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Doctoral thesis

Sophie Kendler

The holistic utilization of European plaice (*Pleuronectes platessa*)

A study of chemical, nutritional and physicochemical quality

NTNU
Norwegian University of Science and Technology
Thesis for the Degree of
Philosophiae Doctor
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Anything's possible if you've got enough nerve.
J.K.R.

Meinen Eltern,
für ihre bedingungslose Unterstützung
auf meinem Weg zum Doktor.

Abstract

European plaice (*Pleuronectes platessa*) is a distinctive marine flatfish species recognized for its morphology and asymmetrical body shape. This species inhabits the European coastlines, including the Norwegian Sea and shares the largest stocks and fisheries around the Northern Sea. Due to rising water temperatures, it is predicted that European plaice will migrate further north within the next decades, making the Norwegian coastline an attractive habitat area. While it is a highly appreciated fish species among customers in Europe, European plaice remains underutilized in the Norwegian market, presenting an unused potential.

The present thesis investigated the quality and potential for the holistic utilization of European plaice, including fillets as the primary product and its by-product fractions. The primary focus lied on the chemical, nutritional, and physicochemical quality of fillets, including factors affecting these attributes. This involved the investigation of fish caught during different seasons, the assessment of chemical contaminants, and the storage stability during different storage conditions. Moreover, the nutritional profile of European plaice was studied and compared to other demersal fish species inhabiting the same area to evaluate the potential as a nutritional food source. Additionally, potential risks and benefits associated with increased consumption of European plaice fillets were evaluated and compared to other demersal fish species. The by-product fractions skins, backbones, and heads were characterized, and their potential for up-cycling into collagen was assessed using ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE), combined with enzymatic hydrolysis or salt-washing as pre-treatments. Furthermore, the feasibility of European plaice in aquaculture was explored, considering the chemical, nutritional, and physicochemical quality parameters from both female and male individuals.

The results revealed that European plaice presents an excellent nutritional value, with good overall fillet quality in September and December. Moreover, it was observed that European plaice is a valuable source of beneficial compounds such as long-chain polyunsaturated fatty acids (LC-PUFA) as well as essential amino acids

(EAA). Hereby meeting daily intake recommendations for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), considering the consumption of a 200 g portion. Fish caught in April showed lower physicochemical and chemical quality attributes, increased ATP metabolites as well as an accumulation of biogenic amines linked to microbial growth, indicating reduced overall quality during this period. Modified-atmosphere packaging (MAP) was found an ideal convenient packaging solution for retail to maintain high product quality during storage, next to whole fish sold on ice.

Investigations of the by-product fractions of European plaice highlighted the potential for holistic utilization. Extraction of collagen was found particularly efficient for skins, independent of extraction method and pre-treatment, and yielded the highest collagen contents. Considering the comparison of farmed European plaice to wild stocks, the quality was found to be comparably high, suggesting its suitability for aquaculture.

With this PhD study, a significant contribution to generating knowledge and uncovering the existing potential for increased holistic utilization of a fish species found along the Norwegian coastline was made. Hereby highlighting that European plaice is a nutritional food resource. Its beneficial nutritional composition and low potential risks of toxins as well as the possibility to sell in convenient packaging, make it a safe and appealing choice for consumers. Moreover, the utilization of green extraction methods to up-cycle by-products contributes to the green shift within the food and biotechnology industry.

Sammendrag

Europeisk rødspette (*Pleuronectes platessa*) er en marin flatfisk anerkjent for sitt tilltalende utseende og asymmetriske flate kroppsform. Arten er utbredt langs den europeiske kystlinjen, samt i Norskehavet. De største bestandene og de viktigste fangstområdene er i Nordsjøen. På grunn av klimaendringer og stigende vanntemperatur har arten begynt å vandre mot nord til ett nytt attraktivt leveområde, den norske kystlinjen. Mens europeisk rødspette er en høyt verdsatt fiskeart blant kunder i Europa, eksisterer det fortsatt ett ubenyttet potensiale i det norske markedet .

I denne avhandlingen ble kvaliteten og potensialet for en helhetlig utnyttelse av europeisk rødspette undersøkt. Dette inkluderte studier på hovedproduktet (filet) og på biproduktene skinn, ryggrad, og hode. For hovedproduktet (filet), ble den kjemiske, ernæringsmessige og fysio-kjemiske kvaliteten undersøkt, samt faktorer som påvirker disse egenskapene. Dette inkluderte undersøkelse av fisk fanget i forskjellige sesonger, undersøkelse av kjemiske fremmedstoffer og evaluering av lagringsstabiliteten under ulike lagringsbetingelser. Ernæringsprofilen til europeisk rødspette ble undersøkt og sammenlignet med andre bunnfiskarter fra samme fangstområde. I tillegg ble potensielle fordeler og risikoer knyttet til økt inntak av europeisk rødspette evaluert og sammenlignet med andre bunnfiskarter. Biprodukter som skinn, ryggrad og hoder ble karakterisert. For å evaluere potensialet til å utvinne kollagen fra disse fraksjonene ble ultralyd og mikrobølgeassistert ekstraksjon testet, kombinert med enzymatisk hydrolyse eller en saltvasking som forbehandling. Videre ble kvaliteten til oppdrettet europeisk rødspette undersøkt. I denne studien var fokuset å undersøke de kjemiske, ernæringsmessige og fysiokjemiske egenskapene til råstoffet. I tillegg ble kjønnsmessige forskjeller undersøkt.

Resultatene viste at europeisk rødspette fisket i september og desember har en utmerket næringsverdi, med en overordnet god filetkvalitet. Studien viste også at rødspette er en verdifull kilde til ernæringsgunstige forbindelser, som langkjedede flerumettede fettsyrer samt essensielle amino syrer. Når man spiser en 200 g porsjon, vil en oppfylle de daglige anbefalingene for eikosapentaensyre og dokosaheksaensyre. Fisk fanget i april viste de dårligste fysiokjemiske og kjemiske egenskapene, inklus-

ivt en raskere nedbrytning av ATP, samt høyere konsentrasjoner av biogene aminer. En økt konsentrasjon av biogene aminer kan relateres til en raskere mikrobiell vekst, noe som indikerer generelt dårligere kvalitet. Ferske produkter pakket i en modifisert atmosfære (MA) ble funnet å ha det største potensialet for å bli distriburt til dagligvarebutikker. Disse produktene holdt en høy kvalitet, en kvalitet som var sammenlignbar med hel fisk lagret på is.

Undersøkelser av bioproduktene understreket potensialet for en helhetlig utnyttelse av råstoffet. Spesielt, ble det funnet et høyt prosessutbytte av kollagen i fraksjonen skinn, uavhengig av ekstraksjonsmetode og forbehandling. Når man sammenligninger oppdrettet europeisk rødspette med ville bestander, ble kvaliteten funnet å være forholdsvis lik, noe som tyder på at arten har et potensiale innenfor oppdrett. Overordnet bidrar denne doktorgradsavhandlingen til å generere kunnskap om, og til syneliggjøre et potensiale for en helhetlig utnyttelse av rødspette. I tillegg viser funnene at europeisk rødspette er en ernæringsmessig god matressurs. Den gunstige ernæringsmessige sammensetning og det lave innhold av kjemiske kontaminanter, samt en god holdbarhet i forbrukervennlige embalasjeløsninger, gjør arten til et trygt og tiltalende valg for forbrukerne. Et godt utbytte av kollagen produsert ved bruk av miljøvennlige ekstraksjonsmetoder synliggjør et potensielt bidrag til det grønne skiftet innenfor mat- og bioteknologiindustrien.

Preface

The PhD work was conducted from August 2020 to September 2023 as a part of the multidisciplinary project OPTiMAT (Optimal Utilisation of Marine Food Resources), funded by the Norwegian University of Science and Technology (NTNU). This research was carried out at the Department of Biotechnology and Food Science, within the Food Science group under the guidance of Professor Jørgen Lerfall as main supervisor and Associate Professor Anita Nordeng Jakobsen as co-supervisor.

During the PhD work, scientific collaborations were established within the Food Science group (Paper I, II, III and V), between the Department of Chemistry, NTNU (Paper I), the Food Institute at the Technical University Denmark (DTU) (Paper IV) and the Institute of Marine Research (IMR; Havforskningsinstituttet HI) (Paper VI), Austevoll.

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List of Papers

I. **S. Kendler**, D. Tsoukalas, A. N. Jakobsen, J. Zhang, A. G. Asimakopoulos and J. Lerfall (2023). Seasonal variation in chemical composition and contaminants in European plaice *(Pleuronectes Platessa)* originated from the west-coast of Norway. *Food Chemistry 2023 Vol. 401*, 134155. `doi.org/10.1016/j.foodchem.2022.134155`

II. D. Tsoukalas, **S. Kendler**, J. Lerfall and A. N. Jakobsen (2022). The effect of fishing season and storage conditions on the quality of European plaice *(Pleuronectes platessa)*. *LWT 2022 Vol. 170*, 114083. `doi.org/10.1016/j.lwt.2022.114083`

III. **S. Kendler**, F. W. Thornes, A. N. Jakobsen and J. Lerfall (2023). Nutritional profiling and contaminant levels of five underutilized fish species in Norway. *Frontiers in Nutrition 2023 Vol. 10*, 1118094. `doi.org/10.3389/fnut.2023.1118094`

IV. **S. Kendler**, S. M. Pires , A. N. Jakobsen and J. Lerfall (2023). Risk-benefit assessment of five underutilized fish species in Norway. *Journal of Food Composition and Analysis 2023 Vol. 123*, 105642. `doi.org/10.1016/j.jfca.2023.105642`

V. **S. Kendler**, S. M. Kobbenes, A. N. Jakobsen, K. Mukhatov and J. Lerfall (2023). The application of microwave and ultrasound technologies for extracting collagen from European plaice by-products. *Frontiers in Sustainable Food Systems 2023 Vol. 7*, 1257635. `doi.org/10.3389/fsufs.2023.1257635`

VI. **S. Kendler**, O. Yilmaz, A. N. Jakobsen, A. M. Jensen, and J. Lerfall (submitted Sept. 2023). European plaice (*Pleuronectes platessa*) in Aquaculture - Its nutritional, chemical, and physicochemical quality compared to wild stocks. *Submitted to Aquaculture on 13.09.2023; Manuscript number: AQUACULTURE-D-23-03246*

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List of Acronyms

A

a*	Redness
ADP	Adenosine-diphosphate
AI	Adequate intake
AMP	Adenosine-monophosphate
APC	Aerobic Plate Count
Apr	April
AS	Alternative scenario
ATP	Adenosine-triphosphate

B

BF	Breaking force
BRAFO	Benefit-Risk Analysis for Food
bw	Body weight

C

CFU	Colony-forming unit

D

Dec	December
DHA	Docosahexaenoic acid
DIAAS	Indispensable Amino Acid Score
DL	Drip loss
DRI	Dietary reference intakes
DRV	Dietary reference value

E

EAA	Essential amino acids
EAR	Estimated average requirement
EFSA	European Food Safety Authority
EPA	Eicosapentaenoic acid

F

FA	Fatty acids
FAA	Free amino acids
FACF	Fatty Acid Conversion Factor
FFA	Free fatty acids

H

$\mathbf{HBV_{Se}}$	Health benefit value of selenium
Hx	Hypoxanthine
HxR	Inosine

I

IAA	Indispensable amino acids
IMP	Inosine-monophosphate

L

L*	Lightness
LC-PUFA	Long-chain-PUFA

M

MAE	Microwave-assisted extraction
MAP	Modified atmosphere packaged
MUFA	Monounsaturated fatty acids

N

n3	Omega-3 fatty acid
n6	Omega-6 fatty acid
NEAA	Non-essential amino acids

P

PC	Psychrotrophic Aerobic Plate Count
PCBs	Polychlorinated biphenyls
PCr	Creatine phosphate
Pi	Inorganic phosphate
PL	Phospholipids
PRI	Population Rreference intake
PUFA	Polyunsaturated fatty acids

R

RBA	Risk-Benefit Assessment
RDA	Recommended dietary allowance
RWI	Recommended weekly intake

S

SD	Standard deviation
Sept	September
SFA	Saturated fatty acids

T

TAA	Total amino acids
TAG	Triacylglycerols
TMA	Trimethylamine
TMAO	Trimethylamine-oxide

U

UA	Uric acid
UAE	Ultrasound-assisted extraction
UL	Tolerable upper intake level

V

VP	Vacuum packaged

W

WHC	Water holding capacity
WI	Whole fish on Ice
ww	wet weight

X

Xa	Xanthine

CHAPTER

ONE

Introduction

1.1 European plaice (*Pleuronectes platessa*)

A marine bio-resource that has not yet been fully discovered in its quality and potential for utilization.

European plaice belongs to the *Pleuronectiformes* family, characterized by its unique morphology and asymmetrical body shape. The flatfish body is oriented horizontally, having an upper body part and a blindside, which is the bottom-facing side, usually recognized due to its white color. Depending on the habitat, the color of the upper body part can vary. In the case of European plaice, it is marked through characteristic bright red-orange spots on a brown background (Figure 1.1). The habitat of European plaice is along almost the entire European coast, from the White Sea, Northeastern Atlantic Ocean, Northern and Baltic Sea, to Spain and Portugal, including the Western Mediterranean Sea. The fish prefers sandy sediments in the inner continental shelf, usually between 10 to 50 m depth, but an occurrence down to 200 m is reported (Grzimek, 2023).

European plaice is a highly appreciated fish species in Europe but is not of significant relevance to the Norwegian market, hereby demonstrating an unused potential. Therefore, more knowledge on its overall quality and possible influencing factors is necessary to promote its consumption and potential for utilization, both as a wild resource and a species having an aquacultural potential.

Intrinsic and extrinsic factors generally affect a bio-resource's chemical and nutritional quality. For European plaice, seasonal fluctuations in the quality due to e.g. differences in food intake, water temperature and pollutants have to be considered to feature its nutritional potential. Little is known about the feasibility of European plaice for cultivation regarding nutritional, chemical, or physicochemical quality attributes. Knowledge generation about its composition is crucial for consumers to

Figure 1.1: Upper body part of European plaice (*Pleuronectes platessa*).

decide whether this fish is a nutritionally valuable food source.

With an increasing world population and more intensive climatic changes to come, it is more important than ever to treat (marine) foods as limited resources. This emphasizes the necessity to investigate European plaice in its entirety to facilitate a sustainable utilization of the species. By doing so, it is possible to up-cycle fractions that are not considered as the primary product for human consumption. In the end, it leads to an increased value of the resource. With the by-product fraction accounting for approximately 50% of the plaice's total weight, there is an excellent potential to valorize these integral parts of the fish.

1.2 Northern flatfish fishery

Next to Greenland halibut and common sole, European plaice is one of the most important flatfish species consumed in the European Union (EU), and with a significant potential in Norway and Iceland. Even though Norway is among the countries listed in Northern Europe to capture European plaice, the annual catch of 718 tonnes (2018) is relatively minor compared to Denmark (16.916 tonnes) and Iceland (8.342 tonnes) in 2018, with stable capture of the past years. Moreover, the total allowable catches (TAC) of European plaice have not been fully exploited in Northern Europe, and together with a non-specified catch quota for Norway, indicates potential for Norwegian fisheries to increase their share in landings within the Nordics. Next to Denmark, the Netherlands (26.378 tonnes) and the United Kingdom (UK) (12.372 tonnes) account for the leading shares of European plaice supply within Europe. Globally, the EU, the UK and the Nordic countries account for 90% of the supply, highlighting the significance of this species (Viðarsson et al., 2022).

Future predictions on climate change and water temperature of the Northern Sea, being the main fishing area for European plaice, indicate that the decline of European plaice stocks is likely. Forecasts suggest that rising water temperatures, particularly in the southern part of the North Sea, may negatively impact fish reproduction (Hamon G. et al., 2019).

Therefore, future climate scenarios indicate that European plaice stocks will migrate to deeper nursing grounds and move further north (Hamon G. et al., 2019). The gradual migration to habitats further north due to climatic changes was already described more than ten years ago by Engelhard et al. (2011). The study reported the long-term distribution patterns of European plaice in over 90 years from 1913 to 2007, with a migration northwards. This underlines the significance of the predicted scenarios from Hamon G. et al. (2019) and highlights the importance of northern habitats for European plaice in the future. Thus, it can be assumed that the Norwegian Sea will become an attractive habitat for the European plaice. This is a significant advantage of Norway over countries with fisheries centered in the Northern Sea that face the challenge of declining stocks.

CHAPTER

TWO

Background

2.1 Quality

What does the term quality mean in fish and seafood? The term quality is complex and multifaceted and linked to many different attributes. This is why this question needs a comprehensive elaboration, rather than a rigid definition to fully understand how e.g. chemical, nutritional, physical, and microbial aspects collectively influence and define "*high quality*".

The quality of fish is highly linked to the chemical, microbial, biochemical, and physicochemical processes occurring in the fish muscle. Listrat et al., 2016 describe meat quality with four different terms, safety, healthiness, satisfaction, and serviceability, and they are related to various quality aspects. These terms are linked to hygienic quality, including microbial and chemical contamination, nutritional quality, organoleptic (sensory) quality, and ease of use, often referred to as technological quality, also connected to the physicochemical quality of a product. Moreover, textural properties linked to firmness and cohesiveness and correlated to high water holding capacity (WHC) of the flesh are associated with a high fish quality and influence consumer's acceptability (Delbarre-Ladrat et al., 2006; Listrat et al., 2016).

Fish and fishery products are regarded as perishable foods, especially when sold as fresh products. Post-harvest fish undergo different stages, such as pre-rigor, rigor mortis, and end of rigor mortis, which lead to a constant change in the initial quality (Ayala et al., 2010; Cheng et al., 2014). For fish, the decline in quality is moreover linked to three major post-mortem degradation processes occurring during the rigor process, being autolysis, microbial degradation, and oxidation (Freitas et al., 2021; Ghaly et al., 2010). These continuously ongoing quality changes start after slaughtering and are caused by a multitude of closely interrelated biochemical changes, all linked to the three major degradation processes (Delbarre-Ladrat et al., 2006).

After slaughter, oxygen (O_2) supply to the muscle cells stops, leading aerobic glycolysis to cease, usually responsible for generating large amounts of adenosine-triphosphate (ATP) as an energy source for the cells. In a post-mortem muscle, glycogen is used for anaerobic glycolysis, using inorganic phosphate (Pi) to first generate glucose-1-phosphate, which isomerizes to glucose-6-phosphate, the starting molecule for glycolysis. Eventually, this leads to lactic acid and a reduced amount of ATP as end products (Hong et al., 2017; Scheffler et al., 2011). Next to anaerobic glycolysis, the creatine phosphate (PCr) pathway is described to convert adenosine-diphosphate (ADP) and PCr to ATP (Hong et al., 2017). At the same time ATP is no longer used as an energy source and with the gradual depletion of PCr and ceasing glycogen availability, a step-by-step de-phosphorylation and subsequent decomposition of the nucleotide is initiated.

Parallel to the ATP degradation, is the formation of actomyosin from actin and myosin fibers (Cheng et al., 2015). At this point, the ATP breakdown exceeds its biosynthesis, and actin and myosin aggregate to actomyosin, the muscle stiffens, and the rigor-mortis state is initiated (Delbarre-Ladrat et al., 2006; Hong et al., 2017; Scheffler et al., 2011; Wu et al., 2019). Subsequently, endogenous and exogenous enzymes slowly start the autolysis process by breaking down myofibrils and collagen fibers and gradual fillet softening begins (Cheng et al., 2014; Cheng et al., 2015; Wu et al., 2019). These textural changes within the muscle structure directly impact the physicochemical quality of the fillet (Cheng et al., 2014; Delbarre-Ladrat et al., 2006). A schematic overview of the subsequent steps occurring in the fish muscle after slaughter is given in Figure 2.1. Moreover, the nucleotide breakdown is further explained in Section 2.1.1, and consequently provides calculations on the freshness indicators K- and H-value (Equations 2.1, 2.2).

Simultaneous to the above-mentioned processes, post-mortem changes within the macromolecular structure of the fish occur. Fish are composed of water, proteins, lipids, nucleic acid, and negligible amounts of carbohydrates (Venugopal and Shahidi, 1996; Wu et al., 2019). Autolysis-initiated muscle softening is connected to the proteolytic and lipolytic activities of enzymes, which cause protein and lipid degradation. The proteolysis leads to the decomposition of the protein (Delbarre-Ladrat et al., 2006). Hereby, the naturally complex structure of the protein is broken down to polypeptides and amino acids, ultimately leading to free amino acids (FAA) (Ayala et al., 2010; Delbarre-Ladrat et al., 2006; Wu et al., 2019). In addition, similar processes occur in lipids. Lipolysis, initiated by endogenous enzymes such as lipases, hydrolyzes triacylglycerols (TAG) and eventually leads to diglycerol and free fatty acids (Shewfelt, 1981). With ongoing autolysis and protein breakdown into small nitrogenous compounds, pH increases. Thus, microbial growth is stimulated, and the spoilage process is accelerated (Cheng et al., 2015; Wu et al., 2019).

With these ongoing alterations within the biochemical structure of the fish muscle and the beginning of microbial growth (Section 2.1.2), physical and chemical changes (hereafter defined as physicochemical) become more visible. Thus, flesh color, texture and WHC decrease. Moreover, off-flavor and off-odor reduce the sensory properties and ultimately lead to reduced product quality (Ayala et al., 2010; Cheng et al., 2014).

Information on microbial degradation processes related to storage stability is enclosed in Section 2.1.2. At this point, further elaboration on oxidation processes occurring in the post-mortem muscle is omitted, as the practical scope of this thesis work did not include this aspect. Instead, readers are encouraged to consult relevant literature for in-depth reading of oxidation processes in muscle foods (Hematyar et al., 2019; Lund et al., 2011; St. Angelo et al., 1996).

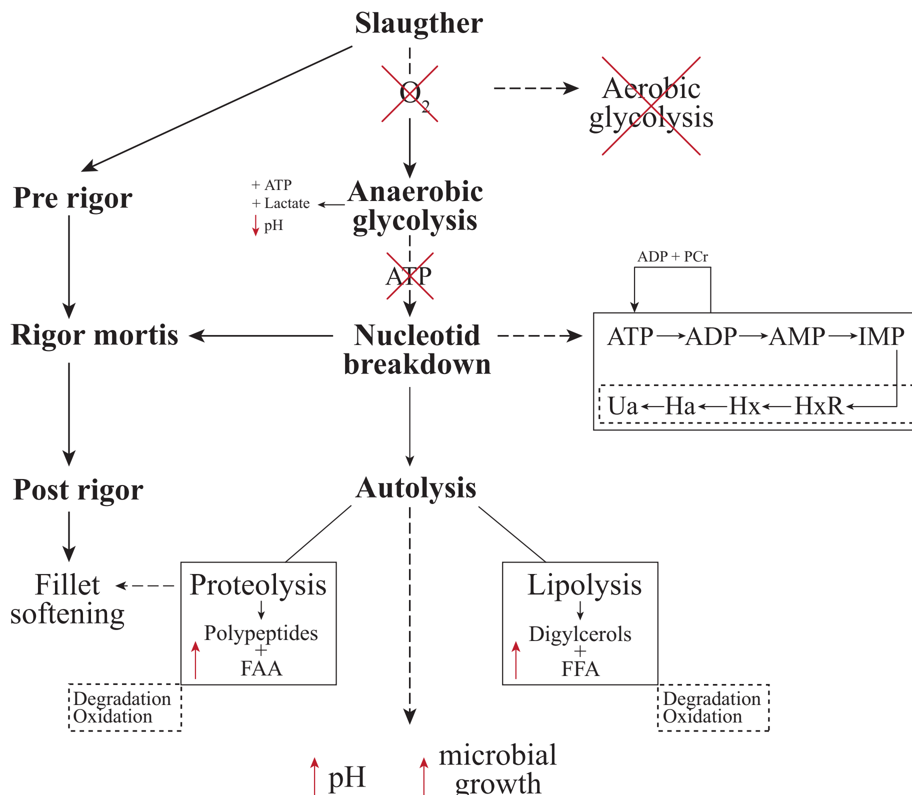

Figure 2.1: Simplified overview of post-mortem reactions in fish muscles. Dashed lines show products signaling significant quality deterioration. Information extracted from Cheng et al. (2015), Delbarre-Ladrat et al. (2006), Hong et al. (2017) and Wu et al. (2019).

2.1.1 Chemical composition

The chemical composition is what defines a food product in general. It includes the structure and availability of compounds present for chemical interactions, which attribute the food to its specific characteristics. Lipids, proteins, water and minerals (ash) form networks and interact with each other at different molecular levels. In this context, macro and micro-components directly affect the quality, safety, processability, and utilization of any type of food (Sethi et al., 2022).

The proximate composition, including proteins, lipids, water and ash within the fish fillet, serves as a reliable basis for an initial assessment of its chemical quality. According to Ahmed et al. (2022), fish fillets in general constitute about 66-81% of water, 0.2-25% of lipids, 1.2-1.5% of minerals (inorganic matter/ ash) and 16-21% of protein. Next to this general division into the four main composites, one has to add that fish can be categorized depending on their fat content, and fish with lower fat content (lean: <2%; low fat: 2-4%) in general show a reversed relationship to the water content, opposed to fattier fish (medium fat: 4-8%; high fat: 8-20%) (Ackman, 1994; Ahmed et al., 2022). Lean and low-fat fish generally comprise a higher water content than fattier fish. For different flatfish species, water contents between 78-82%, protein between 16-21%, lipid between 0.8-4.3%, and ash contents between 0.9-1.5% are reported (Karl et al., 2013).

Moreover, fish contain important chemical elements, some of which are essential for humans. In this context, selenium, copper, iron, zinc, calcium, magnesium, chloride, sodium, and potassium have to be mentioned. These elements can be found in satisfying amounts in fish fillets and present beneficial health effects (Afonso et al., 2013).

During storage, quality changes in the chemical structure are likely to happen. Next to possible protein deterioration and lipid oxidation, the breakdown of nucleotides (ATP) affects the quality characteristics of fish meat. Hereby, ATP, is gradually de-phosphorylated into ADP, adenosine-monophosphate (AMP) and inosine-monophosphate (IMP), which is further broken down to inosine (HxR), hypoxanthine (Hx), xanthine (Xa) and leads to uric acid (UA) as shown in Figure 2.1 (Hong et al., 2017; Huss, 1988). The degradation of ATP and the increase in its relative degradation products are useful indicators for the freshness of fish (Olafsdóttir et al., 1997). Hong et al. (2017) summarize different established values, using ATP and degradation products to indicate freshness and quality for fish. Here, the K-value is widely acknowledged today as one of the most effective freshness indicators concerning the ATP breakdown, which was originally introduced more than half a century ago by Saito et al., 1959. The K-value is calculated as shown in Equation 2.1.

$$K\,[\%] = \frac{[HxR]+[Hx]}{[ATP]+[ADP]+[AMP]+[IMP]+[HxR]+[Hx]} \times 100 \qquad (2.1)$$

In general, K-values of 20% express the supreme quality of fish muscle, implying its freshness, whereas 50% can be regarded as intermediate and 70% as not fresh (Hong et al., 2017; Saito et al., 1959).

Next to the K-value, the H-value has been introduced to relate the ATP degradation with the increased formation of Hx, often linked to microbial spoilage (Section 2.1.2) and can be seen in Equation 2.2.

$$H\,[\%] = \frac{[Hx]}{[IMP]+[HxR]+[Hx]} \times 100 \qquad (2.2)$$

According to Luong et al. (1992), the H-value can be a good taste and freshness indicator for Hx forming species, such as flatfish, including plaice. For these species, HxR is said to be formed only in small amounts, which makes the H-value suitable.

FAA and ATP metabolites are said to be taste-active components in fish and seafood (Sarower et al., 2012). These taste-exhibiting properties are mainly associated with the chemical structure and side chains of the amino acid molecule. According to Diepeveen et al. (2022), side chains, functional groups, hydrophobicity, chirality, or charge can play an important role in the sensory perceptions of amino acids. Two examples are methionine, which can be classified as a hydrophobic amino acid, and phenylalanine, an aromatic amino acid. Both exhibit a bitter taste in seafood (Sarower et al., 2012). Methionine contains a hydrophobic sulfur atom, and phenylalanine contains the ring structure of benzene as a side chain, as illustrated in Section 2.1.3 in Figure 2.3 and 2.4 respectively.

2.1.2 Microbial spoilage

The microbiota of freshly captured fish depends on the microorganisms present in the near environment, and the specific water temperature. This initial microbial community impacts fish's storage stability and spoilage, initiated through microbial growth and metabolism (Anagnostopoulos et al., 2022; Fraser and Sumar, 1998; Ghaly et al., 2010). Especially, the amount of inherent specific spoilage organisms (SSO) present in the microbial community plays a key role in degradation and spoilage (Anagnostopoulos et al., 2022). In this context, *Photobacterium phosphoreum*, *Shewanella putrifaciens*, and *Pseudomonas* spp. are recognized to feature high spoilage activity in seafood (Dalgaard et al., 2006; Ghaly et al., 2010). For European plaice, *Photobacterium phosphoreum* was previously found to be the

primary SSO, independent of external mucosal tissue microbiota, fishing season, and storage conditions (Tsoukalas et al., 2023).

However, the fish muscle itself is initially sterile, and in newly caught fish, bacteria are primarily found on the fish's gills, skins and guts (Ryder et al., 2014; Svanevik et al., 2015). After slaughter, microorganisms can enter the internal system of fish through skins, gills, and body cavities, seeking nutrients to induce growth (Fraser and Sumar, 1998; Ryder et al., 2014). This is accelerated through inappropriate hygiene and safety along the fish production chain. The first threat for microbial contamination is the inappropriate handling and hygiene on board the fishing vessel, followed by insufficient cleaning of contact material like surfaces of processing equipment within the food processing factories (Svanevik et al., 2015). Moreover, storage and processing water can pose a risk of contamination. Here, the autolytical decomposition and breakdown of metabolites and proteins into small molecular weight compounds offer an ideal growth media for bacteria (Fraser and Sumar, 1998).

Chemical changes and breakdown (Section 2.1.1), initiated by autolysis and at some point accelerated by the presence of spoilage bacteria, are closely related to each other. IMP formation is mainly attributed to endogenous enzymes, while the subsequent breakdown to HxR and Hx is initiated by spoilage bacteria (Jeyasanta et al., 2018). The presence of Hx is connected to off-taste, whereas IMP is recognized as a taste-giving component in fish (Luong et al., 1992). This shows how narrow the margin is between a component that exhibits the distinct *umami* taste in seafood to off-flavor related to microbial spoilage (Hong et al., 2017). Both the storage temperature and fish species influence the conversion of HxR into Hx but are also directly affected by the type of spoilage bacteria that is present (Jeyasanta et al., 2018).

Moreover, as mentioned in Section 2.1, FAA are increasingly formed during proteolysis. During storage and ripening processes, FAA are often exposed to decarboxylase-active microorganisms that initiate decarboxylation of FAA into their low molecular weight products biogenic amines which contribute to the typical off-flavor of spoiled seafood (Arulkumar et al., 2023). Hereby, e.g. the amino acid lysine is converted to cadaverine, histidine to histamine, and tryptophan to tryptamine (Visciano et al., 2020). As shown in Figure 2.2, on the example of the formation of cadaverine from lysine, a change in chemical structure, through the removal of the carboxy group leads, from the taste-perception sweet to general fish off-flavor. Histamine-forming microorganisms are e.g. *Enterobacteriaceae*, *Photobacterium* or *Pseudomonas* spp., and in elevated levels, histamine is toxic for humans (Biji et al., 2016; Dalgaard et al., 2006; Visciano et al., 2020).

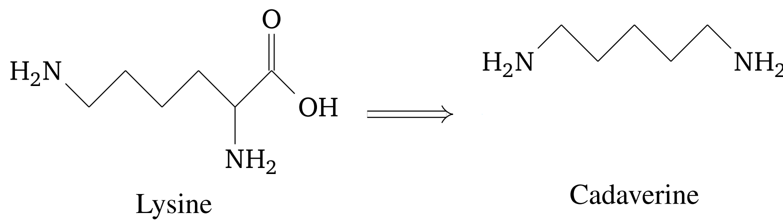

Figure 2.2: Chemical structure of the amino acid lysine and formation of the biogenic amine cadaverine through decarboxylation.

