	0.61 ± 0.04	0.62 ± 0.01	0.63 ± 0.02	0.62 ± 0.03	0.67 ± 0.03	0.028	0.128	0.116
FCR	1.3 ± 0.1	1.5 ± 0.1	1.7 ± 0.2*	1.7 ± 0.3*	1.8 ± 0.0**	0.072	0.185	0.481
PER	1.5 ± 0.1	1.3 ± 0.0	1.1 ± 0.1**	1.2 ± 0.2**	1.1 ± 0.0**	0.017	0.072	0.282

CTRL: commercial-like formulation, formulation concepts (FC): NoPAP: plant-based protein, PAP: processed animal protein, level of fishmeal replacement (FR): 30/60; BW: body weight; SGR: specific growth rate; VFI: Voluntary feed intake; FCR: feed conversion ratio; PER: protein efficiency ratio. Values are expressed as means ± SD (n = 4), significant differences in One-way ANOVA and Multiple comparisons versus CTRL group with the Holm-Sidak method are indicated by asterisks (*$P < 0.050$; **$P < 0.010$), significant differences at $P < 0.050$ in Two-way ANOVA

Table 4 Growth performance parameters of individually tagged turbot (N = 330) fed five experimental diets

	Experimental diets					Two-Way ANOVA P-value		
	CTRL (n = 67)	NoPAP30 (n = 66)	PAP30 (n = 65)	NoPAP60 (n = 66)	PAP60 (n = 66)	FC	FR	FC x FR
Initial BW (g)	302.0 ± 44.4	300.6 ± 45.2	301.4 ± 46.6	299.6 ± 47.0	296.4 ± 52.6	0.840	0.611	0.732
Final BW (g)	511.9 ± 103.9^{a}	484.5 ± 95.3^{ab}	459.8 ± 87.1^{b}	459.0 ± 102.6^{b}	445.7 ± 105.3^{b}	0.117	0.102	0.635
Final CF	2.0 ± 0.2^{ab}	2.0 ± 0.2^{ab}	2.0 ± 0.2^{a}	2.0 ± 0.2^{ab}	1.9 ± 0.2^{b}	0.955	0.001	0.319
WG (g)	209.8 ± 86.5^{a}	184.0 ± 66.2^{ab}	158.4 ± 63.3^{bc}	159.4 ± 74.6^{bc}	149.4 ± 71.7^{c}	0.038	0.050	0.363
SGR (%/day)	0.46 ± 0.17^{a}	0.42 ± 0.11^{ab}	0.37 ± 0.13^{bc}	0.37 ± 0.14^{bc}	0.35 ± 0.13^{c}	0.048	0.033	0.289

CTRL: commercial-like formulation, formulation concepts (FC): NoPAP: plant-based protein, PAP: processed animal protein, level of fishmeal replacement (FR): 30/60; BW: body weight; CF: condition factor; WG: weight gain, SGR: specific growth rate. Values are expressed as means ± SD per experimental diet; different superscript letters (a, b, c) indicate significant differences at $P < 0.050$ in One-way ANOVA and All Pairwise Multiple Comparison Procedures with Holm-Sidak method, significant differences at $P < 0.050$ in Two-way ANOVA.

3.3 Proximate and mineral composition on wet weight basis of the carcass and dress-out loss and filet yield

The proximate composition on wet weight basis of the fish carcass did not significantly differ between the five treatments (One way ANOVA, see Table 5) with a mean of all tanks (N = 20) of 74.9 ± 1.7% moisture, 17.2 ± 0.7% crude protein, 3.7 ± 1.0% crude lipid, 4.7 ± 0.8% ash and 4.6 ± 0.6 MJ/kg gross energy. However, there were significant differences considering the interaction of formulation concepts with level of replacement (Two-way ANOVA 'FC x FR'; P = 0.007) resulting in a significantly higher crude protein content in the fish fed with the NoPAP30 diet (P = 0.006) and in fish from the PAP60 group (P = 0.010) compared to the fish fed the NoPAP60 diet. Furthermore, the energy content of 4.9 ± 0.3 MJ/kg (n = 8) in the fish from the 30% replacement groups was significantly higher than 4.1 ± 0.6 MJ/kg (n = 8) in of the 60% groups (Two-way ANOVA 'FR'; P = 0.009). The mineral composition of the fish carcasses did not significantly differ between all treatments (Table 5). The mean carcass contents of all tanks (N = 20) of 13.7 ± 3.4 g/kg calcium, 0.7 ± 0.1 mg/kg copper, 9.6 ± 8.1 mg/kg iron, 2.8 ± 0.3 g/kg potassium, , 18.1 ± 4.0 mg/kg manganese, 1.9 ± 0.2 g/kg sodium, 7.9 ± 1.6 g/kg phosphorus, 0.5 ± 0.1 mg/kg selenium, 16.4 ± 2.2 mg/kg zinc.

The fish from all experimental groups (N = 60) with a mean total body weight of 534.1 ± 80.8 g resulted in 94.1 ± 0.8% marketable whole fish gutted and a filet yield of 41.5 ± 4.2% with no significant differences between all diets.

3.4 Apparent digestibility of dry matter, crude protein and minerals

Due to a small sample volume of the feces collected from two tanks of the PAP30 group, the mineral analysis including the yttrium levels failed. Hence, the apparent digestibility coefficients (ADCs) from the fish from the PAP30 group (n = 2) are not statistically sound.

The ADC of dry matter (Table 6) in the fish from the CTRL group was significantly (One-way ANOVA, P < 0.001) higher than in fish from the PAP30, NoPAP60 and PAP60 groups, with no significant difference to the fish from the NoPAP30 group. Furthermore the fish from the 30% replacement groups had a significantly higher ADC of dry matter than the fish in the 60% replacement groups (Two-way ANOVA 'FR', P = 0.006). The ADC of crude protein in the fish from the CTRL group was significantly decreased in the fish from the NoPAP60 and PAP60 groups, with no significant differences to the fish from NoPAP30 and PAP30 group.

Table 5 Chemical composition of the fish carcass on wet weight basis of market-sized turbot fed with fed five experimental diets

	Experimental diets					Two-Way ANOVA P-value		
	CTRL	NoPAP30	PAP30	NoPAP60	PAP60	FC	FR	FC x FR
Proximate composition								
Mean body weight (g)	418.6 ± 57.9	427.1 ± 61.6	392.2 ± 64.5	347.6 ± 78.5	341.0 ± 32.8	0.514	0.055	0.654
Moisture (%)	73.7 ± 2.6	74.2 ± 0.5	75.0 ± 0.4	75.6 ± 2.0	75.7 ± 1.4	0.527	0.116	0.626
Crude Protein (%)	17.7 ± 1.3	17.5 ± 0.6	17.0 ± 0.2$^{\$}$	16.5 ± 0.6§	17.4 ± 0.1$^{\$§}$	0.331	0.162	0.007
Crude Lipid (%)	3.8 ± 1.0	4.6 ± 1.1	3.8 ± 0.6	3.0 ± 1.3	3.4 ± 1.6	0.729	0.071	0.266
Ash (%)	4.6 ± 0.3	4.6 ± 0.9	4.7 ± 0.9	5.2 ± 0.9	4.2 ± 0.8	0.293	0.575	0.129
Energy (MJ/kg)	4.8 ± 0.7	5.1 ± 0.4	4.7 ± 0.3	4.1 ± 0.8	4.2 ± 0.5	0.884	0.034	0.682
Mineral composition								
Calcium (Ca, g/kg)	13.4 ± 4.3	13.2 ± 2.5	13.6 ± 4.1	13.2 ± 2.8	14.9 ± 4.7	0.473	0.417	0.790
Copper (Cu, mg/kg)	0.7 ± 0.1	0.8 ± 0.2	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.31	0.347	0.314	0.277
Iron (Fe, mg/kg)	11.1 ± 10.8	14.1 ± 11.8	9.2 ± 9.7	5.3 ± 0.4	8.4 ± 2.3	0.883	0.288	0.340
Potassium (K, g/kg)	2.9 ± 0.1	3.0 ± 0.3	2.8 ± 0.2	2.5 ± 0.4	2.6 ± 0.3	0.705	0.074	0.529
Manganese (Mn, mg/kg)	17.0 ± 5.3	18.6 ± 3.9	16.5 ± 4.0	17.6 ± 2.9	20.8 ± 4.4	0.606	0.146	0.216
Sodium (Na, g/kg)	1.9 ± 0.1	2.0 ± 0.2	1.9 ± 0.1	1.9 ± 0.2	1.9 ± 0.3	0.603	0.542	0.591
Phosphorus (P, g/kg)	7.8 ± 2.0	7.8 ± 1.2	7.8 ± 1.9	7.6 ± 1.3	8.5 ± 2.3	0.449	0.444	0.671
Selenium (Se, mg/kg)	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	0.803	0.585	0.917
Zinc (Zn, mg/kg)	16.6 ± 2.3	18.1 ± 1.5	15.8 ± 1.7	15.0 ± 2.1	16.7 ± 1.3	0.877	0.845	0.067

CTRL: commercial-like formulation, formulation concepts (FC): NoPAP: plant-based protein, PAP: processed animal protein, level of fishmeal replacement (FR): 30/60; Values are expressed as means ± SD (n = 4), same superscript characters ($, §) indicate significant differences at P < 0.050 in Two-way ANOVA.

The ADCs of all evaluated minerals were significantly affected by the experimental diets except for copper, iron and zinc (Table 6). The ADC of calcium and sodium in fish from the control was significantly (One-way ANOVA, $P = 0.003$ and $P < 0.001$, respectively) higher than in the fish from the NoPAP60 and PAP60 groups with no significant differences to fish from the NoPAP30 and PAP30 groups. The fish from CTRL group had a significant (One-way ANOVA, both $P < 0.001$) higher potassium and selenium ADC than the fish from NoPAP30, NoPAP60 and PAP60 groups, with no significant difference to the fish from the PAP30 group. The fish from the CTRL group showed a significantly higher ADC of phosphorus (One-way ANOVA, $P = 0.003$) compared to the fish from the PAP30, NoPAP60 and PAP60 groups with no significant differences to fish from the NoPAP30 group. Furthermore the fish from the CTRL group showed a significantly (One-way ANOVA, $P = 0.017$) higher ADC of manganese than the PAP60 group with no significant differences to the fish from the other groups. The formulation concept affected the ADC for potassium, being significantly higher in the fish from the NoPAP groups compared to the PAP groups (Two-way ANOVA 'FC', $P = 0.026$). The level of fishmeal replacement significantly affected (Two-way ANOVA 'FR') the ADCs of calcium ($P = 0.006$), potassium ($P < 0.001$), sodium ($P < 0.001$) and selenium ($P = 0.006$) being higher in the turbot from the 30% replacement groups compared to fish from the 60% groups. The ADC for selenium was significantly (Two-way ANOVA 'FC x FR') higher in the PAP30 compared to PAP60 ($P < 0.001$) and compared to the NoPAP30 group ($P = 0.005$). Fish from the NoPAP60 group also had a significantly higher selenium ADC than the fish from the PAP60 group ($P < 0.001$).

3.5 Energy reserve parameters

The energy reserve parameters such as hepato-somatic index and glycogen storage in liver and muscle were not significantly affected by the diets. The hepato-somatic index of the sampled fish was on average 1.5 ± 0.4 ($N = 100$ fish). The glycogen content in the liver ranged from 48.8 ± 26.5 mg/g ($n = 20$) in fish from the PAP30 group to 38.4 ± 23.2 mg/g ($n = 20$) in fish from the PAP60 group with a total mean of 43.0 ± 23.9 mg/g ($n = 100$). The glycogen in the muscle ranged from 3.3 mg/g in the fish from the NoPAP30 and PAP30 groups to 2.8 mg/g ($n = 20$) in fish from the NoPAP60 group with a total mean of 3.1 ± 1.0 mg/g ($n = 100$).

Table 6 Apparent digestibility coefficients (ADC) of nutrients and minerals of market-sized turbot fed with fed five experimental diets

	Experimental diets					Two-Way ANOVA P-value		
	CTRL (n = 4)	NoPAP30 (n = 4)	PAP30 (n= 2)	NoPAP60 (n = 4)	PAP60 (n = 4)	FC	FR	FC x FR
ADC Nutrients								
Dry matter (%)	80.1 ± 2.0^{a}	71.6 ± 2.4^{ab}	69.4 ± 0.3^{b}	64.3 ± 7.1^{bc}	56.4 ± 6^{cd}	0.118	0.006	0.353
Crude Protein (%)	94.5 ± 1.0^{a}	88.5 ± 2.0^{ab}	86.6 ± 2.4^{ab}	85.0 ± 4.0^{b}	76.6 ± 5.5^{c}	0.042	0.012	0.180
ADC Minerals								
Calcium (Ca, %)	34.5 ± 5.6^{a}	32.1 ± 10.7^{a}	15.4 ± 0.2^{ab}	-30.6 ± 44^{b}	-22.3 ± 13.8^{b}	0.780	0.006	0.408
Copper (Cu, %)	46.5 ± 15.6	44.2 ± 6.9	28.5 ± 1.8	41.6 ± 8.4	32.4 ± 9.7	0.019	0.876	0.484
Iron (Fe, %)	6.0 ± 6.1	1.9 ± 4.6	12.6 ± 3.3	1.2 ± 4.3	-5.5 ± 11.1	0.618	0.040	0.053
Potassium (K, %)	97.4 ± 0.4^{a}	95.4 ± 0.9^{bc}	95.9 ± 0.0^{abc}	92.5 ± 0.9^{d}	94 ± 1.2^{cd}	0.090	<0.001	0.363
Manganese (Mn, %)	65.1 ± 8.6^{a}	49.9 ± 17.7^{ab}	49.4 ± 1.1^{ab}	12.2 ± 25.6^{ab}	2.6 ± 34.7^{b}	0.730	0.014	0.756
Sodium (Na, %)	65.9 ± 5.6^{a}	44.3 ± 11.3^{a}	38.4 ± 0.4^{ab}	2.7 ± 19.7^{b}	0.8 ± 22.2^{b}	0.700	0.002	0.838
Phosphorus (P, %)	71.2 ± 1.2^{a}	50.9 ± 14.6^{a}	48.4 ± 3.8^{b}	26.9 ± 21.2^{b}	18.8 ± 12.5^{b}	0.560	0.012	0.761
Selenium (Se, %)	80.7 ± 7.0^{a}	62 ± 5.2b	$70.2 \pm 1.2^{ab\$}$	$68.4 \pm 3.3^{b\S}$	$50.8 \pm 5.5c^{\$\S}$	0.096	0.028	<0.001
Zinc (Zn, %)	59.7 ± 11	61.5 ± 6.7	40.3 ± 7.1	50.9 ± 18.8	41.4 ± 7	0.043	0.487	0.391

CTRL: commercial-like formulation, formulation concepts (FC): NoPAP: plant-based protein, PAP: processed animal protein, level of fishmeal replacement (FR): 30/60. Values are expressed as means ± SD (n = 4), different superscript letters (a, b, c, d) indicate significant differences at $P < 0.050$ in One-way ANOVA and All Pairwise Multiple Comparison Procedures with Holm-Sidak method, same superscript characters ($, §) indicate significant differences in the interaction at $P < 0.050$ in Two-way ANOVA.