Marine fish muscle is prone to contain trimethylamine-oxide (TMAO), which can further be reduced to trimethylamine (TMA), a reliable indicator for product spoilage (Huss, 1988). *Photobacterium phosphoreum* (and e.g. *Aeromonas, Shewanella,* etc.) can efficiently reduce TMAO present on fish muscle to TMA (Ringø et al., 1984; Ryder et al., 2014). Moreover, it was found that *Photobacterium phosphoreum* is a main driver of reducing TMAO to TMA in white cold-water fish (Dalgaard et al., 2006). The reduction to TMA is facilitated during conditions with reduced O_2, where bacteria make use of TMAO as an electron acceptor for anaerobic respiration, such as in vacuum packaged (VP) or modified atmosphere packaged (MAP) fish products (Boskou and Debevere, 1998; Ryder et al., 2014; Sørensen et al., 2020). If O_2 is present, aerobic respiration is facilitated, and the production of TMA is reduced (Hoel et al., 2022; Sivertsvik et al., 2002).

Packaging can preserve the quality, increase storage stability and prolong the palatability of a product, next to providing convenience to consumers (Noseda et al., 2014). Two types of packaging dominate the seafood industry, which are MAP and VP goods. Through the modification of the carbon dioxide CO_2, nitrogen N_2 and O_2 concentrations in the food package, antimicrobial effects can be obtained (Noseda et al., 2014; Sivertsvik et al., 2002). In vacuum packaging, the oxygen concentration is significantly reduced in the sealed package, which leads to the inhibition of aerobic bacteria. Moreover, in this context, nitrogen is regarded as a filling gas, exhibiting no antimicrobial effects, whereas CO_2 and reduction of O_2 are featured to actively inhibit microbial growth (Boskou and Debevere, 1997; Daniels et al., 1985; Sivertsvik et al., 2002). Building on this, Devlieghere and Debevere (2000) report an antimicrobial effect of dissolved CO_2 on the growth of typical gram-negative (e.g. *Photobacterium phosphoreum, Shewanella putrefaciens*, etc.) and gram-positive (e.g. *Lactobacillus sake, Brochothrix thermosphacta*, etc.) bacteria in seafood at 7°C. Moreover, by including a reduced amount of O_2 in MAP fish products, the formation of TMA can effectively be reduced (Boskou and Debevere, 1997; Hoel et al., 2022; Lerfall et al., 2022).

2.1.3 Nutritional aspects

The nutritional composition and quality of fish are tightly linked to the chemical composition, as discussed in 2.1.1. While the assessment of the composition and possible changes during storage, handling or processing are associated with chemical quality, nutritional quality is linked to the direct impact of the nutritional composition on the human body. According to Wang et al. (2022) the type and quantity of specific nutrients determine the nutritional value of a food item. Therefore, the nutritional composition itself is important to categorize foods according to their value for human health.

Nutritional quality is often associated with *nutritional profiling* (Fulgoni et al., 2009), which includes specific calculations to assess the nutrient density and relative health benefits of the food itself. When talking about nutritional quality and profiling of foods, terms describing the dietary reference intakes (DRI) are inevitable, as they give indices about how much of the nutrient is required to maintain established nutritional levels in humans (Food and Nutrition Board, 2000). The adequate intake (AI), recommended dietary allowance (RDA), tolerable upper intake level (UL) and estimated average requirement (EAR) are commonly used terms to describe the average daily nutrient intake (Food and Nutrition Board, 2000).

Highly relevant for the assessment of nutritional composition and quality of fish is the fatty acids (FA) composition, particularly the distribution of long-chain-PUFA (LC-PUFA) including omega-3 (n3) and omega-6 (n6) fatty acids. Especially fatty fish is regarded as a good source for LC-PUFA. However, consumption of lean fish species can contribute significantly to the daily intake as well (Tørris et al., 2018).

The health-benefit effects of LC-PUFA, especially n3 on the human metabolism are well described in the literature and are ascribed to their roles in physiological, cellular, and molecular processes (Arab-Tehrany et al., 2012; Calder, 2014; Calder and Yaqoob, 2009; de Lorgeril and Salen, 2012; Kaur et al., 2011). The cellular function of LC-PUFA includes their incorporation into lipid rafts in membranes, which increases the fluidity of the membranes and further alters the functions of membrane proteins (e.g. transporters, receptors, signaling enzymes). Through these alterations on the physical properties of the membrane, effects on signaling pathways, which directly influence and modify the gene expression, are initiated (Calder, 2014; Calder and Yaqoob, 2009). This leads to a cascade of positive changes in the metabolism, including e.g. the regulation of blood pressure, platelet function or plasma TAG. These are all linked to the prevention of cardiovascular diseases, described as one of the main preventive effects of n3 next to being anti-inflammatory (Calder, 2014; Kaur et al., 2011; Khalili Tilami and Sampels, 2018).

To exhibit these health-promoting properties, a sufficient dietary intake is necessary. The recommendations set by authorities for the AI of the two most important n3 FA EPA and DHA differ nationally. However, the European Food Safety Au-

thority (EFSA) gives good guidance in setting an adequate dietary reference value (DRV) for EPA and DHA combined. Therefore, consider an AI of 250 mg as appropriate for the primary prevention of diseases for healthy adults (EFSA Panel on Dietetic Products and Allergies, 2010).

Research data on the FA composition is often received as weight% of total FA or total lipids (Nowak et al., 2014), commonly expressed as weight% of fatty acid methyl esters (FAME). To convert FA to the amount available in 100 g meat wet weight (ww) as the edible portion, different conversion factors (XFA) have previously been proposed. Nowak et al. (2014) has compared different conversion factors for marine fatty acids, such as the conversion factors from Weihrauch, Greenfield, and Southgate, as well as Sheppard. The fatty acid conversion factor (FACF) for fish established from Weihrauch et al. (1977) is an internationally appreciated factor, allowing accurate and continuous data (Nowak et al., 2014). It is commonly used and allows easy comparison between literature.

Weihrauch et al. (1977) differentiates between fish containing less than 5 and above 5% lipids. The calculation of the FACF for lean fish (<5% fat content) can be seen in Equation 2.3. TLC refers to the total lipid content, 0.933 to g FA/g lipid and 0.143 to g FA/100 g lipid as defined by Weihrauch et al. (1977). The FACF is obtained in g FA/g lipid.

$$\text{FACF} = 0.933 - \frac{0.143}{\text{TLC}} \tag{2.3}$$

The FACF is then further used to calculate the g FA/100 g fillets, as shown in Equation 2.4. Here, the FACF is multiplied with the results obtained during FA analysis, usually received as FAME, due to the transesterification of FA as preparation for chromatographic analysis.

$$\text{g FA per 100 g fillets} = \text{weight\% FAME} \times \text{FACF} \times \text{TLC} \tag{2.4}$$

By converting FA research values to the actual available amount in an edible portion, it is possible to assess the nutritional quality of the fish. This makes it possible to evaluate the contribution to the AI of EPA and DHA. Next to providing LC-PUFA, fish is appreciated for its valuable amino acid profile, featuring all 20 proteinogenic amino acids. Moreover, fish can be regarded as a nutritious source for essential amino acids (EAA), containing all nine EAA for the human body. The human body is not able to bio-synthesize valine, methionine, leucine, lysine, isoleucine, histidine, phenylalanine, tryptophan and threonine, which makes the dietary consumption of EAA absolutely essential for a healthy living (Huss, 1988; Nollet et al., 2010). Especially, the consumption of lean fish has been attributed to positive health effects such as lowering the metabolic syndrome, reducing the serum TAG, and prevention of type 2 diabetes and cardiovascular diseases (Khalili Tilami and Sampels, 2018).

Moreover, satiety and postprandial circulating TAG are positively altered through the consumption of lean fish (Tørris et al., 2016; Tørris et al., 2018).

The AI for proteins has previously been defined at 0.66 g/kg body weight (bw) per day for the average adult person (FAO and UNU, 2007). In recent years, the requirements of total protein and amino acids for humans were studied broadly and scrutinized the established 0.66 g/kg bw by FAO and UNU (2007). Pencharz et al., 2016 conclude 1.5 to 2.2 g/kg bw per day to be more appropriate, whereas Humayun et al., 2007 state a mean requirement of 0.91 g/kg bw per day and Tian et al., 2011 a range between 0.91 to 1.09 g/kg bw per day. However, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) agrees with 0.66 g/kg bw per day as average requirement and established 0.83 g/kg bw per day as population reference intake (PRI) which can be considered additionally (EFSA Panel on Dietetic Products and Allergies, 2012). Since the protein quality is primarily defined by its EAA composition, a sufficient intake of EAA has been found more suitable than focusing solely on the total requirement of proteins (FAO, 2013).

Hence, to assess the nutritional quality of fish, the distribution of EAA within the total amino acid composition as well as the relative contribution compared to non-essential amino acids (NEAA), is important. For eleven different fish species, approximate values ranging from 47 to 50% for EAA and NEAA, respectively, were previously reported (McLean et al., 2022). Another term used for EAA is indispensable amino acids (IAA), which is used by FAO, 2013 when assessing the AI for healthy adults. The specific values for each EAA are shown in Table 2.1.

Table 2.1: IAA requirements for adults as defined by FAO and UNU, 2007.

Indispensable amino acid requirements in human nutrition		
Amino acids	Requirements	
	mg/g protein*	mg/kg per day
Histidine	15	10
Isoleucine	30	20
Leucine	59	39
Lysine	45	30
Threonine	23	15
Tryptophan	6	4
Valine	39	26
Methionine (+cysteine)	22	14
Phenylalanine (+tyrosine)	38	25

*assuming a mean total protein requirement of of 0.66 g/kg per day.

Cysteine is not regarded as an EAA. However, as a metabolic product of the methionine catabolism, its synthesis is dependent on the presence of methionine and also explains their similarity in chemical structure as can be seen in Figure 2.3 (FAO, 2013). Phenylalanine and tyrosine underlie the same catabolic connection, with tyrosine being the product of the phenylalanine catabolism, thus being dependent on sufficient phenylalanine dietary intake. Both phenylalanine and tyrosine include an aromatic ring in their chemical structure Figure 2.4. The aromatic ring structure leads to distinct flavor formation, which has been described in Section 2.1.1 (Hakimi et al., 2022; Sarower et al., 2012). Other important taste components have been related to marine FAA (Section 2.1).

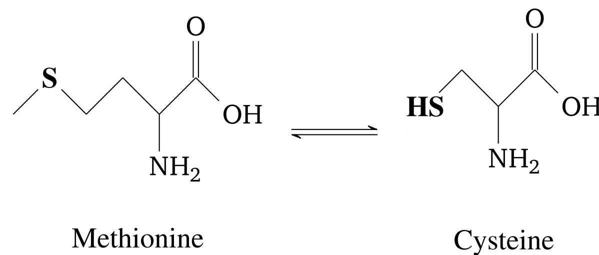

Figure 2.3: Chemical structures of the amino acids methionine, with the distinct sulfur atom and cysteine, with a thiol group.

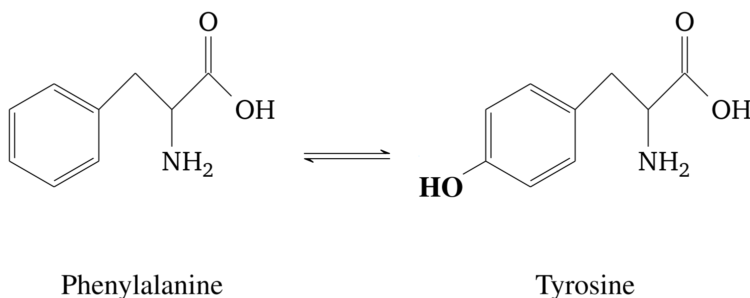

Figure 2.4: Chemical structures of the amino acids phenylalanine, with the distinct benzene ring structure and tyrosine, with an additional hydroxyl group.

With the indispensable amino acid score (DIAAS), the protein quality of foodstuffs can be assessed based on the occurrence of the EAA (FAO, 2013). The amino acid scoring pattern and its individual digestibility combined, build the DIAAS, which is associated with the nutritional requirements set by FAO (2013). Here, the respective digestibility of the amino acids should refer to its true ileal digestibility (FAO, 2013). The FAO (2012) has previously proposed the true ileal digestibility factor for important amino acids in fish, which can be applied in this context or

otherwise the digestibility of the respective crude protein can be used. For fish protein, this factor lies at 91% (FAO, 2012). The calculation for the DIAAS can be seen in Equation 2.5.

$$\text{DIAAS}\ \% = \frac{\text{mg of dietary DIAA in 1 g dietary protein}}{\text{mg of the same dietary IAA in 1 g reference protein}} \times 100 \quad (2.5)$$

Hereby, a high digestible indispensable amino acid (EAA) content that surpasses the dietary needs is what defines a beneficial amino acid content. Generally, the FAO (2013) defines a score below 75% as poor protein quality, 75-100% as good and <100% as excellent protein quality. The protein quality for fish is reported to be excellent (Pyz-Łukasik and Paszkiewicz, 2018; Schaafsma, 2005; Shaheen et al., 2016; Usydus et al., 2009).

Next to the beneficial LC-PUFAs and EAA, other nutrients such as vitamin D, iodine, selenium, or taurine are associated with high amounts in fish (Tørris et al., 2018) and are important indicators for the nutritional quality of fish. Even though micronutrients are only needed in marginal amounts for the human body, they are nevertheless essential for keeping body functions upright and supporting healthy living. Thus, it is important to consider trace elements, including the presence of macro (µg/kg) and microelements (mg/kg), when determining the nutritional profile of foods (Mohanty et al., 2016).

2.1.4 Risk & benefit assessment

The combined holistic view of risks and benefits connected to the consumption of specific foods is called a risk-benefit assessment (RBA) (Watzl et al., 2012). In this context, the Benefit-Risk Analysis for Food (BRAFO)-tiered approach has been introduced through an initiative funded by the European Commission (Hoekstra et al., 2012). This framework allows a stepwise approach and is a useful guide to assess benefits and risks. Furthermore, it allows the comparison to other RBA following the same approach (Hoekstra et al., 2012). An illustration of the BRAFO-tiered approach can be seen in Figure 2.5. Here, the green color visualizes the benefits and the red color the risk factors. In Section 2.1.3 it was highlighted, that the consumption of fish comes along with the intake of beneficial health-promoting compounds. When these beneficial substances are opposed to potential risks arising through consumption, a meaningful comparison of human health risks and benefits concerning the consumption of a certain fish is possible (Thomsen et al., 2021; Watzl et al., 2012). Potential risks are described in Section 2.2.1 and are mainly ascribed to environmental and chemical toxins. In the initial step of an RBA, the potential benefits and risks need to be identified. In this context, the great majority of RBAs that assess the risks and benefits of eating fish, focus on the positive effects of EPA,

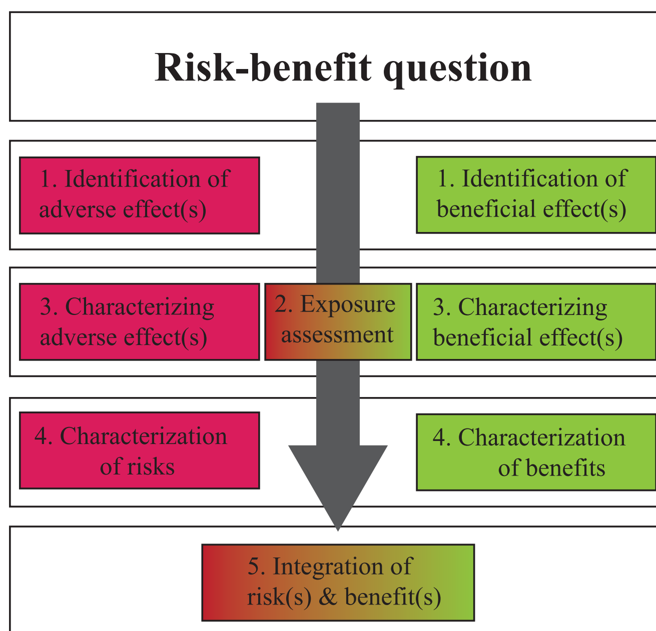

Figure 2.5: The steps of the BRAFO-tiered approach as described by Hoekstra et al. (2012) and Watzl et al. (2012).

DHA, and selenium (Se). These compounds are then opposed to methylmercury (MeHg), dioxins and dioxin-like polychlorinated biphenyls (PCBs) (Afonso et al., 2013; Gao et al., 2015; Prato et al., 2019; Reyes et al., 2017; Sofoulaki et al., 2019; Strandberg et al., 2016).

An example of directly opposing benefits to risk factors is the calculation of health benefit value of selenium (HBV_{Se}) and Se:Hg molar ratios (Ralston et al., 2016; Ralston et al., 2019). MeHg is known for its neurotoxicity and moreover antagonistic effect on Se (Farina et al., 2011; Khan and Wang, 2009). Se, on the other hand, is essential for selenoenzymes, which feature protective characteristics against oxidative stress (Ralston et al., 2016). By calculating HBV_{Se} and Se:Hg molar ratios, the net benefit vs. net risk of these two compounds is assessed (Ralston et al., 2016; Ralston et al., 2019).

Another example is, to oppose the above-mentioned beneficial effects of EPA and DHA to detrimental effects of dioxins and PCBs. Here, it is possible to calculate the net health-promoting effects of the two n3 FAs and the net risk of cancer caused by elevated levels of dioxins and PCBs in the fish (FAO/WHO, 2011).

With the RBA framework and the BRAFO-tiered approach, it is possible to give guidance for setting dietary recommendations, including advice on portion sizes related to safe consumption (Hoekstra et al., 2012; Watzl et al., 2012).

2.1.5 Physicochemical parameters

In food science, physicochemical quality refers to the combined, interacting physical and chemical properties of foods. Here, the chemical composition, described in Section 2.1.1, plays a major contribution. The amount and distribution of water, proteins and lipids in the fillet affect physical properties (Castro et al., 2021; Lazo et al., 2017). The terms physico-chemical and physio-chemical are used interchangeably and both include the following properties: mechanical, optical, thermal, rheological and hydration properties (Igual and Martínez-Monzó, 2022).

For fish fillets, hydration properties, including the water retention capacity, also referred to as water holding properties, are critical. Here, a high WHC is an important quality parameter and opposes the drip loss (DL), which is undesirable. The WHC and DL of fish flesh depend on the chemical state, the water is present in the muscle. A high amount of immobile and free water leads to an increased DL (Chan et al., 2022).

Moreover, mechanical properties, comprising texture attributes are relevant for good fillet quality. Texture is affected by post-mortem enzymatic and chemical reactions, which can lead to softening, and changes in the elasticity or toughness of the fillet (Cheng et al., 2014), as previously mentioned in Section 2.1. A firm fillet texture is preferred for fish flesh and is moreover regarded as an important sensory attribute (Listrat et al., 2016). The comparison of textural properties between species is challenging, due to fillets usually being not uniform in their shape and thickness. However, the texture profile analysis is recognized as a useful approach to give information about firmness, thickness, springiness or elasticity, among others (Cheng et al., 2014; Coppes et al., 2002). Texture profile analyses are often accompanied by using a quantitative descriptive analysis. Hereby, it is possible to compare sensory analysis with data from the physical measurements.

The visual perception of foods is extremely relevant for consumers and an important quality parameter. This is defined by the optical properties, including color appearances (Igual and Martínez-Monzó, 2022). For lean fish, including flatfish species, a white and uniform fillet color is regarded as favorable (Roth et al., 2009). Fillet color can be affected by the choice of capturing methods, pre- and post-mortem handling and storage. Crowding of fish in the net during capture can lead to bruising patterns. In yellowtail flounder, this was found to cause discoloration of the fillet, due to bursting blood vessels, leading to red spots on the fillet. Eventually, it ends with an unappealing fillet for consumers and, causes rapid quality deteriorations (Kenney et al., 2015). In addition, insufficient bleeding can significantly alter the fillet color and quality. It is recommended that exsanguination is carried out rapidly after death and was reported to be ideally within 30 minutes after death for Atlantic cod (Erikson et al., 2021; Olsen et al., 2014b).

2.2 Factors affecting quality

Some factors affecting fillet quality have been touched upon in the sections above, especially related to pre- and post-mortem handling and storage. The following Sections 2.2.1 and 2.2.2 will focus on factors, not directly impacted by human influence.

2.2.1 Extrinsic factors

Extrinsic factors describe external conditions that directly influence the living fish. This is especially related to the near surroundings and habitat area of the fish. It includes fluctuating water temperatures, salinity and daylight, linked to the seasons as well as feed availability (starvation) and pollutants, such as environmental and chemical contaminants (Birnie-Gauvin et al., 2017; Hiddink and Coleby, 2012; Huss, 1988). Birnie-Gauvin et al. (2017) report that these extrinsic factors can contribute to increased oxidative stress and altered metabolism within the fish. The microbial quality and spoilage indicating products (e.g. total volatile basic nitrogen) and free fatty acids (FFA) can differ throughout seasons and are often most contrary between winter and spring/ summer (Cardoso et al., 2021; Durmuş et al., 2014). The chemical composition, particularly the lipid deposit and FA profiles, influence juvenile fish in varying water temperatures and, moreover, impact growth rates in farmed fish (Rotabakk et al., 2018; Tidwell et al., 2003). According to Huss (1988), the lipid composition shows high fluctuations around the year, with especially low contents around the spawning season of the specific fish species. Moreover, species-specific differences in the fluctuation of protein, lipid and FA contents of wild fish around the year are reported (Aidos et al., 2002; Celik, 2008; Gökçe et al., 2004; Küçükgülmez et al., 2010; Suárez et al., 2015). In addition, the location in northern countries like Norway can impact quality parameters such as DL, WHC or water content due to increased daylight up north compared to southern parts, with simultaneously inverted water temperatures (Rotabakk et al., 2018).

In addition, environmental and chemical toxins are other extrinsic components, affecting the quality and directly impacting consumer safety. The many beneficial effects of fish consumption might be compromised due to elevated concentrations present in the living environment of the fish (Rasmussen et al., 2005). Deposits of mercury (Hg) can be traced back to both natural and anthropogenic sources and, once released, start to react and transform. Its most toxic form for humans is MeHg, and demersal fish species such as European plaice, as well as predatory fish, can accumulate high amounts (EFSA, 2012; Parolini et al., 2020; Rasmussen et al., 2005). Both Hg and MeHg accumulate in the fish muscle and bind to proteins (Bosch et al., 2016). Next to MeHg, arsenic, lead and cadmium are harmful compounds that can occur in fish (Bosch et al., 2016). Persistent organic pollutants are a group of chemical toxins previously applied in industrial applications and, due to their

persistent character, circulate and bioaccumulate in nature. These chemical toxins include PCBs and dioxin-like PCBs, which typically accumulate in the adipose tissue of fish due to their lipophilic character (Zhang et al., 2012). Next to location, the fat content and size of the fish influence the concentration within the fillet. In addition, intestines, such as the liver as a lipid storage, are prone to store higher concentrations than fillets (Karl and Lahrssen-Wiederholt, 2009).

2.2.2 Intrinsic factors

Intrinsic factors, on the other hand, are related to the fish itself, including the species, gender, age, maturity and reproduction, as well as the spawning cycle of the fish (Birnie-Gauvin et al., 2017). Female and male European plaice differ significantly in their quality and physical conditions throughout the spawning cycle. Female fish lose more weight during spawning than male fish during the reproductive period, and their energy devoted to gonad development is higher. Moreover, female fish are bigger compared to males when they reach maturity (Bromley, 2000). While female fish are bigger in size, it is important to note that males typically mature earlier, starting at age two, compared to females, who typically begin to mature at age three (Grzimek, 2023). The allocated time for the spawning period is stated differently in sources and depends on locations: From earliest December to March in the Northern Sea, February to March in Danish waters, March to April in Icelandic waters according to Grzimek (2023) and peak spawning season in the Northern Sea from January to March according to Bromley (2000). Post-spawning females need time to recover until they regain high quality connected to fillet yield, flesh color and texture (Ahongo et al., 2021; Bromley, 2000).

2.3 Holistic utilization of fish raw material

In past decades, research has focused a lot on the characterization and on solutions to utilize the whole biomass of fish. Hereby, a lot of knowledge on by-product fractions has been gathered so far (Ferraro et al., 2010; Hassoun et al., 2023; Rustad et al., 2011; Siddiqui et al., 2023). By-products usually comprise fish heads, bones, scales, skins, intestines and, depending on the product, process trimmings (Ozogul et al., 2021). The potential to utilize and up-cycle these fractions into products for livestock, pets or, in the best case, human consumption is significant. However, so are the challenges to enforce a circular approach in the food and biotechnology industries.

2.3.1 Challenges and potentials

For by-products to be suitable for human consumption, the fish processing industry must adapt its infrastructure to specific standards to fulfill given require-

ments. Food regulatory authorities demand a high quality and safety standard, once products are meant for human consumption (Nawaz et al., 2020). Olsen et al. (2014a) state that the implementation of good manufacturing practices (GMP) and Hazard Analysis and Critical Control Point (HACCP) control systems are challenging for existing industries. Changing and adapting processing structures is often connected with additional expenses, as refining by-products requires many additional steps (e.g. extraction of compounds), which in the end is often not economically feasible by today's technology (Caruso et al., 2020).

Next to these challenges, product attributes related to sensory and nutritional quality, also in connection with introducing compounds into different food matrices and possible interactions with other ingredients, have to be considered (Nawaz et al., 2020; Ozogul et al., 2021). Moreover, the bioavailability after digestion of extracted compounds can pose a challenge and is a subject of research.

The potential of fish by-products is attributed to their health-promoting compounds with great nutritional profiles and good functionality. Moreover, these compounds can find utilization in different branches and applications, such as the food and nutraceutical industry, from fortified food products and supplements to skin care and medical products (Mutalipassi et al., 2021). The protein fraction of by-products can be refined to generate bioactive peptides, collagen, or gelatine (Abuine et al., 2019; Gao et al., 2021; Ozogul et al., 2021). The lipid fraction can be up-cycled to produce high-quality n3 oil and supplements enriched with EPA+DHA (Mutalipassi et al., 2021; Pateiro et al., 2021).

In addition, skins, as a valuable fraction with high amounts of collagen, have been spotlighted lately. Here, research was previously also conducted on flatfish skins to up-cycle bioactive peptides (Fang et al., 2017; Hua, 2013). Collagen has multifaceted applications and offers many beneficial properties that are attributed to its ability to restore and support the physiology and tissue healing of skin cells (Alves et al., 2017; Mutalipassi et al., 2021; Sibilla et al., 2015). The digestibility and bioavailability of bioactive peptides can be enhanced with modern techniques. Here, nanoencapsulation is a targeted technology with promising features to support the exhibition of the attributed health-promoting effects (Aguilar-Toalá et al., 2022; Chatterjee et al., 2022; Hanachi et al., 2022; Hosseini et al., 2017; Hosseini et al., 2021).

2.3.2 Green side-stream processing methods

According to Armenta et al. (2019), the focus on green analytical chemistry started already in 1995, with subsequent development of alternative methods to reduce the environmental impact. Armenta et al. (2019) summarize five critical points, that have to be considered for green sample preparation and extraction, being 1) miniaturization, 2) automation, 3) energy saving, 4) consumable saving,

and 5) waste treatment. In other words, the idea is that through scale-down (1) and automated routines (2), the amount of reagent, solvent and required samples (4), as well as the amount of waste produced (5), is significantly reduced and hereby increase the method's environmentally friendliness (Armenta et al., 2019). According to Chemat et al. (2012), the definition and purpose of green extraction methods for natural products are to focus on developing extraction methods that require less energy, including the use of renewable natural resources and alternative solvents, and produce extracts and products that are both safe and of a satisfying quality.

In this context, different principles should be followed to guarantee a green process (Chemat et al., 2012). Concerning the extraction of marine bio-materials derived from by-products, enzymatic hydrolysis has been applied and optimized widely in the past (Ivanovs and Blumberga, 2017; Rustad et al., 2011). Today, technologies such as pulsed electric field, high-pressure, ultrasound, microwave, supercritical fluid or deep eutectic solvent extractions are gaining more attention (Chemat et al., 2019; Mohamad Razali et al., 2023; Pal and Suresh, 2016; Tiwari, 2015). Both ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) can be used without solvents, making them highly attractive due to significant waste reduction (Chemat et al., 2019). Given the relevance of UAE and MAE to the research conducted in this thesis work, the subsequent paragraph will briefly explain the principles of these two extraction methods.

The main effect behind UAE is acoustic cavitation, a phenomenon caused by ultrasonic waves. The term describes the effects of the alternating pressure fluctuations in a liquid medium, which leads to the generation, growth, and implosive collapse of microbubbles in liquids exposed to ultrasound waves (Tiwari, 2015). Acoustic cavitation is the primary factor that drives the extraction effects of sonication. The bubble implosion during UAE leads to a sudden increase in temperature and pressure, ultimately disrupting the membrane and cell walls. Thus, the solvent can diffuse into the sample structure and effectively transfer the target compounds into the solvent matrix (Chemat et al., 2017; Mohamad Razali et al., 2023; Tiwari, 2015). MAE operates with electromagnetic waves that lead to heating and evaporation of the water in the sample core and hereby disrupt the cell walls. The heat transfer leads to intermolecular and intramolecular deformations and releases the compound, in principle similar to UAE. The target compound can dissolve in the solvent and consequently be extracted (Destandau et al., 2013; Mohamad Razali et al., 2023).

CHAPTER
THREE

Research Objectives

The overall aim of the present thesis was to perform a comprehensive and holistic investigation of wild European plaice, which involved studying factors affecting its quality. Hereby, the fillet quality, including chemical, nutritional, and physicochemical aspects, was studied. Additionally, the product's stability under different storage conditions, the nutritional profile as well as potential risks and benefits linked to an increased consumption of European plaice were investigated. The final part of the thesis included the study of the by-product fractions to evaluate their potential for extraction of collagen to follow a holistic utilization of European plaice. Moreover, the feasibility of utilizing European plaice in aquaculture based on an evaluation of the fillet quality as compared to wild fish was assessed.

The specific research objectives were covered in Paper I-VI and are as follows:

I. To study the chemical and nutritional composition, including environmental and chemical contaminants and the physicochemical quality of European plaice during three different fishing seasons.

II. To investigate the effect of two different fishing seasons and three different storage conditions on the physicochemical and microbial quality and the breakdown of metabolites of European plaice.

III. To evaluate the nutritional profile and contaminant levels of European plaice and compare it to four other demersal fish species, all captured in the same habitat area. In this context, to assess the contribution of potential health-promoting components and relate to dietary reference values to conclude its nutritional potential.

IV. To assess risks and benefits coming along with increased consumption of European plaice and four other demersal fish species and feature dietary suggestions for these.

V. To characterize by-product fractions derived after filleting of European plaice and to investigate the potential of green extraction methods to up-cycle these fractions to generate collagen.

VI. To study the nutritional, chemical and physicochemical composition of farmed European plaice, including both female and male individuals and to compare the quality with wild fish stocks.

CHAPTER
FOUR

Results and Discussion

The amount of protein, lipids, moisture (water), and inorganic matter defines and highly influences the nutritional value of foodstuffs in general. When considering fish for human consumption, the proximate composition, but also the distinctive nutritional composition of fats and proteins with their specific unsaturated and saturated fatty acids as well as essential and non-essential amino acids, is of great relevance (Ahmed et al., 2022). Intrinsic and extrinsic (external) factors can affect the proximate and nutritional composition, physicochemical attributes as well as storage stability. Thus, directly affect the quality and, in the end, have an impact on consumer choices. Especially maturity and sex (intrinsic), as well as environment and season, including feed availability, water temperature, microbiota and pollutants (extrinsic), affect the overall quality of fish (Birnie-Gauvin et al., 2017).

The presented research, which is centered around the quality attributes of European plaice and factors affecting these, is further discussed in the following sections. Hereby, seasonal variations were examined, which come along with changes in the food composition and availability in winter, changing water temperatures, microbiota, and environmental pollutants. Moreover, sex (including maturity) and family, as well as species (European plaice vs. other demersal fish), were considered as intrinsic factors. Moreover, different storage conditions were chosen to find an optimal and safe retail solution for European plaice. The focus hereby lies on the possible effect on the overall quality and how these factors affect the potential for increasing the utilization of European plaice as a nutritional food resource. An overview of the research areas covered in Papers I to VI is shown in Figure 4.1.

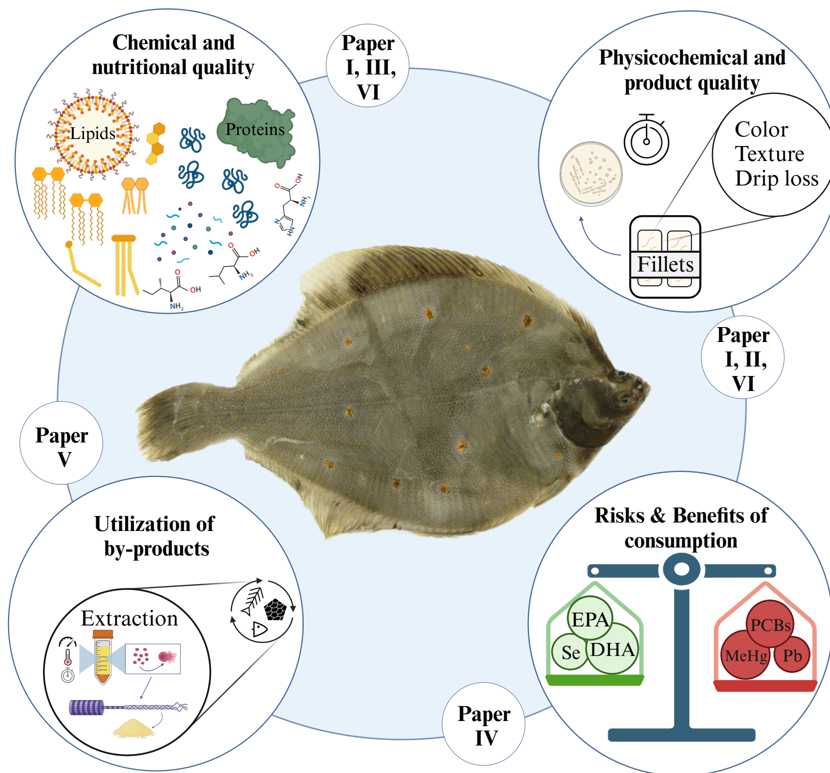

Figure 4.1: Graphical overview of the project work, showing the holistic approach followed throughout the PhD project. The investigated areas are covered in Papers I to VI. Created with BioRender.com

4.1 Proximate composition

The proximate composition of fish depends on species, size, maturity as well as season (Ackman, 1994). In this thesis work, respective analyses on wild or farmed European plaice and other wild demersal fish species were conducted on the fillets. As a first basis to evaluate fillet quality, the proximate composition was investigated.

The present work found changes in the proximate composition depending on the season (Paper I), between sexes, and farmed vs. wild European plaice (Paper VI). Wild European plaice showed significantly higher fillet lipid contents in December (Dec) compared to the very lean fish caught in April (Apr) (Paper I). In addition, the low lipid content in post-spawning (Apr) goes along with a significantly lower protein content (compared to September (Sept)) and significantly higher water content (compared to Sept and Dec) (Paper I). The water content of wild European plaice was significantly affected by seasons (Paper I) and correlated with the water

holding capacity (WHC), as highlighted in Paper I. An inversed relationship of lipid and water content was previously observed in common sole (*Solea solea*) caught along the Mediterranean coast during the four different seasons of the year (Gökçe et al., 2004). When comparing wild vs. farmed immature halibut (*Hippoglossus hippoglossus*) throughout different seasons, Olsson et al. (2003) found greater fluctuations in the proximate composition in farmed fish. However, in the study by Olsson et al. (2003), wild captured halibut was not followed throughout the whole year, unlike studied farmed individuals. The fluctuations within the proximate composition as well as overall fillet quality of fish can depend on its life-cycle stage, but also on the season, connected to feeding and starvation, daylight and water temperature (Foss et al., 2009; Olsson et al., 2003; Rotabakk et al., 2018). Post-spawning, female fish need time to rebuild muscle mass, to regain satisfying filleting yield and quality, which is connected to the proximate composition, texture and other quality attributes (Ahongo et al., 2021; Bromley, 2000). It can be assumed that most European plaice individuals investigated as part of this thesis, were in the post-spawning state in Apr, with the peak spawning time being January to March (Bromley, 2000; Rijnsdorp, 1989). This can be correlated to a significantly smaller fish size in European plaice captured in Apr, compared to Dec (0.93 kg±0.34 vs. 1.23 kg±0.40). However, in the present thesis work, wild European plaice was not separated according to sex, nor age/ maturity status. Thus, only assumptions correlating fillet quality related to maturity can be made.

Moreover, the presented results suggest that the sex of farmed European plaice individuals has a strong impact on the proximate composition, as differences were observed in the protein, lipid, water and ash contents between genders (Paper VI). Interestingly, next to sex, the factor family shows to influence the protein and water content of farmed fish but not the lipid and ash content. Future research might be necessary to study the effect of family on the proximate composition of farmed European plaice. Previous studies reported higher lipid but lower water contents of farmed fish when compared to wild individuals of the same species (Fuentes et al., 2010; Olsson et al., 2003; O'Neill et al., 2015; Rodríguez-Barreto et al., 2012). This is in accordance with observed differences in the proximate composition in Paper VI and indicates that the constant food supply and controlled environment for reared fish throughout the year influences their proximate composition (Olsson et al., 2003). Thus, farmed European plaice shows, on average, significantly higher lipid contents compared to those captured wild. Specifically, differences were found for farmed European plaice compared to those captured wild in the Sept and Apr catch but were not different compared to Dec. A significant correlation between lipid and water content ($r = -0.629$; $p < 0.001$) for wild European plaice was found in the smaller Dec group (n=7) presented in Paper VI, but not in the entire wild captured population of Dec (n=21; Paper I). Moreover, no correlations were

found for wild captured European plaice for the two other seasons (Sept, Apr), and also not for female or male farmed individuals. This indicates no observed relationship between the lipid and water content in the European plaice sampled as part of the present work. Zhang et al. (2014) found an inverse relationship between lipids and water for nine freshwater fish species and calculated the relationships between the lipid and water contents of fish previously examined in other studies. In the study from Zhang et al. (2014), relationships were found between different fish species. However, not within one fish species, and the correlation between lipid and water contents is generally stronger in fattier fish than lean species.

When comparing European plaice with other flatfish species, European plaice can be seen as relatively similar in its proximate composition, with comparable protein content to flounder and lemon sole as well as corresponding lipid contents to flounder and megrim (Paper III). Barbosa et al. (2018) report similar results of the central fillets of megrim, compared to results obtained in Paper III. However, observations made in this thesis work show that wild European plaice has significantly higher ash contents than other investigated wild captured flatfish species (Paper III), with the highest ash contents in Sept, compared to Dec and Apr (Paper I). Karl et al. (2013) reported ash concentrations of around 0.9% for European plaice, being lower than values featured in Paper I (1.07% Dec, 1.17% Apr and 1.28% Sept) and in Paper III (1.25%). Values of 1.1 to 1.5% of other flatfish studied by Karl et al. (2013) are within the range of the values reported for European plaice in the presented thesis.

4.2 Nutritional quality

As previously mentioned, the nutritional value of fish is largely determined by the amount of essential amino acids (EAA) in comparison to non-essential amino acids (NEAA) as well as the amount of important polyunsaturated fatty acids (PUFA) compared to saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) (Sarma et al., 2013; Wang et al., 2022). Nutritional profiling as described in 2.1.3 was partly applied in Paper III. Here, calculations on the fatty acids (FA) composition and assessment of the contribution of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) to the daily requirements, as well as for the amino acid profile of five different demersal fish species including European plaice, were conducted. Moreover, in Paper IV, values obtained through nutritional profiling were utilized to calculate health benefits and compare them to health risk factors.

4.2.1 Amino acids

Fish muscles are an important source of amino acids, and the composition and distribution of amino acids within the protein structure are highly important to evaluate the nutritional quality of fish and other foodstuffs (Sarma et al., 2013). The

distribution of total amino acids (TAA), including the ratios of EAA vs. NEAA, gives important information about the nutritional quality of fish.

To objectively evaluate the protein quality, the FAO (2013) introduced the indispensable amino acid score (DIAAS), which can be used to calculate the distinctive amino acid profiles and was applied in Paper III. Combining the DIAAS with the amino acid requirements in human nutrition established by FAO and UNU (2007) revealed a DIAAS of 110% for fillets of wild European plaice (Paper III). Moreover, the findings in Paper III show that a 200 g portion of European plaice covers the total required daily amount of EAA for a 80 kg person to 123%, despite covering phenylalanine only to 53%.

The TAA distribution was investigated throughout the seasons, and results have shown only minor variety between wild captured European plaice in Sept, Dec, and Apr (Paper I). No differences were determined for the total EAA composition between seasons. However, significantly higher amounts of the branched-chain amino acids leucine, lysine, and valine were found for Apr samples (Paper I). Higher amounts of the three listed amino acids were also observed in spring samples of zander (given in g/100 g) by Çağlak and Karsli (2017). Moreover, the TAA distribution of wild European plaice differs significantly to farmed individuals for 12 of the 16 investigated amino acids (Paper VI). The results obtained in this work show that wild European plaice has a significantly higher ratio of EAA than their farmed counterparts (Paper VI). This was also observed in a study on wild Ussuri catfish *Pseudobagrus ussuriensis*, where higher values for TAA, EAA, as well as delicious amino acids (glutamic acid, aspartic acid, alanine and glycine), were found (Wang et al., 2014). The distribution of the four amino acids previously categorized as *delicious* by Wang et al. (2014) was also higher in the results on wild European plaice presented in Paper VI.

Next to being important indicators for nutritional quality, amino acids in their free unbound form as free amino acids (FAA) give information about the taste and flavor of fish (Ruiz-Capillas and Moral, 2004; Sarower et al., 2012). On the other hand, their increase during storage signals degradation processes initiated by enzymatic and microbial activity and leads to spoilage of the product (Section 4.4). The distributions and concentrations of FAA are influenced by seasons (Paper I) and sex (Paper VI) and differ between wild and farmed European plaice (Paper VI). Moreover, variations in the composition were identified between different flatfish species captured in the same habitat area, as reported in Paper III. Higher essential FAA were found in Apr compared to the two other seasons, with additionally overall high contents of total FAA (Paper I). Figure 4.2 displays the distribution of FAA grouped according to taste perceptions and highlights the significant differences between the seasons for individual FAA. It can be seen that fish caught in Apr has significantly higher concentrations for most of the FAA,

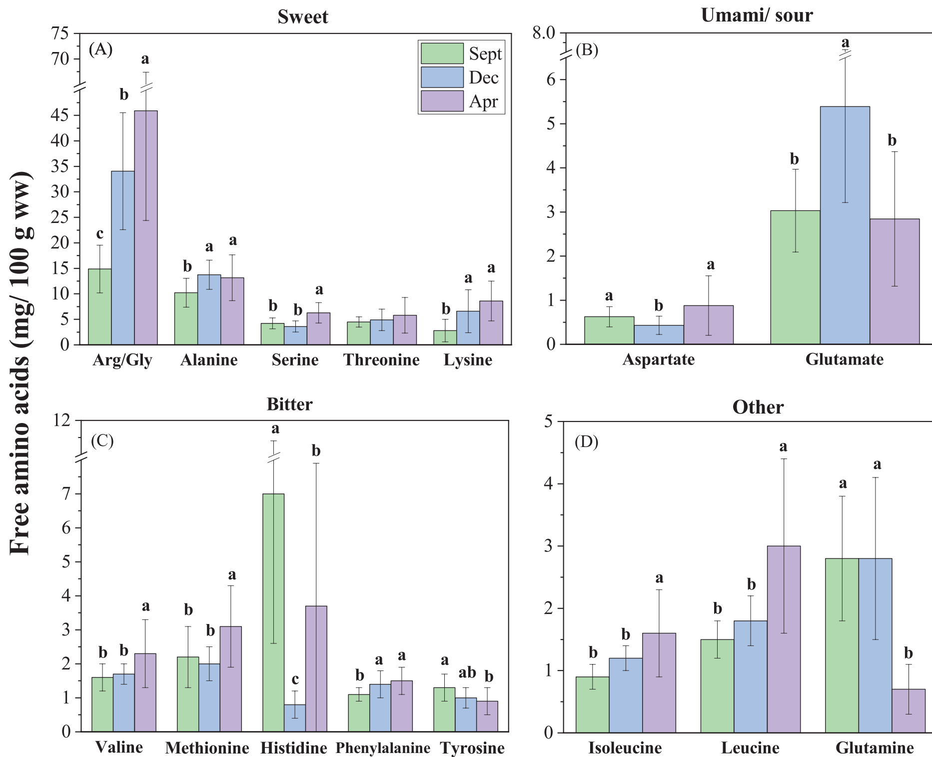

Figure 4.2: Extracted results from Paper I (Table 2). FAA in mg/100 mg sample ww, distribution grouped in taste perceptions sweet (A), umami/ sour (B), bitter (C), and other (D). Error bars show standard deviation (SD). ANOVA was applied on seasons and each FAA; where significant difference between species was detected ($\alpha < 0.05$), a Tukey HSD *post hoc* test was applied. Values with different superscript letters (a, b) are significantly different ($p < 0.05$). Asparagine is not visualized due to low contents (> 0.1 mg/100g).

which could indicate different taste perceptions of fish caught in the spring season (post-spawning).

Interestingly, farmed European plaice showed significantly higher total FAA compared to those captured wild, with values being two to three times higher (Paper VI). In particular, the two amino acids arginine and glycine[1] are substantially higher in concentration compared to wild European plaice (Paper III). This is especially interesting, as amino acids are associated with different sensory attributes when available in their free unbound form as previously mentioned (Figure 4.2, Paper III + VI). The classification of FAA into groups of sweet, umami/ sour, and bitter-tasting amino acids in Figure 4.2 was based on Kirimura et al. (1969), Fuke

[1] Arg/Gly are co-eluted in the method used from Osnes and Mohr (1985)

and Konosu (1991) and Sarower et al. (2012).

The amino acid glycine can be linked to sweetness. In contrast, arginine is linked to bitterness (Sarower et al., 2012), and values found for these two amino acids in farmed European plaice were approximately five times higher than those observed among wild captured European plaice (Paper I, Paper VI). It has to be mentioned that due to the co-elution of arginine and glycine in the applied method in the present thesis work, separation into the respective taste classes was not feasible. When comparing these results to the study of Karl et al. (2013) on European plaice and other flatfish, values for Arg/Gly found in Paper I and Paper VI were higher, but the total FAA concentrations were lower (265 mg/100g compared to 460 mg/100g). In general, large variability was observed between individuals, both for farmed and wild European plaice, visualized with a large SD in the results of the respective papers.

In fact, the taste perception of foods is complex, and taste-producing components like FAA vary between species and are affected by environmental factors (Sarower et al., 2012). Furthermore, understanding a product's flavor is complex and involves both complementary and antagonistic effects of various taste compounds. E.g. arginine is linked to a bitter taste. However, it is also supposed to exhibit a slightly sweet note (Kirimura et al., 1969), and tyrosine and phenylalanine are bitter-tasting amino acids, yet can improve the umami flavor in seafood (Sarower et al., 2012). Moreover, it has also been demonstrated that the pH and the relative amounts of the flavor compounds influence how taste is experienced (Sarower et al., 2012). This makes a general comparison with literature difficult and rather suggests sensory evaluation with consumers to identify possible differences and preferences of farmed and wild European plaice. Previous studies have compared the sensory properties of farmed and wild individuals of e.g halibut, common sole, and seabream (Olsson et al., 2003; Parma et al., 2019; Rincón et al., 2016). Some results indicate significant differences in taste perception and rate sensory evaluation as an important quality parameter as part of the optimization of fish farming.

4.2.2 Fatty acids and lipid classes

Even though wild European plaice can be regarded as a lean fish with a maximum of 1.55 g/100g fat measured in fish captured in Dec (Paper I), the FA distribution is relevant for human nutrition. Especially the contribution of the two important omega-3 (n3) FA EPA and DHA to the adequate intake (AI) set by the EFSA Panel on Dietetic Products and Allergies (2010) are of importance as highlighted in Paper III.

Consuming a 200 g portion of wild European plaice not only meets but surpasses the AI of 250 mg EPA and DHA for a regular adult person (Paper III) (EFSA Panel on Dietetic Products and Allergies, 2010). On average, wild European plaice (n=10)

contributes 136% to the AI per day (Paper III). The same calculations on the FA distribution as established by Weihrauch et al. (1977) and previously described in Paper III and Paper IV were applied to farmed individuals (data from Paper VI) as well as the whole population of wild fish (data from Paper I). Hereby, it was found that farmed European plaice contributes considerably to the daily AI of the two n3 FA as indicated in Figure 4.3.

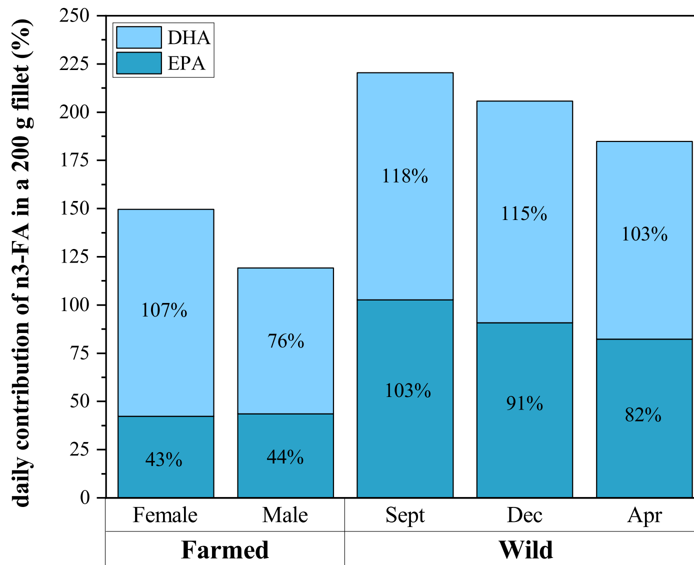

Figure 4.3: Average contribution of a 200 g fillet portion to cover the daily AI of 250 mg DHA and EPA by the EFSA Panel on Dietetic Products and Allergies (2010) for female (n=6x2) and male (n=6x2) farmed and wild European plaice caught in Sept (n=6x2), Dec (n=6x2) and Apr (n=6x2)[2]

On average, farmed European plaice contributes between 120% and 150% (female vs. male fish, respectively) to the AI of the n3 FA. This means a person needs to consume between 134 g and 168 g of farmed European plaice fillets to cover 250 mg of EPA and DHA. Moreover, Figure 4.3 highlights the contribution of DHA, which is distinctively higher than EPA, both for farmed and wild European plaice. To put the daily contributions from Figure 4.3 into absolute numbers, a consumer would need to either consume 151 g (average farmed) or 98 g (average wild) fillet to fulfill a minimum of the suggested AI of 250 mg n3 FA or consume 5.3x (average farmed) or 3.5x 200 g portions per week to reach the recommended weekly intake (RWI) of 1.75 g (250 mgx7). This is comparable to other lean flatfish like flounder (3.2), megrim (3.8), and lemon sole (5.4 portions of 200 g per week)(Paper III).

[2] n=6x2 indicates that two fillets per sample were chosen for analyses, being in total 12 measurements per group

Higher relative values of EPA and DHA in Sept (Figure 4.3) indicate an effect of season on the contribution of the two n3 FA, which is in accordance with results featured in Paper I. Moreover, when considering the whole population per season, 200 g of plaice fillets caught in Sept, contribute up to 220% to the AI (Figure 4.3).

In Paper IV, alternative intake scenarios were calculated, with a weekly portion size of 250 g in alternative scenario (AS) 1 and 450 g of European plaice fillets in AS 2, simulating an increased consumption of European plaice, opposing to no consumption (reference scenario). Here, it was estimated that the net IQ points[3] gained can be increased when consuming higher amounts of wild European plaice. Moreover, the intake of n3-LC-PUFA would increase more than 20% from AS 1 to AS 2. However, it is still recommended to additionally consume a portion of fatty fish per week to guarantee a sufficient intake of EPA and DHA, as suggested in AS 1 (Paper IV).

In contrast to generally higher lipid content in wild European plaice captured in Dec, the proportion of PUFA, including n3 FA, did not increase in results obtained in Paper 1, and Sept showed significantly higher ratios of PUFA compared to Dec. Moreover, it was found that farmed European plaice contain higher overall lipid concentrations than the wild individuals investigated in Paper VI. However, no significantly higher PUFA ratios were observed compared to those captured wild (Paper VI). When comparing farmed European plaice to wild individuals of each season, the wild captured European plaice in Sept, featured significantly higher PUFA values than the farmed individuals. When looking closer at the overall FA distribution of wild vs. farmed European plaice, significant differences for some of the long-chain unsaturated FA were observed. Thus, C20:1 (Eicosenoic acid), C20:4 n6 (Arachidonic acid), and C22:6 n3 (DHA) were found in higher concentrations in wild fish (Paper VI). No significant differences due to a high variety indicated with a large SD were identified for wild European plaice. Nevertheless higher values for PUFA, MUFA and C20:5 n3 (EPA) (15.3±3.3% in wild vs. 7.4±3.3% in farmed) were seen. Olsson et al. (2003) observed the same trend for wild halibut, in contrast to farmed individuals.

In the present thesis work, phospholipids (PL), triacylglycerols (TAG), and cholesterol (Paper I) were identified as the three main lipid classes for wild European plaice. The composition of lipids plays an important role in identifying how FA are stored in the fish muscle. No investigations have been conducted on the FA composition of the specific lipid classes, and in general, it is difficult to identify and allocate FA to a respective lipid class, leaving room for uncertainty when applying the calculations established by Weihrauch et al. (1977). Schuchardt and Hahn (2013) describe that fish stores EPA and DHA mainly in the form of TAG,

[3] net IQ points gained/lost, being the effect on the neurodevelopment of unborn infants as opposed to detrimental effects due to methylmercury (MeHg) intake of the mother

specifically at the sn-2 position of the TAG molecule, or in the unbound free form as free fatty acids (FFA). Moreover, Sushchik et al. (2020) observed storage of LC-PUFA mainly in TAG in different fish.

In addition, it has to be mentioned that in the computation of the fatty acid conversion factor (FACF) of Weihrauch et al. (1977), it is assumed that the PL concentration increases with increased total lipid content. This FACF was used as the basis for calculations on the contributions of EPA and DHA to the AI in Paper III, Paper IV and shown in Figure 4.3. This assumption on the PL opposes the results found in Paper I on the seasonal changes of lipid classes. The FA, as well as the lipid class composition, differ throughout the investigated seasons, with TAG predominating in Dec (77%) and Sept (68%) compared to Apr (46% TAG and 48% PL) (Paper I). This suggests that plaice stores FA mainly as part of TAG either in adipocytes incorporated into the muscle or directly as fat droplets in the muscle, but also to some extent in cell membranes, attached to PL, especially when it is found to be leaner (Apr) (Sushchik et al., 2020). Moreover, this leads to the assumption, that the contribution of EPA and DHA in the higher fat samples of farmed males (total fat of 2.35%) and wild Dec samples (1.55%) as shown in Figure 4.3 is underestimated compared to the other investigated groups and might actually provide higher n3 FA, important for human health.

4.3 Physicochemical quality

Physicochemical quality parameters of fish and seafood often include the analysis of its textural properties, color attributes, WHC as well as its drip loss (DL) (Chan et al., 2022; Cheng et al., 2014). These parameters define the initial quality of fish, next to the chemical and nutritional quality and are susceptible to deterioration during storage, packaging and processing. Moreover, physicochemical parameters are interdependent and influenced by the chemical composition, making it an important quality attribute for assessing the nutritional potential and utilization of European plaice (Castro et al., 2021; Chan et al., 2022; Lazo et al., 2017).

4.3.1 Water holding capacity

In the present work, the WHC was found to be influenced by storage conditions (Paper II), seasons (Paper I), and sex (Paper VI). Significant differences were found between the seasons Sept and Apr for wild European plaice stored as whole fish on ice (WI), vacuum packaged (VP), and modified atmosphere packaged (MAP) fillets in Paper II. But no difference in WHC of wild European plaice fillets was found between the three observed seasons (Paper I), as indicated by a high SD. In Paper II however, the seasons (Sept vs. Apr) did seem to influence the capacity of retaining the water in fillets during different storage conditions.

In general, the WHC of fresh fillets of farmed European plaice was lower compared to wild captured plaice (Paper VI), agreeing with other comparative studies on different farmed and wild fish species (Fallah et al., 2011; Olsson et al., 2003). Olsson et al. (2003) suggests higher liquid loss (hence a lower WHC) with decreasing pH. However, in the present study on farmed European plaice (Paper VI), only weak correlations between the WHC and pH (males: $r = -0.590$, females: $r = -0.481$) and no significant differences were observed. Moreover, gender seems to affect the WHC, with male European plaice featuring significantly higher WHC compared to females when farmed (Paper VI). Thus, the present results suggested that European plaice from different seasons, stored under different conditions and packaging, show different physicochemical properties, which causes changes in the overall quality. Moreover, the results in this work indicate differences in the physicochemical attributes of farmed European plaice, including differences between female and male farmed individuals, compared to those captured wild.

4.3.2 Texture

There are many external (e.g. storage, packaging, etc.) and internal factors (e.g. chemical changes, adenosine-triphosphate (ATP) degradation, etc.) that affect the quality and hereby influence the structure and texture of fish (Cheng et al., 2014). The storage time and conditions have a significant impact on the texture of wild European plaice fillets (Paper II). Thus, results of wild European plaice collected in Sept and stored as WI indicate significantly firmer texture compared to fish stored in MAP after 9 days of storage, as indicated by a higher breaking force (BF). Furthermore, Paper II revealed differences in the texture, storage time, and condition among the fish caught during the two seasons Sept and Apr. Here, MAP European plaice individuals collected in Apr differ significantly from all other storage conditions for European plaice collected in Sept as well as for VP fish from Apr after a storage time of 9 days. According to Castro et al. (2021), a higher lipid content can lead to softening of fillet texture, which would conversely mean a firmer texture in fillets with lower lipid content. Results reported in Paper I indicated significantly lower lipid content combined with the highest fillet BF in wild European plaice captured in Apr.

According to Paper VI, there is no difference in the texture between female and male individuals of European plaice. However, results on fresh wild European plaice carried out in Paper I and VI have to be considered with caution, as both measurements on BF and 60% fillet compression featured high overall SD. This indicates a high variety between the individuals, but also that the method for measuring texture could potentially be improved.

4.3.3 Drip loss

In Paper II, an increased DL during the storage period of 16 days was reported for wild European plaice, stored as a whole (WI; 0°C) or in fillets as VP or MAP (4°C). The storage conditions significantly impact the DL, with MAP-groups, independent of season, featuring the highest loss. This is followed by the lowest water content in MAP fillets between the three different storage conditions (Paper II).

Although Paper II did not find any significant differences in the DL between Sept and Apr, unpublished data from the experimental work conducted in Paper I, indicated significantly higher DL of fish captured in Apr during 9 days and 16 days of storage as whole fish on ice. In general, wild European plaice captured in Apr showed significantly higher water content and the lowest protein content (Paper I) of the three investigated seasons. Both the water and protein content are highly related to the fish muscle's ability to retain water (WHC) and the muscle DL (Huff-Lonergan and Lonergan, 2007). It can be assumed that the high amount of water in the Apr catch of wild European plaice was mainly in the form of free water, not being bound to proteins and therefore being more prone to leaking out from the fillet (Chan et al., 2022). Thus, the water left in the fillet is most probably bound to proteins, which can explain a high WHC of Apr fillets.

The results on the WHC and DL of stored wild European plaice under different conditions and seasons (Paper I and Paper II) are somewhat ambiguous and difficult to interpret. This demonstrates how intricate physicochemical quality attributes are, and at the same time, how interconnected they are with the chemical composition in general.

4.3.4 Color

Visual attributes give a first important impression on a product and can alter consumer decisions (Byrne, 2020). Depending on the species, the flesh color will differ, and for flatfish such as halibut or turbot (or European plaice), the fillet whiteness is an essential quality attribute (Roth et al., 2009).

Guillerm-Regost et al. (2006) noticed color changes of Atlantic halibut throughout storage, with the whiteness, or lightness (L*) increasing with prolonged storage, but simultaneously coming along with a negative redness (a*) indicating green color. For European plaice, independent of storage condition, L* significantly increased over the storage period of 16 days (Paper II), similar to what was observed by Guillerm-Regost et al. (2006). Moreover, MAP fish from Apr featured the significantly highest L* after 16 days of storage. Previously, it was also reported that farmed fish is lighter (increased L*), compared to wild individuals (Olsson et al., 2003). Similar to DL, it was observed that the water content plays a significant role in an increased L* in the present study. Moreover, a significant correlation ($r = 0.68$; $p < 0.001$) between L* and DL was found,

explaining the significantly higher L* in MAP fish compared to other storage conditions (Paper II).

In Paper VI, fresh farmed European plaice individuals were significantly lighter in color when compared to wild fish captured in all three investigated seasons (Sept, Dec, Apr). Interestingly, in aquaculture, females feature significantly lighter (increased L*) flesh colors, but also greater a* than males.

4.4 Storage stability

The following section will concentrate exclusively on results indicating product storage stability, which were investigated in Paper II. Although these results are inherently correlated with previously discussed results in Section 4.3 on the physicochemical quality, the objective is to highlight the significant influence of ATP breakdown, the formation of biogenic amines, and microbial growth on the storage stability. By doing so, the aim was to provide an enhanced and clear overview of results that are interconnected with quality changes throughout the storage.

Three storage conditions were compared to each other in Paper II: whole fish on ice (WI) stored at 0°C opposing modified atmosphere packaged (MAP) and vacuum packaged (VP) European plaice fillets stored at 4°C, as previously referred to in Section 4.3.

The season (Sept vs. Apr) and storage time (up to 20 days) were found to significantly affect the ATP breakdown. Here, it was observed that European plaice fillets caught in Sept kept a higher quality throughout the storage period when compared to Apr, which is indicated by overall significantly lower K- and H-values, independent of storage condition. Moreover, a significant correlation between aerobic plate count (APC), psychrotrophic aerobic plate count (PC) and hydrogen sulfide (H_2S)-producing bacteria and an increased H-value for European plaice caught in Apr was found. The increased microbial growth observed in Apr can be directly linked to a faster ATP degradation in this season. Regarding the storage conditions, it is important to mention that VP fish exhibited significantly higher K- and H-values in both seasons when compared to MAP fish and WI. This can be attributed to significantly lower IMP and HxR, but higher Hx values and indicates reduced quality in VP European plaice fillets. In previous studies, a sensory rejection threshold at a K-value of 70% was identified for different flatfish species (Özogul et al., 2006; Rodríguez et al., 2006). The investigation in Paper II showed higher K-values after 10 days of storage for European plaice caught in Apr for both MAP and VP fillets, and a close value (68%) for WI.

Moreover, the formation of biogenic amines during the three above-mentioned storage conditions of fillets from wild European plaice captured in Sept and Apr was investigated. The amount of biogenic amines significantly increased during the storage time in all three storage conditions. Moreover, all investigated metabolites,

despite tryptamine, were affected by the storage condition or the fishing season. Cadaverine was found to be the predominant metabolite with the highest value of 7.34 mg/100g for VP samples of Apr at the end of storage but stayed below the sensory threshold of 9.1 mg/100g through the storage period of 20 days (17 days for VP) as suggested by Vallé et al. (2020). Cadaverine, spermidine, and spermine were most abundant in VP-groups overall, followed by WI and MAP-groups and can be positively correlated to increased APC and a higher PC.