4 Discussion

In this study, we observed adverse effects on growth and feed performance as well as the nutrient utilization of marked-sized turbot fed with diets of different levels of processed animal proteins (PAP) and a high level of sustainable feed ingredients without the inclusion of PAPs (NoPAP) compared to the turbot from the control group (CTRL) fed with a commercial formulation. On an individual fish basis, due to a higher sample number (N = 330) these effects were more comprehensive and could be traced either to the formulation concept or to the fishmeal (FM) replacement level.

There are only a few studies, which investigate the effects of alternative feed ingredients on the growth and feed performance of larger sized turbot with an initial body weight (BW) of more than 100 g. In order to classify the observed growth performance, we first compare the growth performance of the fish from the CTRL group (initial BW: 303 g; weight gain (WG): 210 g; specific growth rate (SGR): 0.47%/day; mean water temperature: 17.5°C) with growth data and models for turbot in similar aquaculture settings. Compared to the modeled growth performance of strain B (WG: 215 g and SGR: 0.48%/day at 16.5°C water temperature) in Lugert et al. (2019) and to the data from Baer et al. (2011) the fish from the CTRL group had a normal growth performance. However, the growth was lower compared to the models from Arnason et al. (2009) (WG: 385 g and SGR: 0.74%/day at 17.5°C) and Lugert et al. (2019) for strain A (WG: 270 g and SGR: 0.57%/day at 16.5°C) and to the data from Weiß and Buck (2017) (initial BW: 201 g; SGR: 0.82%/day at 16.4°C). This comparably lower growth performance of the fish from the CTRL group might be explained by the origin of the turbot strain used in the present study. Turbot is known to exhibit counter gradient variation (Imsland et al., 2000) and strains from lower latitudes, such as France, show generally lower growth compared to populations from higher latitudes, such as Norway and Iceland (Imsland et al., 2001).

The feed performance of the fish from the CTRL group measured as voluntary feed intake (VFI: 0.61% ABW/day) and feed conversion ratio (FCR: 1.3) was slightly better than modeled for strain B at 16.5°C (0.56% ABW/day and 1.47, respectively) in Lugert et al. (2019). Arnason et al. (2009) calculated the optimum temperature at 16.1°C for the FCR for large turbot (499 g) at a value of 0.77, which is similar to the water temperature in this study.

The growth and feed performance the turbot from the CTRL group exhibited, it can be considered as normal for a strain from France and the diet-dependent effects should be comparable to other studies using smaller turbot.

There were no diet-dependent effects on the proximate composition (N = 20) of the carcass with values within the range of turbot (Dietz et al., 2012, Bonaldo et al., 2015, Fuchs et al., 2015, Bian et al., 2017, Hoerterer et al., 2022b). However, this is expected since the proximate composition of growing fish is mainly determined by fish size, life cycle stage and energy intake (Shearer, 1994), which did not differ in this study. Similarly for the mineral composition of the carcass, where data for turbot is rare but similar to previous studies (van Bussel et al., 2014, Hoerterer et al., 2022b), and is similar to those of other species (Antony Jesu Prabhu et al., 2016). Differences in the mineral composition are only expected when there are strong deficiencies in the diet (Shearer, 1994) and are further influenced by the mineral concentrations in the rearing water (van Bussel et al., 2014, Antony Jesu Prabhu et al., 2016). Both factors are not present in this study, since the diets were formulated and manufactured to meet the species-specific demands for macro- and micronutrients and all fish were kept in the same rearing water.

The nutritional status (N = 100) indicated by the hepato somatic index (HSI) and glycogen content in liver and muscle did not show any diet-dependent effects. The HSI of the turbot in this study were on the lower range of compared to other studies (Dietz et al., 2012, Bonaldo et al., 2015, Nagel et al., 2017) it can be considered as normal. The in general lower HSI might be attributed to the French strain used in this study, as similar to growth turbot strains from France exhibit lower BW and lower HSI compared to strains from other countries such as Norway (Schlicht et al., 2019), Great Britain (Arfsten et al., 2010) and Iceland (Imsland et al., 2001). Positively correlated to the HSI is the hepatic glycogen as it is stored in the liver as an energy reserve and therefore influences the liver weight (Hemre et al., 2002). The glycogen levels in liver and muscle are also within the expected range (Liu et al., 2014a, Guerreiro et al., 2015a, Zeng et al., 2015, Miao et al., 2016a).The nutritional status indicators show that at the end of the study, the fish could be considered as healthy and the energy allocation not negatively affected.

4.1 Effects of the formulation concepts and fish meal replacement level

Similar to salmonids (Hua and Bureau, 2012), this study suggest that the tolerance to FM replacement depends on the group of alternative ingredients. At lower levels of FM replacement, these effects cannot be detected, as shown in the preceding study with smaller turbot (initial BW: 20 g), there were no significant differences between the PAP and PLANT (analogue to NoPAP) formulation concepts (Hoerterer et al., 2022b), which might be contributed to the comparably low FM replacement level of 20%. In order to further explain the differences in protein digestibility and growth further analysis of the amino-acid profile of the diets and a more detailed evaluation of the variability of the nutritional value for the specific fish species could be helpful to optimize the formulation concepts (Glencross et al., 2020). The results from this study suggest that the inflection point of FM replacement level for turbot lies between 30% and 60% and might be higher for plant protein ingredients that for animal protein ingredients. This means that more fishmeal could be replaced with plant protein ingredients in the diets and maintain similar performance as the currently used practical diets. This could lead to an economic advantage since the calculated costs of the NoPAP formulation is lower than of the commercial diets.

Growth and feed performance

The growth performance of the market-sized turbot was better in the fish from the NoPAP groups as well as the 30% FM replacement groups, without any interactions between those factors. A similar pattern was observed for the protein efficiency ratio (PER). These performance indicators obtained in the present study showed that the market-sized turbot could better utilize the NoPAP than the PAP formulations, independent from the FM replacement level. Even though the fish from the PAP groups consumed more of the diets on a daily basis, they were not able to compensate the growth and feed utilization. Similar to this study, plant proteins allowed a higher level of FM replacement of 35% and more without negatively affecting growth rate (Burel et al., 2000a, Fournier et al., 2004, Bonaldo et al., 2015, Hermann et al., 2016, von Danwitz et al., 2016, Bian et al., 2017). Whereas, turbot growth reacts more sensitive to FM replacement by terrestrial animal derived proteins such as insect meal (20%, Kroeckel et al., 2012) and feather meal (30%, Cao et al., 2020). Looking at the possible interactions between the formulation concepts and the level of FM replacement, only the crude protein content of the carcass was affected. The fish from the PAP60 group had a significantly higher crude protein content in the carcass than in fish from the NoPAP60 group, reflecting the low crude protein content in the NoPAP60 diet (50.5%). This is in contrast to Shearer (1994) who suggested that fish size is the main factor influencing the crude protein of the carcass.

Apparent digestibility of nutrients

The effects on the growth and feed performance by the formulation concepts and by the level of fishmeal replacement might be contributed to the fact that the digestibility of the single ingredients used to replace the fish protein fraction differs greatly and these effects accumulate at higher replacement levels. The ADC of protein was higher in the turbot from the NoPAP groups than in the PAP groups. This reflects the results from previous studies reporting a higher digestibility of plant protein (70 - 96%; Burel et al., 2000b, 84 - 86%; Bonaldo et al., 2011) than the digestibility of insect meal (63%; Kroeckel et al., 2012) or of processed animal proteins (75 - 78%; Davies et al., 2009, 70-74%; Cao et al., 2020). Similar to growth and in line with previous studies the ADC of crude protein in turbot decreases with FM inclusion level (Regost et al., 1999, Bonaldo et al., 2011, Liu et al., 2014a, Bai et al., 2019, Li et al., 2019a). Furthermore, the fishmeal from by products and the fish protein hydrolysate might have a lower protein digestibility (47%; Davies et al., 2009) as well, but the effects only accumulate in the fish fed the diets with the higher replacement. The level of FM replacement also had an effect on the energy content of the carcass and ADCs of dry matter and of some minerals. The ADC of dry matter manly depends on composition of macronutrients present in the diets, and can depend on the carbohydrate levels in plant-based diets. In order to get a more detailed picture into the causes of decreased digestibility of protein, amino acid composition and digestibility and possible anti-nutritional factors (Hua et al., 2019)present further analysis of the single ingredients and diets need to be done to evaluate the variability of the nutritional value for the specific fish species (Glencross et al., 2020).

Apparent digestibility of minerals

The digestibility of minerals strongly depends on the bioavailability in the ingredients (Lall and Kaushik, 2021). In this study, the mineral composition of the fish carcass was not affected by the formulations concepts nor by the level of FM replacement indicating no deficiencies. However, the ADCs of most minerals measured, except for copper and zinc, were correlated to the level of FM in the diets, being highest in the CTRL group and lowest in the 60% replacement groups. This indicates a lower bioavailability of the minerals in the alternative feed ingredients, due to different mineral composition leading to interaction between minerals in by-product fish meals, the presence of mineral binding compounds such as phytate in plant ingredients (Lall, 2022). Especially in the case of calcium and sodium, the low levels might indicate an increased uptake of calcium and sodium from seawater, due to low dietary levels (Lall, 2022). Copper and zinc availability was lower in the PAP groups than in the NoPAP groups indicating lower availability in animal derived feed ingredients. Besides the correlation to the FM replacement level, the low selenium digestibility in the PAP60 indicates a higher availability in the low fishmeal replacement levels within the PAP formulation and the 60% replacement levels compared to the NoPAP formulation. This might be contributed to the fact, that the dietary selenium level was lowest in the PAP60 group.

4.2 Performance of the sustainable formulations concepts in comparison to a commercial formulation

On a tank basis, fish from the CTRL and NoPAP30 groups (n = 4) had similar final BW, biomass gain, SGR, FCR and PER, whereas feeding the PAP and NoPAP60 formulations negatively affected these performance indicators in the fish. A similar pattern was observed on an individual basis, in final BW, WG and SGR, with fish from the PAP60 group having a significantly lower performance than the fish from the NoPAP30 group. The ADCs of dry matter and crude protein as well as the macro minerals calcium, potassium, sodium and phosphorus and of the trace minerals manganese and selenium follow the same pattern. Even though no diet-dependent effects on growth performance and VFI could be detected in the preceding study on smaller turbot fed with the same formulation concepts with 20% fishmeal replacement, the PAP formulation concept negatively affected feed utilization (FCR, PER, ADC) (Hoerterer et al., 2022b).

At final sampling, the condition factor (CF) of the individually tagged fish from the PAP60 (1.9) group was significantly lower compared to the fish from the CTRL group (2.0). The difference is very small and the CFs presented in the fish from all groups is considered as normal (Fuchs et al., 2015, von Danwitz et al., 2016, Nagel et al., 2017, Weiß and Buck, 2017, Wanka et al., 2019), whereas turbot strains from France appear to have a lower CF (Imsland et al., 2000, Arfsten et al., 2010). In smaller turbot the CF was reduced at FM replacement levels of 45% by fermented soybean and blood meal in turbot (Dan et al., 2021, Zheng et al., 2022) more than 55% with plant-based diets (Bonaldo et al., 2015) and insect meal-based diets (Kroeckel et al., 2012). Similar to previous studies with smaller turbot, the CF of larger turbot has a higher tolerance at high FM replacement levels with plant protein ingredients than with animal protein ingredients.

The present results suggest that the NoPAP30 formulation could be an adequate alternative for the practical diets currently used in commercial turbot aquaculture. The PAP60 formulation seems to be the least suitable alternative to the practical diet, having the lowest overall performance, also compared to the NoPAP30 formulation.

4.3 Marketable product and prediction of feed and production costs

For a fish farmer's revenue and overall production costs the growth and feed performance, price and amount of marketable product and feed costs are variable and therefore offer the opportunity to lower costs or/and increase profit. Turbot is considered a high value species in Europe and, depending on the market and country, sold as live/fresh or frozen fish (unit price between 7.30 €/kg to 17.18 €/kg) starting from 0.5 kg to larger fish of up to 3 kg (EUMOFA, 2022a). In this study, the different diets did not affect the amount of the marketable products gutted fish (94%) and filet (42%) and lying within the commercial range (Arfsten et al., 2010, Schlicht et al., 2019).

The present results on growth and feed performance indicate that the NoPAP30 formulation has the highest potential to be used in the grow-out phase of commercial turbot aquaculture. Compared to the commercial diet, the NoPAP30 formulation is cheaper, therefore offering the opportunity to increase profits (Tirano et al., 2021). The lower formulation costs of the PAP formulation and being the more sustainable choice considering environmental impacts in life cycle assessment (Maiolo et al., 2020) will not outweigh the lower growth performance in this study. However, the price and competitiveness of the today's standard feed ingredients might change due to shortages, thus further increasing the economic benefits of the alternative ingredients. Furthermore, to make sustainable fish products more attractive, communication efforts should include and target the preferences for sustainable lifestyle and products of the different consumer groups, which are known to be influenced by age, education and location of stakeholders/consumers (Maesano et al., 2020, Hoerterer et al., 2022a).

5 Conclusion

Comparing the growth and feed performance among all experimental diets as well as the potential costs, we can conclude that the NoPAP30 formulation is an environmentally sustainable and economically viable alternative to the current commercial/standard formulation. Furthermore, the performance of the fish fed the PAP30 and NoPAP60 formulations was similar showing that turbot has the potential for further fishmeal reduction with the alternative feed ingredients used in the NoPAP formulation concept. The fish fed with the PAP60 formulation had overall the lowest performance leading to the conclusion that processed animal protein is not a suitable feed ingredient for market-sized turbot at high inclusion and when compared to other sustainable alternatives such as plant protein. Further experimental studies in combination with meta-analyses and/or nutritional model simulations could find the inflection point. We conclude that for plant-based ingredients investigated here this breaking point is at a higher FM replacement level than for processed terrestrial animal protein ingredients used in the PAP.

In addition to the performance of the fish, the sustainability of the feed ingredients and the economic benefits for the fish farmer, other factors influence the sustainable development of European aquaculture. Consumers are becoming increasingly interested in the production processes of their food (health and welfare issues concerning animal husbandry, feeding and slaughter), making the communication of the life cycle analysis of a product necessary, thus warranting more transdisciplinary aquaculture research.