The initial microbial loads of wild European plaice (Sept vs. Apr) were found to be low, demonstrated by less than 3 log colony-forming unit (CFU)/g for PC, 2.3 log CFU/g for APC and 1 log CFU/g for H_2S-producing bacteria. This indicates a high initial quality of the fresh raw material. Moreover, neither *Pseudomonas* spp., *Brochothrix thermosphacta*, Enterobacteriaceae, nor *Listeria monocytogenes* were detected in fresh European plaice muscle in either of the two investigated seasons (Sept vs. Apr). The three different storage conditions WI, MAP and VP, as well as storage time, significantly influenced the microbial growth of PC, APC, H_2S-producing bacteria, lactic acid bacteria and *Pseudomonas* spp.. However, a seasonal difference was only detected for PC and H_2S-producing bacteria, not for APC. VP fish fillets show reduced quality in both seasons, as demonstrated by the significantly shorter lag phase and higher maximum specific growth rate for PC and APC compared to the other storage conditions. Similar results on reduced quality of VP fish compared to MAP packaged fillets were observed by Babic Milijasevic et al. (2023). Moreover, VP-groups in both seasons showed PC values exceeding 7 log CFU/g at storage day 17, which is why the storage trial was stopped at this point, instead of after 20 days as for MAP and WI. Whereas MAP and WI samples show comparable high quality. Previously, the bacteriostatic effect of CO_2 on APC bacteria (Rodrigues et al., 2016) and other typical seafood bacteria (e.g. *Photobacterium phosphoreum*, *Shewanella putrefaciens*, *Brochothrix thermosphacta* etc.) (Devlieghere and Debevere, 2000) of packaged fish fillets has been reported. Therefore, MAP European plaice fillets can be considered as a convenient retail option.

Concerning the microbial spoilage, formation of biogenic amines and ATP breakdown, results reported in Paper II indicate a better quality of wild European plaice captured in Sept compared to Apr. This is due to significantly higher contents of biogenic amines, together with differential microbial growth progress at the end of storage in Apr.

4.5 Risks and benefits of trace elements

To represent a holistic investigation of the nutritional potential and utilization of European plaice, it is crucial to evaluate its potential risks next to the benefits of its consumption. To generate an objective comparison of risks vs. benefits of

consuming certain foodstuffs, the risk-benefit assessment (RBA) has become an eligible framework. The application of RBAs has shown to be useful, especially for evaluating the impact of fish and seafood on human health (Thomsen et al., 2021). The Benefit-Risk Analysis for Food (BRAFO)-tiered approach, as applied in Paper IV, has previously been used to evaluate risks and benefits connected to the consumption of different fish species (Gao et al., 2015; Hoekstra et al., 2012; Watzl et al., 2012).

4.5.1 Micronutrients

The investigations on wild European plaice in this thesis work show especially high contributions of selenium (Se) (Paper III, Paper IV). A dietary reference value (DRV) of 114% for Se was reported in Paper IV. This corresponds to 0.40±0.12 mg/kg, compared to the AI of 0.070 mg/day set by EFSA, 2014. In both suggested alternative scenario (AS) in Paper IV, the high concentrations of Se did not exceed the RWI and are expected to exhibit beneficial effects (Ralston et al., 2016). These findings for Se in wild European plaice, flounder, lemon sole, and megrim (Paper I, Paper III) were on average higher than the results found from Karl et al. (2013) and Barbosa et al. (2018) on European plaice and other flatfish, but similar to results obtained on megrim and other demersal fish by Afonso et al. (2008) and Cardoso et al. (2015).

Moreover, to determine whether the accessible Se concentration exceeds the mercury (Hg) concentration and whether Se reduces or even eliminates the toxicity of Hg and therefore methylmercury (MeHg), one can utilize the Se:Hg molar ratio and health benefit value of selenium (HBV_{Se}) (Barone et al., 2021; Ralston et al., 2016). Positive Se:Hg ratios were found for European plaice and other investigated demersal fish species, showing that Se predominates over Hg (Paper IV). Additionally, positive HBV_{Se} were determined, establishing a connection between Se's health advantages and the consumption of European plaice. Positive Se:Hg molar ratios as well as HBV_{Se} for plaice originating from the Northeast Atlantic were observed by Azad et al. (2019). Moreover, Barone et al. (2021) found comparable Se:Hg molar ratios and HBV_{Se} for different flatfish species. High selenium values in demersal fish like, e.g., European plaice are especially important, as environmental toxins (MeHg) tend to accumulate closer to the seabed (Parolini et al., 2020).

The results in Paper IV feature the potential positive effects of Se on human health. Especially the possible antagonistic effects on MeHg have to be mentioned (Barone et al., 2021) as further discussed in Section 4.5.2.

With regards to other micronutrients (e.g. sodium (Na), potassium (K), magnesium (Mg), etc.), Paper III shows satisfying concentrations of the 11 investigated elements. A 200 g fillet of European plaice and other flatfish feature potassium (K) concentrations that contribute approximately 20% to the daily AI (EFSA Panel on

Dietetic Products et al., 2016). These results indicated in Paper III are comparable to results found by Karl et al. (2013) on European plaice and other flatfish. Moreover, Paper III features higher values of calcium (Ca) and Na and comparable values of Mg to Karl et al. (2013).

4.5.2 Environmental and chemical toxins

Bioaccumulation of trace elements is influenced by both endogenous and exogenous factors. Those factors include different sizes, and sexes, as well as feeding grounds, and migration behaviors of individuals (Afonso et al., 2013; Paper I). In Paper I, no significant difference in trace element concentrations (note: only significance found for molybdenum) of the investigated essential and toxic elements between wild European plaice captured in three different seasons were discovered. A reason for this can be the small sample sizes, but also the large variety (indicated with high SD) which was observed between the sample concentrations (Paper I). In general, concentrations of environmental and chemical contaminants in wild European plaice were low, and an elevated accumulation was not detected for any of the investigated seasons (Paper I), nor investigated fish species (Paper III).

Regarding MeHg[4], the concentrations found for European plaice were generally low and below the maximum allowed levels in seafood (EFSA, 2012) for all three seasons (Paper I). A similar result was also seen for other demersal species captured in the same area (Paper III). MeHg exhibits neurotoxicity, and elevated levels can be very critical to the neurological development of the fetus. This is attributed to its manipulating effect on the synthesis of selenoproteins, causing oxidative stress in neurons (Farina et al., 2011; Khan and Wang, 2009). Beneficial components can be opposed to the risk factors emerging through contaminants and often balance these detrimental factors. The RBA performed in Paper IV concluded that the beneficial effects of increased consumption of European plaice and other studied demersal fish outweigh the risks caused by MeHg and chemical contaminants (dioxins and polychlorinated biphenyls). Similar results were obtained in previous studies investigating the risks and benefits related to the intake of different fish species and connected to Se, MeHg as well as EPA and DHA concentrations (Afonso et al., 2013; Gao et al., 2015; Sofoulaki et al., 2019; Strandberg et al., 2016).

4.6 Utilization of the whole biomass

Approximately 50% of European plaice's weight[5] can be regarded as discards, and those by-products are not considered primary products for human consumption. When considering European plaice as a potential food source, both as wild captured

[4]MeHg is expressed as Hg; approximately 90% of Hg is found in the form of MeHg in seafood (EC, 2006)

[5]unpublished data from sampling of Paper I

fish and suitable for aquaculture cultivation (Paper VI) and thus increasing its production, it is important to investigate the whole biomass utilization.

In Paper V, the three main by-product fractions, skins, heads, and backbones, were utilized for the extraction of collagen. By doing so, microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE), were applied. Moreover, salt-washing or enzymatic hydrolysis was performed prior to extraction and opposed to untreated material. Results obtained in Paper V feature that the extraction of collagen with these two green extraction procedures can be realized. The highest yield of collagen was achieved from the extraction of skins, independent of pre-treatment, extraction method, and time. Especially promising results of a collagen yield of 77% were achieved for skins when combined with enzymatic hydrolysis as a pre-treatment.

The FAA composition of the three fractions differed but showed more similarity between backbones and heads compared to skins. Moreover, the time and extraction type seem to influence the amount of certain FAA, with significant differences between by-product fractions as well as extraction times and types. This is important as FAA exhibit different taste perceptions as mentioned in Section 4.2.1, especially when considering the extracted collagen for further utilization in food or pharmaceutical applications (Mutalipassi et al., 2021).

The main identified FAA in all three fractions were glycine and arginine (combined), similar to the highest values in the fish fillets (Paper I, Paper III, Paper VI). It might be possible to regulate the taste attributes by choosing an extraction method + time, which could lead to e.g. less bitterness exhibiting FAA. This could be further investigated in future research and was not investigated in this thesis work.

Ali et al. (2018) previously investigated the application of UAE on fish skins and reported greater collagen yield connected to longer extraction time and amplitude. Moreover, Shaik et al. (2021) have effectively applied UAE to generate collagen from Sharpnose stingray (*Dasyatis zugei*) skins. Underlining these results, Lu et al. (2023) concluded in a review on marine by-product derived collagen through UAE in combination with conventional treatments, that the additional application of UAE gives higher yield and better quality. Moreover, decreased extraction times were reported for UAE compared to extraction processes without additional ultrasonification (Lu et al., 2023).

MAE on the other hand, seems to be a more unexplored, novel approach to extracting collagen from marine fish by-products, indicated by limited available studies. However, previous research conducted by El Knidri et al. (2019) and Santos et al. (2019), on extraction of chitosan and chitin from shrimp shells has been successful. Moreover, Pal and Suresh (2016) and Mohamad Razali et al. (2023) list MAE (and UAE) among other green extraction methods, to increase the process efficiency of collagen extraction. In addition, Mohamad Razali et al.

(2023) highlight the effectiveness of MAE in combination with acid or enzymatic treatments to open up the collagen structure, hereby efficiently improving the extraction yield.

With Paper V, the potential to extract collagen from the three main fractions of European plaice by-products was demonstrated. Moreover, it was highlighted that it is generally possible to up-cycle the discards into higher-value products. The crucial next step for future implementations and scale-up is to test the methods in a pilot scale setup to provide information on the industrial feasibility. Moreover, the literature suggests the many positive attributes of marine collagen in a variety of potential applications, as previously described in Section 2.3. However, the actual implementation and functionality in such products need to be executed to be able to verify their actual function. Here, research on application technologies in different products is demanded. These steps are crucial to lean towards a sustainable, green shift of blue bio-resources to achieve the 2030 Sustainable Development Goals from the European Commission (2020).

CHAPTER

FIVE

Conclusion

In this thesis, comprehensive knowledge circling around the holistic potential of European plaice as a bio-resource was gathered. Particularly the chemical composition and beneficial nutritional profile were depicted. European plaice can be regarded as a nutritional food source with good overall fillet quality throughout autumn (Sept) and winter (Dec). Comparatively, the physicochemical attributes were found to be lower, and biogenic amines, ATP metabolites, and microbial growth were accelerated in the post-spawning season, highlighted by the results obtained from the Apr catch. Therefore, captures in this period should be reconsidered and more data be gathered.

European plaice contains beneficial compounds, with the amounts of EPA+DHA directly contributing to reaching the daily intake recommendations from food authorities. The excellent fat composition in combination with it being a lean fish species, makes European plaice an attractive food source. Building upon this, the benefits of consuming European plaice, including its low potential risks of toxins and good storage stability, make it a safe and appealing choice for consumption. Moreover, the storage and packaging of European plaice in MAP gives good quality and offers a convenient retail option, next to fish sold as whole fish on ice.

The high amount of skins and other by-product fractions were successfully utilized to generate collagen. This opens up the potential for a holistic utilization of the entire fish as a valuable bio-resource. Future work can focus on the assessment of the chemical structure and functionalities of the up-cycled fraction. Moreover, it was possible to highlight the feasibility of two green extraction methods, both being relevant for accelerating the green shift in the food and biotechnology industries.

The nutritional, chemical and physicochemical quality of farmed European plaice was found to be comparable to the wild stocks. This is a first step towards understanding whether European plaice is a suitable species for farming or not.

In conclusion, European plaice can be promoted as a nutritional, high in quality, and safe food resource with the potential to be utilized holistically.

CHAPTER

SIX

Outlook

This thesis has demonstrated that European plaice is a valuable food resource with good overall quality. Moreover, it showed that farmed European plaice obtained similar quality to its wild counterpart, making it an interesting species for aquaculture.

Considering European plaice as an aquaculture species will come with potentially higher production costs than what the market is willing to pay at the moment. However, to meet the challenges we face today, including climate changes, it is crucial to expand the knowledge and increase the number of species having a potential in aquaculture. Future research should therefore focus on optimizing the aquaculture conditions for European plaice. This should include the investigation of optimal growth conditions, with the development of specialized feed for this species, and determining the ideal rearing duration. A part of the commercialization of farmed European plaice should include sensory studies. Through these, it should be possible to highlight the standardized high quality of farmed fish and promote its similar/ higher sensory properties compared to wild individuals. Moreover, if European plaice fillets should be available as convenient retail products, comparable to cod or salmon in Norwegian supermarkets, the value chain needs to be optimized. Next to an established aquaculture system, this includes slaughter and post-harvest handling and filleting. Here, it will be necessary to optimize and standardize the filleting process to obtain a good filleting yield in addition to convenient packaging solutions.

Simultaneously, the introduction of the flatfish industry in Norway should involve the establishment of processing facilities that include the efficient handling and utilization of by-products. This will lead to additional products for food and biotechnology and add value to these industries.

This emphasizes the need for future research to find cost-effective solutions along the value chain of potentially farmed European plaice, while also addressing global challenges such as the rising world population, steady demand for proteins, potentially declining wild fish stocks and climate change.

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Paper I

Kendler, S., Tsoukalas, D., Jakobsen, A. N., Zhang, J., Asimakopoulos, A. G., & Lerfall, J. (2023d). Seasonal variation in chemical composition and contaminants in European plaice (*Pleuronectes Platessa*) originated from the west-coast of Norway. *Food Chemistry*, *401*, 134155. https://doi.org/10.1016/j.foodchem.2022.134155

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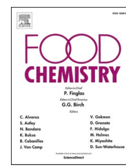

Seasonal variation in chemical composition and contaminants in European plaice (*Pleuronectes Platessa*) originated from the west-coast of Norway

Sophie Kendler [a,*], Dionysios Tsoukalas [a], Anita Nordeng Jakobsen [a], Junjie Zhang [b], Alexandros G. Asimakopoulos [b], Jørgen Lerfall [a]

[a] Department of Biotechnology and Food Science, Norwegian University of Science and Technology (NTNU), NO-7491 Trondheim, Norway
[b] Department of Chemistry, Norwegian University of Science and Technology (NTNU), NO-7491 Trondheim, Norway

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ABSTRACT

The seasonal effect on chemical composition and contaminants in European plaice (*Pleuronectes platessa*) captured in Norwegian waters was investigated in three seasons (September, December, April). Fillets were compared by analysing proximate composition, lipid and protein profile, including fatty acids, lipid class distribution, free and total amino acids. Trace elements and polychlorinated biphenyls (PCB) were determined to assess the potential health risk of consumption. Results on proximate composition reveal differences in water, ash, lipid, and protein content between the seasons. Beneficial levels of n3 fatty acids, including a sufficient n3/n6 ratio (6.1–8.7), were observed. Phospholipids and cholesterol were negatively correlated with total lipid content, adding to high triacylglycerol concentrations observed in December samples. No elevated levels for toxic trace elements, yet detrimental concentrations of dioxin-like PCB-118 were found. Results show that European plaice is highly nutritious with desirable amino and fatty acid profile throughout the year, and with few potential risks.

1. Introduction

European plaice *(Pleuronectes platessa)* has been of great commercial interest in Europe for the past decades, with its leading capture being the Northern Sea (EC, 2020). The Netherlands hold a significant part of the flatfish capture, followed by Denmark, United Kingdom, Belgium, France and Germany (EC, 2020). The Annual Economic Report on the EU Fishing Fleet of 2020 states that it is expected that stocks of European plaice will decrease in the Northern Sea as fish move further north (EC, 2020). With these changes in migration patterns, increased stock volumes of European plaice in the Norwegian sea and evolving significance for Norwegian fisheries can be expected in the future. Data on catch and quota collected from the Norwegian Directorate of Fisheries shows slight increases from 2020 to 2021 of 699 to 794 tons (whole fish equivalent) for European plaice captured in Norwegian waters (Directorate of Fisheries, 2022). Data on human consumption of plaice in Norway is limited, as consumer studies only include regularly consumed fish.

Seafood is known to provide significant amounts of bioavailable nutrients like proteins, lipids, and micronutrients such as vitamins, iron, selenium, or zinc with well-recognized health benefits (Ahern et al., 2021; EFSA, 2014). The recommended intake for seafood, including fish, ranges from 100 g (FAO/WHO, 2011) to 300 g (EFSA, 2014) per week to ensure a satisfying intake of certain nutrients. Fish is a good source of long-chain polyunsaturated fatty acids (LC-PUFAs), supporting human health by various metabolic functions. Next to providing essential fatty acids, fish delivers considerable amounts of proteins. With its excellent digestibility and sufficient distribution of essential amino acids, fish is a source of high-quality proteins. Moreover, free amino acids contribute to fish's flavour and taste development, relevant for sensory perception during consumption (Ruiz-Capillas & Moral, 2004).

The lipid and protein content of fish can vary throughout the year, as fish must manage environmental conditions fluctuations. Due to changes in nutrient content and feed availability, changes within the proximate composition of fish have been observed (Celik, 2008; Gökçe, Taşbozan, Çelik, & Tabakoğlu, 2004). Furthermore, factors such as water temperature, maturity, and sex can influence the nutritional composition and hence the fish quality (Aidos, van der Padt, Luten, & Boom, 2002). Several studies have been conducted to investigate the differences related to the proximate composition, especially focusing on distribution of fatty acids of fish and other seafood (Aidos et al., 2002;

* Corresponding author.
E-mail address: sophie.kendler@ntnu.no (S. Kendler).

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Gökçe et al., 2004; Küçükgülmez, Celik, Ersoy, & Yanar, 2010; Mateos, Lewandowski, & Su, 2010).

However, the likelihood of elevated levels of contaminants, such as mercury in the form of methylmercury or dioxins and polychlorinated biphenyls (PCBs), must be considered when consuming fish. Contaminants can bioaccumulate in organisms, especially in predatory fish at the top of the aquatic food chain (Costa, Korn, Brito, Ferlin, & Fostier, 2016) and demersal fish (Parolini et al., 2020). European plaice is a demersal fish species inhabiting muddy or sandy areas near the seabed (Parolini et al., 2020). According to Parolini et al. (2020), concentrations of toxins like PCBs are elevated closer to the sea bottom than in the upper sea column, increasing the risks of accumulating chemical toxins in groundfish species such as the European plaice.

Literature on the chemical composition of European plaice and its compositional changes throughout the year are limited. So far, European plaice is of no great commercial relevance in Norway with low overall catching volumes. Considering the ongoing changes in migration patterns due to climatical changes, leading to bigger stock volumes further north, European plaice will probably gain increased relevance in Norway in coming years. Therefore, this study investigated the chemical composition of European plaice captured in Norwegian coastal water. Following the fishing seasons of the Norwegian west coast with samplings in autumn (September), winter (December) and spring (April), the study aimed to identify seasonal variations within the proximate and nutritional composition, including determination of lipid classes, free amino acids, and the fatty acid profile of European plaice fillets. In addition, physiochemical quality parameters such as texture and water holding capacity (WHC) were studied on the fresh fillet. Moreover, analyses on trace elements, including toxic elements, and PCBs, were conducted to assess chemical contaminants and consequently the potential health risk of consuming European plaice captured in Norwegian waters.

2. Material and methods

2.1. Raw material

European plaice individuals were caught at three different times of the year by local fishermen on the Norwegian west coast using purse seine. The samplings took place in autumn (September 2020), winter (December 2020), and spring (April 2021). A total of 59 individuals ($n_{(sept)} = 17 \times 2$; $n_{(dec)} = 21 \times 2$; $n_{(apr)} = 21 \times 2$) were collected and muscle samples were taken from two fillets per individual ($n \times 2$) to get substantial knowledge on the chemical composition and to detect possible variations in the nutritional composition within different body parts. The upper belly fillet and bottom loin fillet were chosen, and each analysed in duplicates, giving four values per individuals. Following the FAO Major Fishing Areas (FAO, 1990–2021), the capture took place in area 2.a.2. with varying sampling locations in this area. The water temperature at catch was 13 °C, 8.7 °C and 5.4 °C, respectively. The pH was measured before the fish was gutted, weighed, and thereafter stored as a whole on ice until the end of rigor mortis. The average pH value of gutted fish was between 6.6 and 6.8 in all three seasons and gutted fish weighed 0.91 ± 0.29 kg (September), 1.17 ± 0.36 kg (December) and 0.84 ± 0.33 kg (April). The fish were filleted 41 to 57 h post-mortem, depending on the release of rigor mortis. For physiochemical analyses, fresh fillets were used and for chemical analyses, fillets were immediately frozen and stored at –80 °C. Parts of the samples were freeze-dried for further analyses on total amino acid distribution, as well as trace elements and polychlorinated biphenyls.

2.2. Proximate composition

Dry matter and ash content were determined gravimetrically. For dry matter analysis, 2–3 g wet weight (ww) samples were dried for 24 h at 105 °C (ISO:6496, 1999). To determine the inorganic matter, the samples were put in a muffle furnace at 550 °C for 24 h. Samples were weighed before and after heating.

Total lipids (%) were determined as described by Bligh and Dyer (1959). Around 2 g (ww) sample was weighed into chloroform-stable tubes. To achieve phase separation, thorough homogenizing and centrifugation were applied on the solvent-sample mixture. The chloroform-lipid phase was separated from the aqueous phase. Chloroform was evaporated with nitrogen, and the total amount of lipids was calculated (Bligh and Dyer, 1959). Additional oil extracts were kept, to investigate the lipid distribution and lipid classes.

Total crude protein (%) was determined by a Kjeldahl apparatus (K-449 and K-375. Büchi Switzerland) following the application manual No. 114/2013 of Büchi by applying 15 mL sulfuric acid H_2SO_4 and two titanium tablets (3.5 g K_2SO_4/0.105 g $CuSO_4{\cdot}5H_2O$/0.105 g TiO_2) for digestion of the samples. For titration, boric acid H_3BO_3 was used, and a conversion factor of 6.25 × Nitrogen (%) was applied according to NMKL (2003) to calculate the total protein content.

2.3. Lipid profile

2.3.1. Fatty acid composition

To determine the fatty acid composition, six individuals per catching season (in total 18 samples) were analysed and thereof approximately 100 mg of extracted oil per sample was used to prepare fatty acid methyl esters (FAMEs). To obtain FAMEs, the method of Metcalfe, Schmitz, and Pelka (1966) was followed. Hence 3 mL KOH (0.5 M) was added to the sample tubes and samples heated up to 60–80 °C for 20 min, with mixing in between. After cooling down, 5 mL of methanolic BF_3 was added, and samples were heated for another 5 min before 2 mL of *n*-butylacetat was added, tubes filled up with saturated NaCl (~1.5 mL) and 1–2 scoops of sodium sulfate were added. Finally, 200 μL Hexane was added. The organic phase containing the lipid fraction was transferred and used to detect the fatty acid composition with gas chromatography. A FAME mixture (Supelco 37 Component FAME Mix, Merck Life Sciences AS, Oslo, Norway) composed of 37 fatty acid methyl esters was used as an external standard.

Fatty acid methyl ester analyses were performed on a gas chromatography (GC) apparatus (Agilent 6850, Agilent Technologies, Santa Clara, USA), equipped with a polyethylene glycol column (HP-INNOWAX, i.D.: 250 μm; film: 0.25 μm), a flame ionization detector (FID) adjusted to 310 °C and an evaporation injector (inlet: 260 °C, pressure: 18.1 psi). Hydrogen was used as carrier gas. The oven program was set to a constant temperature of 160 °C for 3 min, with an increase of 3 °C/min to 240 °C and held for 3 min.

The identification of fatty acids was based on the relative retention times (RRTs) of the external FAME standards. Chromatogram peaks were considered for determination, when the retention time of the peak matched the peak of the external standard compound. The percentage distribution of each individual fatty acid was determined by calculating the intensity of each specific peak against the total intensity of FAMEs in each specific sample.

2.3.2. Lipid class distribution

Lipid classes were analysed on three pooled samples per season (in total 9 samples). Each pooled sample contained the oil fraction of three individuals to guarantee a representative size of the sample population per season as well as to provide enough oil for analysis. The extracted oil phase as described in 2.2 was used, chloroform was evaporated with liquid nitrogen using a heating block (Stuart™ block heater type: SBH130D/3; Cole-Parmer, United States) set to 40 °C equipped with a sample concentrator (Stuart™ concentrator type: SBHCONC/1; Cole-Parmer, United States) and thereafter samples got mixed. The prepared samples were analysed externally by Innolipids AS, Norway with an HPLC system coupled to an ELSD detector and equipped with a ReproSil-Pur CN column (i.D.: 4.6 mm; length: 200 mm; particle size: 3 μm; Dr Maisch GmbH, Germany). A mixture of polar and non-polar

eluents was chosen and a flow rate of 1.0 mL/min was applied. The samples were analysed for phospholipids (PL), lyso-PL, cholesterol, cholesterol-esters, free fatty acids (FFA), monoacylglycerols (MAG), diacylglycerols (DAG) and triacylglycerols (TAG). Polar lipids (phosphatidylcholine and phosphatidylethanolamine) were quantified from a standard curve prepared from a herring roe PL concentrate. Refined moisture-free cod liver oil was used to prepare the standard curve for TAG.

2.4. Protein profile

2.4.1. Amino acid distribution

The amino acid profile analysis was done as described previously by Blackburn (1978). A total of 17 essential and non-essential amino acids, excluding tryptophan, which is destroyed during acid hydrolysis, are detected with this method. The freeze-dried muscle samples (50 mg protein) were hydrolysed with 1 mL 6 M HCl at 105 °C for 22 h. The samples got neutralized through titration using NaOH. The solutions were filtered through glass microfiber filters (GF/C) applying suction, diluted, and filtered through a 0.2 µm polyethersulfone filter (VWR International; United States), before analysed on an ultra-HPLC (UltiMate 300, Thermo Scientific).

The HPLC system included a TSP P400 pump, ultimate 3000WP injector, RF2000 detector, and a Nova-Pak C18 column (WAT086344, particle size: 4 µm, 3.9 mm*150 mm, Waters Corp., USA). Methanol and 0.08 M Sodium acetate with 2 % tetrahydrofuran were applied as mobile phases. The system's flow rate was adjusted to 0.9 mL/min.

2.4.2. Free amino acid distribution

The free amino acid profile was determined according to Osnes and Mohr (1985). A water-soluble protein extract (2 g ww in 10 mL) was prepared by homogenizing and centrifuging muscle samples in distilled water. 1 mL extract was transferred to a microcentrifugation tube, and 0.25 mL 10 % sulphosalisylic acid was added. The samples were shaken and left at + 4 °C for 30 min before centrifuging for 10 min. The supernatant was diluted and filtered through a 0.2 µm polyethersulfone filter (VWR International; North America) before analyses were done on an ultra-HPLC (UltiMate 300, Thermo Scientific). The same system parameters were applied as in 2.4.1.

2.5. Physiochemical characteristics

Water holding capacity (WHC) was measured according to Skipnes, Østby, and Hendrickx (2007) on the fresh upper belly fillets of chosen individuals. The analysis was performed by punching each fillet three times with a round sharp cutting tool (diameter: 31 mm). Two pieces were weighed and placed into a metal carrier (Part No.4750. Hettich Lab Technology, Germany) and centrifuged with 1800 rpm (4 °C, 15 min). The third portion was weighed and dried to analyse dry matter, hence water content (WC) at 105 °C for 24 h. WHC was calculated using following equations from Skipnes et al. (2007): $WHC = \left(\frac{(W-\Delta W)}{W}\right) \times 100\%$, where $W = \left(\frac{m_W}{(m_W+m_D)}\right) \times 100\%$ and $\Delta W = \left(\frac{\Delta m_W}{(m_W+m_D)}\right) \times 100\%$. Where m_w being the mass of water, m_D the mass of dry matter and Δmv being the separated water in the sample, being removed during centrifugation.

Texture Profile Analysis (TPA) was performed on fresh fillets with skin by a puncture test using a Texture Analyser (TA-XT Plus, Stable Micro Systems, UK), including a 5 kg load cell. The texture was determined by poking the fillets three times using a flat-ended cylindrical probe (diameter 25 mm, P/0.5). The muscle samples were compressed to 60 % of fillet thickness (F60), measuring the fillet firmness. Moreover, breaking force (Bf), being the force (N) employed until the breakage of the fillet's surface, was detected. The data analysis was carried out with the texture analysis software Exponent (www.stablemicrosystems, Stable Miro Systems ltd.).

2.6. Trace elements and polychlorinated biphenyls

For analyses on trace elements and polychlorinated biphenyls (PCBs), the fish were pooled together representing different catching locations and seasons. Each sample contained three individuals of same size, equally distributed and homogenized. In total, two pooled samples per season (in total 6 samples) were analysed for trace elements including toxic elements and PCBs.

2.6.1. Trace elements

Samples were analysed for Zn, V, Se, Pb, Ni, Mo, Mn, Hg, Fe, Cu, Cr, Co, Cd, As, and Ag using an 8800 Triple Quadrupole inductive coupled plasma mass spectrometry (ICP-MS) system (Agilent, USA) equipped with a prepFAST M5 autosampler (ESI, USA). Accuracy of the analysis was determined using certified reference materials cod (MODAS-5, CodTis: Nr. 0496) and herring tissues (MODAS-3, HerTis: Nr. 0958). Each sample, containing 400 mg freeze-dried muscle and 5 mL 50 % nitric acid (HNO_3 v/v), was decomposed in a high-pressure microwave digestion reactor (UltraClave, Milestone GmbH, Leutkirch,Germany) followed by dilution with ultrapure water (~18.2 MΩ·cm) to achieve a final HNO_3 concentration of 0.6 M before being analysed with ICP-MS system following methods as described by Sørmo et al. (2011). System parameters during analysis include general parameters: RF Power (1550 W), Nebulizer gas (0.75 L/min), Makeup gas (0.35 L/min), sample depth (8.0 mm), O_2 Mode: O_2 gas flow (0.675 L/min) and NH_3 mode: NH_3 gas flow (1.5 L/min), He gas flow (1.5 L/min).

2.6.2. Polychlorinated biphenyls (PCBs)

The analysis for PCBs included: PCB-3, 8, 28, 52, 101, 118, 138, 153, 180, 195, 206 and 209. The fish samples were extracted as described by Teunen et al. (2021). Approximately 0.5 g freeze-dried samples were fortified with 10 µL IS (F-PCB-mix 1 mg/L). Thereafter, the samples were extracted 2 consecutive times with 6 mL hexane: acetone (3:1, v/v). For each extraction, the mixture was ultrasonicated for 20 min, vortex mixed for 2 min, and centrifuged at 3500 rpm (Eppendorf centrifuge 5804). The supernatants were combined, and the extract was further evaporated to ~ 0.5 mL under a gentle nitrogen (N_2)-stream, purified through a 6 g acid silica (44 %, Sigma, Oslo, Norway) cartridge, from which the analytes were eluted with 20 mL hexane (MS SupraSolv®; Sigma, Oslo, Norway) followed by 15 mL dichloromethane (>99 % v v^{-1}, VWR, Oslo, Norway). The extract was evaporated to a volume of 1–2 mL using a rotary evaporator (Rota vapor R-200. Büchi Switzerland). Furthermore, the extract was evaporated to dryness under the gentle N_2-stream, redissolved in 100 µL hexane and analysed by GC–MS.

A GC–MS system (7890A, Agilent Technologies, Santa Clara, USA) with split liner injection, a Thermo TG 5MS column (length: 30 m; i.D.: 250 µm; film: 0.5 µm) and an inert mass selective detector (5975, Agilent Technologies, Santa Clara, USA) was applied to detect PCBs. The injector was set at 290 °C and the column oven temperature was programmed as follows (accounting for a total run time of 34.75 min): start temperature 50 °C held for 2 min; increased up to 250 °C at a rate of 25 °C/min and held at 250 °C for 1 min; second increase up to 286 °C at a rate of 3 °C/min and held for 3 min; third increase up to 308 °C at a rate of 8 °C/min and held for 1 min. The final temperature was reached at 310 °C at a rate of 1 °C/min and held for 3 min. The GC/MS interface was heated at 290 °C. An ionization energy was applied at 70 eV. and the electron ion source was kept at 230 °C. The MS was run in full scan (from 50 *m/z* to 550 *m/z*) as well as SIM mode. Standards were used to confirm the signal identification, while the identified target analytes of the full scan electron ionization mass spectra were compared to the available MS Library spectra (NIST MS library).

Multi-level calibration curves in the linear response interval of the detector were created for the quantification to cover the whole range of concentrations measured in the samples, and a good correlation (r^2 >

0.99) was achieved for all target analytes. The identification of analytes was based on the relative retention times to the internal standards used for quantification, the ion chromatograms and the intensity ratios of the monitored ions. The chromatographic peaks were determined when: (1) the retention time matched that of the standard compound within ± 0.05 min and (2) the signal-to-noise ratio (S/N) was higher than 3:1. For each target analyte, the limit of quantification (LOQ) was calculated as 10 times the signal from the baseline noise (S/N ratio). The quality control was performed by regular analyses of procedural blanks and by random injection of standards and solvent blanks. Recoveries for individual PCB congeners ranged between 75 and 120 % (Relative standard deviation, RSDs < 15 %).

2.6.3. Calculations on health effects of contaminants

The European Commission (EC), as well as the European Food Safety Authority (EFSA), regularly update on the maximum allowed level of specific contaminants as well as tolerable weekly intake (TWI) for safe consumption (EC, 2006, 2011; EFSA, 2009, 2012, 2014). The results obtained from section 2.6.1 and 2.6.2 were further used to evaluate whether these values exceed human intake recommendations set by the EC (EC, 2006, 2011). Furthermore, benchmark values for TWI of specific contaminants specified by the EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) were considered (EFSA, 2009, 2012). To determine the effect of the analysed components on a healthy adult, a person with 60 kg body weight was chosen as standard for all calculations as previously suggested by FAO/WHO (2011).

2.7. Statistical analysis

Statistical analyses were carried out using Minitab 19 (www.minitab.com, Minitab Inc., USA). A Grubbs Outlier test with a significance level of $\alpha < 0.05$ was performed to detect outliers within the data set. One-way ANOVA was applied to determine the differences between seasons and chemical parameters of the samples. A Tukey HSD post-hoc test was applied when significance was detected to investigate the differences between groups. A significance level of $\alpha < 0.05$ was chosen. A Pearson correlation was conducted between variables that were naturally dependent to each other. The correlation coefficient (r) and significant levels are indicated at the individual measurements. All analyses were carried out in 2 × 2 parallels (2 parallels per fillet; 4 in total per sample) and are presented as means ± standard deviation (SD) if not other stated.

3. Results & discussion

Fish (n = 59) was studied by analysing both the bottom loin and upper belly fillet of each individual separately. The results gave no significant differences ($P < 0.05$) between the two fillets of individual fish. Therefore, results of the two fillets were combined, generating one average value for each individual. The results shown in this section include the combined values of the two fillets.

3.1. Proximate composition and physiochemical characteristics

The analysis on proximate composition of European plaice revealed significant differences throughout the three investigated seasons as can be seen in Table 1. The lowest protein (14.17 %) and lipid (0.75 %) levels were found among fish captured in April, while the highest lipid content (1.55 %) was observed in December. The protein decreased significantly from September to April (17.64–14.17 %, $P < 0.001$), going along with an increasing water content (80.55–84.47 %, $P < 0.001$) from September to April. Furthermore, differences in the lipid ($P < 0.001$) and ash ($P < 0.001$) content were detected. Karl, Manthey-Karl, Ostermeyer, Lehmann, and Wagner (2013) studied European plaice captured in the Northern Sea between March and June. According to this study, European plaice contains about 81 % water, 16.6 % protein, 0.8 % fat and 0.9 % ash (Karl et al., 2013). In a study on common sole captured during four seasons in the Mediterranean sea, fluctuations in proximate composition were observed (Gökçe et al., 2004). Furthermore, Gökçe et al. (2004) found that common sole captured during the spawning season shows decreased lipid levels, while the protein content remained stable. Results in this study show a significantly lower lipid content in the post-spawning season in April compared to September ($P < 0.001$), however, the protein composition was also affected by spawning ($P < 0.001$). The reproductive season for European plaice from the Norwegian sea varies from North (December-May) to South (January-March) (Huse, 2018). The peak is from February to March, when fish barely feed and use up their lipid storage. This was also observed by Rijnsdorp (1989), studying European plaice in the Northern sea and reporting an average spawning peak from January to March. Dawson and Grimm (1980) describe quantitative changes in protein, lipid and energy content of European plaice carcasses (including fillet). According to Dawson et al. (1980), European plaice stops feeding from December to March, which is reflected by a constant decline of proteins and lipids, which agrees with the results in the present study. The collected data indicate that fish captured in December has built a lipid storage before entering the spawning season. Consequently, results on proximate composition in the present study show that fish captured in April have a relatively poor nutritional quality compared to the two other pre-spawning seasons. Moreover, fish captured in April were significantly lower in weight compared to December ($P = 0.006$). The high protein content and intermediate lipid level in September may further be a result of a higher water temperature (13 °C vs 8.7 °C and 5.4 °C) as well as longer day length, which has been reported to impact the feeding habits of fish (Olsson, Olsen, Carlehog, & Ofstad, 2003).

Table 1
Proximate composition of European plaice captured during three different seasons. Results presented as mean values ± SD.

Composition (%)	Seasons September n = 17	December n = 21	April n = 21	P-value*
Ash	1.28 ± 0.06^{a}	1.07 ± 0.09^{c}	1.17 ± 0.09^{b}	<0.001
Water	80.55 ± 1.2^{c}	82.56 ± 1.4^{b}	84.47 ± 2.4^{a}	<0.001
Proteins	17.64 ± 0.9^{a}	15.13 ± 1.2^{b}	14.17 ± 2.4^{b}	<0.001
Lipids	1.20 ± 0.4^{a}	1.55 ± 0.6^{a}	0.75 ± 0.2^{b}	<0.001

*ANOVA was applied to detect differences in proximate composition; where significant difference was detected ($\alpha < 0.05$), a Tukey.
PostHoc test was applied. Values with different superscript (^{a,b}) within a row are significantly different ($P < 0.05$).

Furthermore, seasonal variations in physiochemical parameters have been observed in this study. Analysis of the texture and water holding capacity showed certain differences in the physiochemical quality of fillets. No significant difference was found in the WHC between the three seasons ($P = 0.059$). However, a correlation in the WHC of fillet was observed from September to April (87.8 %, 85.7 % to 80.1 %, r = -0,619, $P = 0.038$), which can be negatively correlated to higher water contents (r = -0,755, $P = 0.009$). The Bf showed identical values (approximately 50 N) for each season. The fillet's firmness was measured by compressing the samples to 60 %. The results show significant textural changes between the three seasons ($P = 0.001$). The fillets had intermediate firmness in September (59.2 N) compared to April (67.7 N). Whereas fish captured pre-spawning in December showed increased softness (45.7 N). Studies have reported that fillet texture can vary during the reproductive cycle of female fish (Aussanasuwannakul et al., 2012). Aussanasuwannakul et al. (2012) described softer fillet texture during egg development and growth, and increased firmness during spawning of rainbow trout (*Oncorhynchus mykiss*). It must be considered that relatively high standard deviations for all physiochemical parameters were observed. In this study, both male and female fish were used for experiments. Therefore, great individual differences can be

expected, explaining the discrepancy in values.

3.2. Nutritional composition

The average levels of total amino acids (total-AA) and free amino acids (free-AA) in muscle tissues of European plaice captured during three seasons are shown in Table 2. The fish contain a generous distribution of amino acids recognized as essential (EAA) for the human body. The exogenous intake of these amino acids through diet is crucial to ensure human health. Hence, European plaice can be regarded as a good source for all EAA, with a prevalence of lysine (10.6–11.3 %) and leucine (9.1–9.4 %) in all three seasons. Wu (2010) states a class of functional amino acids, which are important regulators for a variety of metabolic functions in the body. Within this group, arginine, glycine, leucine and methionine are prevalent in all three seasons. The distribution between total-EAA did not vary significantly between seasons ($P = 0.103$), however the total level of free-EAA differed ($P = 0.003$). A substantially lower level of total free-AA was found in September (59.0 mg (100) g^{-1}, $P < 0.001$) with increasing numbers from December (82.3 mg (100) g^{-1}) to April (100.1 mg (100) g^{-1}). April shows the highest amount of free-EAA, but levels fluctuated considerably between individuals in all three seasons. Histidine was the dominant free-EAA in September and lower values were found in December (P=<0.001). Free leucine, methionine and valine show similar results for September and December and are significantly lower to values found in April. This pattern was not observed in the total-AA composition. All samples contained further high concentrations of free arginine and glycine, being considerably different in all three seasons (P=<0.001). Glutamine and asparagine are found in low amounts due to the conversion to aspartic and glutamic acid during acid hydrolysis. Free-AA such as free alanine, glutamic acid and glycine are known to contribute to the distinct flavour and taste of fish and fishery products (Hayashi, Yamaguchi, & Konosu, 1981; Ruiz-Capillas et al., 2004; Wu, 2010). Karl et al. (2013) reported in average 5-fold higher total free-AA values for European plaice and other flatfish compared to this study. The level of free-AA can be elevated depending on the quality, storage conditions and freshness before freezing (Calanche et al., 2019). To guarantee freshness, fish in this study was frozen at −80 °C immediately after filleting hence explaining the relatively low free-AA levels.

The boxplots in Fig. 1 visualize the percentage distribution and variation of EAA in all three seasons. Fish caught in December shows a high variation with more extreme discrepancy in the lowest and highest measured values of isoleucine, leucine, lysine, phenylalanine, threonine, and valine. Furthermore, a bigger interquartile range was observed emphasising that fish in pre-spawning state (December samples) are heterogenous in their nutritional composition. A higher SD of the AA concentrations was observed in December compared to September and April (Table 2), probably linked to differences in the spawning state of indivuals (Huse, 2018; Rijnsdorp, 1989).

The fatty acid composition, including relevant saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids have been investigated for all three seasons (Table 3). A significantly lower concentration ($P = 0.021$) of myristic acid (C14:0) was found in September (1.63 %) compared to April, (2.96 %) while stearic acid (C18:0) showed a significantly higher value in September (3.97 %) compared to December (2.94 %, $P = 0.02$) and April (2.79 %, $P = 0.007$). High values of palmitic acid (C16:0) were found in all three seasons, which is in accordance with other findings studying flatfish species (Gökçe et al., 2004; Karl et al., 2013; Olsson et al., 2003). The percentages of the two main n3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were between 14.4 and 16.8 % and 18.2–19.3 % respectively, with no significant differences (P-EPA = 0.152, P-DHA = 0.587) between the three seasons. In a study on common sole captured during different seasons, Gökçe et al. (2004) found comparable values of DHA (18.8–20.2 %), but distinctively lower values in EPA (3.36–4.26 %) to this study. In a study of Karl et al. (2013) a comparable DHA distribution for European plaice of 19.4 % was found, whereas the reported values of 22.5 % and 1.2 % for EPA and docosapentaenoic acid (DPA) differ to the present study. DPA distributions of 2.9–3.29 % were observed in the three investigated seasons. The reported values can be considered as high concentrations for both EPA and DHA. Nevertheless, European plaice is a lean fish, composing of relatively low overall lipid levels. In addition to a beneficial n3 distribution, excellent ratios of n3/n6 (6.1–8.8) were observed, emphasising the

Table 2
Total-AA (%) and free-AA distribution (mg (100)g^{-1} ww) of European plaice during different seasons. Results presented as mean values ± SD.

Amino Acids	Seasons: September n = 17	December n = 21	April n = 21		September n = 17	December n = 21	April n = 21	
Essential*	Total-AA%			P-value***	Free-AA mg (100)g^{-1}			P-value***
Histidine	2.5 ± 0.3	2.8 ± 0.3	2.5 ± 0.2	0.597	7.0 ± 4.4^{A}	0.8 ± 0.4^{C}	3.7 ± 4.2^{B}	<0.001
Isoleucine	4.8 ± 0.5	5.2 ± 0.9	4.9 ± 0.3	0.450	0.9 ± 0.2^{B}	1.2 ± 0.2^{B}	1.6 ± 0.7^{A}	<0.001
Leucine	9.4 ± 1.1^{ab}	9.1 ± 2.8^{b}	9.4 ± 0.6^{a}	0.027	1.5 ± 0.3^{B}	1.8 ± 0.4^{B}	3.0 ± 1.4^{A}	<0.001
Lysine	11.3 ± 1.2^{a}	10.6 ± 3.4^{b}	11.2 ± 0.8^{a}	0.010	2.8 ± 2.2^{B}	6.6 ± 4.2^{A}	8.6 ± 3.9^{A}	<0.001
Methionine	3.5 ± 0.5^{b}	4.1 ± 0.4^{a}	3.7 ± 0.3^{a}	0.006	2.2 ± 0.9^{B}	2.0 ± 0.5^{B}	3.1 ± 1.2^{A}	<0.001
Phenylalanine	4.8 ± 0.6^{a}	4.7 ± 1.0^{a}	4.6 ± 0.3^{b}	<0.001	1.1 ± 0.2^{B}	1.4 ± 0.4^{A}	1.5 ± 0.4^{A}	0.001
Threonine	5.5 ± 0.8	5.6 ± 1.2	5.4 ± 0.4	0.247	4.5 ± 1.0	4.9 ± 2.1	5.8 ± 3.5	0.291
Valine	5.3 ± 0.6	5.4 ± 1.2	5.2 ± 0.4	0.108	$1.6 \pm 0.4A^{B}$	1.7 ± 0.3^{B}	2.3 ± 1.0^{A}	0.001
Σ EAA*	47.2 ± 2.5	47.6 ± 8.5	46.6 ± 2.6	0.103	21.5 ± 7.4^{B}	20.5 ± 6.2^{B}	29.6 ± 12^{A}	0.003
Non-essential								
Asparagine	<0.03	<0.03	<0.03	/	$<0.05^{A}$	$<0.03^{B}$	$<0.03^{B}$	<0.001
Glutamine	<0.06	<0.06	<0.06	/	2.8 ± 1.0^{A}	2.8 ± 1.3^{A}	0.7 ± 0.4^{B}	<0.001
Arg/Glyc**	6.7 ± 1.0^{b}	7.1 ± 1.6^{b}	7.4 ± 0.5^{a}	0.003	14.9 ± 4.7^{C}	34.1 ± 11.5^{B}	45.9 ± 22^{A}	<0.001
Tyrosine	4.2 ± 0.6	4.5 ± 0.6	4.2 ± 0.3	0.664	1.3 ± 0.4^{A}	1.0 ± 0.3^{AB}	0.9 ± 0.4^{B}	0.005
Alanine	6.4 ± 0.9	6.5 ± 1.9	6.5 ± 0.5	0.107	10.2 ± 2.8^{B}	13.7 ± 2.9^{A}	13.2 ± 4.5^{A}	0.008
Aspartic acid	12.2 ± 1.7^{ab}	12.3 ± 2.2^{b}	12.1 ± 0.8^{a}	0.022	0.6 ± 0.2^{A}	0.4 ± 0.2^{B}	0.7 ± 0.2^{A}	<0.001
Glutamic acid	17.7 ± 2.0^{a}	16.5 ± 3.3^{b}	17.2 ± 1.1^{a}	<0.001	3.0 ± 0.9^{B}	5.4 ± 2.2^{A}	2.8 ± 1.5^{B}	<0.001
Serine	5.4 ± 0.6^{ab}	5.5 ± 1.3^{b}	5.6 ± 0.4^{a}	0.042	4.2 ± 1.1^{A}	3.6 ± 1.1^{A}	6.3 ± 2.0^{B}	<0.001
Σ NEAA	52.8 ± 3.7^{ab}	52.4 ± 9.2^{b}	53.5 ± 4.4^{a}	0.010	37.48 ± 7.7^{B}	61.9 ± 17.1^{A}	66.6 ± 26.2^{A}	<0.001
Σ Free-AA					59.0 ± 10.7^{B}	82.3 ± 22.0^{A}	100.1 ± 38.6^{A}	<0.001

*Tryptophan is not detected due to acid hydrolysis.
**Arginine/Glycine could not be separated.
***ANOVA was applied to detect differences in total AA and free AA; where significant difference was detected ($\alpha < 0.05$), a Tukey PostHoc test was applied. Values with different superscript within a row are significantly different ($P < 0.05$); small letters (a,b):total-AA; big letters (A,B): free-AA.

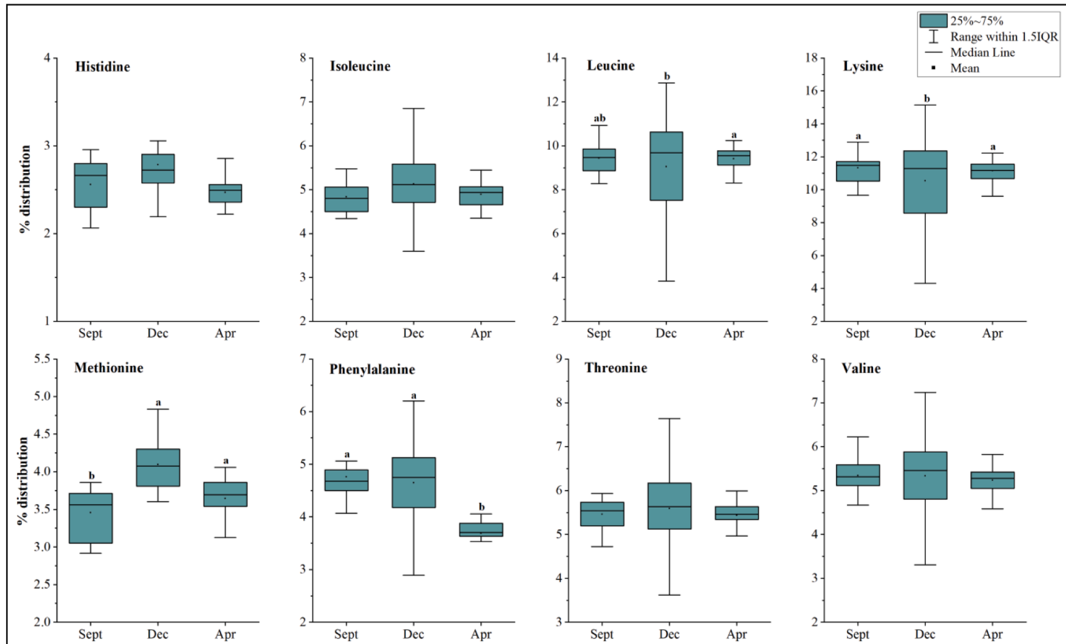

Fig. 1. Boxplots of essential amino acids (%) of European plaice captured during three different seasons (September, Sept; December, Dec; and April, Apr). Boxes visualize 25–75 % of sample values; whiskers the 1.5 interquartile range (IQR); line (median), dot (means); small letters (a,b,c) show significant differences between groups ($P < 0.05$).

importance of fish to provide sufficient levels of n3 and its role in human nutrition. Karl et al. (2013) reported a n3/n6 ratio of 4.9 for European plaice and ranges of 1.89 to 3.84 for sole were observed in the study of Gökçe et al. (2004). In the study of Olsson et al. (2003) on wild and farmed halibut (total lipid < 1 %), similar values of 40.4 % of n3 fatty acids were found, whereas values for n6 fatty acids were lower (3.5 %).

Furthermore, statistical analyses showed a negative correlation between the content of MUFA and n6 fatty acids ($r = -0.987$, $P = 0.05$). This is demonstrated by significantly lower MUFA distributions in September and April (26.08 ± 1.0 and 27.78 ± 0.7, $P = 0.001$), compared to higher levels of n6 fatty acids (6.29 ± 1.8 and 6.1 ± 1.2) in these two seasons. A significantly higher concentration ($P < 0.001$) of MUFA was found in December compared to the two other seasons. Although no significant statistical difference was found for n3 fatty acids ($P = 0.235$), a similar trend in negative correlation ($r = -0.740$) was observed. September shows the highest percentage distribution (42.15 ± 1.2), compared to the lowest MUFA portion in all three seasons. Moreover, the high concentration of PUFA in September (49.10 ± 1.3) differs considerably ($P = 0.023$) to the lower concentration in December (45.35 ± 1.1).

The analysis on lipid classes revealed abundance of three main lipid classes: phospholipids (PL), cholesterol (Chol) and triacylglycerols (TAG). Other classes such as diglycerides, monoglycerides, lysophospholipids or free fatty acids were below the limit of detection (LOD). For the three identified classes, no significant differences ($P > 0.05$) between the seasons were found. A reason for this can be the small sample size that was analysed, consisting of only three samples per season. It was not possible to analyse more samples, as the oil fraction of the fish contributed to only 0.75–1.55 % (Table 1), hence individuals needed to be merged to get enough oil for analysis. Even though no statistically significant difference ($P > 0.05$) was found, a clear trend can be seen in Fig. 2. The PL fraction (48 %) increased noticeable in April, followed with a higher cholesterol (6 %) and lower TAG (46 %) composition compared to the other two seasons. Fish in December had the highest amount of TAG. This agrees with the higher overall lipid content. TAG are storage lipids, which are used during the cold winter season to provide energy, hence fish is feeding up until winter to guarantee an adequate storage. The change in lipid class distribution can be associated with the change in total lipid concentration in the samples. The higher TAG content in December is significantly correlated to the higher total lipid content ($r = 0.988$; $P = 0.05$). With regards to total lipid content, a negative correlation has been found for the PL ($r = -0.988$; $P = 0.05$) as well as the Chol fraction ($r = -0.993$; $P = 0.039$). These correlations indicate an increase in cholesterol and phospholipids, with decreasing total lipid content. Whereas triacylglycerides are predominant at a higher total lipid content.

The amount of essential amino acids, PUFA and n3 fatty acids in the results emphasise the high nutritional value of European plaice. Moreover, the n3:n6 ratio can be regarded as very sufficient in all three studied seasons. The EAA distribution, distribution of overall PUFA as well as important n3 fatty acids such as EPA, DPA and DHA do not differ significantly between the three seasons. Differences were only observed with regards to proximate composition, showing higher lipid contents in winter, followed by low lipid and protein content in April. However, no correlation between total fat content and fatty acid distribution of SFA, MUFA or PUFA was observed in any of the three seasons. Moreover, no correlation between fatty acid composition and lipid classes was found. This indicates a relative stable composition of EAA, n3 fatty acids and total PUFA throughout the three fishing seasons.

3.3. Trace elements and polychlorinated biphenyls

European plaice, being a demersal fish species can accumulate higher amounts of toxic trace elements as well as contaminants like PCBs (Parolini et al., 2020). Hence the aspect of food safety must be considered when consuming European plaice regularly. Nevertheless, the

Table 3
fatty acid composition (% of total fatty acids w w^{-1}) of European plaice at different seasons. Results presented as mean values ± SD.

Fatty Acids	Seasons: September n = 6	December n = 6	April n = 6	
	%	%	%	P-value**
SFA				
C14:0	1.63 ± 1.4^{b}	2.71 ± 1.4^{ab}	2.96 ± 0.5^{a}	0.018
C15:0	0.28 ± 0.4	0.13 ± 0.2	0.34 ± 0.5	0.339
C16:0	15.86 ± 2.8	15.20 ± 2.0	14.33 ± 1.3	0.223
C17:0	0.28 ± 0.3	0.10 ± 0.2	0.13 ± 0.2	0.171
C18:0	3.97 ± 0.7^{a}	2.94 ± 1.1^{b}	2.79 ± 0.8^{b}	0.005
Σ SFA	22.03 ± 1.0	21.08 ± 0.8	20.55 ± 0.4	0.192
MUFA				
C14:1	0.05 ± 0.2	0.01 ± 0.04	0.03 ± 0.07	0.720
C16:1 n7	5.57 ± 1.3^{ab}	6.67 ± 0.7^{a}	4.68 ± 2.3^{b}	0.011
C17:1	0.29 ± 0.4	0.33 ± 0.4	0.34 ± 0.3	0.935
C18:1 n7	3.31 ± 2.6	2.86 ± 0.8	2.50 ± 0.6	0.480
C18:1 n9	7.66 ± 2.9^{b}	10.40 ± 0.8^{a}	9.37 ± 1.3^{ab}	0.004
C20:1 n9	0.42 ± 0.5	0.27 ± 0.3	0.33 ± 0.3	0.651
C20:1 n11	5.50 ± 1.4	6.03 ± 0.7	5.97 ± 0.5	0.342
C22:1	3.28 ± 1.9	4.87 ± 2.0	4.6 ± 1.0	0.071
Σ MUFA	26.08 ± 1.0^{b}	31.54 ± 0.6^{a}	27.78 ± 0.7^{b}	0.001
PUFA				
C16:2 n4	0.66 ± 0.6	0.88 ± 0.5	1.03 ± 0.4	0.216
C18:2 n6 (LA)	0.67 ± 0.6^{b}	1.24 ± 0.6^{a}	1.34 ± 0.6^{a}	0.001
C18:3 n3	0.35 ± 0.5^{b}	0.93 ± 0.6^{a}	0.90 ± 0.6^{a}	0.020
C18:4 n3	0.88 ± 1.0^{b}	2.17 ± 1.4^{a}	1.95 ± 1.0^{ab}	0.023
C20:2 n6	0.14 ± 0.3	0.10 ± 0.2	0.17 ± 0.2	0.748
C20:4 n6 (AA)	5.48 ± 3.5	3.76 ± 2.4	4.41 ± 2.2	0.313
C20:4 n3	1.58 ± 2.8	0.65 ± 0.5	0.5 ± 0.5	0.254
C20:5 n3 (EPA)	16.79 ± 3.1	14.37 ± 3.6	14.75 ± 2.8	0.152
C22:5 n3 (DPA)	3.29 ± 1.1	3.04 ± 0.5	2.9 ± 0.3	0.346
C22:6 n3 (DHA)	19.27 ± 3.2	18.21 ± 2.3	18.40 ± 2.4	0.587
Σ PUFA*	49.10 ± 1.3^{a}	45.35 ± 1.1^{b}	46.14 ± 1.0^{ab}	0.026
Σ n3	42.15 ± 1.2	39.37 ± 1.3	39.37 ± 1.1	0.108
Σ n6	6.29 ± 1.8	5.10 ± 1.2	6.1 ± 1.2	0.529
n3/n6	6.1 ± 2.5	8.8 ± 3.3	7.5 ± 3.3	0.130
Others	2.79 ± 3.5	2.03 ± 2.0	5.1 ± 5.6	0.154

*including n3 and n6.
**ANOVA was applied to detect differences in fatty acid composition; where significant difference was detected (α < 0.05), a Tukey.
PostHoc test was applied. Values with different superscript ([a,b]) within a row are significantly different (P < 0.05).

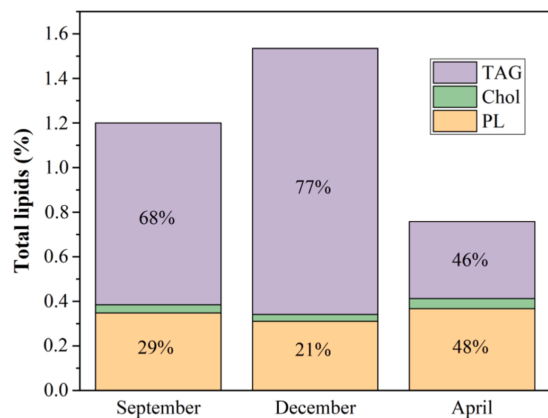

Fig. 2. Total lipid content and lipid class distribution (total distribution of 100%) of European plaice captured in September, December and April.

bioaccumulation of trace elements, including both essential elements and toxic elements depends on endogenous and exogenous factors (Afonso et al., 2013). It is possible to have higher variations of trace element concentrations between individuals of different size, sex, but also feeding ground and migration habits must be considered. In this study, different locations for the toxic screening of toxic trace elements and PCBs of fish captured during three seasons were considered. Statistical analyses have shown no significant differences (P > 0.05) between the fishing locations. It must be mentioned that the sample size for one-way ANOVA of trace elements and PCBs was rather small, as individuals were pooled together to represent the population. The data is therefore grouped in seasons (Table 4).

Although the concentration of elements varied largely between individuals, the levels are within the suggested intake of the EC (EC, 2006). The guidelines for the maximum levels of contaminants in fishery products and fish meat of the EC for mercury (0.5 mg kg^{-1} ww), lead

Table 4
Trace elements and PCBs of European plaice during three different seasons. n = pooled samples constituting of 3 individuals per sample. Results presented as mean values ± SD.

Trace elements	Season: September n = 2	December n = 2	April n = 2	
	μg kg^{-1}	μg kg^{-1}	μg kg^{-1}	P-value***
Vanadium*	24.0 ± 21.3	6.5 ± 3.3	6.7 ± 1.7	0.580
Chromium*	8.2 ± 3.3	6.3 ± 0.6	8.4 ± 2.1	0.621
Manganese	31.0 ± 2.3	44.8 ± 28.0	44.0 ± 4.3	0.680
Nickel*	2.2 ± 0.4	1.4 ± 0.1	2.4 ± 0.7	0.229
Molybdenum	0.65 ± 0.3^{a}	0.65 ± 0.06^{a}	1.3 ± 0.02^{b}	0.033
Silver*	0.01 ± 0.01	0.1 ± 0.1	0.3 ± 0.08	0.221
Cadmium*	0.16 ± 0.03	0.19 ± 0.1	0.2 ± 0.1	0.939
Cobalt	2.7 ± 1.6	1.8 ± 0.09	3.0 ± 1.2	0.597
Lead*	0.53 ± 0.2	0.76 ± 0.5	1.1 ± 0.9	0.637
	mg kg^{-1}	mg kg-1	mg kg-1	
Arsenic*	45.3 ± 11.8	51.8 ± 11.2	79.7 ± 49.0	0.544
Iron	1.0 ± 0.3	0.95 ± 0.2	0.82 ± 0.3	0.771
Zinc	3.4 ± 0.02	4.0 ± 0.3	4.3 ± 0.5	0.142
Selenium	0.3 ± 0.09	0.4 ± 0.08	0.5 ± 0.2	0.504
Copper	0.1 ± 0.007	0.1 ± 0.002	0.13 ± 0.03	0.229
Mercury*	0.11 ± 0.1	0.15 ± 0.02	0.09 ± 0.06	0.642
Σ toxic elements	45.4 ± 11.9	51.9 ± 11.2	79.8 ± 49.1	0.545
*toxic elements				
PCBs	September n = 2 ng g^{-1}	December n = 2 ng g^{-1}	April n = 2 ng g^{-1}	P-value***
PCB-3	0.012 ± 0.01	0.123 ± 0.17	<LOD	0.493
PCB-8	<LOD	<LOD	<LOD	/
PCB-28*	<LOD	<LOD	<LOD	/
PCB-52*	0.010 ± 0.01	<LOD	0.015 ± 0.02	0.631
PCB-101*	<LOD	0.147 ± 0.07	<LOD	/
PCB-118**	0.076 ± 0.01	0.141 ± 0.001	0.092 ± 0.065	0.347
PCB-138*	0.180 ± 0.027	0.420 ± 0.154	1.886 ± 2.22	0.455
PCB-153*	0.138 ± 0.02	0.362 ± 0.11	0.563 ± 0.36	0.289
PCB-180*	<LOD	<LOD	0.706 ± 0.99	/
PCB-195	<LOD	<LOD	<LOD	/
PCB-206	<LOD	<LOD	<LOD	/
PCB-209	<LOD	<LOD	<LOD	/
Σ ICES-6*	0.368 ± 0.058	0.698 ± 0.326	0.658 ± 3.55	0.449
Σ DL-PCBs**	0.076 ± 0.01	0.141 ± 0.001	0.092 ± 0.065	0.347
Σ total PCBs	0.416 ± 0.03	1.192 ± 0.15	3.262 ± 3.62	0.467

*ICES-6: non dioxin-like PCBs (NDL-PCBs).
**dioxin-like PCBs (DL-PCBs; Σ of non-ortho + mono-ortho PCBs).
***ANOVA was applied to detect differences in trace elements and PCBs; where significant difference was detected (α < 0.05), a Tukey PostHoc test was applied. Values with different superscript ([a,b]) within a row are significantly different (P < 0.05).