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Author contributions

CH is lead author, conceptualized and designed the study, conducted the experiment, lab and data analysis, wrote and reviewed the manuscript, and prepared it for submission.

JP conceptualized and designed the study, conducted the experiment, reviewed the manuscript.

GL provided input to the methodology and analysis, provided laboratory resources and supervision.

JJ conceptualized the study, provided input to data analysis, results and discussion, reviewed the manuscript, responsible for funding acquisition.

LC: conceptualization and design of the study, provided resources, and responsible for project administration and funding acquisition.

BB conceptualized and designed the study, provided input to results and discussion, and reviewed the manuscript, responsible for project administration and funding acquisition.

All authors contributed to the article and approved the submitted version.

Ethics statement

The experiments were performed under the guidelines of the local authority 'Food surveillance, animal welfare and veterinary service (LMTVet)' of the state of Bremen with the permission to carry out animal experiments (500–427-103–1/2019–1-19).

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Manuscript IV

Informed Choice: The role of knowledge in the willingness to consume aquaculture products of different groups in Germany

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Manuscript IV

Abstract

Translating the agricultural eco(logical)-intensification model to European aquaculture hosts the potential for sustainably providing local food for local communities. Using online and printed surveys, we investigated the relationship between social factors such as age, gender, and education to seafood consumption behavior and the perception of aquaculture production. The frequency of seafood consumption was significantly lower in young and female respondents, whereas respondents with a higher level of education consume more frequently. Furthermore, high-frequency seafood consumers had a significant preference for wild-caught fish. Young and female respondents also perceived sustainability of aquaculture lower, whereas the level of education had a significantly positive relation to the attitude towards aquaculture. To foster the acceptance of eco-intensified aquaculture production, we suggest that communication efforts need to be group-tailored, focusing on the reduced environmental impacts, increased animal welfare, and novel products like seaweed to meet the values of the German consumer groups.

Keywords: Perception; Sustainability; Seafood; Consumption; Generation

1 Introduction

Sustainability, defined in the Brundtland report by the United Nations Commission in 1987 as the use of resources to meet "the needs of the present without compromising the ability of future generations to meet their own needs", has become an overarching concept in all aspects of contemporary human life: ranging from mobility to resource production and consumption. In the light of the climate crisis, younger generations, as seen in the 'Fridays for Future' movement, are reinforcing this former call for a stronger balance by asking for more mindfulness for their future among politicians and the older generations. As part of this sustainability movement, people in developed countries are increasingly choosing food according to its environmental (e.g. organic, carbon footprint, recyclable packaging), social (e.g. improvement of worker's welfare, access to health services, and school education), and economic (e.g. guaranteed minimum price and access to international markets) sustainability criteria that include aspects of animal welfare and local production (Annunziata and Scarpato, 2014, Lucas et al., 2021). Consumers' attitudes towards sustainable food are often based on personal values, perceived barriers and the confidence of information received (Corrin and Papadopoulos, 2017, Sanchez-Sabate and Sabate, 2019). Scientists have observed that especially ecology-oriented, female and young consumers are more likely to shift to a meat-reduced, vegetarian, or vegan lifestyle in western countries (Pribis et al., 2010, Gvion, 2020, Kymalainen et al., 2021). However, the effects of sustainability concerns among different consumer groups in relation to their seafood consumption are rarely studied.

Seafood is often linked to cultural preferences (coastal communities vs. land), health beliefs, and consumption habits driven by respective cultural settings (childhood) (Carlucci et al., 2015, Jacobs et al., 2015). Furthermore, it is very diverse in terms of production method (wild vs. farmed) and in relation to the accessible variety of available species groups (finfish, shellfish, algae) (Carlucci et al., 2015, Laborde et al., 2020). Food from the sea contributes 17% to the globally available animal protein and in contrast to fisheries, aquaculture hosts a great potential for sustainable growth (Costello et al., 2020). To achieve this in Europe, where food production is dominated by agriculture, aquaculture production needs to be sustainably boosted, without compromising social and economic benefits while reducing the impact on the environment. This is timely, as for instance from the economic perspective the European Union (EU, 28 member states) has a trade deficit of 33% to date and relies heavily on the import of seafood from non-EU countries (EUMOFA, 2020) that renders the EU vulnerable in terms of marine food security. However, concepts on how to implement sustainable growth in aquaculture are rare and criticized for focusing too much on economic growth and not meeting the environmental and social Sustainable Development Goals (SDG) (Cisneros-Montemayor et al., 2021, Farmery et al., 2021). Eco(logical)-intensification, an agricultural model, "to feed the world now and in the future, while maintaining and enhancing ecosystems functions" (Tittonell, 2014). This concept includes different models but basically applies the "harnessing ecosystems services for food security by using e.g. nutrient cycling or biological pest control (Bommarco et al., 2013). Translated to aquaculture it might be a solution for such a sustainable growth of the EU's aquaculture sector. This could mean, e.g. applying circular economy in a farm-to-fork value chain (Schebesta and Candel, 2020, Maiolo et al., 2021) reusing valuable resources such as cuts from fish processing for fish diets (Vazquez et al., 2019, Hoerterer et al., 2022c). These sustainable aquaculture products will most likely cost extra for consumers therefore it is necessary to highlight the benefits in audience tailored communication efforts, assuming that consumers can make an informed choice when purchasing seafood. For instance, socio-economic interests, environmental concerns, aesthetic aspects as well as moral, emotional, and personal values all influence the public's acceptance and perception of aquaculture to a different extent (Mazur and Curtis, 2008, Freeman et al., 2012, Alexander et al., 2016, Thomas et al., 2018). Furthermore, the majority of consumers are often uninformed about contemporary aquaculture practices and the benefits of aquaculture products in terms of environment, health, and quality of the products (Feucht and Zander, 2015, Bronnmann and Hoffmann, 2018).

The aim of the study was to identify the socio-demographic factors that influence seafood consumption behavior, the knowledge base on and attitude towards aquaculture of different groups on a showcase basis in Germany. To achieve high relevance and applicability of this study, the authors addressed especially younger age groups (25 years and younger and 26 to 39 years) by placing questionnaires at a conference for young scientists and by a citizen science project with high school students.

2 Material and Methods

2.1 Participants

The study was set at two international conferences and in a large online survey addressing the different consumer groups characterized by different age groups and different presumed knowledge about aquaculture. At first, we attended the 'International Conference for YOUNG Marine Researchers' (ICYMARE) 2019 in Bremen which was characterized by participants who were all aged under 40 years (see table 1). At the Aquaculture Europe Conference AE2019 in Berlin, we conducted a subsample addressing specifically research experts and practitioners from the aquaculture sector with higher average age (26 years and older). At last, we included high school scholars following a citizen science approach under the 'HIGH school of Science and Education at the AWI' (HIGHSEA at the Alfred Wegener Institute for Polar and Marine Research, Bremerhaven) program. The students translated and adapted the existing questionnaire to German used at the ICYMARE and distributed it as an online survey among the public with a lower average age (25 years and younger).

2.2 Questionnaires

The study's methods resemble a set of potential quantitative and qualitative approaches from a social science stance (Kelle, 2014, Levitt et al., 2018). All methods were pre-tested and outcomes of the first surveys were further refined. The foundation for this study were 442 online and printed questionnaires with the same design and questions, which were distributed in English at the ICYMARE and AE2019 conferences and via email in German language by the HIGHSEA scholars following the snowball principle. The questionnaires were used as an explorative survey method to collect self-reported qualitative (Thronicker et al., 2019) and quantitative data within different social groups in a national context by combining predetermined and open-ended questions (Altintzoglou et al., 2017).

The first part consisted of five predetermined questions of which three were based on the concept of the 5-point Likert-scale and adapted to the ordinal data collected in this study (Allen and Seaman, 2007) and two based on categorical data which were ranked later. After Almeida et al. (2015), the respondents were asked to indicate their frequency of seafood consumption on a 5-point scale ranging from 1 = "never" to 5 = "at least once a week" and the options "I don't know" and "Prefer not to answer". The respondents were asked to self-assess their knowledge about aquaculture production based on a 5-point scale ranging from 1 = "no experience" to 5 = "excellent knowledge" as well as to state their perception of sustainability of fish farming based on a 5-point scale ranging from 1 = “not sustainable” at all to 5 = “very sustainable”. Furthermore, the respondents were asked to give their preference in seafood origin in the in the categories “wild”, “aquaculture”, “unknown”, “no preference”) and their attitude towards aquaculture by agreeing to positive, neutral and negative statements. The general attitude towards aquaculture was based on specific positive (n = 3), negative (n = 4), or neutral (n = 1) statements the respondents were asked to agree with. The statements addressed social, economic, and environmental aspects of aquaculture practices (see figure 1).

Open-ended questions were used to capture the attitude towards aquaculture, as respondents were able to comment on “other”, and in addition in the HIGHSEA survey “How do you define sustainability?”. The question about the country of origin was excluded in the HIGHSEA survey because of the language and focus on German respondents. To analyze these open-ended questions, we applied a qualitative content analysis (Bryman, 2004), which can be used on digitized survey data, protocols, and interview transcripts that are the output of the semi-structured interviews, focus groups, workshops ,and questionnaires.

In the second part, socio-demographic characteristics were collected and evaluated, since we expected that distance to the sea, level of education (Anacleto et al., 2014), gender, and age (NSC, 2019) affect the frequency of seafood consumption as well as knowledge and perception of aquaculture. The county of origin was asked in the ICYMARE and AE2019 questionnaires but was excluded from the HIGHSEA questionnaire, due to the German language and distribution range. The level of knowledge is related to the proximity to aquaculture farms (Mazur and Curtis, 2008, Freeman et al., 2012, Thomas et al., 2018) and frequency of seafood consumption (Aarset et al., 2004, Almeida et al., 2015), thus points out to the role of prior exposure (Ladenburg and Krause, 2011).

Overall, the reach and response rate differed strongly between the addressed audiences. At the ICYMARE both printed and online versions of the questionnaire were provided but whereas 46 of 50 printed versions were filled, only six respondents used the online version (N = 52) and 29 respondents stated Germany as their country of origin. At the AE2019 also both versions were provided but due to logistic reasons, we only were able to retrieve the online versions (N = 5), whereas only two respondents stated Germany as their country of origin. The HIGHSEA questionnaires had a high response rate (N = 385). However, 51 questionnaires were incomplete and therefore excluded from the data. The number of analyzed questions differs between questionnaires because some respondents choose not to answer one or two of the demographic characteristics (n = 17).

2.3 Regression and data analysis

A generalized linear regression model with a significance level of $P < 0.050$ was used to test the relationship between continuous response variables and predictors such as consumer demographics (Agresti, 2007). Continuous response variables were defined as preference of the origin of consumed seafood (ranked: 1 = "aquaculture", 0 = "no preference", -1 = "wild", answers with "unknown" were not included), the frequency of seafood consumption (5-point scale: 1= "never" to 5 = "at least once a week") and the attitude towards aquaculture (ranked: 1 = positive, 0 = neutral or -1 = negative). The ordinal data on the respondents' self-assessment on knowledge about aquaculture (5-point scale 1= "no experience" to 5 = "excellent knowledge") and the perception sustainability of aquaculture (5-point scale 1 = "not sustainable" at all to 5 = "very sustainable") in relation to the demographic groups was analyzed with Kruskal-Wallis One Way ANOVA on ranks based on the medians and 25% and 75% percentiles using the Dunn's method for All Pairwise Multiple Comparison Procedures with an overall significance level of $P < 0.050$. The demographic groups were defined as age groups of 25 *years and younger*, *26-39 years* and *40 years and older*, gender identification as *female* or *male* and education in *school*, *vocational training*, and *academic.* Furthermore, the questionnaires were categorized by the presumed level of knowledge about aquaculture from low in the *public* (HIGHSEA respondents) to high in the *science* community (ICYMARE and AE2019 respondents). The linear regression model was fitted with all potential predictors. Predictors with no correlations to the response variables were sequentially eliminated from the results based on p-values ($P >= 0.050$). Analysis was conducted using SigmaPlot statistical software (12.5, Free Software Foundation, 2020).

3 Results

We focused on how the socio-demographic factors age, gender, and education (see table 1) and frequency of seafood consumption affect the knowledge about, perception of, and attitude towards aquaculture in Germany. The age distribution in the study was slightly skewed towards the *25 years and younger* age group (42%), whereas the age groups *26-39 years,* and *40 years and older* represent a similar amount of respondents (27% and 31%, respectively). Further identified as *female* (53%) and had a high level (55%) of education. Due to the geographical focus of this study, in the following results, we present only the data from respondents, who stated Germany as their country of origin.

Table 1 Socio-demographic characteristics of survey respondents of the HIGHSEA online survey and at the ICYMARE and AE2019 conferences

Socio-demographic characteristic	Subclassification	Public	Science		Total
		HIGHSEA	ICYMARE	AE2019	
Age	n	331	29	2	362
	25 years and younger	44%	27%	0%	42%
	26-39 years	22%	73%	100%	27%
	40 years and older	34%	0%	0%	31%
Gender	n	326	28	2	356
	Female	50%	89%	100%	53%
	Male	50%	11%	0%	47%
Level of Education	n	322	27	2	351
	School	49%	0%	0%	45%
	Vocational	13%	0%	0%	12%
	Academic	38%	100%	100%	43%
Distance to sea	n	332	26	2	360
	Close (walking distance)	17%	31%	0%	18%
	Relatively close (by car)	65%	58%	100%	64%
	Relatively far	14%	12%	0%	13%
	My country is landlocked	5%	0%	0%	4%

ICYMARE: International Conference for Young Marine Researchers September 24-27 2019 in Bremen; AE2019: Aquaculture conference of the European aquaculture society October 7-10 2019 in Berlin; HIGHSEA: 3-year scholar program of the Alfred Wegener Institute in Bremerhaven.

Respondents from the questionnaire addressing the public answered, "How do you define sustainability?" in 95 of 385 questionnaires and we counted how often keywords were used. 'Resources', 'protect', and 'nature' or related words were mentioned most often and each occurred in 25% of the answers. 'Lasting' and 'intrusion' occurred in 20% and 17% of the answers given by the respondents, respectively. ‘Balance’, ‘food’, ‘damage’ and ‘consume’ occurred in 9% of the answers. ‘Generation’, ‘production’ and ‘environment’ were used in 7% of the answers. ‘Life’, ‘regeneration’ and ‘handling’ were used in 6% of the answers.