(0.3 mg kg^{-1} ww), and cadmium (0.1 mg kg^{-1} ww) are not exceeded in fish examined in this study (EC, 2006). According to EC (2006) around 90 % of total mercury is present in the form of methylmercury, which is most alarming in fish and seafoods. Methylmercury is the predominant form of inorganic mercury, which can origin from the earth's crust, but also from anthropogenic sources (EFSA, 2012). The EFSA CONTAM Panel sets a TWI of 1.3 μg kg^{-1} body weight (b.w.) for methylmercury, expressed as mercury (EFSA, 2012). Taking the maximum value of 170 μg kg^{-1} total mercury found in fish of December as a basis for calculating the TWI for a person with 60 kg body weight, 459 g European plaice can be consumed per week. This would account for approximately 2.5 portions (180 g portion) of European plaice and is higher than the recommendation of EFSA of 300 g (EFSA, 2014) seafood per week. The analysis of variance showed no significant differences for each element with regards to both locations and seasons ($P > 0.05$). Only the concentration of the essential element molybdenum varied significantly ($P = 0.033$) between the three seasons, being double the concentration in April (1.3 ± 0.02 μg kg^{-1}) compared to September (0.65 ± 0.3 μg kg^{-1}) and December (0.65 ± 0.06 μg kg^{-1}). Afonso et al. (2013) studied different trace elements in five fish, among those megrim (*Lepidorhombus whiffiagonis*) and four spotted megrim (*Lepidorhombus boscii*), two flatfish species with comparable proximate composition to European plaice. Comparable values of Zn with slightly higher values in Fe compared to values found in this study are shown by Afonso et al. (2013). High values of total arsenic were found in all three seasons, with a particular high SD in April. Seafood in general has higher concentrations of total arsenic compared to other food commodities. The greatest part is in the form of organic, non-toxic arsenobetaine, whereas inorganic forms such as arsenite (As + 3) and arsenate (A + 5) are highly toxic and carcinogenic for humans (Sloth, Larsen, & Julshamn, 2005). The CONTAM Panel points out the limited amount of published data on inorganic arsenic levels of seafood and announced that the previously established provisional TWI of 15 μg kg^{-1} can be regarded as too high (EFSA, 2009). Furthermore, EFSA (2009) sets a benchmark dose lower confidence limit ($BMDL_{01}$) of 0.3 to 8 μg kg^{-1}b.w. per day, where BMDL01 explains benchmark dose concentrations for a 95 % lower confidence limit of 1 % extra risk for lung, skin or bladder cancer or skin lesions. Sloth et al. (2005) analysed inorganic arsenic in different marine species, including fish and pointed out that among all samples, the amount of inorganic arsenic constituted to<1 % of the total arsenic content. Expecting a maximum of 1 % inorganic arsenic in fish captured for this study, this would mean a maximum of 1.29 mg kg^{-1} (April) and a minimum of 0.34 mg kg^{-1} (September). Following the recommendation of 8 μg kg^{-1} as an upper $BMDL_{01}$b.w. per day for a person with 60 kg b.w., this would lead to a recommended dietary allowance (RDA) of 372 g European plaice per day considering the maximum values of inorganic Arsenic in April. This leads to a PTWI of at least 2.6 kg European plaice for a safe consumption.

The analysis of several PCBs included both non dioxin-like PCBs (NDL-PCBs) as well as one dioxin-like PCB congener (DL-PCB; PCB-118). Several concentrations of the targeted PCBs were lower than the detection limit. This indicates a non-significant concentration, hence no major exposure to these PCB congeners when consuming this fish. According to the regulation of the European Commission (EC, 2011), present results of NDL-PCBs do not exceed the recommended limits of 75 ng g^{-1} wet weight for fish and fishery products. Furthermore, the total amount of detected PCBs in the samples is at a low level, being around 0.4, 1.19 and 3.26 ng g^{-1}, respectively. No statistical significance between the three seasons could be found. However large differences in standard deviations indicate large discrepancies in the pooled samples, hence imply larger variations between individuals. Unexpectedly, the concentration of PCB-118 (0.14–0.07 ng g^{-1}), being a DL-PCB exceeds the recommended upper limit 6.5 pg g^{-1} (EC, 2011) in all three seasons. Particularly high concentrations (0.141 ± 0.001 ng g^{-1}) were found in pooled samples of December. Previously, foods with <1 % fat have been excluded from regulations on the maximum level of dioxins and DL-PCBs (EC, 2011), as these chemicals tend to accumulate in the adipocytes of organisms. The accumulation of DL-PCBs could be a result of the higher lipid content compared to the other two seasons.

Concerning the analysis of toxic trace elements and PCBs, values exceeding the recommended levels for DL-PCBs (PCB-118) were obtained, while values corresponding to e.g., lead, total mercury or cadmium can be considered within the acceptable limit. The risk of possibly elevated levels of DL-PCBs can be opposed to the positive nutritional aspects, including a good distribution of essential amino acids as well as n3 fatty acids of European plaice. To our knowledge, so far, no risk–benefit assessment on increasing the consumption of European plaice has been carried out.

4. Conclusion

In this study, chemical analyses and screening of toxic elements and PCBs in European plaice throughout three seasons showed predominantly positive results to promote the consumption of the species in all three seasons. The results indicate stable total essential amino acid distributions in all three investigated seasons, but a variance in PUFA content, with higher amounts of PUFA in fish caught in September. Changes in the total lipid content and lipid composition imply higher lipid storage during December and more membrane lipids in fish caught in April. Values on toxic trace elements revealed no harmful elevated values and the overall PCB content in fish was below the maximum accepted limit for all three seasons. Nevertheless, elevated levels of DL-PCBs (PCB-118) were found in all three seasons, with highest values in December which must be considered. Moreover, more data on inorganic arsenic on fish must be collected to set up a concentration limit for European plaice as well as fish and fishery products in general. Future work should focus on the assessment of possible negative and beneficial effects that come along with an increased consumption of European plaice in Norway.

CRediT authorship contribution statement

Sophie Kendler: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Dionysios Tsoukalas:** Investigation, Conceptualization. **Anita Nordeng Jakobsen:** Conceptualization, Methodology, Supervision. **Junjie Zhang:** Formal analysis, Methodology, Investigation. **Alexandros G. Asimakopoulos:** Resources, Methodology. **Jørgen Lerfall:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Paper II

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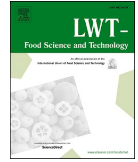

The effect of fishing season and storage conditions on the quality of European plaice (*Pleuronectes platessa*)

Dionysios Tsoukalas *, Sophie Kendler, Jørgen Lerfall, Anita Nordeng Jakobsen

Department of Biotechnology and Food Science, Norwegian University of Science and Technology (NTNU), NO-7491, Trondheim, Norway

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ABSTRACT

The combined effect of fishing season (September/April) and storage conditions on the quality of European plaice (*Pleuronectes platessa*) was investigated. Investigated storage conditions were; fillets packaged in vacuum or a modified atmosphere (MA; 70% CO_2, 20% N_2, 10% O_2) and stored at 4 °C. As a control, whole fish on ice (0 °C) was used, representing the commercial standard. Plaice showed in general lower quality in April than September, demonstrated by a faster microbial evolution, higher content of biogenic amines, and higher rate of ATP degradation representing by K- and H-values. MA-fillets and whole fish on ice showed lower K- and H-values and biogenic amines content than fillets packaged in vacuum. VP-fillets had the lowest microbial quality as demonstrated by the shortest lag-phase of psychrotrophic aerobic plate count and aerobic bacteria, and the highest maximum specific growth rate of H_2S-producing bacteria. MA packaging significantly reduced microbial growth; however, MA-fillets showed the lowest water content and the highest drip loss and water holding capacity. No differences between groups were observed in colour intensity or hue angle at the storage end. Despite the significant seasonal variations, MA packaging stands out as the best solution to maintain the freshness of convenient retail plaice products.

1. Introduction

European plaice (*Pleuronectes platessa*) is one of the most widespread flatfish species in the North Sea (Madsen et al., 2013) and is a significant by-catch in many North-Western European fisheries (Bayse et al., 2016). The most significant catches are landed in the Netherlands, followed by Denmark and the United Kingdom (EUMOFA, 2016). The agreed quota in the North Sea and Skagerrak was 162,607 tons in 2021 (ICES, 2021), while the total landings of plaice in Norway were 794 tons (whole fish equivalent) (Directorate of Fisheries). Compared to the quota, the low percentage of caught plaice in Norway is due to its low commercial value. Although there is no official record, one part of the caught plaice is traded as a fresh whole fish on ice in the Norwegian fish market, and the rest is exported to Europe as fresh or frozen whole fish. The trend of consumers preferring convenient seafood products (Carlucci et al., 2015) represent a potential for value-added product development of plaice to increase its commercial use, domestic consumption, and exportable quantity.

Modified atmosphere-packaging (MAP) and vacuum-packaging (VP) combined with low temperature are used to prolong the shelf life of raw seafood, counteracting deteriorative effects during storage due to microbial and endogenous enzymatic activity (Bouletis et al., 2017). However, seafood's physicochemical and microbial quality can be affected differently by implementing these packaging technologies, as demonstrated in many studies (Esteves et al., 2021; Silbande et al., 2018). Moreover, it is well known that seasonal variation affects the quality of fresh fish (Cardoso et al., 2021; DurmuŞ et al., 2014; Grigorakis et al., 2003; Ntzimani et al., 2022; Papaharisis et al., 2019; Parlapani et al., 2021; Tzikas et al., 2007).

Plaice, like other seafood, is highly perishable, and the quality deteriorates fast due to enzymatic and microbial activity and oxidations (Gram & Huss, 1996). To our knowledge, there is no research on the physicochemical and microbial quality of plaice caught in Northern Europe. Moreover, literature on optimal packaging and storage conditions of plaice as well as seasonal quality variations, are limited. Therefore, the aim of this study was to investigate the combined effect of fishing season and storage conditions on microbial growth, metabolites, and physicochemical parameters during cold storage. Plaice was caught in September and April. Plaice fillets were packaged in either vacuum or modified atmosphere (70% CO_2, 20% N_2, 10% O_2) and stored at 4 °C.

* Corresponding author.
E-mail address: dionysios.tsoukalas@ntnu.no (D. Tsoukalas).

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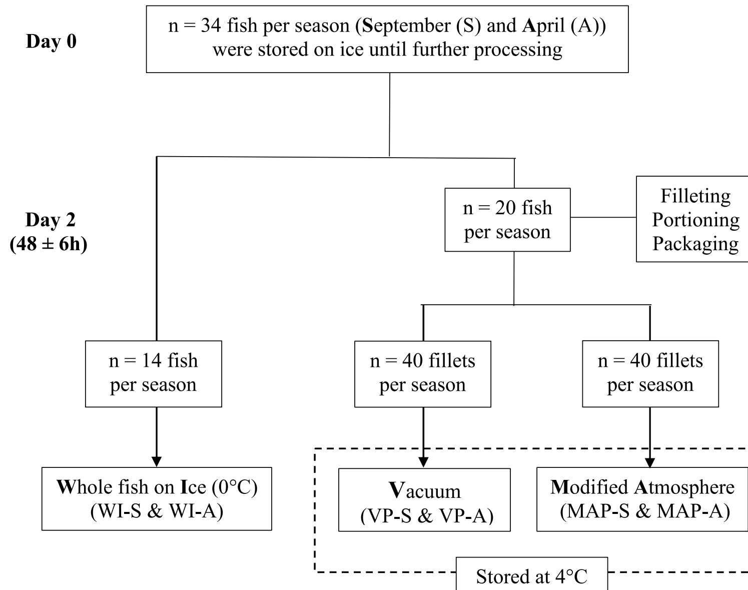

Fig. 1. Experimental overview. Thirty-four fish per season were stored on ice until further processing. On day 2 (48 ± 6 h), 14 fish per season were randomly selected, wrapped in plastic film, and stored as a whole fish on ice (WI–S and WI-A). The remaining fish (n = 20 per season) were filleted, deskinned, randomly distributed, and packaged in either vacuum (VP–S and VP-A) or modified atmosphere (70% CO_2, 20% N_2, 10% O_2) (MAP-S and MAP-A).

Whole fish stored on ice (0 °C) was selected as a commercial standard since it is the most predominant form of trading plaice in the Norwegian market. Increased knowledge of quality measures of plaice can increase the utilisation and commercial value through the production of high-quality, convenient retail plaice products.

2. Material and methods

2.1. Raw material and experimental design

Plaice used in the present study was caught in September 2020 and April 2021 by local fishermen using purse seine on the west coast of Norway (approximately 62.75°N, 6.51°S). The seawater temperature was 13 °C in September and 5.4 °C in April. The average weight of whole fish was 0.98 ± 0.31 kg in September and 0.93 ± 0.38 kg in April. The fresh-caught plaice (measured within 1 h after catch) had a pH value of 7.6 ± 0.1 in September (S) and 7.7 ± 0.2 in April (A). The fish (n = 34 per season) were instantly killed before being gutted, packaged in expanded polystyrene boxes with ice slurry, and transported within 12 h to the laboratory and stored until further processing.

A factorial design was set up to study seasonal variations and storage conditions on the physicochemical characteristics, microbial growth kinetic parameters, and autolytic and microbial deterioration of European plaice (Fig. 1). Plaice fillets were packaged in vacuum (VP–S and VP-A) and modified atmosphere (MAP-S and MAP-A) and stored at 4 °C. Whole fish on ice (WI–S and WI-A) was selected as a control group representing the commercial standard (0 °C).

On day 2 (48 ± 6 h), 14 fishes per season (WI–S and WI-A) were randomly selected, wrapped in plastic film, and stored on ice. The rest of the fish (n = 20) were filleted. Due to the plaice's morphology, four fillets were obtained from each fish (upper loin, upper belly, bottom loin, bottom belly). The fillets were deskinned, randomly distributed, and packaged in either vacuum (VP–S and VP-A) or modified atmosphere (MAP-S and MAP-A).

2.2. Packaging

VP-S and VP-A were packaged in 20-μm polyamide (PA)/70-μm polyethylene (PE) bags (160 × 200 mm, oxygen transmission rate (OTR) 50 cm^3/m^2 × 24 h × bar at 23 °C, Star-Pack Productive, Boissy-l'Aillerie, France) with a Webomatic Supermax-C vacuum machine (Webomatic, Bochum, Germany). Air was evacuated to an end pressure of 10 mbar before sealing. MAP-S and MAP-A were placed in 230 mL semi-rigid crystalline polyethylene terephthalate (CPET) trays (C2125–1B, OTR 66–78 cm^3 × 25 $μm/m^2$ × 24 h × bar at 23 °C, Færch Plast, Holstebro, Denmark) with an absorbent underneath using a semi-automatic tray sealing packaging machine (TL250, Webomatic, Germany) to obtain a gas:product ratio of 1:2. The air was evacuated to an end pressure of 30 mbar before the trays were filled with the pre-set packaging gas mixtures before heat sealing of the top film (40-μm PE, ethylene vinyl alcohol (EVOH), PA and PET) (Topaz B-440 AF, OTR 2.5 cm^3 × 40 $μm/m^2$ × 24 h × atm at 23 °C, Plastopil, Almere, The Netherlands). CO_2, N_2 and O_2 (food grade quality) were mixed using a MAP Mix 9000 gas mixer (Dansensor, Ringsted, Denmark) to obtain a packaging atmosphere of 70% CO_2, 20% N_2 and 10% O_2. To ensure the correct settings, the gas composition was measured in six dummies (sealed packages without products) with an O_2 and CO_2 analyser (Checkmate 9900 analyser, Dansensor). A rubber septum (Nordic Supply, Skodje, Norway) was placed on the sealing foil before inserting the syringe for headspace gas collection to avoid the introduction of the surrounding atmosphere. The initial packaging gas composition (day 2) of the MAP-S fillets was CO_2 = 70.3 ± 0.4%, O_2 = 10.3 ± 0.1%, and N_2 = 19.4 ± 0.1%, and MAP-A fillets was CO_2 = 70.3 ± 0.3%, O_2 = 10.0 ± 0.1% and N_2 = 19.7 ± 0.5%. To control the packages' quality (MAP-S and MAP-A), the gas composition was also measured on day 4, 7, 9, 10, 14, 16, 17, and 20. The packaging gas composition analysis was performed in triplicates (n = 3) for each group at each sampling point.

2.3. Microbial analysis

Microbial analysis was performed in triplicates (n = 3) for each group on days 2, 4, 7, 10, 14, and 17 (VP) and days 2, 4, 7, 10, 14, 17, and 20 (WI and MAP). *Listeria monocytogenes* and Enterobacteriaceae were determined at day 2 and at the end of the storage trial (VP: 17th day; WI/MAP: 20th day). A 10-g sample of fish muscle was aseptically transferred to a sterile stomacher bag and diluted 1:10 with sterile peptone water (1.0 g bacteriological peptone (Lyngby, Oxoid, Oslo, Norway) and 8.5 g/L NaCl (AnalaR NORMAPUR® ACS)) and homogenised vigorously for 120 s in a Stomacher 400 Lab Blender (Seward Medical Ltd., UK). Appropriate serial dilutions were made in sterile peptone water and spread on or moulded in their respective agar plates.

Aerobic bacteria (APC) and H_2S-producing bacteria were quantified as total and black colonies, respectively, by pour plating on iron agar (Oxoid) supplemented with 0.04% L-cysteine (Sigma-Aldrich, Oslo, Norway). Plates were incubated at 22 °C for 72 h. Psychrotrophic aerobic plate count (PC) was quantified by spreading on Long and Hammer agar (LH) with 1% NaCl, in order to support the growth of the salt requiring *Photobacterium phosphoreum* (NMKL No. 184, 2006). Plates were incubated at 15 °C for six days. Lactic acid bacteria (LAB) were quantified by spreading on de Man, Rogosa and Sharp agar (MRS) (Oxoid) that was incubated under anaerobic conditions at 25 °C for five days. Enterobacteriaceae was quantified using violet-red-bile-glucose agar (VRBGA) (Oxoid) by pour plating and incubated at 37 °C for 24 h. *Pseudomonas* spp. was quantified on Pseudomonas agar base (CM0559, Oxoid) supplemented with Pseudomonas CFC selective supplement SR0103 (Oxoid) by spread plating and incubated aerobically at 25 °C for 48 h. *Brochothrix thermosphacta* was quantified using STAA agar base (CM0881) supplemented with STA selective supplement SR0162 (Oxoid) by spread plating and incubated aerobically at 22 °C for 48 h. Presumptive *Listeria monocytogenes* were quantified on Brilliance Listeria agar (Oxoid) supplemented with Brilliance Listeria Supplement (Oxoid). The plates were incubated at 37 °C and read after 24 and 48 h. Microbial counts were expressed as log CFU/g.

2.4. Metabolites analysis

Metabolite analysis samples were taken on days 2, 10, 17 (VP) and days 2, 10, 20 (WI and MAP) and kept at −80 °C until extraction and further analysis. All analyses were performed in triplicates (n = 3) for each group at each sampling point. Frozen samples were shredded using a kitchen grater, and approximately 2.0 g (exact weight listed) was homogenised with trichloroacetic acid (TCA, 6%, 10 mL) for 2 min with an Ultra Turrax T25 Basic (Janke & Kunkel IKA®-Labortechnik, Staufen, Germany). The sample solution was thereafter added 1.5 mL of potassium hydroxide (KOH, 1 M), shaken lightly, and centrifuged (12,000 rpm, 4 °C, 10 min) in a Kubota 1700 centrifuge (Kubota corporation, Tokyo, Japan) before the supernatant was filtered through a nylon filter (0.45 μm). One part of the supernatant was transferred to HPLC vials (Agilent, 862-09-16, 2 mL) for ATP-degradation products analysis, while another part was used for biogenic amines analysis. The degradation products of ATP were determined as described by Lerfall et al. (2018). The K-value and H-value were calculated by using the formulas (Hong et al., 2017); K-value (%) = [(HxR + Hx)/(ATP + ADP + AMP + IMP + HxR + Hx)] × 100, and H-value (%) = [Hx/(IMP + HxR + Hx)] × 100, where HxR is inosine, Hx is hypoxanthine, ATP is adenosine triphosphate, ADP is adenosine diphosphate, AMP is adenosine monophosphate, and IMP is inosine monophosphate.

The supernatant for biogenic amines analysis was neutralised by using potassium hydroxide (KOH, 1 M) and derived with benzylchloride (99%, Sigma-Aldrich, CAS: 98-88-4) according to Özogul et al. (2002). The reaction time was set to 20 min at room temperature. Benzyl-amines were thereafter extracted two times with diethyl ether. The upper organic layer was transferred to a glass tube and evaporated to dryness (N_2, 30 °C) before the residue was dissolved in a mixture of acetonitrile and water (90:10). The biogenic amines were quantified according to Lerfall et al. (2018).

2.5. Physicochemical parameters

2.5.1. Muscle pH, drip loss, water holding capacity and water content

The muscle pH was measured on days 0, 2, 4, 7, 9, 10, 14, 16, 17, and 20 of the storage trial while samples for drip loss (DL), water content (WC), and water holding capacity (WHC) were taken on days 2, 9, and 16 of the storage trial. All analyses were performed in triplicates (n = 3) for each group at each sampling point, except for the drip loss of WI-S and WI-A where n = 7 for all sampling days. Muscle pH was measured using a portable pH meter (Hach HQ40d multi–Portable Meter, Hach, USA) equipped with a puncture pH electrode (Hach Intellical™ PHC108, Hach, CO, USA). DL was calculated gravimetrically as the percentage difference (%) of the weighed sample (g) to its initial weight (g) (initial average weight ± SD, WI-S: 810 ± 89.2; VP-S: 122.6 ± 15.9; MAP-S: 85.9 ± 1.3; WI-A: 694 ± 106.2; VP-A: 135.4 ± 22.9; MAP-A: 81.2 ± 1.5). WHC and WC was measured based on the low-speed centrifugation method of Skipnes et al. (2007). The muscle sample (~5 g) was punched with a metal cylinder (diameter 31 mm, height 6 mm) and transversally sliced into 2 pieces. Weighed top piece was placed in metal carriers (Part No. 4750, Hettich Lab Technology, Germany) and centrifuged (Rotina 420 R, Hettich Lab Technology, Germany) at 1800 rpm (15 min, 4 °C). The bottom piece was weighed and dried to analyse contents of dry matter, thereby WC, by drying at 105 °C for 16–18 h to constant weight.

2.5.2. Colour analysis

Colour was measured on days 2, 9, and 16 of the storage trial. Colorimetric analyses were performed in n = 34 (September) and n = 42 (April) replicates on day 2, and in triplicates (n = 3) for each group for the rest of the sampling days. The surface colour (CIE Lab) was measured on a DigiEye full system, VeriVide Ltd., Leicester, UK. The samples were placed in a standardised light-box (daylight, 6400 K) and photographed using a digital camera (Nikon D80, 35 mm lens, Nikon Corp., Japan). The software DigiPix (version 2.8) was used to calculate L*a*b* values from RGB values obtained from the fillet image where L* represents lightness, a* redness and b* yellowness (CIE, 1994). Chroma (C*) and hue angle (h*) was calculated by using the formulas; $C^* = (a^{*2} + b^{*2})^{1/2}$ and $h^* = \arctan(b^*/a^*)$. Total colour difference (ΔE) was calculated based on CIE (1994).

2.5.3. Texture analysis

Texture analysis was performed on days 2 and 9. Texture was measured in n = 16 (September) and n = 21 (April) replicates on day 2, and in triplicates (n = 3) for each group on day 9. Instrumental textural analyses were performed on each sample using a Texture Analyser TA-XT plus (Stable Micro Systems Ltd, England) equipped with a 5-kg load cell and a flat-ended cylindrical probe (12.7 mm P/0.5). Force-time graphs were recorded and analysed by Texture Exponent light software for Windows (version 4.12, SMS). The breaking force was measured as the force (N) recorded when the breakage of the sample surface was observed. Textural measurement was performed with a constant speed of 2 mm/s.

2.6. Statistics and estimation of growth kinetics parameters

Statistical analyses were performed using an IBM SPSS statistics software (release 28, IBM Corporation, USA). Statistical analysis of microbial counts was done on log-transformed data. Log-transformed counts of PC, APC and H_2S-producing bacteria were fitted to the primary growth model of Baranyi and Roberts (1994) by applying the DMFit program available at www.combase.cc. Bacterial counts and estimated growth kinetics parameters are presented as mean values ± SE. All the other results are given as mean values ± SD. The data were analysed by a general linear model (GLM) with storage conditions and

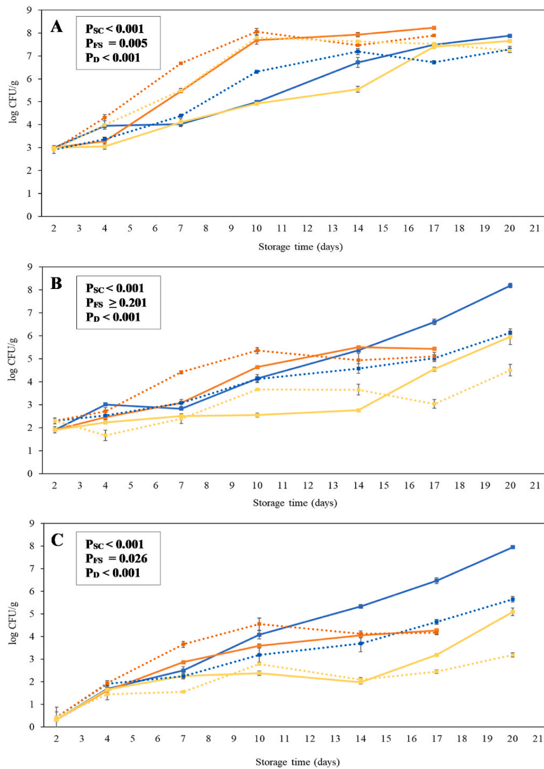

Fig. 2. Evolution of (**A**) psychrotrophic aerobic plate count (PC), (**B**) aerobic bacteria (APC) and (**C**) H_2S-producing bacteria in plaice caught at different seasons and stored at different storage conditions. Legends: Whole fish on ice-September (WI–S) (—■—); Vacuum packaged fillets-September (VP–S) (—■—); Modified atmosphere packaged fillets-September (MAP-S) (—■—); Whole fish on ice-April (WI-A) (··■··); Vacuum packaged fillets-April (VP-A) (··■··); Modified atmosphere packaged fillets-April (MAP-A) (··■··). Bars represent one standard error (n = 3). P_{SC}, P_{FS} and P_D are the significant levels (GLM) for the effects of storage conditions, fishing season and storage time, respectively.

season as fixed factors and storage time (days) as a covariate. Significant differences between experimental groups were analysed by one-way ANOVA combined with Tukey's pairwise comparison test. The significance level was set to 5% ($P < 0.05$). Pearson's correlation coefficient (r) was used to calculate the linearity dependence between variables X and Y.

3. Results & discussion

3.1. Seasonal variations in the quality of raw material

The reproductive season of plaice is usually from December to March in the North Sea (Hufnagl et al., 2013), indicating the plaice caught in September and April to be in a pre- and post-spawning condition, respectively. The initial pH of freshly caught plaice ranged between 7.6 and 7.7 at both seasons showing careful handling and good capturing routines (Morzel et al., 2003). No significant seasonal variations were found in the initial microbial loads of fillets. The initial microbial counts indicated high quality of the raw material and satisfactory hygiene conditions of the fishing vessel since the mean initial PC, APC and H_2S-producing bacteria counts were lower than 3 log CFU/g, 2.3 log CFU/g and 1 log CFU/g, respectively. Additionally, no *Pseudomonads* spp., *Brochothrix thermosphacta*, Enterobacteriaceae and *Listeria monocytogenes* were detected in the fresh plaice muscle in either season.

The plaice caught in September had significantly lower initial K-value and H-value (14.9% and 3.3%, respectively) compared to the April catch (26% and 6.9%, respectively). In the present study, K-values were in the range of 6–33% which have been previously reported in various flatfish species (Özogul et al., 2006, 2011). The initial WC, WHC and breaking force did not significantly differ between the seasons, while significant differences were found in the colorimetric characteristics of freshly caught fish. Fillets from the September catch were less translucence, more reddish, less yellowish, and had a lower colour intensity, and a hue angle closer to the orange side of the spectra compared to those from April.

Table 1
Growth kinetic parameters (maximum specific growth rate (μ_{max}, day^{-1} ± SE), lag-phase duration (days) and maximum population density (Y_{max}, log CFU/g ± SE) in plaice caught at different seasons and stored at different storage conditions for psychrotrophic aerobic plate count (PC), aerobic bacteria (APC) and H_2S-producing bacteria.

Treatments	Lag-phase (days)	μ_{max} (day^{-1})	Y_{max} (log CFU/g)	R^2	SE
PC					
WI-S	3.25 ± 0.17^a	0.38 ± 0.04^d	7.88 ± 0.12^{bc}	0.97	0.31
VP-S	2.08 ± 0.18^b	0.92 ± 0.09^a	8.08 ± 0.10^{ab}	0.99	0.35
MAP-S	2.17 ± 0.31^b	0.32 ± 0.02^d	8.24 ± 0.12^a	0.96	0.35
WI-A	2.50 ± 0.22^b	0.61 ± 0.04^c	7.07 ± 0.04^e	0.98	0.26
VP-A	0.17 ± 0.10^d	0.86 ± 0.03^{ab}	7.63 ± 0.05^{cd}	0.98	0.31
MAP-A	1.12 ± 0.10^c	0.69 ± 0.02^{bc}	7.55 ± 0.03^d	0.97	0.32
*P-value**	*< 0.001*	*< 0.001*	*< 0.001*		
APC					
WI-S	3.89 ± 0.74^b	0.40 ± 0.03	NA^x	0.97	0.37
VP-S	2.47 ± 0.55^c	0.47 ± 0.03	5.49 ± 0.08^b	0.98	0.19
MAP-S	11.10 ± 0.36^a	0.55 ± 0.07	6.05 ± 0.39^a	0.95	0.33
WI-A	1.93 ± 0.66^{cd}	0.35 ± 0.08	NA^x	0.95	0.29
VP-A	1.53 ± 0.33^d	0.63 ± 0.05	5.14 ± 0.16^b	0.94	0.32
MAP-A	3.12 ± 1.29^b	0.41 ± 0.07	3.74 ± 0.20^c	0.74	0.55
*P-value**	*< 0.001*	*0.385*	*< 0.001*		
H_2S-producing bacteria					
WI-S	ND	0.41 ± 0.04^{bc}	NA^l	0.98	0.34
VP-S	ND	0.48 ± 0.07^b	4.07 ± 0.35^a	0.95	0.32
MAP-S	ND	0.37 ± 0.06^{cd}	NA^l	0.74	0.72
WI-A	ND	0.26 ± 0.02^d	NA^l	0.91	0.51
VP-A	ND	0.79 ± 0.08^a	4.25 ± 0.31^a	0.94	0.39
MAP-A	ND	0.27 ± 0.06^d	2.62 ± 0.35^b	0.71	0.50
*P-value**		*< 0.001*	*< 0.001*		

R^2, coefficient of determination; SE, standard error of fit to the model; $NA^{x,l}$, not analysed due to no asymptote, linear model, respectively; ND, not detected.
Whole fish on ice-September (WI–S); Vacuum packaged fillets-September (VP–S); Modified atmosphere packaged fillets-September (MAP-S); Whole fish on ice-April (WI-A); Vacuum packaged fillets-April (VP-A); Modified atmosphere packaged fillets-April (MAP-A).
*Anova was applied to detect differences in lag-phase, maximum specific growth rate (μ_{max}) and maximum population density (Y_{max}); where significant differences were detected ($P < 0.05$), a Tukey's pairwise comparison test was applied.
[a–e]Different superscript letters within each column and each parameter indicate significant differences ($P < 0.05$) between treatments.

3.2. Storage quality

3.2.1. Microbial evolution

The evolution of microbial counts and growth kinetics as a function of storage conditions, storage time and fishing season are presented in Fig. 2 and Table 1. The storage conditions and storage time significantly affected the microbial evolution of PC, APC, H_2S-producing bacteria, LAB, and *Pseudomonas* spp. ($P < 0.001$). The fishing season significantly affected the PC, H_2S-producing bacteria, and LAB counts ($P < 0.027$), whereas APC ($P \geq 0.201$) and *Pseudomonas* spp. ($P \geq 0.428$) were not affected.

The WI-S was the only treatment that surpassed the upper acceptable APC limit of 7 log CFU/g for marine species suitable for human consumption (ICMSF, 1986) on the last day of the storage trial. However, in the present study, higher PC than APC counts were found at all sampling points, and all groups reached PC counts higher than 7 log CFU/g at the end of storage (Fig. 2A and B). The high PC loads can be attributed to the high prevalence *Photobacterium* spp., as *Photobacterium* spp. are a common psychrotrophic spoilage organism in marine cold-water fish species (Dalgaard et al., 1993). *Photobacterium* spp. counts higher than 7 log CFU/g indicate microbial spoilage and sensory rejection of seafood (Dalgaard et al., 1997; Kuuliala et al., 2018). Based on the PC counts, the storage trials of the VP-groups were terminated earlier than the others (17 days vs 20 days). In general, PC counts might be a better indicator for microbiological spoilage in marine cold-water fish species than APC.

The shortest lag-phase and the highest μ_{max} of PC (Table 1) were observed for VP-groups in both seasons, reaching Y_{max} (7–8 log CFU/g) on the 10th day (Fig. 2A). The μ_{max} of PC in VP-S was 2.9–2.4 times higher than for MAP-S and WI-S, respectively. However, a lower effect on the PC growth rate was seen for the April samples. Randell et al. (1999) and Hansen et al. (2009) have also reported faster evolution of PC counts in VP compared to MAP salmon. The presence of CO_2 in the MAP-groups resulted in significantly lower μ_{max} of PC than observed for VP-groups and was almost comparable to WI-groups. Devlieghere and Debevere (2000) stated the linear relationship between the concentration of dissolved CO_2 in the food matrix and the μ_{max} of Gram-negative bacteria, including *P. phosphoreum*. However, the psychrotrophic *P. phosphoreum* is regarded as CO_2-tolerant as high *P. phosphoreum* counts (>10^7 log CFU/g) have been found in CO_2 equilibrum between 38 and 50% (Dalgaard et al., 1993, 1997; Kuuliala et al., 2018). The significantly longer lag-phase in WI-groups was expected due to lower storage temperature (0 °C vs 4 °C) and whole fish deteriorate slower than the fish fillets (Paleologos et al., 2004).

H_2S-producing bacteria constitute one of the most relevant specific spoilage organisms in aerobically stored and vacuum packaged marine fish (Skjerdal et al., 2004). The absence of lag-phase confirms that H_2S-producing bacteria proliferated quickly ($P < 0.001$) and were dominant among the APC (Table 1, Fig. 2C). The proliferation of APC and H_2S-producing bacteria in WI-groups was slower than in VP-groups until the middle of the storage trial but WI-groups had the highest APC and H_2S-producing bacteria loads compared to VP and MAP-groups at the end of storage. A possible explanation for the slower growth in WI-groups at the first days of storage is the lower storage temperature (0 °C) due to the use of ice. However, Zotta et al. (2019) have reported the absence of H_2S-producing bacteria in thawed plaice fillets stored at 0 °C in air. Also, Baixas-Nogueras et al. (2007) have found lower counts of H_2S-producing bacteria in frozen-thawed than unfrozen Mediterranean hake stored on ice for 15 days. The possible sensitivity of H_2S-producing bacteria to freezing and thawing could explain the higher H_2S-producing bacteria in our study than thawed plaice. Moreover, Zotta et al. (2019) found higher μ_{max} and load of APC compared to our study.

The presence of CO_2 significantly extended the lag-phase of APC and reduced the μ_{max} of H_2S-producing bacteria, resulting in significantly lower APC and H_2S-producing bacteria counts in MAP-groups than in all other treatments. The bacteriostatic effect of CO_2 on APC have also been stated in other studies (Masniyom et al., 2002; Rodrigues et al., 2016). Although initial CO_2 headspace concentrations and gas:product ratio higher than 50% and 0.5, respectively, inhibit the growth of the H_2S-producing *Shewanella putrefaciens* (Lerfall et al., 2018), several studies have shown no complete inhibition of H_2S-producing bacteria in high initial CO_2 concentrations (Dalgaard et al., 1993; Hovda et al., 2007; Stamatis & Arkoudelos, 2007). Despite, *P. phosphoreum* is not commonly recognised as H_2S-producing bacteria (Dalgaard, 1995), its potential contribution to H_2S production would need to be further investigated.

The MAP-groups had the lowest *Pseudomonas* spp. counts ($P < 0.05$), while the WI-A and WI-S had the highest numbers of 5.6 ± 0.1 and 6.4 ± 0.1 log CFU/g, respectively, on the last day of the storage. Chilled marine cold-water fish stored aerobically are primarily spoiled by *Shewanella* and secondarily *Pseudomonas* species (Gram, 2009). Our study is in accordance with these results since *Pseudomonas* spp. followed the population of H_2S-producing bacteria at WI-S and WI-A treatments. *Pseudomonas* spp. are known to be CO_2-sensitive (Devlieghere & Debevere, 2000).

The VP and MAP-groups had the highest and lowest LAB concentrations ($P < 0.05$), respectively. Similar results have been reported in saithe fillets (Lerfall et al., 2018) and farmed Atlantic cod fillets (Hansen et al., 2007) packaged in vacuum and CO_2 equilibrium concentrations above 40%. Despite high CO_2 headspace concentration of MAP favouring LAB's growth (Devlieghere & Debevere, 2000), a delay in the proliferation of LAB has also been found at initial CO_2 headspace concentration higher than 40% (Kostaki et al., 2009; Sørensen et al., 2020; Yesudhason et al., 2014).

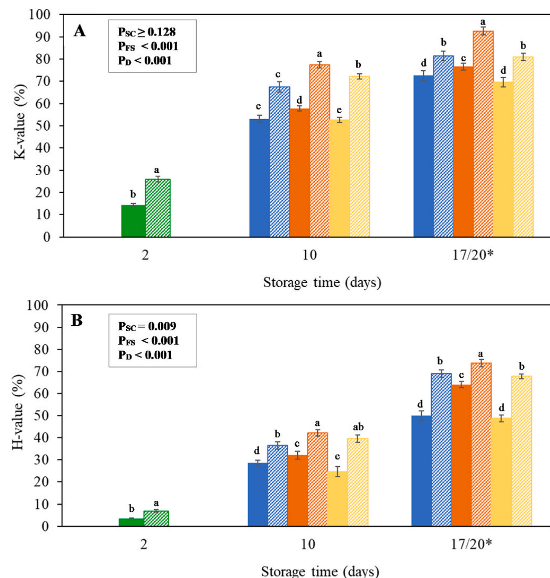

Fig. 3. K-value and H-value evolution in plaice caught at different seasons and stored at different storage conditions. Legends: Freshly caught fish-September (■); Freshly caught fish-April (▨); Whole fish on ice-September (WI–S) (■); Vacuum packaged fillets-September (VP–S) (■); Modified atmosphere packaged fillets-September (MAP-S) (■); Whole fish on ice-April (WI-A) (▨); Vacuum packaged fillets-April (VP-A) (▨); Modified atmosphere packaged fillets-April (MAP-A) (▨). a–eDifferent letters within each storage time (days) indicate significant differences ($P < 0.05$) between treatments. Bars represent one standard deviation (n = 3). P_{SC}, P_{FS} and P_D are the significant levels (GLM) for the effects of storage conditions, fishing season and storage time, respectively. *Comparison of K-value and H-value at the end of storage (17 days for VP-groups while 20 days for WI and MAP-groups).

Table 2
Average content (mg/100g ± SD) (n = 3) of the biogenic amines cadaverine, tryptamine, spermidine and spermine in plaice caught at different seasons and stored at different storage conditions.

		Treatments						
Amine	**Day**	**WI-S**	**VP-S**	**MAP-S**	**WI-A**	**VP-A**	**MAP-A**	*P-value***
Cadaverine	2	0.09 ± 0.02^{C}	0.09 ± 0.02^{C}	0.09 ± 0.02^{C}	0.1 ± 0.03^{C}	0.1 ± 0.03^{C}	0.1 ± 0.03^{C}	*0.904*
	10	1.90 ± 0.22^{Bc}	2.32 ± 0.15^{Bc}	1.8 ± 0.3^{Bc}	2.45 ± 0.36^{Bbc}	4.30 ± 0.15^{Ba}	3.02 ± 0.37^{Bb}	*< 0.001*
	17/20*	4.53 ± 0.28^{Ab}	6.73 ± 0.5^{Aa}	3.7 ± 0.52^{Ac}	5.23 ± 0.10^{Ab}	7.34 ± 0.31^{Aa}	4.99 ± 0.19^{Ab}	*< 0.001*
	*P-value***	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	
Tryptamine	2	ND	ND	ND	ND	ND	ND	
	10	0.12 ± 0.02^{Bc}	0.17 ± 0.02^{Ba}	0.13 ± 0.01^{Bbc}	0.16 ± 0.01^{Bab}	0.15 ± 0.01^{Bab}	0.13 ± 0.03^{Bab}	*0.01*
	17/20*	0.26 ± 0.01^{A}	0.27 ± 0.03^{A}	0.26 ± 0.04^{A}	0.24 ± 0.04^{A}	0.26 ± 0.01^{A}	0.24 ± 0.04^{A}	*0.825*
	*P-value***	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	
Spermidine	2	0.06 ± 0.01^{C}	0.06 ± 0.01^{C}	0.06 ± 0.01^{C}	0.07 ± 0.01^{C}	0.07 ± 0.01^{C}	0.07 ± 0.01^{C}	*0.533*
	10	0.42 ± 0.02^{Bd}	0.63 ± 0.01^{Bb}	0.37 ± 0.02^{Bd}	0.50 ± 0.06^{Bc}	0.75 ± 0.01^{Ba}	0.53 ± 0.03^{Bc}	*< 0.001*
	17/20*	0.89 ± 0.05^{Acd}	1.03 ± 0.05^{Aab}	0.86 ± 0.04^{Ad}	1.01 ± 0.02^{Ab}	1.12 ± 0.06^{Aa}	0.97 ± 0.04^{Abc}	*< 0.001*
	*P-value***	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	
Spermine	2	0.04 ± 0.01^{C}	0.04 ± 0.01^{C}	0.04 ± 0.01^{C}	0.04 ± 0.01^{C}	0.04 ± 0.01^{C}	0.04 ± 0.01^{C}	*1.000*
	10	0.26 ± 0.01^{Bc}	0.36 ± 0.03^{Ba}	0.23 ± 0.05^{Bc}	0.24 ± 0.01^{Bc}	0.34 ± 0.04^{Bab}	0.28 ± 0.03^{Bbc}	*0.002*
	17/20*	0.57 ± 0.01^{Acd}	0.62 ± 0.01^{Abc}	0.55 ± 0.02^{Ad}	0.63 ± 0.02^{Aab}	0.67 ± 0.03^{Aa}	0.64 ± 0.04^{Aab}	*< 0.001*
	*P-value***	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	

Whole fish on ice-September (WI–S); Vacuum packaged fillets-September (VP–S); Modified atmosphere packaged fillets-September (MAP-S); Whole fish on ice-April (WI-A); Vacuum packaged fillets-April (VP-A); Modified atmosphere packaged fillets-April (MAP-A).
*Comparison of the biogenic amines content at the end of storage (17 days for VP−groups while 20 days for WI and MAP-groups)
**Anova was applied to detect differences in biogenic amines content; where significant differences were detected (P < 0.05), a Tukey's pairwise comparison test was applied.
$^{a-d}$Different superscript letters within each row and each parameter indicate significant differences (P < 0.05) between treatments.
$^{A-C}$Different superscript letters within each column and each parameter indicate significant differences (P < 0.05) throughout the storage time.
ND, not detected.

The fishing season significantly affected the PC evolution (P = 0.005). The PC counts were significantly higher in the April than September-groups until the middle of storage due to the higher μ_{max} and shorter lag-phase (Table 1, Fig. 2A). The seawater temperature was lower in April (5.4 °C vs 13 °C, respectively). Therefore, higher growth rates and shorter lag-phases were expected as the microbiota of fresh fish coming from colder water suffers a less intensive thermal shock when the fish is stored at low temperatures (Grigorakis et al., 2003).

Similarly, the lag-phase of APC was shorter in April than for September-groups, resulting in lower APC counts until the middle of the storage trial (Fig. 2B). On the other hand, no lag-phase was detected for H_2S-producing bacteria in either of the seasons. While no significant difference was found in either APC or H_2S-producing bacteria counts of VP-groups at the end of the storage trial, the APC and H_2S-producing bacteria counts were significantly higher in WI-S and MAP-S compared to the WI-A and MAP-A (Fig. 2B and C). The higher μ_{max} following these groups confirms the higher APC and H_2S-producing bacteria counts observed in WI-S and MAP-S samples. The apparent faster microbial deterioration of April-groups agrees with the results of other studies (DurmuŞ et al., 2014; Ntzimani et al., 2022; Papaharisis et al., 2019) which stated that the lower seawater temperatures at catching led to higher microbial deterioration.

No seasonal variations in the *Pseudomonas* spp. proliferation were found. The LAB concentration ranged between 2.4 and 3.2 log CFU/g in fillets from fish captured in September during the storage trial. Moreover, their counts remained significantly higher than those observed in April throughout storage. Dalgaard et al. (1993) also stated low levels of LAB populations (<4 log CFU/g) during VP and MAP storage of cod fillets.

Brochothrix thermosphacta was analysed at all sampling points but not detected in any sample. Potentially harmful bacteria, such as Enterobacteriaceae and *Listeria monocytogenes*, were analysed but not detected.

3.2.2. Metabolites

K-value and H-value as a function of storage conditions, storage time and fishing season are presented in Fig. 3. The storage conditions significantly affected the samples H-value (P = 0.009) but not the K-value (P ≥ 0.128). Both measures were affected by season and storage time (P < 0.001). The VP-groups had significantly higher K and H-values in both seasons due to the significantly lower IMP and HxR content and higher Hx content in these groups (data not shown). The results indicate a similar delay in autolytic and microbial degradation of nucleotides for storage of plaice fillet in MAP (70% CO_2, 20% N_2, 10% O_2) at 4 °C as for the WI-groups (0 °C).

The K- and H-value observed in fillets from plaice caught in September remained significantly lower than April's catch through the entire storage period, indicating the comparative higher quality of plaice caught in September. Grigorakis et al. (2003) also reported higher K-values in winter than summer caught seabream after the middle of storage when microbial counts were significantly increased. A correlation (P < 0.001) was found between PC, APC, H_2S-producing bacteria and *Pseudomonas* spp. and the H-value (r = 0.92, 0.84, 0.84, 0.68, respectively), indicating increased microbial counts in April-groups have contributed to higher nucleotides' degradation.

Previous studies of various flatfish species reported a K-value above 70% at sensory rejection (Rodríguez et al., 2006; Özogul et al., 2006, 2011). The April treatments had already surpassed (VP-A and MAP-A) or was close (WI-A) to the above-mentioned sensory rejection limit in the middle of storage trial (Fig. 3A) when the PC counts were also quite high (>6.3 log CFU/g) (Fig. 2A). However, the VP-S had also high PC counts (7.2 ± 0.2 log CFU/g) on day 10, but its K-value was 58%.

The content of biogenic amines as a function of storage conditions, storage time and fishing season are given in Table 2. The storage time had a significant effect on the content of biogenic amines (P < 0.001). Tryptamine was the only biogenic amine that was not affected by the

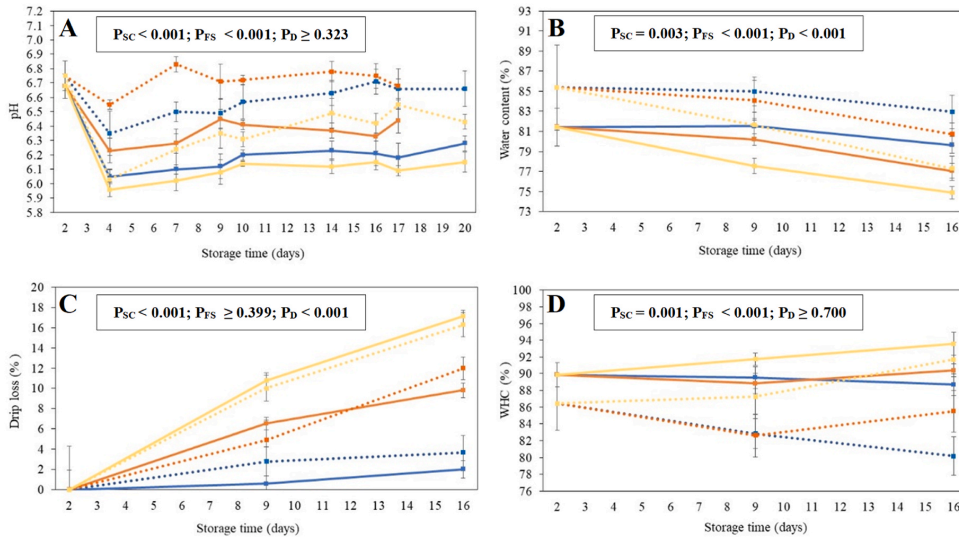

Fig. 4. Evolution of (**A**) muscle pH, (**B**) water content (WC) (%), (**C**) drip loss (DL) (%) and (**D**) water holding capacity (WHC) (%) in plaice caught at different seasons and stored at different storage conditions. Legends: Whole fish on ice-September (WI–S) (—■—); Vacuum packaged fillets-September (VP–S) (—■—); Modified atmosphere packaged fillets-September (MAP-S) (—■—); Whole fish on ice-April (WI-A) (··■··); Vacuum packaged fillets-April (VP-A) (··■··); Modified atmosphere packaged fillets-April (MAP-A) (··■··). Bars represent one standard deviation (n = 3, except for the DL of WI-S and WI-A where n = 7). P_{SC}, P_{FS} and P_D are the significant levels (GLM) for the effects of storage conditions, fishing season and storage time, respectively.

storage conditions and fishing season ($P \geq 0.226$ and 0.670, respectively). Cadaverine became the dominant amines in all treatments during the storage trial, however lower than sensory rejection limit of 9.1 mg/100g (Vallé et al., 2020).

In general, VP-groups had the highest content of cadaverine, spermidine and spermine followed by WI and MAP-groups. A correlation ($P < 0.001$) was found between cadaverine, spermidine, spermine, and PC ($r = 0.87$, 0.92 and 0.87, respectively) as well as APC ($r = 0.80$, 0.86 and 0.86, respectively). A similar strong correlation between biogenic amines and PC or APC has been found in other seafood (Hu et al., 2012). The observed inhibitory effect of CO_2 on biogenic amine production agrees with other studies (Rodrigues et al., 2016; Özogul et al., 2002). The April catch had significantly higher average biogenic amines content than the September catch at the end of storage which could be attributed to the different patterns in the microbial evolution of PC and APC. Given that the formation rate and the final concentration of biogenic amines is considered a chemical indicator of seafood quality and decomposition (Laly et al., 2017; Prester, 2011; Ruiz-Capillas & Herrero, 2019), September catch seems to maintain better quality than April.

3.2.3. Physicochemical quality deterioration

3.2.3.1. Muscle pH, drip loss, water content and water holding capacity. Changes in muscle pH, WC, WHC, and DL of plaice as a function of storage conditions, storage time and fishing season are illustrated in Fig. 4. No significant effect of storage time was found on muscle pH ($P \geq 0.323$) and WHC ($P \geq 0.700$). The DL was the only parameter that was not affected by the fishing season ($P \geq 0.399$), while the storage conditions significantly affected muscle pH, WC, WHC, and DL ($P < 0.004$).

Several studies have reported no seasonal variations in muscle pH (Grigorakis et al., 2003; Tzikas et al., 2007). Contrastingly, in the present study, the muscle pH was significantly affected by season ($P < 0.001$). The pH of postmortem muscle depends on the glycogen content, and fish accumulate higher amounts of glycogen during the pre-spawning period (Kumari & Ahsan, 2011) and mature plaice hardly fed during the spawning period (Rijnsdorp, 1989). The muscle pH significantly decreased up to day 4 in all groups and remained significantly lower for September-groups (Fig. 4A) due to the possible production of higher amounts of lactic acid during anaerobic glycolysis. A correlation analysis was performed including the time interval from the recording of the lowest muscle pH in all groups (day 4) to the end of the storage trial. From this analysis, a significant correlation was found between the muscle pH and either fishing season ($r = 0.68$, $P < 0.001$) or microbial counts ($r = 0.49$ (PC), 0.35 (APC), 0.27 (H_2S-producing bacteria), $P < 0.006$). The possible accumulation of higher amounts of alkaline compounds due to higher microbial spoilage could contribute to higher pH in April-groups through the storage time which advocates the lower quality of April-groups. Moreover, the lower pH in September-groups could partially be attributed to higher LAB counts. MAP-groups had the lowest pH at all sampling points in either of the seasons ($P < 0.05$), due to the dissolution of CO_2 in the aqueous phase of the muscle tissue (Kostaki et al., 2009; Lerfall et al., 2018; Masniyom et al., 2002).

A linear increase in DL and a subsequent decrease in the WC was observed in all treatments as the storage days increased (Fig. 4B and C). The WC of VP and MAP-groups was significantly reduced during the storage. The MAP-groups had the highest DL and the lowest WC in all sampling points at both seasons, followed by VP and WI-groups. Moreover, a significant correlation was found between the WC and DL ($r = -0.63$, $P < 0.001$). Increased DL in MAP-groups compared to VP-groups has also been reported by Dalgaard et al. (1993) and Randell et al. (1999). The differences in pH between VP and MAP-groups as well as the significant correlation between the muscle pH and either DL ($r = -0.49$, $P < 0.001$) or WC ($r = 0.64$, $P < 0.001$) indicates that the capacity of fish proteins to hold water is reduced at lower pH. Although higher DL was expected in September-groups due to lower pH, no seasonal variation was found. The increased proteolytic activity caused by higher bacterial counts in April-groups could induce a faster muscle protein degradation as visualised with highest DL of April samples (Olsson et al., 2003). Moreover, April-groups have lower protein levels (Kendler et al., 2023) which probably creates a less strong protein matrix that, combined with

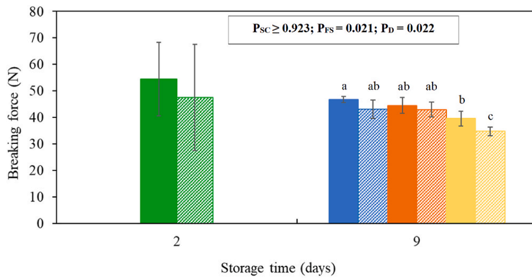

Fig. 5. Breaking force (N) changes in plaice caught at different seasons and stored at different storage conditions. Legends: Freshly caught fish-September (■); Freshly caught fish-April (▨); Whole fish on ice-September (WI–S) (■); Vacuum packaged fillets-September (VP–S) (■); Modified atmosphere packaged fillets-September (MAP-S) (■); Whole fish on ice-April (WI-A) (▨); Vacuum packaged fillets-April (VP-A) (▨); Modified atmosphere packaged fillets-April (MAP-A) (▨). [a–c]Different letters within each storage time (days) indicate significant differences ($P < 0.05$) between treatments. Bars represent one standard deviation (n = 3, except for day 2 where n = 16 (September) and n = 21 (April)). P_{SC}, P_{FS} and P_D are the significant levels (GLM) for the effects of storage conditions, fishing season and storage time, respectively.

higher water concentrations, can lead to similar DL as the September-groups.

Independent of season, the WHC of WI-groups was continuously decreasing throughout the storage. However, increased WHC was observed in VP and MAP-groups on day 16 compared to day 2 of storage trial. These increased measures can be attributed to the “leaking-out” effect according to which the reduced WC can cause an increase in WHC during storage since the DL removes a large amount of free water. Similar observations have been reported in Atlantic cod (Herland et al., 2010) and halibut (Olsson et al., 2003). The WHC on the last sampling day of April-groups was significantly lower than those observed in September. As WHC is an important quality parameter (Chan et al., 2021), the higher ability of September-groups' muscle to retain water might indicate a better fish quality. Moreover, an inverse relationship between WHC and WC ($r = -0.47$, $P = 0.001$) was found.

3.2.3.2. Texture and colour parameters. The textural and colorimetric properties of plaice as a function of storage conditions, storage time, and fishing season are shown in Fig. 5 and Table 3. The breaking force ($P = 0.022$) as well as the L*, h*, and ΔE ($P < 0.001$) were significantly affected by the storage time. The ΔE was the only parameter that was not affected by the fishing season ($P \geq 0.760$), while the storage conditions did significantly affect the L* and ΔE ($P = 0.041$ and <0.001, respectively).

The observed decrease in the surface breaking force implies that the fillet structure tenderised during storage. The post-mortem softening could be associated with the degradation of myofibrillar proteins and the weakening of connective tissue due to the activity of autolytic enzymes (e.g. collagenase, ATPase) (Viji et al., 2015).

The fish fillets became less translucent, less reddish, and retained a slightly more yellowish colour throughout the storage time. No significant changes were found in the colour intensity, C* ($P \geq 0.203$), while the significant increase in h* value indicates that the fish muscle gradually leaned from orange-yellowish on day 2, to be more green-yellowish on day 16.

The MAP-groups were observed to be the least translucent, followed by VP and WI-groups ($P < 0.05$) while no significant difference was found in the C* and h* on the last day of analysis. The correlation between L* and both DL and WC ($r = 0.68$ and -0.46, respectively, $P < 0.001$) confirms that larger water deposits on fish surfaces could cause increased lightness (Duun & Rustad, 2008). Moreover, the dissolution of CO_2 in the fish muscle of MAP-groups caused the denaturation of sarcoplasmic proteins and increased flesh's lightness (Ruff et al., 2002). Poli et al. (2006) did also report higher lightness in European sea bass MAP-fillets compared to whole fish on ice at the end of storage.

According to C* and h*, the effect of the fishing season seemed to

Table 3
Changes in lightness (L*), chroma (C*), hue angle (h*) and total colour difference (ΔE) (mean ± SD) (n = 3, except for day 2 where n = 34 (September) and n = 42 (April)) in plaice caught at different seasons and stored at different storage conditions.

		Treatments						
	Day	WI-S	VP-S	MAP-S	WI-A	VP-A	MAP-A	*P-value**
L*	2	65.9 ± 3.5^{a}	65.9 ± 3.5^{Ba}	65.9 ± 3.5^{Ca}	59.7 ± 4.7^{Bb}	59.7 ± 4.7^{Bb}	59.7 ± 4.7^{Cb}	*< 0.001*
	9	65.6 ± 3.5^{c}	75.9 ± 1.0^{Aab}	81.0 ± 1.6^{Ba}	66.5 ± 4.5^{ABc}	69.8 ± 0.7^{Abc}	73.9 ± 3.5^{Bb}	*< 0.001*
	16	69.9 ± 1.6^{c}	78.9 ± 1.8^{Ab}	87.21 ± 0.8^{Aa}	70.7 ± 2.5^{Ac}	73.9 ± 2.2^{Ac}	80.8 ± 2.1^{Ab}	*< 0.001*
	*P-value**	*0.158*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	
C*	2	11.8 ± 1.3^{Bb}	11.8 ± 1.3^{Bb}	11.8 ± 1.3^{Bb}	15.2 ± 2.2^{Aa}	15.2 ± 2.2^{Aa}	15.2 ± 2.2^{Aa}	*< 0.001*
	9	12.1 ± 1.4^{AB}	12.2 ± 1.4^{B}	13.7 ± 1.7^{AB}	12.6 ± 1.3^{B}	13.2 ± 2.0^{B}	12.3 ± 1.1^{B}	*0.763*
	16	14.2 ± 0.8^{A}	15.9 ± 1.9^{A}	15.7 ± 1.4^{A}	13.6 ± 1.6^{B}	13.9 ± 1.0^{B}	14.3 ± 1.6^{A}	*0.308*
	*P-value**	*0.017*	*< 0.001*	*< 0.001*	*0.040*	*0.226*	*0.011*	
h*	2	56.1 ± 4.9^{Cb}	56.1 ± 4.9^{Bb}	56.1 ± 4.9^{Bb}	73.2 ± 6.1^{Ca}	73.2 ± 6.1^{Ba}	73.2 ± 6.1^{Ca}	*< 0.001*
	9	85.4 ± 6.7^{Bb}	106.0 ± 8.8^{Aa}	102.9 ± 1.1^{Aa}	102.4 ± 8.5^{Ba}	114.6 ± 5.5^{Aa}	118.5 ± 0.5^{Ba}	*< 0.001*
	16	111.9 ± 4.3^{A}	117.2 ± 7.1^{A}	120.0 ± 19.5^{A}	123.3 ± 7.0^{A}	123.9 ± 5.6^{A}	125.5 ± 2.8^{A}	*0.518*
	*P-value**	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	
ΔE	2	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	
	9	5.4 ± 1.7^{Bc}	10.1 ± 1.3^{Bab}	11.5 ± 0.2^{Ba}	7.2 ± 1.4^{Bbc}	9.6 ± 0.9^{Bab}	11.4 ± 1.1^{Ba}	*< 0.001*
	16	10.6 ± 0.4^{Ad}	13.8 ± 1.4^{Abc}	16.4 ± 2.2^{Aa}	11.4 ± 0.5^{Ad}	12.4 ± 0.5^{Acd}	14.9 ± 0.3^{Aab}	*< 0.001*
	*P-value**	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	*< 0.001*	

Whole fish on ice-September (WI–S); Vacuum packaged fillets-September (VP–S); Modified atmosphere packaged fillets-September (MAP-S); Whole fish on ice-April (WI-A); Vacuum packaged fillets-April (VP-A); Modified atmosphere packaged fillets-April (MAP-A).
*Anova was applied to detect differences in colour parameters; where significant differences were detected ($P < 0.05$), a Tukey's pairwise comparison test was applied.
[a–d]Different superscript letters within each row and each parameter indicate significant differences ($P < 0.05$) between treatments.
[A–C]Different superscript letters within each column and each parameter indicate significant differences ($P < 0.05$) throughout the storage time.

weaken as the muscle aged since no significant differences were found between treatments on day 16 (Table 3). Roth et al. (2009) stated a negative correlation between the L* value and muscle pH, which agrees with our observations ($r = -0.56$, $P < 0.001$).

The MAP-groups had the highest total colour difference (ΔE) followed by VP and WI-groups. On the other hand, no significant seasonal variations were found according to ΔE. Although no correlation was observed between ΔE and C* ($r = 0.04$, $P \geq 0.541$), a strong correlation was found between ΔE and L* ($r = 0.66$, $P < 0.001$), indicating changes in L* to be the parameter of highest significance to ΔE observed. However, the final colour perception could be assumed similar in all treatments on the last day of analysis as no significant difference was found in the C* and h* on day 16.

4. Conclusion

The fishing season, storage conditions and storage time were found to affect the quality of European plaice. The observed seasonal variations in the metabolites, microbial and physicochemical parameters lead us to the conclusion that the overall quality is lower of plaice caught in April than September. Moreover, this study shows that MAP (70% CO_2, 20% N_2, 10% O_2) combined with cold storage (4 °C) resulted in comparable quality as the whole fish on ice (0 °C). However, the production of MAP-fillets has a comparative advantage over the whole fish on ice in terms of convenient use and easier transport. Given that the quality measures showed a satisfactory quality of plaice, increasing utilisation and commercial value could be achieved through the production of modified atmosphere-packaged plaice fillets. Nevertheless, future research is needed to investigate how seasonal variations in the initial microbiome affects microbial spoilage of plaice. This knowledge can contribute to the extent of the shelf life of plaice and in the production of high-quality, convenient retail plaice products.