3.1 Seafood consumption behavior, preference and attitude towards aquaculture in relation to age, gender, and education

Overall (N = 385), 60% of respondents consume seafood “at least once a month” (high-frequency). However, the frequency of seafood consumption significantly differs in relation to age, gender, and level of education (see table 2) and increases with age (linear regression, $t = 7.024$; $P < 0.001$). It is noticeable that 18% of the respondents aged *25 years and younger* stated that they “never” consume seafood and 52% of the respondents aged *40 years and older* consume seafood “at least once a week”. In relation to gender, *female* respondents consume seafood less frequently compared to *males* (linear regression, $t = -3.103$; $P = 0.002$). Similar to age, the frequency of seafood consumption increases with the level of education (linear regression, $t = 4.110$; $P < 0.001$), whereas 16% of respondents with *school* education “never” or rarely consume seafood. Regarding respondents with a higher level of education, more respondents with *academic* background consume seafood at a higher frequency than respondents with *vocational* training.

Table 2 Frequency of stated seafood consumption in relation to age, gender, and education

		n	Never	Less than once a year	Less than once a month	At least once a month	At least once a week	P
Total		385	12%	7%	20%	31%	30%	
Age	25 years and younger	152	18%	10%	26%	27%	19%	< 0.001 (S)
	26-39 years	96	8%	11%	22%	40%	19%	
	40 years and older	114	5%	0%	10%	33%	52%	
Gender	Female	166	16%	10%	23%	25%	25%	0.002 (S)
	Male	159	7%	5%	18%	37%	33%	
Education	School	159	16%	10%	23%	25%	25%	< 0.001 (S)
	Vocational	42	7%	10%	17%	45%	21%	
	Academic	150	7%	4%	17%	37%	36%	

n = number of answers given per group; linear regression was used to identify statistical differences with significance level $P < 0.050$ (S) and $P >= 0.050$ (NS)

Interestingly, overall (N = 364), only 7% of the respondents prefer "aquaculture" products compared to 31% who prefer "wild" products (see table 3). However, 37% have "no preference" or it depends on the type of seafood product they buy (e.g. smoked salmon, fish fingers, etc.). One quarter stated that they do not know whether the products they buy are from aquaculture or the wild ("unknown"). Noteworthy, the preference for "aquaculture" or "wild" products was not correlated to age group, gender, or education (linear regression; P = 0.050). However, respondents that consume seafood "at least once a month" (high-frequency) have a lower preference for "aquaculture" products and at the same time prefer i.e. "wild" seafood compared to respondents that consume "less than once a month seafood" (low-frequency) (linear regression, t = -2.537, P = 0.012). Moreover, 64% of low-frequency consumers state not to prefer a certain origin compared to high-frequency consumers (31%).

Table 3 Stated preference of production method of seafood in relation to age, gender, education, and frequency of seafood consumption

		n	Aquaculture	No Preference	Wild	Unknown+	P
Total		364	7%	38%	30%	25%	
Age	25 years and younger	139	5%	35%	27%	34%	0.287 (NS)
	26-39 years	91	9%	44%	25%	22%	
	40 years and older	111	9%	29%	41%	21%	
Gender	Female	175	7%	34%	33%	26%	0.527 (NS)
	Male	161	7%	35%	30%	27%	
Education	School	146	7%	29%	30%	34%	0.243 (NS)
	Vocational	40	8%	44%	31%	17%	
	Academic	144	5%	30%	38%	28%	
Seafood consumption	Less than once a month	119	9%	44%	22%	25%	0.012 (S)
	At least once a month	225	6%	32%	35%	27%	

n = number of answers given per group; linear regression was used to identify statistical differences with significance level P < 0.050 (S) and P >= 0.050 (NS); + the category unknown was not included in the linear regression analysis

3.2 Knowledge and perception of aquaculture production in the public and science community

A central issue of the questionnaires was placed on capturing the existing knowledge about aquaculture, the perceived sustainability of aquaculture (table 4), and the plurality of attitudes on aquaculture (table 5)

Overall, the knowledge about aquaculture from survey participants' self-assessment based on a 5-point scale of 1 ("no experience") to 5 ("excellent knowledge") had a median of 2.0 (1.0 – 3.0) among all German respondents (N = 300), with no differences between the three age groups (One-way ANOVA on ranks, P = 0.730) and gender (One-way ANOVA on ranks; P = 0.136). Furthermore, the self-assessment of the existing knowledge base significantly increased (One-way ANOVA on ranks, P < 0.001) with the level of education from *school* education and *vocational* training (2.0 (1.0 – 3.0) and 2.0 (1.0 – 2.25), respectively) to *academic* education (2.0 (2.0 – 4.0)) and from the *public* (2.0 (1.0 – 3.0) to the *science* community (4.0 (2.0 – 4.0)).

Table 4 Self-assessed knowledge base and the perception of the sustainability of aquaculture production

		Knowledge		Sustainability	
		n	Median	n	Median
Total		300	2.0 (1.0 - 3.0)	339	3.0 (2.0 - 3.0)
Age	25 years and younger	125	2.0 (1.0 - 3.0)	146	2.0 (2.0 -3.0)[a]
	26-39 years	81	2.0 (1.0 - 3.0)	90	2.0 (2.0 - 3.0)[ab]
	40 years and older	92	2.0 (1.0 - 3.0)	101	3.0 (2.0 - 3.0)[b*]
Gender	Female	162	2.0 (1.0 - 3.0)	174	2.0 (2.0 - 3.0)[a]
	Male	134	2.0 (1.0 - 3.0)	157	3.0 (2.0 - 4.0)[b**]
Education	School	129	2.0 (1.0 - 3.0)[a]	149	2.0 (2.0 - 3.0)
	Vocational	30	2.0 (1.0 - 2.25)[a]	36	3.0 (2.0 - 3.0)
	Academic	131	2.0 (2.0 - 4.0)[b**]	143	3.0 (2.0 - 3.0)
Audience	Public	269	2.0 (1.0 - 3.0)[a]	311	3.0 (2.0 - 3.0)
	Science	31	4.0 (2.0 - 4.0)[b**]	28	2.5 (2.0 - 3.0)

n = number of answers given per group; Kruskal-Wallis One-way ANOVA on ranks, values given as medians and the 25% and 75% percentiles, values with different letters within the same columns of one group are significantly different (Dunn's method, P >= 0.050), *P < 0.050; ** P < 0.001

The rating of the sustainability of aquaculture among all German respondents had a median of 3.0 (2.0 – 3.0) (N = 339) with significant differences between the age groups and gender. The age group of *40 years and older* ranked aquaculture with a median of 3.0 (2.0 – 3.0) significantly more sustainable (One-way ANOVA on ranks, P = 0.033) than the median of

2.0 (1.0 – 3.0) in the age groups *25 years and younger* and *26-39 years*. *Male* respondents rank sustainability of aquaculture production with a median of 3.0 (2.0 – 4.0) significantly higher (One-way ANOVA on ranks, P < 0.001) than *female* respondents (2.0 (2.0 – 3.0)). The level of education and audience (*public*, *science* community) did not affect the sustainability ranking (One-way ANOVA on ranks, P = 0.918).

Overall, 52% of respondents (N = 338) have a positive attitude towards aquaculture, which was not influenced by age, gender or the audience. However, respondents with a high level of education have a significantly more positive attitude towards aquaculture than those with *school* education (linear regression, t = 2.414, P = 0.016). However, the overall attitude did not differ among the *public* (HIGHSEA, n = 277), and the *science* community (n = 31) (linear regression, t = 0.155, P = 0.908).

Table 5 Attitude towards aquaculture in percentage

		Attitude				P
		n	*positive*	*neutral*	*negative*	
Total		338	52%	22%	26%	
Age	25 years and younger	124	52%	19%	29%	0.650 (NS)
	26-39 years	81	53%	30%	17%	
	40 years and older	103	56%	15%	29%	
Gender	Female	151	50%	24%	26%	0.487 (NS)
	Male	151	58%	17%	26%	
Education	School	132	48%	20%	33%	
	Vocational	32	56%	25%	19%	0.016 (S)
	Academic	135	60%	20%	20%	
Audience	Public	277	53%	20%	26%	0.908 (NS)
	Science	31	52%	25%	23%	

n = number of answers given per group; linear regression was used to identify statistical differences with significance level P < 0.050 (S) and P > 0.050 (NS)

Interestingly, 39% of respondents from the *science* community (n = 31) agree with the positive statement that 'aquaculture is important for social welfare in the region', whereas only 19% of public respondents (n = 277) agree (total 21%). The majority (61%) of the respondents from all groups agree with the positive statement that 'aquaculture is an economic way to produce seafood'. Comparing terrestrial livestock production with aquaculture, 35% of the science community and 30% of the *public* agree with the positive statement that 'aquaculture is more sustainable than terrestrial livestock production'. More public than *science* respondents agree with the negative statement that 'aquaculture products are less healthy than capture fisheries' (19% and 10% respectively). In contrast, more respondents from the *science* community agree with the negative statements that 'aquaculture is a highly environmentally negative form of producing fish' (19% and 14% respectively) and 'aquaculture consumes more fish than it actually produces and therefore threatens the oceans' (16% and 10% respectively). Approximately one-third (30%) of all respondents agree with the negative statement that 'aquaculture production is associated with the use of toxins and chemicals'. *Public* respondents agree in 40% of the answers with the statement that 'aquaculture is not good for fish welfare, but it is the only way to ensure seafood availability', whereas fewer *science* respondents agree with this statement (26%).

In the option "other", the respondents were able to give their statement, which 29% of the *science* community and 7% of *public* respondents did. The answers given in the option "other" could be grouped into four categories (see table 6). Categories (1) 'The sustainability of aquaculture depends on the culture system (IMTA, RAS, intensity), cultured species and regionality.' and (3) 'Aquaculture is not sustainable because of pollution by antibiotics and the spread of parasites, impacts on wild populations.' are centrally addressing environmental issues. In contrast, category (2) 'Aquaculture is necessary to ensure food security.' addresses primarily societal and economic issues. Only a few respondents in the *public* survey stated that they (4) '[…] are uninformed'. Respondents mentioned that aquaculture "can be sustainable if…" or "some aquaculture practices are sustainable, others need to be improved…" showing that both, positive and negative attitudes of aquaculture are centrally correlated to the production method, scale, and environment (Category 1). The respondents are also aware that aquaculture is important for food security (Category 2) "…if done right…" and "necessary to other regions". The *public*, as represented by the HIGHSEA survey, displays an overall more negative attitude and agrees more with the negative aspects, i.e., focusing on the negative environmental impacts, the use of toxins and chemicals associated with aquaculture production, pollution and the threat to wild populations (Category 3).

Table 6 Categorized comments on the option "other" in the question about the attitude towards aquaculture

Category	Statement
(1) The sustainability of aquaculture depends on the culture system (IMTA, RAS, and intensity), cultured species and regionality.	*"Aquaculture, if done in a multi-trophic and local scale can be a very sustainable alternative for seafood"* *"for some species already very sustainable and good; but improvements needed for other species"* *"It all depends on the methods/type of aquaculture"* *"There are semi-intensive AQ systems. AQ can be a sustainable way for fish production, more research and improvement of nutrition, animal welfare has to be done"* *"[...] I think it depends on the manner in which it is done. [...]"* *"Aquaculture is a diverse field; I prefer some production methods to others."* *"Aquacultures are only ecological reasonable as organic aquacultures"*
(2) Aquaculture is necessary to ensure food security.	*"Aquaculture can be necessary to other regions"* *"it's a necessity"* *"Aquaculture if done right can be beneficial to feeding humans. [...]"* *"The main point is that the fish price and the quality is right"* *"Aquaculture is a useful addition to traditional fishing)"*
(3) Aquaculture is not sustainable because of pollution by antibiotics and the spread of parasites, impacts on wild populations.	*"negative effects due to use of antibiotics and spreading of diseases and parasites"* *"they use antibiotics in aquaculture and thus pollutes the ocean"* *"spread of parasites, farmed fish are fed fish"* *"[...] If toxins, overpopulation, wrong waste management occurs, aquaculture can be detrimental to the environment"* *"Aquaculture must be ecologically compatible, otherwise it damages and threatens wild fish, for example, salmon in western Canada"*
(4) Respondents are uninformed	"There is too little information on the subject." "No knowledge available"

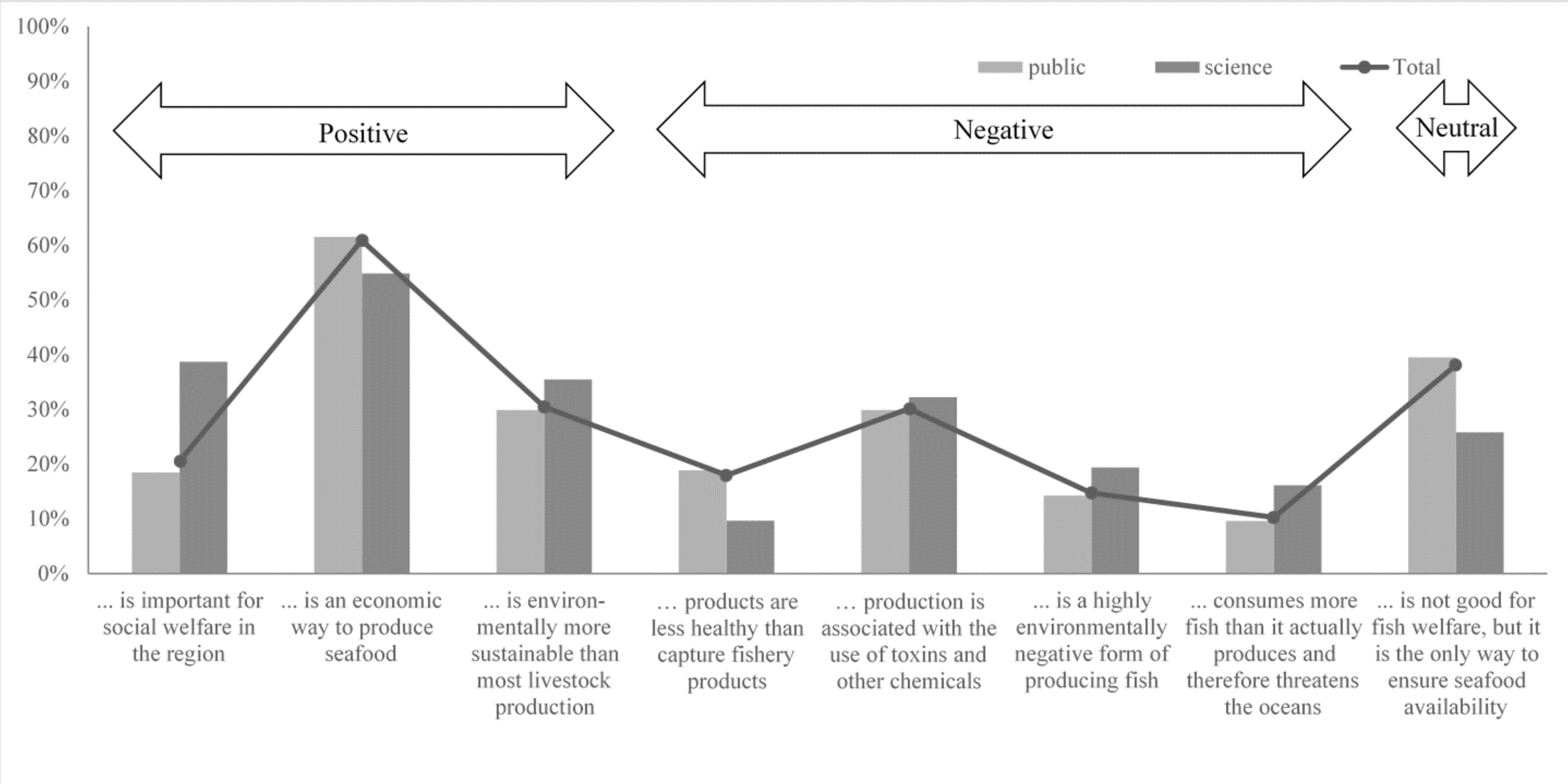

Figure 1 Percentage of agreement to statements "Aquaculture…" of questionnaire respondents from public (n = 277) and science community (n = 31). Arrows with "positive", "negative" and "neutral" indicate the statements' connotations. Multiple answers possible (n = 724 of 308 answered questionnaires).

4 Discussion

The questionnaire was developed to look in more detail at various aspects that relate to the social acceptance of aquaculture in Germany. In addition, these questionnaires also enquired about the common understanding of sustainability to achieve a better understanding, of what consumers and stakeholders assume what sustainability should entail. The central focus was placed on younger generations (70% of respondents were under the age of 40 years) and their levels of acceptance, which does not represent the age group distribution in Germany as a whole, where 43% contribute to the under the age of 40 years groups and 57% to the 40 years and older group (DESTATIS, 2021). With these results, we derived recommendations on how to potentially improve and tailor information availability for fostering acceptance of eco-intensification measures of aquaculture. As relevant social factors, we identified age group, gender, and educational level that are discussed in more detail according to their influence on the response parameters below. Moreover, we discuss the implications of the subsample in relation to the direct influence of increased knowledge on the change of attitude towards aquaculture.

4.1 Influence of social factors on seafood consumption behavior

There appears to be a discrepancy between the voiced preference for wild fish and the actual higher consumption rate of farmed fish that somewhat mirrors the findings of Lopez-Mas et al. (2021). Indeed, our results showed that preference for seafood of a certain production method (wild vs. farmed) was not influenced by age, gender, or education, but rather by the frequency of seafood consumption. High-frequency seafood consumers (respondents that consume seafood more than once a month) prefer wild seafood, while low-frequency seafood consumers (respondents that consume less than once a month) are more likely to have no preference. However, as seen in this study, Germans consume less frequently seafood (65% at least once a month) than the average European (70%), but expose the same preference for wild (31%) and aquaculture (9%) products (Eurobarometer, 2018). The younger age group of under 40 years (born after 1980) stated to consume seafood less frequently than the 40 years and older group, which is in contrast with the NSC (2019) report that stated that the fish consumption was higher in the younger age groups. By large, female respondents show similar preferences as the younger age groups, consuming less seafood. Furthermore, respondents with a high level of education are more likely to eat seafood at least once a month. This can be explained by seafood usually being associated with a healthy lifestyle and especially more educated and older aged people have a better understanding of the health benefits of certain products (Bjørndal et al., 2014), which leads in turn to a higher seafood consumption rate (Heuer et al., 2015).

The result that younger and female respondents consume less seafood might be related to the increased awareness of environmental issues of food production and the modern lifestyle of Europeans (Verbeke et al., 2007b, Kymalainen et al., 2021). Several young and noteworthy especially female respondents stated that they do not consume seafood at all, which reflects the outcomes of the NSC (2019) report and the global trend of meat reduction due to moral and environmental reasons (Pribis et al., 2010, Koch et al., 2019, Gvion, 2020). In contrast to this observable trend among young age groups, it is noteworthy that especially in the older and more educated consumers, possible health benefits, taste, and consumption habits might be an underlying motivation for a prevailing high seafood consumption (Carlucci et al., 2015, Eurobarometer, 2018, Cantillo et al., 2021).

4.2 Perception of sustainability and the attitude towards aquaculture

In order to communicate the benefits of seafood produced in eco-intensified aquaculture production, we need to understand how the different consumer groups perceive and interpret sustainability and the positive and negative dimensions of aquaculture production. Scientists are much more aware of the tradeoffs between the benefits and costs in the ecological, social, and economic dimensions of aquaculture production than the public (Chu et al., 2010, Bacher et al., 2014). The current prevailing societal narrative of aquaculture to date focuses more on the environmental dimensions of sustainability and to a much lesser extent on the social and economic domains (Freeman et al., 2012). The diversity of responses in this study showed that not only academic but also all social groups within a society (e.g. politicians, decision-makers, ordinary citizens, children, etc.) need a better (common) understanding of sustainability. 'Resources' and 'nature' were most often mentioned as central definitions for sustainability, and surprisingly little attention was voiced on social (and economic) factors, rather only related to 'generation', 'food' and 'impact'. In this regard, science is expected to support and become involved in processes of social learning to comply with these new demands (Siebenhüner, 2004). However, the concept of sustainability, its dimensions, and its definition is complex and often viewed one-sided by different stakeholder groups (Risius et al., 2017, Béné et al., 2019, Lawley et al., 2019). For instance, economic stakeholders often focus on economic and environmental sustainability whilst neglecting the social dimension (Hoerterer et al., 2020). Similar to the findings of Lawley et al. (2019), the assumed greater involvement in the topic of seafood production of the scientific community was positively related to the ranking of sustainability.

This somewhat persistent narrow perception of sustainability in the public is reflected in the respondents' agreement with aquaculture statements. Public and science respondents alike mainly voiced negative environmental concerns such as the degree of pollution of the marine environment, use of antibiotics and other chemicals. This coincides with other studies, where environmental risks and impacts are noted to be a major concern and act as an ethical and moral barrier for consumption of aquaculture products (Mazur and Curtis, 2008, Chu et al., 2010, Bacher et al., 2014, Bergleiter and Meisch, 2015, Feucht and Zander, 2015). As shown in this study, the public is not as aware of social benefits of aquaculture such as social welfare (see figure 1) and food security (see table 6) as the informed groups of scientists (Schlag and Ystgaard, 2013, Bacher et al., 2014, Krause et al., 2020). In Whitmarsh and Wattage (2006) the public perceived minimizing environmental damage as the most important objective in the salmon farming industry, whereas maintaining employment, improving product quality, avoiding conflicts with other resource users, and ensuring fair prices were perceived as less important with very little variations between the surveyed areas. Indeed, Aarset et al. (2004), Verbeke et al. (2007b), and Feucht and Zander (2015) showed that there is a perception-reality gap between actual environmental impacts of aquaculture production and the health benefits and nutritional value of aquaculture products, rendering attitude towards aquaculture products more negative, especially fish.

In this study, the public respondents from Germany stressed the importance of health issues ("wild-caught fish is healthier than aquaculture fish") and animal welfare as well as the price for the product. This links to the findings across Europe that health benefits and higher animal welfare standards are a central driver for seafood purchase and consumption, but often negatively associated with aquaculture products (Feucht and Zander, 2014, Carlucci et al., 2015, Rickertsen et al., 2017, Cantillo et al., 2021). Concerning the price of seafood, previous studies have shown that high prices can be a barrier to seafood consumption (Carlucci et al., 2015). However, consumers of southern countries such as Portugal appear to be more willing to pay for sustainable salmon, compared to consumers from Norway (Misund et al., 2020). In contrast, German consumers are less willing to pay more for sustainable products or will not purchase a product if the price is higher (Bronnmann and Hoffmann, 2018). However, improved information about animal welfare (Stubbe Solgaard and Yang, 2011), local, domestic, or European production (Zander and Feucht, 2017), or 'natural' production methods (Risius et al., 2017), such as pond aquaculture could increase the willingness to pay extra for sustainable aquaculture products.

Despite that the younger age groups and female respondents from the public audience ranked the sustainability of aquaculture as low, the attitude towards aquaculture was overall positive (> 50%). This is in contrast to previous studies where consumers from different countries and backgrounds had a more negative attitude towards aquaculture and aquaculture products (Verbeke et al., 2007a, Rickertsen et al., 2017). Respondents with higher education or science background have an even more positive attitude towards aquaculture. This suggests that a higher level of knowledge might lead to a positive attitude towards aquaculture, but its ripple effects on sustainable consumption behavior are not clear (Almeida et al., 2015, Feucht and Zander, 2015, Richter and Klockner, 2017).

In summary, the public needs improved knowledge on aquaculture production and the interwoven plurality of sustainability dimensions therein to order to understand the manifold processes that take place and how these are embedded in our economies, environment, and societies. Such systemic worldviews offer scope towards transformative pathways of future marine food production across Europe. In its wake, forming linkages between different mindsets, worldviews, cultural belief systems of sustainability create both conceptual and cultural challenges.

4.3 Does information lead to informed choice?

More often, consumers are rather driven by moral and ethical reasons in their seafood purchasing and consumption behavior, such as values and (culturally rooted) daily habits, than by scientific reasoning that acknowledges environmental, social, and economic benefits of local, domestic or European aquaculture (Schlag and Ystgaard, 2013). That said it is crucial to know how and in what ways improved scientific knowledge affects seafood-purchasing decisions. This will allow tailoring better communication pathways to inform about the benefits of eco-intensified aquaculture products that are based on scientific findings as well as endorsing the respective consumer's values, culture, and habits.

In this study, the majority (77%) of the public respondents self-assessed to have a low level of knowledge about aquaculture, whereas the knowledge of the science community respondents was higher. Furthermore, the level of education can be positively correlated to the knowledge about aquaculture, which might be related to a higher general level of knowledge including knowledge about aquaculture. However, some public respondents voiced that they are uninformed and are not able to agree with the statements about aquaculture. Pretesting showed that the degree of knowledge about aquaculture did not affect the perception of aquaculture. In contrast, previous studies showed that the level of knowledge is related to the proximity to aquaculture farms (Mazur and Curtis, 2008, Freeman et al., 2012, Thomas et al., 2018) and the frequency of seafood consumption (Aarset et al., 2004).

In the exploratory survey at the ICYMARE aquaculture session, three respondents changed their attitude more positive due to improved knowledge about sustainable aquaculture, while the other eight respondents did not change their attitude. No one changed the perception towards more negative, suggesting that improved information about the sustainability of aquaculture practices and its products could only have positive effects on the perception. However, previous studies have shown that information and improved knowledge could also lead to a shift in consumers' decisions against aquaculture products (Feucht and Zander, 2015, Claret et al., 2016). Due to the small number of respondents, the results offer only on a very exploratory scale that there are potential shifts possible in the perceived impacts of aquaculture. These exploratory results indicate that more research is warranted to fully understand the role of improved scientific information in everyday decision-making of food consumption. However, the engagement with trustworthy knowledge holders (scientists presenting aquaculture-related research results) led to a topical perception shift, indicating a learning process on the individual level.

4.4 Implications for a future acceptance of aquaculture products from the eco-intensification approach in Germany

The premise of this study was that social change towards acceptance of eco-intensification measures in aquaculture would benefit from a better understanding of sustainability thinking among ordinary citizens and especially younger age groups. It is not sufficient for only experts to be knowledgeable about eco-intensification measures in aquaculture. Research insights need to be tailored to the specific needs of the respective audiences in order to develop relevant or meaningful outputs (Krause and Schupp, 2019). What constitutes relevance or meaningfulness is part of an ongoing negotiation process between academia and society and may vary widely for different social groups and contexts, and different scientific disciplines alike (Hornidge, 2014). For contextualization of research findings towards the social realities of stakeholders, the requirements of actors from scientific and societal realms need to be understood in order to design a targeted output (Regeer and Bunders, 2003).

In the case of communicating the benefits of eco-intensified aquaculture production, this study's results conform to previous studies (Schlag and Ystgaard, 2013, Risius et al., 2017, Zander and Feucht, 2017). Tailored communication per consumer group should highlight research insights on new developments reducing environmental impacts, animal welfare, and nutritional and health benefits of locally produced seafood products addressing values and habits of the respective groups. In the German context, it might be crucial to communicate the benefits of the application of circular economy in the production of feeds for Europe's most popular fish species like trout (Maiolo et al., 2021), salmon (Vazquez et al., 2019), sea bream and sea bass and the technological advancement for monitoring environmental interaction (O'Donncha and Grant, 2019, Burke et al., 2021). Indeed, the current pandemic and the recognition of how vulnerable globalized food systems are has acted as an accelerator for regional, circular economy thinking (Kaiser et al., 2021b). However, communication alone will not be sufficient, since consumers want to rely on the aquaculture industry to follow sustainable standards (Feucht and Zander, 2015, Banovic et al., 2019), produce reliable labeling (Carlucci et al., 2015, Risius et al., 2017), without giving too complex information (Reinders et al., 2016, Bronnmann and Hoffmann, 2018, Cantillo et al., 2021).

It is noteworthy that this study revealed that especially the younger age groups consume less frequently or no seafood than the older groups. This reduction might be mainly due to moral and ethical reasons (Verbeke et al., 2007b), and emphasizing benefits of eco-intensifications measures for animal welfare, no pollution, and absence of drugs and hormones as well as sustainable fish feed might be crucial for communication for this respective age group (Schlag and Ystgaard, 2013, Zander and Feucht, 2017). Aquaculture advocates, belonging mostly to the older age groups, should leave preconceived notions such as assumed positive consumer behavior changes if messaging health benefits of seafood consumption (Jacobs et al., 2015), but rather uptake young and critical consumers' interests that revolve more strongly around vegetarian or vegan lifestyle. Scherer and Holm (2020) proposed that advocating eating lower trophic levels of seafood might tap into the potential of locally produced marine resources, which acknowledges the raising demand for regionalization of food production. In order to accommodate the trend of a plant-based diet among the "consumers of tomorrow", aquaculture advocates should promote the production and consumption of novel plant/algae based aquaculture products, such as seaweed. At the ICYMARE aquaculture session some respondents stated that the sea grapes (*Caulerpa* *lentillifera*) presented by Stuthmann et al. (2019) were interesting to them as a novel food. Production of seaweed is in many ways considered sustainable by not using fished resources as finfish production, as its reputation as a functional food, and its potential for ecosystem services (Buchholz et al., 2012, Garcia-Poza et al., 2020).

5 Conclusion

The presented findings mirror previous studies, in which age, education, and location of stakeholders influenced the preferences towards a more sustainable lifestyle (Black and Cherrier, 2010, Schoolman et al., 2014, Kapferer and Michaut-Denizeau, 2019) and the willingness to accept higher prices of sustainable products (De Pelsmacker et al., 2005, Stubbe Solgaard and Yang, 2011).

However, the results of this and previous studies do not clearly indicate that consumers will choose a more sustainable product based on provided information on the benefits of aquaculture products from eco-intensified production. Even though consumers state that sustainability is important for them, their purchase behavior is often run along by values, habits, lifestyle, convenience, and trust in information sources and not (solely) by scientific reasoning (Gaviglio and Demartini, 2009, Carlucci et al., 2015, Feucht and Zander, 2015, Jacobs et al., 2015). Instead of relying only on a bottom-up transformation through consumers' decision to purchase and consume sustainable aquaculture products, the aquaculture industry should also intrinsically aim for a successful transformation to an eco-intensified European aquaculture sector (Almeida et al., 2015, Bergleiter and Meisch, 2015, Richter and Klockner, 2017, Lawley et al., 2019). This might enhance the trust of the consumers in sustainable and especially environmentally friendly production of food from the seas.

Overall, more factors have to be considered when the aquaculture industry wants to boost sustainable production in Europe. Current and unforeseen developments such as the COVID-19 pandemic host the potential to change environmental awareness, sustainable consumption, and social responsibility (Kaiser et al., 2021b, Severo et al., 2021).

Furthermore, the aspiration for economic growth and increased consumption should be seen more critically, especially in the light of the younger generations having other values than the older generations. Wanting to produce more to sell more, might be the wrong strategy facing lower seafood consumption rates among the younger age group now and in the future. Initiatives like the Blue Growth Agenda launched by the EU are very important. However, these risk delivering only a part of the promise as they focus strongly on economic dimensions but overlooking other aspects necessary for sustainable seafood production (Eikeset et al., 2018). Scientists (see Ertör and Hadjimichael, 2019) and organizations such as the High Level Panel for a Sustainable Ocean Economy (HLP or the Ocean Panel), which was created in 2018 advocates blue degrowth in order to reduce environmental impacts, securing a future worth living for generations to come.

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Author contributions

CH: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization.

JP: Conceptualization, Methodology, Validation, Investigation, Writing – review & editing.

GK: Supervision, Project administration, Funding acquisition.

Ethics statement

This study was in accordance with the regulations of the German Research Foundation (DFG) and the Council for Social and Economic Data (RatSWD), as all collected information was anonymous and non-sensitive and participants are not identifiable. Participants were explicitly informed about and consented to the aim of the study, the methodology and about what data will be collected, processed, stored and published. All data were collected, stored and processes in compliance with the General Data Protection Regulation (GDPR).

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4 Synthesis

This thesis evaluated eco-efficient feed formulations at graded fishmeal replacement levels for turbot in the juvenile (Manuscript I and II) and in the grow-out phase (Manuscript III). These eco-efficient diets have the potential to enhance the environmental performance of turbot farming, while addressing public concerns on environmental impacts of aquaculture, which are one constraint of choosing aquaculture products (Manuscript IV).

As the turbot's live stage determined the response to the replacement of FM by alternative protein sources, this thesis will discuss the role of replacement and incorporation of these protein sources in the physiological, environmental and economic performance of turbot from the juvenile and grow-out phase.

4.1 What matters more? – The amount of replaced fishmeal or the alternative ingredient used.

Previous studies have shown that growth rates are significantly reduced in turbot, if more than 35% of FM in the control diet are replaced by alternative feed ingredients (Bonaldo et al., 2015, Hermann et al., 2016, Bai et al., 2019, Zheng et al., 2022). Unexpectedly, neither the level of FM replacement (20% and 40%) nor the feed formulation (PLANT/NoPAP, PAP and MIX) significantly affected growth performance of juvenile turbot (Manuscript I and II). In contrast, the level of FM replacement (30% and 60%) and the feed formulation (NoPAP and PAP) significantly affected growth performance of turbot in the grow-out phase (Manuscript III). Interestingly, turbot fed the PAP formulation with 30% FM replacement level had a similar performance to turbot fed the NoPAP formulation with 60% FM replacement level. To further highlight the correlation between specific growth rate (SGR) of turbot and the dietary manipulation factors (FM replacement or ALT incorporation) the results of this thesis are put into perspective by comparing them to a meta-analysis of 30 publications on turbot feeding trials (see Appendix Methodology and Tables A.1, A.2 and A.3) as proposed by Hua and Bureau (2012).

Comparing the response in the SGR of juvenile turbot (Manuscript I and II) to the meta-analysis data, the effects of the NoPAP and PAP formulations on the SGR are within the expected range, whereas turbot fed with the MIX formulation had a higher SGR than expected (Figure 6, bright red data points). Although the FM replacement level of 40% was within the range of expected significant decrease (yellow area in Figure 6 A) the emerging feed ingredients (insects, bacteria, yeasts and microalgae) seem to have sufficiently covered the nutritional demands allowing a high SGR. This means that using the right alternative feed ingredients; the level of FM replacement is not obligatory the limiting factor in feed formulations for juvenile turbot. In contrast, the growth performance of the grow-out turbot (Manuscript III) fed the PAP formulations and the NoPAP formulation with 60% FM replacement was lower than expected for > 100 g turbot (see Figure 6 dark red data points). This emphasizes the role of the alternative protein source used to replace the fish-derived protein in the grow-out phase of turbot. However, the meta-analysis data show that turbot in the grow-out phase seem to be more tolerant to the level of incorporation (Figure 6 B; 101-400 g), which is in contrast to this thesis (Manuscript III; Figure 6 B dark red data points). Even though, the SGR was correlated to the level of FM replacement on the individual basis in grow-out turbot (Manuscript III), the meta-analysis data suggest that the level of FM replacement is less relevant in the grow-out phase of turbot than in the juvenile phase. This could allow the turbot farmer to feed a low FM diet in the grow-out phase, and presumably save feed costs without having a relevant decrease in the growth performance.

Supporting the growth results from the meta-analysis, feed utilization was influenced rather by the incorporated protein source than by the level of FM replacement. Feed conversion ratio and protein efficiency ratio were reduced in both juvenile and grow-out turbot when fed the PAP formulations (Manuscript I and III). Variations in the nutritional composition and lower digestibility of PAPs (Davies et al., 2009, Hua et al., 2019, Glencross et al., 2020, Oliva-Teles et al., 2022) might have caused multiplication effects of the level of FM replacement and the ALT incorporation level. In contrast, the apparent digestibility of nutrients and minerals is influenced by the level of FM replacement, as in both trials, the dry matter and crude protein digestibility as well as the digestibility of various minerals was highest in the control group and decreased with increasing level of FM replacement.

The metabolic assessment evaluated if and how the decreased digestibility of nutrients could lead to an energy deficiency in turbot caused by the FM-reduced diets (Roques et al., 2020b). This relationship was only observed in juvenile turbot, where the hepato-somatic-index (HSI) and hepatic glycogen were significantly reduced when the fish were fed the FM-reduced diets (Manuscript I and II). In this context, the 1H-NMR-spectroscopy indicated a reduced metabolic activity due to energy deficiency caused by the diets (Kullgren et al., 2010, Casu et al., 2017, Melis et al., 2017) based on the reduced metabolite concentrations in liver and muscle tissue of juvenile turbot (Manuscript II).

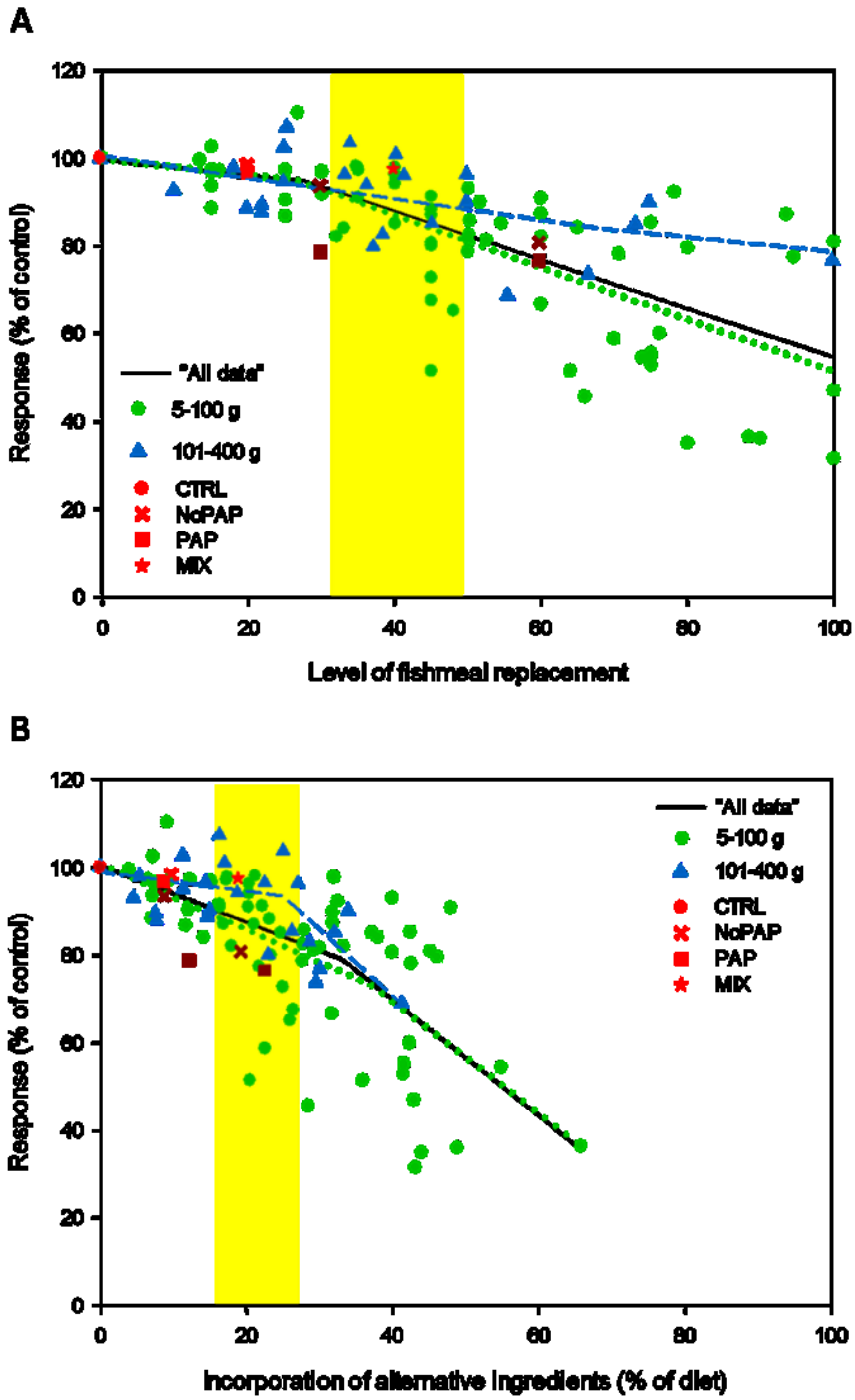

Figure 6 Relationship between the specific growth rate response (% of control) and the level of fishmeal replacement (A) and level of incorporation of alternative ingredients (B) in turbot. Data from the meta-analysis of 30 publications are presented as 2-segenemt-line regression of "All data" points (5-400 g turbot), juvenile turbot (5-100 g; green dotted line), and grow-out turbot (101-400 g; blue dashed line). The yellow area represents the range of the dietary manipulation factor in which these publications found significant change of SGR. Red data points represent means SGR response of juvenile (bright red) and grow-out turbot (dark red) from Manuscript I, II and III fed with the feed formulations: CTRL: commercial-like, NoPAP: plant- protein based formulation, PAP: animal protein based; MIX: balanced mixture of plant based, animal based and emerging protein sources.

The energy deficiency might have been caused by the 9% lower gross energy content in the FM-reduced diets for the juvenile turbot (Manuscript I and II). However, the decreased protein digestibility might have been another driving factor for the energy deficiency, as protein is the main energy source in juvenile fish (Cho and Kaushik, 1990). In grow-out turbot, the decreased protein digestibility (Manuscript III) might not have affected the energy status as lipid becomes more important as an energy source in fish reaching maturity (Cho and Kaushik, 1990). Additionally, the diets in the feeding trial with grow-out turbot can be considered to be isoenergetic (4% difference). Further metabolomic studies in grow-out turbot could help to elucidate the role of fish and alternative proteins in the energy metabolism of turbot.

4.2 What is the most promising formulation concept for turbot farming?

The main goal of making aquafeeds more sustainable is to increase environmental and economic performance (eco-efficient) through the replacement of wild-fish protein with alternative protein sources. This thesis proved that high-quality FM (wild-fish) can be fully replaced with by-product FM without compromising performance of turbot (Manuscript I, II, and III). This is substantial progress for turbot, because turbot diets contain high amounts of protein (Oliva-Teles et al., 2022) and marine ingredients. The nutrient content of by-product FM (low-quality) differs slightly from that of high-quality FM with 10-20% less crude protein content and up to twice as much ash content (Hua et al., 2019). This is expected since the whole fish, fillets and other parts of the body differ in the nutrient composition, and the proportions used to produce the fishmeal will contribute to the nutrient variability (Malcorps et al., 2021).

This thesis demonstrated that plant protein and emerging ingredients are the better alternative protein source than PAPs, resulting in a better growth and feed performance of juvenile (Manuscript I) and grow-out turbot (Manuscript III). Interestingly, the juvenile turbot fed with the MIX diets, had a good overall performance despite higher level of FM replacement (Manuscript II). The balanced mixture of emerging feed ingredients with higher incorporation of insect meal, microbial meals (bacteria, yeast, and microalgae) and less animal and plant proteins, enabled the higher inclusion without negative effects on the growth performance as it was predicted by the model (see section 4.1). These emerging feed ingredients have a good nutritional profile, with high protein content and a similar amino acid profile to high-quality fishmeal (Kroeckel et al., 2012, Makkar et al., 2014, Gamboa-Delgado and Márquez-Reyes, 2018, Hua et al., 2019). Furthermore, they can act as functional ingredients, increasing the health of farmed fish and crustaceans (Refstie et al., 2010, Vallejos-Vidal et al., 2016, Dineshbabu et al., 2019). The ^{1}H-NMR spectroscopy based metabolic profile of liver and muscle tissue, identified the MIX diet as over-spanning the other two formulations, supporting the growth results (Manuscript II). The overall reduced performance of turbot fed with PAP (Manuscripts I and III) was most likely caused by imbalances in the nutritional composition, which were not apparent at the time the diets were formulated. Feed utilization, reduced lipid content of the carcass (Manuscript I) and the reduced betaine concentration in the liver of juvenile turbot (Manuscript II) are indicators for an unbalanced lipid composition or supply of other methyl donors such as methionine (Maruhenda Egea et al., 2015, Roques et al., 2020b). This is in contrast to previous studies where animal by-products (feather meal, blood meal and meat and bone meal) allowed a FM replacement of up to 35% without negative effects on growth in juvenile turbot (< 100 g) (Dong et al., 2016, Cao et al., 2020). However, more research is needed to determine the effects of different animal by-products as protein sources in diets for turbot.

For turbot in the grow-out phase (> 100 g) replacing 30% or more of the FM in the standard formulation with the NoPAP feed ingredients could be a sustainable alternative to the current commercial diets (Manuscript III). Using the NoPAP formulation, in commercial turbot aquaculture can contribute to the sustainable growth of the European aquaculture sector balancing fish (see Manuscript I, II, III), environmental (Maiolo et al., 2020) and economic performance (Tirano et al., 2021). Higher FM replacement might be possible with applying the MIX formulation to diets for turbot in the grow-out phase, increasing the amount emerging feed ingredients (microalgae, microbial biomasses and insect meal). However, the production of the emerging feed ingredients is not at industrial scales, not competitive in price, or do not have a constant quality to be included into commercial aquafeeds (Matos et al., 2017, Hua et al., 2019, FAO, 2022c).

Suitable alternative ingredients for turbot include FM and fish protein hydrolysates from fishery and aquaculture by-products, microbial meals, insect meal, plant protein concentrates, macroalgae, microalgae, salmon oil (from aquaculture by-products), algae oils, and rapeseed oil. Instead of Antarctic krill meal as used in the diets for grow-out turbot (Manuscript III), which sustainable use is questionable (Hewitt et al., 2002); locally sourced by-products from brown shrimp fisheries (small individuals, processing remains) could be more sustainable choice. The brown shrimp processing remains have a good nutritional profile, a high nutrient digestibility (Fricke et al., 2022a) and enhanced growth in white leg shrimp (Fricke et al., 2022b)

Furthermore, this thesis' results suggest focusing on larger turbot giving feeding trials a more practical approach. It is crucial to evaluate the effects of dietary manipulations for the grow-out phase, which makes up approx. 70% of the overall production cycle (FAO, 2022b) and has the highest feed input.

4.3 The real life potential and applicability of the formulation concepts

Moving away from traditionally testing alternative feed ingredients one-by-one, this thesis followed a practical approach of evaluating "ready-to-use" feed formulations for commercial turbot farming. The feeding trials with turbot (this thesis) and other important aquaculture species in the GAIN2020 project (see Petereit et al. 2022a), confirmed that it is possible to produce high-value fish using eco-efficient formulations and with sustainable ingredients that fit into a circular economy framework (Conceição et al., 2021, Pereira et al., 2022).

However, not only the fish performance is important in the success of aquaculture production with sustainable aquafeeds. Supply and price for the raw materials, and other production costs, such as personnel, energy and licensing costs (Maiolo et al., 2020, Tirano et al., 2021) as well as the acceptance of consumers (Manuscript IV) play an important role in the success of eco-efficient diets.

4.3.1 Future of turbot farming in Europe

The NoPAP formulation proved to be a suitable alternative to commercial formulations for turbot (Manuscript I and III) to improve the environmental performance, yet the sustainable growth potential of turbot farming is uncertain. The overall production volume and value is low compared to Atlantic salmon or Rainbow trout (EUMOFA, 2022a) and growth has slowed due to uncertainties in legislative and institutional frameworks restraining the planning the future development of the industry (Fernandez-Gonzalez et al., 2021). Producers prefer good growth performance and product quality, marketing turbot fed with high FM diets (60% marine ingredients) as high quality (Label rouge standards see Aqualabel, 2022, Aubin et al., 2006,). In this case, the NoPAP formulation, high in plant–based protein sources, might not convince producers. Therefore, diets containing high levels of FM and fish protein hydrolysates from by-products could be used instead. Fish-derived proteins from by-products are an economic and environmental option for sustainable aquafeeds (Whiteman and Gatlin, 2005, Bendiksen et al., 2011, Hua et al., 2019) and already make up almost half of the used fishmeal in diets for salmon and marine fish (Naylor et al., 2021). To increase production volume and profitability of turbot farming, Fernández-

González et al. (2022) suggest that differentiation of the product in terms of quality (e.g. Label rouge in France) and orienting small production to local markets (restaurants) and self-marketing (e.g. Seafarm B.V. in the Netherlands). Furthermore, governance and legislative frameworks as well as public support (e.g. funding) are key factors for the success of turbot farming in the EU (Fernández-González et al., 2022).

4.3.2 Acceptance of the consumer for sustainably produced fish

The "*Blue Transformation*" of aquaculture (FAO, 2022a) aims to increase productivity, while reducing environmental impacts and ensuring food security pursuing the UN's SDGs. Eco-efficient aquafeeds can contribute to this sustainable intensification by producing fish with a good environmental performance and that have a good sensory acceptance by consumers (Conceição et al., 2021, Petereit et al., 2022a). However, when looking at the top-5-consumed fish products in the EU (total 10 kg per capita), the majority (7.6 kg per capita) originates from fisheries (tuna, Alaska Pollock, cod, and herring), and only 2.3 kg per capita are of aquaculture origin (salmon) (EUMOFA, 2022a). This apparent consumption was also reflected by the voiced preference for wild-caught fish of consumers in Germany (see Manuscript IV). However, there is a discrepancy between voiced preference and actual consumption (Lopez-Mas et al., 2021), since aquaculture species (Atlantic salmon, Rainbow trout, Gilthead seabream, European seabass, and mussels) dominate the household consumption in Spain, Italy, and France (EUMOFA, 2022a). Aquaculture still has a "bad image" based on environmental concerns (Mazur and Curtis, 2008, Feucht and Zander, 2015) and personal values often associated with animal welfare (Feucht and Zander, 2014, Rickertsen et al., 2017, Cantillo et al., 2021). Furthermore, the price for sustainable seafood will be one of the biggest constraints to overcome, as this is one of the main drivers to purchase a certain product (Zander and Feucht, 2017, Bronnmann and Hoffmann, 2018, Petereit et al., 2022b). To be able to offer fish from sustainable intensified aquaculture, European consumers need to shift their preferences and consumption towards aquaculture products. Furthermore, decreasing seafood consumption in younger age groups due to a shift towards a vegetarian or vegan food choice should be taken into consideration (see Manuscript IV). For this to happen, communication efforts should include emphasizing on the sustainable transition in the aquaculture sector reducing environmental impacts, while increasing animal welfare and promoting aquaculture products for a vegan lifestyle (see Manuscript IV, O'Donncha et al., 2021, Petereit et al., 2022b).

4.3.3 Future-proofness of eco-efficient formulations under current events

A major risk for aquafeed producers are rapid changes in supply chains, affecting availability and price (Glencross et al., 2020) as recently experienced during the COVID19 pandemic, Brexit and the Ukraine-Russia conflict. The COVID19 pandemic had tremendous effects on aquaculture worldwide (Sarà et al., 2022) and on the EU's turbot aquaculture (EUMOFA, 2022b). This highlights the need for local supply chains and the application of a circular economy framework over-spanning the food production sector making eco-efficient formulations competitive to aquafeeds based on imported ingredients. Especially, short-term changes pose a risk to local companies and producers; whereas long-term changes like the climate change are more predictable and allow for adaption to these changes (Hoerterer et al., 2020). Economic reasons are often applied not to implement innovations, and the aquafeed industry is often waiting to be pushed, rather jumping into the future (Glencross et al., 2020). Long-term strategies with ambitious domestic production goals (Pernet and Browman, 2021) for aquaculture are needed to account for "climate change and an increasing gap between future production and consumption" (Froehlich et al., 2021).

4.4 "Feedomics" - the future of nutritional research in aquaculture

Fish and other aquatic foods such as shellfish, crustaceans and algae are considered healthy food due to their essential fatty acids, high protein content and other valuable nutritive substances such as micronutrients. These "blue foods" have the potential to deliver beneficial components to various consumer groups increasing health and well-being and contribute to food security of human populations worldwide (Wei et al., 2022). Especially, the amount and the composition of fatty acids can be influenced trough the fish's diet, contributing to the consumer's health and well-being and making the product more attractive. Taping on this relationship, Cifuentes (2009) defined "*Foodomics as a discipline that studies the Food and Nutrition domains through the application and integration of advanced –omics technologies to improve consumer's well-being, health and knowledge*". Sun and Guan (2018) transferred this approach to agriculture as *"Feedomics" i*ntegrating the –omics approach to the sustainable livestock production. This thesis commenced integrating feed chemistry, fish performance, metabolomics, and nutritional model simulations in a practical approach. Further investigations on the mechanisms how alternative feed ingredients influence the organismic response are crucial to understand the effects and to improve feed formulations in the future. With -omics studies on the rise in nutritional aquaculture research (Balasubramanian et al., 2016, Figueras et al., 2017, Alfaro and Young, 2018, Casu et al., 2019, Carrera et al., 2020, Wei et al., 2021) in combination with nutritional model simulations as developed by Hua and Bureau (2012), "Feedomics" could be used to predict the effects of FM replacement and the incorporation of alternative feed ingredients on the targeted fish species. Furthermore, this would allow reduction of costs per feeding trial; refine the amount of treatments and therefore the amount of fish used in the trials.

5 List of manuscripts and explanation of contributions

Manuscript I: Sustainable fish feeds: potential of emerging protein sources in diets for juvenile turbot (*Scophthalmus maximus*) in RAS

Christina Hoerterer, Jessica Petereit, Gisela Lannig, Johan Johansen, Gabriella V. Pereira, Luis E. C. Conceição, Roberto Pastres, Bela H. Buck

Published in *Aquaculture International*, doi:10.1007/s10499-022-00859-x, 16 March 2022

Contribution of the PhD candidate in % of the total work load (100% for each of the following categories):

Experimental concept and design:	ca. 30%
Acquisition of experimental data:	ca. 65%
Data analysis and interpretation:	ca. 75%
Preparation of Figures and Tables:	ca. 100%
Drafting the manuscript:	ca. 100%

Manuscript II: NMR-based metabolic profile in muscle and liver tissue of juvenile turbot (*Scophthalmus maximus*) fed with plant and animal protein sources

Christina Hoerterer, Jessica Petereit, Gisela Lannig, Christian Bock, Bela H. Buck

To be submitted to *Metabolites*

Contribution of the PhD candidate in % of the total work load (100% for each of the following categories):

Experimental concept and design:	ca. 75%
Acquisition of experimental data:	ca. 60%
Data analysis and interpretation:	ca. 80%
Preparation of Figures and Tables:	ca. 100%
Drafting the manuscript:	ca. 100%

Manuscript III: Effects of dietary plant and animal protein sources and replacement levels on growth and feed performance and nutritional status of market-sized turbot (*Scophthalmus maximus*) in RAS

Christina Hoerterer, Jessica Petereit, Gisela Lannig, Johan Johansen, Luis E. C. Conceição, Bela H. Buck

Published in *Frontiers in Marine Science*, doi:10.3389/fmars.2022.1023001, 14 November 2022

Contribution of the PhD candidate in % of the total work load (100% for each of the following categories):

Experimental concept and design:	ca. 60%
Acquisition of experimental data:	ca. 50%
Data analysis and interpretation:	ca. 85%
Preparation of Figures and Tables:	ca. 100%
Drafting the manuscript:	ca. 100%

Manuscript IV: Informed choice: The role of knowledge in the willingness to consume aquaculture products of different groups in Germany

Christina Hoerterer, Jessica Petereit, Gesche Krause

Published in *Aquaculture*, doi:10.1016/j.aquaculture.2022.738319, 3 May 2022

Contribution of the PhD candidate in % of the total work load (100% for each of the following categories):

Experimental concept and design:	ca. 40%
Acquisition of experimental data:	ca. 30%
Data analysis and interpretation:	ca. 75%
Preparation of Figures and Tables:	ca. 100%
Drafting the manuscript:	ca. 100%

Co-Author Publications associated with this thesis

Petereit, J., **Hoerterer, C.**, Bischoff-Lang, A. A., Conceição, L. E. C., Pereira, G., Johansen, J., Pastres, R. & Buck, B. H. 2022. Adult European Seabass (*Dicentrarchus labrax*) Perform Well on Alternative Circular-Economy-Driven Feed Formulations. Sustainability, 14, 7279,doi:10.3390/su14127279.

Petereit, J., **Hoerterer, C**. & Krause, G. 2022. Country-specific food culture and scientific knowledge transfer events – Do they influence the purchasing behaviour of seafood products? *Aquaculture,* 560**,** 738590, doi:10.1016/j.aquaculture.2022.738590.

6 Appendix

Methodology – Meta-analysis and 2-segment-line regression

The effects of fishmeal (FM) replacement by alternative feed ingredients on the growth of turbot were investigated through a meta-analysis based on standardized growth data. The approach was adapted from Hua and Bureau (2012) and instead of the thermal-unit growth coefficient (TGC) the specific growth rate (SGR) was chosen for the standardized growth performance of turbot. Large temperature variations are uncommon in turbot trials, making it unnecessary to correct the growth with temperature.

The available publications were selected for the following criteria adapted from Hua and Bureau (2012):

a. A control diet containing FM.
b. Experimental diets investigating alternative protein sources to replace FM.
c. The incorporation level of alternative protein sources that were present in the control and experimental diets was corrected show only the increase of the incorporation level.
d. The experimental diets were isonitrogenous and isoenergetic.
e. The SGR was reported or could be calculated from the reported information on initial and final bodyweight and the experimental duration.

The dataset includes 30 studies (see Table A.1 and A.2) with incorporation levels of alternative ingredients in the diets of 0% to 65.8%. The size range of the turbot was from 5 g to 201 g initial body weight and 15 g to 400 g final body weight. Alternative feed ingredients included a variety of meals, protein concentrates, protein isolates and hydrolysates from plants (soybean, corn, cottonseed, wheat, lupine, rapeseed, peanut, pea, sunflower), animal by-products (poultry, feather, meat and bone, blood) and emerging ingredients (insects, brewer's yeast, blue mussel, shrimp, krill). The meta-analysis was performed for the entire dataset, or sub-grouped data according to the size-class. The size-classes were defined by the final body weight into two sub-groups reflecting the size-classes/production phases of turbot used in this thesis: juvenile phase: 5-100 g and grow-out phase: 101-400 g.

The SGR was either taken directly from the original publications or calculated from the reported information on initial and final body weight (BW) and number of feeding days according to the following equation (Charles Bai et al., 2022):

(1) $SGR = 100 \times (\ln(final\ BW) - \ln(initial\ BW))/feeding\ days$

The response ratio for SGR of the mean from the experimental group to the control group (% of control) was used to measure the effects of the proportional dietary manipulation (Hedges et al., 1999). The two factors for dietary manipulation were (1) the level of FM replacement (% of control) and (2) the incorporation level of alternative (ALT) ingredient (% of diet) in the experimental and control groups.

(2) $SGR\ response\ (\%\ of\ control) = 100 \times SGR_{treatment}/SGR_{control}$

(3) $Level\ of\ FM\ replacement\ (\%\ of\ control) = 100 \times (FM\ content_{control} - FM\ content_{treatment})/FM\ content_{control}$

(4) $\text{Level of incorporation of ALT ingredient } (\%\text{ of diet}) = 100 \times (\sum ALT\ ingredient\ content_{treatment} - \sum ALT\ ingredient\ content_{control})/1000$

Where content of the FM and alternative ingredients was standardized to g/kg.

Based on the broken-line model of Robbins et al. (2006), the relationship between the independent factor of dietary manipulation (t) and the dependent SGR response (y) were analyzed by 2 segment linear regression using the SigmaPlot's Piecewise Nonlinear Regression extension (StarCom, 2022) following the equation using T1 as the breaking point of the 2 segment linear regression.

t1 = min(t)

t2 = max(t)

region1(t) = (y1(T1-t) + y2*(t-t1))/(T1-t1)*

region2(t) = (y2(t2-t) + y3*(t-T1))/(t2-T1)*

f = if(t <= T1; region1(t); region2(t))

The resulting values for T1 and the respective R^2 and P-values are presented in Table A.3.

Table A.1 Literature data on response of specific growth rate (SGR) to fishmeal (FM) replacement and incorporation of alternative (ALT) ingredients for turbot in the 5-100 g size-class

Reference	Body weight in control (g)		Fishmeal (g/kg)		Alternative ingredients (g/kg)			Response SGR (% of control)			Level of FM replacement (%)		Level of ALT incorporation (%)	
	initial	final	Max	Min	source	Max	Min	Max	Min	sign.	↔	↓	↔	↓
Bai et al. (2017)	7.6	34.2	600	330	Plant	400	0	-	-	80.7	-	45%	-	40%
Bai et al. (2019)	11.4	50.3	620	182	Plant	426	0	98.1	78.1	89.8	35%	52%	21%	32%
Bian et al. (2017)	5.3	63.1	620	341	Plant	335	0	102.5	91.2	97.2	25%	35%	12%	17%
Bonaldo et al. (2015)	9.7	69.0	500	50	Plant	732	243	91.6	36.0	82.0	30%	60%	16%	33%
Cao et al. (2020)	37.5	81.6	650	130	Plant and PAP	640	20	97.0	35.0	82.1	16%	32%	9%	18%
Chen et al. (2018)	9.6	57.7	680	408	Plant	379	0	-	-	85.0	-	40%	-	37%
Dan et al. (2021)	7.2	46.3	500	275	Plant and PAP	476	21	-	-	67.47	-	45%	-	26%
Dong et al. (2016)	8.6	36.7	620	124	Plant and PAP	741	280	91.0	79.6	81.6	35%	50%	20%	30%
Gu et al. (2017)	8.5	40.2	600	150	Plant	705	280	-	-	85.2	-	75%	-	43%
Li et al. (2019)	-	73.9	600	240	Plant	592	249	97.2	87.2	88.2	30%	45%	15%	23%
Liu et al. (2014)	8.6	39.0	600	240	Plant & Emerg	505	25	97.8	90.7	93.0	40%	50%	32%	40%
Nagel et al. (2012)	32.0	98.4	476	0	Plant and PAP	776	344	84.0	31.5	45.5	33%	66%	14%	29%
Nagel et al. (2014)	32.4	106.2	621	148	Plant & Emerg	721	297	91.0	56.0	63.7	52%	75%	29%	42%
von Danwitz et al. (2016)	17.7	85.3	360	108	Plant	774	548		-	58.7	-	70%	-	23%
Wang et al. (2016a)	5.1	37.7	600	240	Plant	634	317	96.7	66.7	80.0	30%	45%	15%	23%
Wang et al. (2016b)	-	37.8	630	378	Plant & Emerg	452	248	97.8	94.2	96.3	35%	40%	17%	20%
Wanka et al. (2019)	20.2	42.5	463	101	Plant, PAP & Emerg	722	397	-	92.2	n.s.	n.s.		n.s.	
Xu et al. (2016)	9.2	28.8	600	330	Plant	516	266	-	-	72.7	-	45%	-	25%
Zhang et al. (2019)	-	39.2	300	220	Plant & Emerg	634	542	110.3	99.5	110.0	sign. ↑ at 27%		sign. ↑ at 9%	
Zheng et al. (2022)	7	30.8	500	275	Plant and PAP	584	379	-	-	51.4	-	45%	-	21%
Zhou et al. (2016)	-	59.4	600	33	Plant	503	285	93.6	77.4	90.3	15%	25%	7%	12%

PAP: processed animal protein, Emerg: emerging ingredients, sign.: significant different; ↔: not significantly different to control group, ↓: significantly decreased compared to control group, n.s.: not significant

Table A.2 Literature data on response of specific growth rate (SGR) to fishmeal (FM) replacement and incorporation of alternative (ALT) ingredients for turbot in the 101-400 g size-class

Reference	Body weight in control (g)		Fishmeal (g/kg)		Alternative ingredients (g/kg)			Response SGR (% of control)			Level of FM replacement (%)		Level of ALT incorporation (%)	
	initial	final	Max	Min	source	Max	Min	Max	Min	sign.	↔	↓	↔	↓
Bonaldo et al. (2011)	24.4	126.6	550	250	Plant	461	204	97.7	85.0	93.9	18%	36%	19%	25%
Burel et al. (2000)	48.6	122.9	590	370	Plant	460	230	107.1	79.9	87.6	22%	37%	23%	8%
Dietz et al. (2012)	48.0	149.6	748	493	Plant	330	80	103.5	-	n.s.	n.s.		n.s.	
Fournier et al. (2004)	26.0	130.7	400	0	Plant	802	350	96.0	76.0	n.s.	n.s.		n.s.	
Fuchs et al. (2015)	95.8	299.5	585	320	Plant	437	175	-	-	85.2	-	45%	-	26%
Hermann et al. (2016)	73.1	147.5	450	150	Plant and PAP	665	370	96.4	73.5	73.5	33%	67%	15%	30%
Kroeckel et al. (2012)	55.0	139.0	687	8	Plant, PAP & Emerg	876	22	88.4	36.4	88.4	-	20%	-	15%
Regost et al. (1999)	65.9	133.7	520	0	Plant	780	35	100.9	46.9	85.0	40%	73%	17%	32%
Weiß and Buck (2017)	50.0	131.0	300	0	Plant & Emerg	695	395	90.1	76.6	90.1	-	50%	-	15%
Weiß and Buck (2017)	5.1	37.7	600	240	Plant	634	317	96.7	66.7	80.0	30%	45%	15%	23%

PAP: processed animal protein, Emerg: emerging ingredients, sign.: significant different; ↔: not significantly different to control group, ↓: significantly decreased compared to control group, n.s.: not significant.

Table A.3 Breaking points (T1) for two dietary manipulation factors of the response in the Specific growth rate of turbot from different size classes

	All data (N=122)			5-100 g (n=88)			101-400 g (n=34)		
	R^2	T1	P-value	R^2	T1	P-value	R^2	T1	P-value
FM replacement	0.581	27.7	0.0139	0.604	26.7	0.0617	0.445	66.7	0.4709
ALT incorporation	0.570	33.0	<0.0001	0.56	37.3	0.0007	0.533	25.0	<0.0001

FM: fishmeal, ALT alternative ingredient

Questionnaires – Manuscript IV

ICYMARE and EAS questionnaire

ALFRED-WEGENER-INSTITUT HELMHOLTZ-ZENTRUM FÜR POLAR- UND MEERESFORSCHUNG

HELMHOLTZ GEMEINSCHAFT

GAIN Green Aquaculture Intensification

Aquaculture Knowledge Survey

The following questionnaire aims at developing a better understanding of seafood consumption and the role of fish farming. This is important information and your feedback contributes to the EU's effort towards sustainability.

Your responses to this questionnaire are anonymous, and will be treated confidentially. Data will be aggregated and summarized from across all responses and further used for improving knowledge transfer at the science-society interface in the GAIN Project and beyond.

1. How frequently do you eat seafood at home, restaurants and other food outlets?

- At least once a week
- At least once a month but less than once a week
- Several times a year but less than once a month
- Less than once a year
- Never
- Don't know
- Prefer not to answer

2. Seafood can be farmed or originates from wild. What would you say?

- I prefer wild products
- I prefer farmed products
- I have no preference
- I don't know if the products I buy or eat are wild or farmed
- It depends on the type of product
- Don't know
- Prefer not to answer

3. How do you rank your knowledge on aquaculture production?

1 = No experience *Excellent knowledge = 10*

1	2	3	4	5	6	7	8	9	10
○	○	○	○	○	○	○	○	○	○

4. What attitude do you have to aquaculture in general (multiple answers possible)?

- Aquaculture is important for social welfare in the region
- Aquaculture is an economic way to produce seafood
- Aquaculture is environmentally more sustainable then most livestock production
- Aquaculture products are less healthier then capture fishery products
- Aquaculture production is associated with the use of toxins and other chemicals
- Aquaculture is a highly environmentally negative form of producing fish
- Aquaculture is not good for fish welfare, but the only way to ensure seafood availability
- Aquaculture consumes more fish than it actually produces and therefore threatens the oceans.
- Other (Please comment)

This project has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement N° 773330

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greenaquaculture2020

5. **How do you rank the sustainability of fish farming?**

1 = Not sustainable at all *Very sustainable = 10*

1	2	3	4	5	6	7	8	9	10
O	O	O	O	O	O	O	O	O	O

6. **Do you consider yourself living close to the sea**
   - Close (walking distance)
   - Relatively close (by car)
   - Relatively far
   - My country is landlocked
   - Prefer not to answer

7. **What is your level of education?**
   - Primary education
   - Secondary education
   - Further education (e.g. training or vocational education)
   - Higher education
   - Prefer not to answer

8. **What is your gender?**
   - Female
   - Male
   - Diverse
   - Prefer not to answer

9. **What is your age?**
   - Under 14
   - 15-24
   - 25-39
   - 40-54
   - 55+
   - Prefer not to answer

10. **What is your country of origin?**
    - Germany
    - Northern EU
    - Mediterranean/South EU
    - Eastern EU
    - Western EU
    - Non-EU Europe
    - Other
    - Prefer not to answer

Please ask three friends, family members, colleauges etc. to fill the questionnaire on paper or online.

Follow the shortlink
http://bit.ly/ICYMAREGAIN

Or the QR-quode

For more information visit

www.awi.de/maq

https://gain2020.blog/

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Translated questionnaire as distributed by the HIGHSEA scholars

GAIN Green Aquaculture Intensification — AWI ALFRED-WEGENER-INSTITUT HELMHOLTZ-ZENTRUM FÜR POLAR- UND MEERESFORSCHUNG — HELMHOLTZ GEMEINSCHAFT

1. Wie oft essen Sie zuhause, in Restaurants oder bei anderen Lebensmittelausgaben Fisch und Meeresfrüchte?

- Mindestens einmal die Woche
- Mindestens einmal im Monat
- Mehrfach im Jahr aber seltener als einmal im Monat
- Seltener als einmal im Jahr Never
- Niemals
- Keine Angabe

2. Fische und Meeresfrüchte können als Wildfang oder aus Zuchtanlagen im Einzelhandel landen. Welche Art der Produkte bevorzugen Sie?

- Ich bevorzuge natürliche Produkte.
- Ich bevorzuge gezüchtete Produkte
- Ich habe diesbezüglich keine Vorliebe
- Ich weiß nicht, ob die Produkte, die ich kaufe, natürliche oder gezüchtete Produkte sind.
- Keine Angabe

3. Wie schätzen Sie Ihr Wissen über die Produktion von Aquakultur ein?

1 = keine Erfahrung *Expertenwissen = 10*

1	2	3	4	5	6	7	8	9	10
○	○	○	○	○	○	○	○	○	○

4. Wie würden Sie Ihre Einstellung zu Aquakultur beschreiben?

- Aquakultur ist wichtig für das soziale Wohlergehen in der Region.
- Aquakultur ist eine ökonomische Methode zur Produktion von Fischen u. Meeresfrüchten.
- Aquakultur ist ökologisch nachhaltiger als die meisten Produktionen durch Tierhaltung.
- Aquakultur-Produkte sind weniger gesund als Fischfangerzeugnisse.
- Aquakultur-Produktion wird mit der Verwendung von Giften und Chemikalien assoziiert.
- Aquakultur ist eine ökologisch sehr schlechte Methode.
- Aquakultur ist nicht gut für das Wohlergehen der Fische, aber die einzige Möglichkeit, die Verfügbarkeit von Fischen sicherzustellen.
- Aquakultur verbraucht mehr Fische als es tatsächlich produziert und bedroht damit die Meere.
- keine Angaben
- Andere (Bitte kommentieren)

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5. **Wie schätzen Sie die Nachhaltigkeit der Fischzucht ein?**

1 = Überhaupt nicht nachhaltig *Sehr nachhaltig = 10*

1	2	3	4	5	6	7	8	9	10
O	O	O	O	O	O	O	O	O	O

6. **Was bedeutet der Begriff Nachhaltigkeit für Sie?**

7. **Wie nah leben Sie am Meer?**

- Nah (fussläufig)
- Relativ nah (mit dem Auto erreichbar)
- Relative weit entfernt
- Ich lebe in einem Binnenland (von Land umschlossen)
- Keine Angabe

8. **Was ist Ihr höchster Bildungsabschluss?**

- Grund-/Hauptschulabschluss
- Realschule (Mittlere Reife)
- Gymnasium (Abitur)
- Abgeschlossene Ausbildung
- Fachhochschulabschluss
- Hochschulabschluss
- kein Schulabschluss
- Keine Angabe

9. **Welches Geschlecht haben Sie?**

- Weiblich
- Männlich
- Divers
- Keine Angabe

10. **Wie alt sind Sie?**

- Unter 14
- 15-20
- 21-25
- 26-30
- 31-40
- 40-50
- Älter als 50
- Keine Angabe

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