CRediT authorship contribution statement

Dionysios Tsoukalas: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Sophie Kendler:** Investigation, Conceptualization. **Jørgen Lerfall:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Anita Nordeng Jakobsen:** Conceptualization, Methodology, Supervision, Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Paper III

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EDITED BY
Miroslava Rossenova Atanassova,
Møreforsking AS,
Norway

REVIEWED BY
Karl-Erik Eilertsen,
UiT The Arctic University of Norway, Norway
Edgar Pinto,
Polytechnic Institute of Porto, Portugal
Shengjun Chen,
South China Sea Fisheries Research Institute (CAFS), China

*CORRESPONDENCE
Sophie Kendler
✉ Sophie.kendler@ntnu.no

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Nutritional profiling and contaminant levels of five underutilized fish species in Norway

Sophie Kendler*, Frida Walle Thornes, Anita Nordeng Jakobsen and Jørgen Lerfall

Department of Biotechnology and Food Science, Norwegian University of Science and Technology (NTNU), Trondheim, Norway

Exploring and making use of underutilized marine resources can be a sustainable approach to achieve future demands of fish consumption by the ever-growing population. Five species, namely European plaice (*Pleuronectes platessa*), European flounder (*Platichthys flesus*), lemon sole (*Microstomus kitt*), megrim (*Lepidorhombus whiffiagonis*), and thornback ray (*Raja clavate*), often captured as by-catch in Norway, were characterized for their nutritional value and potential accumulation of hazardous components. The proximate composition, protein profile, fatty acid profile as well as essential and toxic trace elements and polychlorinated biphenyls (PCBs) were analyzed. Digestible indispensable amino acid (DIAA) ratios and scores (DIAAS) and contributions of omega-3 fatty acids to the diet were calculated. Analysis on proximate composition revealed low fat contents of 0.74 to 1.25% and sufficient protein contents between 16.9 and 24% in the five species. Results of DIAA indicate a profitable distribution, with contributions exceeding the daily intake recommendations for an adult person related to a 200 g fillet. Moreover, findings on the distribution of eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) showed remarkable results, considering that the investigated species are lean fish. All five investigated fish exceed the recommended average daily intake level (AI) of EPA+DHA in a 200 g portion. As to toxic trace elements and PCBs, no significantly elevated levels were found considering a portion size of 200 g. Consequently, the nutritional quality of the investigated fish can be regarded as profitable with overall low potential health risks.

KEYWORDS

nutritional profile, flatfish, underutilized fish, DIAAS, omega 3 fatty acids, risks and benefits, healthy

1. Introduction

By 2050, the demand for food is projected to increase by 50% as the world's population is expected to reach 9.7 billion people (1, 2). At the same time, food production accounts for a quarter of total greenhouse gas emissions, and immediate action is needed to reduce climate gas emissions and counter climate change (3). Finding ways to produce more food, while at the same time reducing the climate impact of food, is a tremendous challenge in the years to come. Norway, being the country with the second-longest coastline globally shows a variety of marine species. Moreover, Norway is the second largest fish exporter (4). Nevertheless, only around 10%

of Norway's 220 marine species have been commercially utilized as food (5). A sustainable approach to achieve the future demand for fish can be to explore species that are considered as underutilized or classified as little utilized resources (LUR). A report on LUR species in Norway was published in 2011 and concluded that flatfish were among the species with the most significant potential for successful commercialization (6). Further research and investigations on flatfish were recommended to enable commercialization (6).

The excellent nutritional composition of fish and seafood in general has been reported in various studies and reviewed amongst others by Khalili Tilami et al. (7). Moreover, authorities have set recommendations to guarantee a satisfying intake of certain nutrients. FAO/WHO (8) and the European Food Safety Authority (EFSA) recommend a seafood consumption of 100 g and up to 300 g of fish per week, respectively, accounting for at least two meals a week to cover the recommended intake. High amounts of important long chain polyunsaturated fatty acids (LC-PUFAs) in fish are recognized for promoting overall human health due to their activities in physiological, molecular as well as cellular processes (9). Furthermore, marine proteins are recognized for their favorable nutritional value, due to high bioavailability and abundance of important peptides and essential amino acids. Studies focusing on the potential health benefits of marine proteins and hydrolysates are becoming increasingly prevalent due to their beneficial digestibility (7). Khalili Tilami et al. (7) mention that results indicate that fish proteins, peptides, and hydrolysates give improved health benefits somewhat comparable to marine lipids.

Next, to being a source of valuable macronutrients, fish contain essential trace elements like calcium and selenium. As a key indicator of bone density, calcium is crucial for the health of the skeleton and plays a vital role in many metabolic processes. Moreover, selenium deficiencies can lead to several diseases (7). Next to the importance of maintaining metabolic health in humans, selenium in fish is especially important because of its potential counter effects on methylmercury (10). Methylmercury is the methylated form of mercury, which naturally occurs in, e.g., volcanos and the atmosphere, but can also end up in environmental cycles if human caused sources like, e.g., fungicides, antiseptics or batteries are inappropriately discarded (11). Methylmercury is known to have several harmful impacts on human health. It is particularly problematic for pregnant women, as it can migrate across the placenta walls. High methylmercury exposure in pregnant women directly affects the neurodevelopment of the fetus (12). Moreover, bioaccumulation of persistent organic pollutants such as, e.g., polychlorinated biphenyls (PCBs) can pose serious health issues due to their persistence and toxicity to the human body (13).

The aim of the present study was to characterize four different flatfish (*Pleuronoectiformes*) species, and a ray (*Rajiformes*), whereof all are often captured as by-catch in Norway and regarded as underutilized species. More specifically, the study presents the chemical and nutritional profile of European plaice (*Pleuronectes* platessa), European flounder (*Platichthys flesus*), lemon sole (*Microstomus kitt*), megrim (*Lepidorhombus whiffiagonis*), and thornback ray (*Raja clavate*), including comparisons between species related to health promoting nutritional components. The analyses contained the proximate composition, total and free amino acids as well as fatty acid profile. Marine food sources in general are regarded as the main contributors to the intake of contaminants, which pose a potential risk for the consumers. Therefore, PCBs and trace elements, including both essential and toxic elements, were determined to support the safe consumption of these species.

2. Materials and methods

2.1. Raw material

The five fish species of interest were captured with purse seine between September 2020 and April 2021 by local fishermen at the Norwegian west coast. The catch occurred in area 2.a.2, according to the FAO Major Fishing Areas (14). The fish was gutted immediately after capture and kept on ice until the end of rigor mortis (41–57 h post-mortem). Fish were either filleted directly or frozen as whole (−80°C) and subsequently thawed before filleting. In order to have a thorough understanding of the chemical composition and identify any potential changes in the nutritional content within different body regions, muscle samples were taken from two fillets per flatfish. The two fillets, being the lower loin (LL) and upper belly (UB), of the flatfish samples were considered for analyses ($n_{\text{Flounder}} = 7 \times 2$, $n_{\text{Lemon sole}} = 5 \times 2$, $n_{\text{Plaice}} = 10 \times 2$, $n_{\text{Megrim}} = 5 \times 2$), as shown in Figure 1A. In contrast, only one fillet was kept for thornback ray ($n = 5$), as it consists of two central fillets (Figure 1B). All fillets were frozen directly and stored at −80°C until further use.

2.2. Proximate composition

Dry matter and ash content was determined following the AOAC 925.10 method (15). Samples were homogenized and between 1 and 2 g were weighed in duplicates in porcelain crucibles. The samples were placed in a dehydrator at 105°C for 24 h (TS8056; Termaks, Norway). After 24 h, the samples were placed in a desiccator to cool down to room temperature, weighed and water content was calculated according to equation 1. The dried samples were transferred to an ash oven and burned at 550°C for 20 h (B410; Nabertherm,Germany). The samples were placed in a desiccator to cool down, weighed and the inorganic matter was then determined following the principle of equation 1.

$$Water\ content\,(\%) = \frac{Sample_{wet} - Sample_{dried}}{Sample_{wet}} \times 100 \qquad (1)$$

Total crude protein content (%) was determined using the Kjeldahl method (15). A Kjeldahl apparatus (K-449 and K-375, Büchi Labortechnik, Schwitzerland) was used for measurements. The sample digestion and titration were carried out following the application manual No: 114/2013 of Büchi Switzerland. Briefly, sulfuric acid (H_2SO_4, 95–97%) and two Kjeldahl Tablets Eco (3.5 g K_2SO_4/0.105 g $CuSO_4 \times 5H_2O$/0.105 g TiO_2) were added to samples (1.5 g) before digestion. Digested samples were first neutralized with NaOH (32%, 15–90 ml) and H_2SO_4 (0.25 mol/l) was used as the titration solution subsequently. To determine the total protein concentration, a conversion factor of 6.25 × nitrogen (%) was applied (16).

Total lipids (%) were determined following the method of Bligh et al. (17). Samples (2 g ww) were weighed into chloroform-resistant tubes and chloroform was added. The solvent-sample mixture was

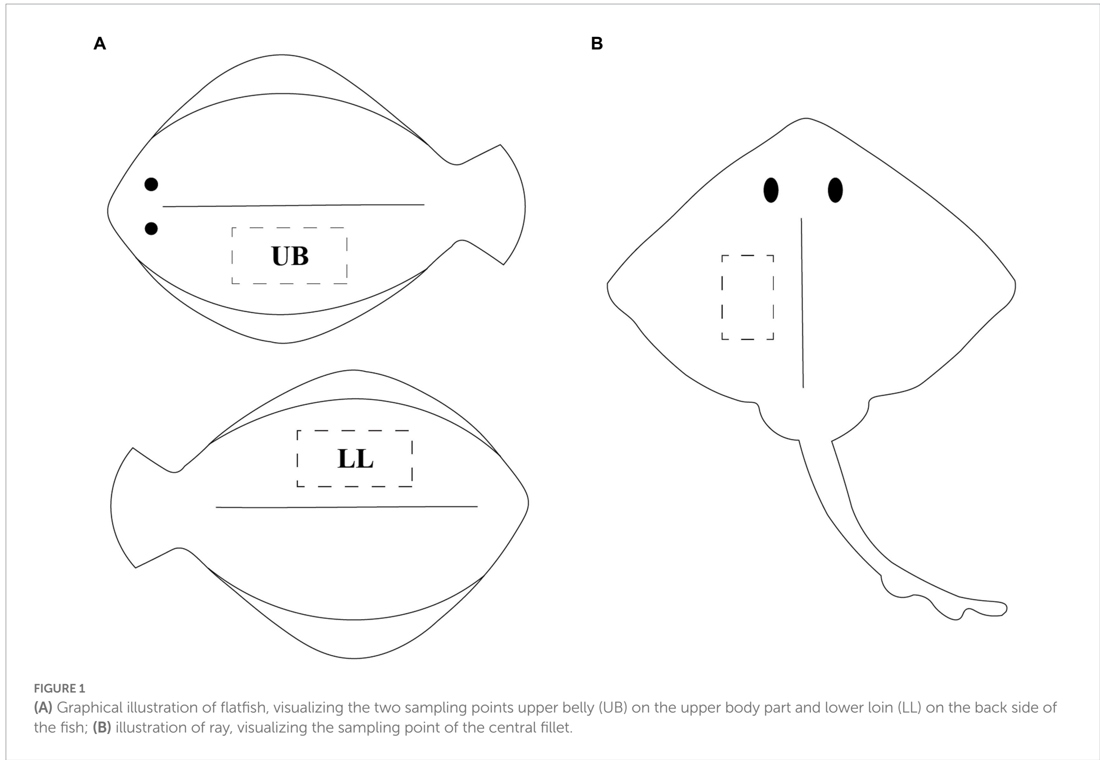

FIGURE 1
(A) Graphical illustration of flatfish, visualizing the two sampling points upper belly (UB) on the upper body part and lower loin (LL) on the back side of the fish; **(B)** illustration of ray, visualizing the sampling point of the central fillet.

subjected to extensive homogenization and centrifugation to achieve phase separation. The aqueous phase and chloroform-lipid phase were separated. Chloroform was evaporated from samples by applying liquid nitrogen. To accelerate the evaporation, the samples were placed on a heating block turned to 40°C (Stuart™ block heater type: SBH130D/3, Cole-Parmer, United States). The total lipids (%) were calculated according to Bligh et al. (17). The remaining chloroform phase containing lipids was frozen and stored at −80°C for fatty acid analysis.

2.3. Fatty acid composition

Fatty acids were prepared as methyl esters for analysis by gas chromatography. For fatty acid methyl ester (FAME) preparation, the method of Metcalfe et al. (18) was used. Chloroform phases containing lipids from individual fish were systematically merged to obtain five samples for thornback ray ($n=5$), four samples for megrim ($n=4$), and three samples each for lemon sole ($n=3$) and flounder ($n=3$). As these fish are very lean and individual fish were limited in size, it was necessary to merge samples to obtain at least 0.02 g of lipids per sample. Samples from European plaice were not merged due to bigger fish sizes, and 6 individuals ($n=6$) were chosen for analysis. Nitrogen evaporation was conducted at 30°C until all chloroform was removed from the samples, and 3 ml KOH in methanol (0.5 M) was added to the samples and vortexed to saponify the lipids. Samples were incubated in a water bath at 70°C for 20 min, vortexed, and cooled on ice. Afterwards, 5 ml of boron trifluoride-methanol (14%, BF_3) was added to allow acid-catalyzed esterification of the fatty acids. The samples were re-incubated in the water bath at 70°C for 5 min and cooled on ice. *N*-butyl acetate (2 ml) was added, and the samples were shaken. Subsequently, saturated NaCl (around 1.5 ml), and two spatulas of powdered sodium sulfate (Na_2SO_4) were added to the samples, and the samples were rested at room temperature (21°C) to allow phase separation. Around 0.5 ml of hexane was added, and the lipid phase was then pipetted out and filtered using a 0.2 μm PTFE membrane (VWR International, United States) into GC vials.

The FAMEs were analyzed by gas chromatography (GC) using a GC apparatus (Agilent 6850, Agilent Technologies, United States). The samples (2–3 μl) were introduced by an evaporation injector (inlet: 260°C, pressure: 18.1 psi). Hydrogen was used as a carrier gas to pass the samples onto a polyethylene glycol column (HP-INNOWAX, i.D.: 0.25 mm; film: 0.25 μm, Merck Life Sciences, Norway), where FAMEs were separated at different times along the stationary phase. A flame ionization detector (FID) adjusted to 310°C was used to detect the samples. The oven program was set to a constant temperature of 160°C for 3 min, with an increase of 3°C/min to 240°C and held for 3 min.

Fatty acids were identified by comparing relative retention times (RRTs) of the external FAME standard mix containing 37 fatty acid methyl esters (Supelco 37 Component FAME Mix, Merck Life Sciences, Norway) with sample peaks. Chromatogram peaks, showing similar RRTs to the external standard were considered for determination. The intensity of each peak was calculated against the total intensity of FAMEs, to determine the percentage distribution of the individual fatty acids in each sample.

2.4. Protein profile

2.4.1. Amino acid distribution

Total amino acids were extracted from the samples following the method of Blackburn (19). Samples were freeze-dried for 22 h at −40°C and 13.3 Pa as a preparation for the analysis. Freeze-dried samples (80 mg) were weighed up in duplicates, and 1 ml of HCl (6 M) was added. The tubes were incubated for 22 h at 105°C to allow protein hydrolysis. Hydrolyzed samples were pH-neutralized by adding NaOH. The samples were filtered through a glass microfiber filter GF/C using suction, subsequently filled up to 10 ml with deionized water and suitably diluted. Diluted samples were filtered through 0.22 µm polyethersulfone filters (VWR International, United States) and transferred into HPLC vials.

Both free and total amino acids were analyzed by ultra-high-performance liquid chromatography (HPLC, UltiMate 300, Thermo Fisher Scientific, United States). As mobile phase, methanol and sodium acetate (0.08 M) with 2% tetrahydrofuran were applied. The HPLC was equipped with a Nova-Pak C18 column (WAT086344, particle size: 4 µm, 3.9 mm*150 mm, Waters Corp., United States), a TSP P400 pump and an injection valve (ultimate 3000WP injector). A pre-column derivatization step using the *o*-phtalaldehyde (OPA) method was applied and the flow rate was adjusted to 0.9 ml/min. After passing through the column, the amino acids were detected by fluorescence and recognized by a Dionex RF2000 detector. Alpha-aminobutyric acid (Aba) was used as an internal standard. Three amino acids were not analyzed: cysteine, proline, and tryptophan. The amino acids glycine and arginine were co-eluted in the analysis.

2.4.2. Free amino acid distribution

Free amino acids were extracted from the samples following the method of Osnes et al. (20). Approximately 2 g of frozen grated sample was placed into centrifuge tubes. Deionized water (10 ml) was added to the tubes, and the mixture was homogenized for 45 s to disrupt cells and release proteins (Ultra Turrax T25, Ika, Germany). The tubes were centrifuged for 3 min at 500 g at 4°C to obtain two phases (1700, Kubota, Japan). The soluble protein extract phase was taken out, and 1 ml of the extract was mixed with 0.25 ml of sulphosalicylic acid (10%, $C_7H_6O_6S$) to allow protein breakdown. The samples were shaken vigorously and placed in a fridge (4°C) for 30 min. After protein breakdown, the tubes were centrifuged for 10 min at 2700 g and 4°C (Megafuge 8R, Thermo Fisher Scientific, United States). The supernatant containing the free amino acids was suitably diluted. The diluted samples were filtered through 0.2 µm polyethersulfone membrane filters and 0.205 ml of the samples were transferred to vials before performing HPLC analysis as described in section 2.4.1.

2.5. Trace elements and polychlorinated biphenyls

For analyzing potentially elevated levels of contaminants in the samples, a variety of trace elements and polychlorinated biphenyls (PCBs) were chosen, and samples were pooled together. Each sample contained two individuals (three for European plaice) of same size, equally distributed and homogenized. Per species, two pooled samples ($n = 2$) were examined.

The samples were analyzed for 20 elements, including both toxic and essential trace elements such as Ag, Al, As, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Mo, Na, Ni, Pb, Se, V, and Zn. An inductive coupled plasma mass spectroscopy (ICP-MS, 8800 Triple Quadrupole; Agilent Technologies, United States) system was used. The system was linked to an autosampler (prepFAST M5, ESI, United States). To test the accuracy of the analysis, certified reference materials (CRM) were used, namely MODAS-5 (cod tissue, Nr. 0496) and MODAS-3 (herring tissue, Nr. 0958). The procedure of sample preparation, including microwave digestion and subsequent steps were previously described in detail by Kendler et al. (21) following the method of Sørmo et al. (22).

The analysis for PCBs included PCB-3, 8, 28, 52, 101, 118, 138, 153, 180, 195, 206, and 209, including the ICES-6 PCBs (PCB: 28, 52, 101, 138, 153, 180) and the dioxin-like PCB 118. A GC–MS system (7890A, Agilent Technologies, United States) was employed to detect PCBs. The system included split liner injection, an inert mass selective detector (5,975, Agilent Technologies, United States) and a Thermo TG 5MS column (length: 30 m; i.D.: 250 µm; film: 0.5 µm). A detailed description of the procedure can be found in Kendler et al. (21). The sample extraction followed the method described by Teunen et al. (23).

2.6. Nutritional quality parameters

2.6.1. Digestible indispensable amino acid score

The protein quality of a foodstuff can be determined by calculating the digestible indispensable amino acid score (DIAAS). For calculations on the quality of the distinct amino acid profiles of the five investigated fish species, the DIAAS as proposed by FAO (24) was considered and calculated.

The score is a product of the amino acid scoring pattern of the protein and the digestibility of these amino acids. The amino acid scoring pattern is related to how the amino acids in the protein correspond to the nutritional requirements set by FAO (24). A beneficial amino acid content is characterized by a high digestible indispensable amino acid (DIAA) content, which exceeds the nutritional requirements.

The DIAA reference ratios can be calculated for each DIAA from the amino acid content and the ileal digestibility, as seen in equation 2. IAA ratios above one are characterized by a high content of DIAA, which exceeds nutritional recommendations. DIAA ratios below one mean that the DIAA in the protein does not meet the recommendations. The lowest DIAA reference ratio is multiplied by 100 to obtain the DIAAS (24). Food with scores above 100 can be classified as "excellent" protein quality sources, scores between 75 and 100 can be classified as "good" protein quality sources, while scores below 75 can be regarded as "low" protein quality sources (24). Previous investigations on DIAAS in fish have determined them to be of excellent protein quality (25, 26).

$$DIAA\ reference\ ratio = \frac{mg\ of\ AA\ in\ 1\ g\ sample\ protein \times df}{mg\ of\ AA\ in\ 1\ g\ of\ reference\ protein} \quad (2)$$

Where:

df: true ileal digestibility factor for specific amino acids in fish as proposed by FAO (27). When specific digestibility factors were not

available for the given amino acid, the general digestibility factor for the protein was used.

reference protein: nutritional requirements set by FAO et al. (28).

2.6.2. Fatty acids

An estimation of the total amounts of fatty acids per 100 g edible fillet wet weight (ww) of the investigated species was conducted using equation 3. For the assessment of total fatty acids, published work from Weihrauch et al. (29) considering different lipid conversion factors in fish was applied, following the fatty acid conversion factor (FACF) as shown in equation 4.

$$g\ fatty\ acid\ per\ 100\ g\ fillets = weight\%\ FAME \times FACF \times TLC \qquad (3)$$

Where:

Weight% FAME: results from FAME analysis, assuming the same as weight%-FA since marine lipids mainly consist of long-chain fatty acids (29).

FACF: fatty acid conversion factor (g FA/g lipid), from conversion factors proposed by Weihrauch et al. (29) calculated as in equation 4.

$$FACF = \frac{0.933 - 0.143}{TLC} \qquad (4)$$

TLC: total lipid content as measured in g lipid per g fillet ww from the analysis on total lipids (17).

2.7. Statistical analysis

All statistical analyses were performed using Minitab 19[1] (Minitab Inc., United States). A Grubbs Outlier test with a significance level of $\alpha < 0.05$ was conducted to find outliers in the data set. Data were analyzed using univariate analysis of variance (ANOVA) combined with Tukey HSD *post hoc* test when significance was detected to investigate the differences between groups. Statistical differences were reported at the level of $\alpha < 0.05$. For flatfish representatives, analyses were carried out in 2×2 parallels (2 parallels for each UB and LL fillet; 4 in total per sample) and are presented as means ± standard deviation (SD) if not other stated. For thornback ray, the same analyses were performed in duplicates.

3. Results and discussion

In addition to species comparison, differences in the proximate and total and free amino acids composition among the UB and LL fillets were investigated for the four flatfish species. The UB fillets did not significantly differ from the LL for any of the species ($p > 0.05$). Hence, the data of the UB and LL fillets were combined, giving one mean value for each flatfish individual, which was further considered when presenting the results. This leads to the assumption that nutrients are equally distributed throughout the body regions of the investigated flatfish species. The findings correspond with our previous study on European plaice (21), where no significant difference in proximate and nutritional composition between muscle samples from upper and lower body fillets was found. Moreover, the results are in accordance with the study of Barbosa et al. (30) on megrim, which found no differences in lipid content between the upper and lower body fillets. The differences between fillets of upper and lower body were not investigated for thornback ray as its morphology differs from flatfish species, having only two main fillets.

3.1. Proximate composition

The proximate composition of flounder, lemon sole, megrim, plaice and thornback ray are shown in Table 1. Significant differences were observed for the species' ash, water, protein and lipid content. Megrim was found to have the lowest average water content of 79.2%, being significantly lower than flounder ($p = 0.003$). The measured water content for megrim equaled the values found by Afonso et al. (31), and Barbosa et al. (30), who showed values from 75–79%. Thornback ray showed a similar water content (80.1%) compared to lemon sole (81.4%) and plaice (80.5%), but differed from previous investigations by Colakoglu et al. (32) and Turan et al. (33) with water contents of 77%. The water contents of all investigated species are similar to those found by Karl et al. (34), investigating different flatfish species with average values ranging from 78.1 to 82.1%.

The ash content of the four flatfish representatives was between 1.0–1.25%, while fillets from thornback ray (0.9%) showed lower values of inorganic material. This was in line with previous investigations on flatfish, although previous studies on thornback ray found slightly higher ash values (1.1–1.4%) for this species (32, 33). Ash content in plaice is higher than previously investigated by Karl et al. (34) of 0.9% but similar to results from three different seasons by Kendler et al. (21) of values ranging from 1.07 to 1.28%. Plaice has a significantly higher ash content compared to flounder ($p = 0.005$) and thornback ray ($p < 0.001$).

For protein content, the differences were more significant between the investigated species. Megrim had a significantly higher protein content than lemon sole ($p = 0.036$), flounder ($p = 0.003$) and plaice ($p = 0.024$) with an average of 19.6%, being marginally higher than the studies by Barbosa et al. (30) with 16.6 to 18.6%. Lemon sole and flounder had a protein content of 16–18%, in line with previous investigations of plaice (16.6%) and yellowfin sole (16.0%) of Karl et al. (34). Significantly higher protein values were observed for thornback ray with 24.0%, being considerably higher than all investigated flatfish species ($p < 0.001$). The measured protein content of thornback ray was not in correspondence with previous studies and was 4–5% higher than observed in studies by Colakoglu et al. (32) and Turan et al. (33) with 18.6 and 20%, respectively. It must be mentioned that the proximate composition of thornback ray exceeds a total of 100%, which indicates an overestimation of the protein content measured by the Kjeldahl method using a conversion factor of 6.25. The Kjeldahl method measures the total nitrogen content, assuming approximately 16% nitrogen in proteins. However, other non-proteins in the cells also contain nitrogen, which can lead to an overestimation of the protein nitrogen in the food (35). Ray tissue contains around 350–400 mM urea, a nitrogen containing non-proteinaceous

1 www.minitab.com

TABLE 1 Proximate composition of central fillets of flounder, lemon sole, megrim, plaice, and thornback ray.

	Species					
Composition (%)	Flounder	Lemon sole	Megrim	Plaice	Thornback ray	*p*-value*
	$n = 7$	$n = 5$	$n = 5$	$n = 10$	$n = 5$	
Ash	1.11 ± 0.07^{b}	1.04 ± 0.05^{bc}	1.10 ± 0.04^{b}	1.25 ± 0.07^{a}	0.94 ± 0.10^{c}	<0.001
Water	82.1 ± 1.2^{a}	81.4 ± 1.0^{ab}	79.2 ± 1.4^{b}	80.5 ± 1.4^{ab}	80.1 ± 0.8^{ab}	0.004
Proteins	16.9 ± 1.0^{c}	17.5 ± 0.7^{c}	19.6 ± 1.2^{b}	17.6 ± 1.1^{c}	24.0 ± 1.4^{a}	<0.001
Lipids	0.94 ± 0.08^{ab}	0.74 ± 0.16^{b}	0.98 ± 0.16^{ab}	1.25 ± 0.47^{a}	0.76 ± 0.08^{b}	0.015

Results presented as mean values ± SD. *ANOVA was applied to detect differences in proximate composition; where significant difference was detected ($\alpha < 0.05$), a Tukey HSD *post hoc* test was applied. Values with different superscript (a, b) within a row are significantly different ($P < 0.05$).

component, which might have been an interfering substance in the Kjeldahl analysis (36). Moreover, as the relative nitrogen content of amino acids fluctuates and the amino acid composition depends on the protein source, the assumption of 16% nitrogen content is rather general. Studies have shown that using a conversion factor of 6.25 can overestimate the total protein content of some foods (35, 37, 38). For this reason, attention has been given to creating conversion factors that are species/ food-specific (37, 38). Nevertheless, the established conversion factor of 6.25 is still widely used being officially recognized by the AOAC as a standard analytical method for protein determination, which makes results better comparable (39). The results suggest that protein contents of the four flatfish species are not overestimated, as the proximate composition (100%) is not notably exceeded and standard deviations are acceptable (1%).

Regarding the lipid content, all species can be considered as lean species, having values below 2%. Plaice was found to have significantly higher lipid values of in average 1.25% compared to lemon sole ($p = 0.025$) with 0.74% and thornback ray ($p = 0.036$) with 0.76% total fat content. The findings in this study show lower lipid contents for megrim (0.98%) compared to the study of Pastoriza et al. (40) finding a lipid content of up to 1.9%. Karl et al. (34) found lower lipid values for plaice of around 0.8%, but similar values of around 1% of other investigated flatfish. In a previous study of Kendler et al. (21), significant differences in the lipid content of plaice depending on fishing season were discovered ranging from 0.75 to 1.55%. For thornback ray, the measured lipid content of 0.76% was marginally higher than the finding of Turan et al. (33) of 0.5%, while much lower than the finding of Colakoglu et al. (32) of 3.4%. Two previous studies on deep-sea fish found that general deep-sea elasmobranchs like thornback ray had a lipid content of around 0.7 to 1.0%, which support the findings of the present study (41, 42).

3.2. Protein profile

Total amino acids (TAA) were investigated to determine the nutritional value of the proteins in the fish. The TAA results for the species are given in Table 2. The most abundant TAA for all species were leucine and lysine, as well as glutamic and aspartic acid, under physiological conditions in the form of glutamate and aspartate. The same abundant amino acids were found in an investigation of three flatfish species by Kim et al. (43), although showing higher amounts of glycine than in the present study. The contents of glutamate and aspartate can be regarded as overestimated as glutamine and asparagine were converted to these two amino acids during acid analysis (44). Consequently, asparagine and glutamine were detected in the lowest amounts in all investigated samples. Significant differences ($p < 0.05$) were found for most of the amino acids between the five species. All species have a preferable distribution of indispensable amino acids (IAA), accounting for more than 50% of the TAA distribution. Megrim was found to have significantly higher amounts of IAA (7.24 g/ 100 g; $p = 0.002$) compared to flounder (6.14 g/100 g), lemon sole (6.07 g/100 g), plaice (5.62 g/100 g), and thornback ray (5.88 g/100 g). Furthermore, megrim has comparably more total amino acids (13.80 g/100 g; $p = 0.002$) than the other species. This was also found in three of the four species for the total protein content, with the exception of thornback ray, where an overestimated protein content is suspected. The amino acid determination by Blackburn (19) directly calculates the protein amount by considering only amino acid residues in the analysis and does not take into account possible interfering non-proteinaceous components. Amino acid hydrolysis can therefore be regarded as a good approach to determine the total amino acid content and gives an indication of the protein content of foods. However, the concentration of some of the amino acids might be lowered significantly due to the hydrolysis step prior to HPLC-analysis, which leads to an underestimation of the total protein content (35). Furthermore, it has to be mentioned that due to their instability during acid hydrolysis, cysteine and tryptophan were not determined in the applied method. Consequently, these two amino acids should be analyzed separately if the TAA measurement is used to indicate the total protein content.

The DIAA ratios were calculated and the DIAAS was calculated based on the DIAA ratios. The DIAA reference ratios are displayed in Table 3, and indicate whether all DIAA were present in the protein in adequate amounts to meet the requirements for adults set by FAO et al. (28). To calculate the ratios, the measured TAA levels were converted to mg/g protein for the different species as described in section 2.6.1. Scores above 1 indicate sufficient IAA levels, while ratios below 1 indicate insufficient levels. In addition, the DIAAS (%), was calculated, being the lowest amino acid ratio per species multiplied by 100 to convert the ratio to a percentage score. As indicated in Table 3, all amino acids show ratios above 1, despite methionine and cysteine (as combined values) in lemon sole (0.7), implying an excellent overall protein quality of the species. Cysteine and tryptophan were not analyzed in this study, which explains the low methionine + cysteine ratio, as it only consists of methionine. Ratios up to 2.5 for threonine were observed for thornback ray, megrim and lemon sole, pointing out the relevance of these species for a sufficient intake of indispensable amino acids. Moreover, DIAAS of over 100% were discovered for flounder (120%), megrim (120%), plaice (110%), and thornback ray

TABLE 2 Total amino acid contents (total-AA) in g/100g central fillets of flounder, lemon sole, megrim, plaice and thornback ray, showing indispensable (IAA) and non-indispensable amino acids (non-IAA).

Amino acids	Species					
	Flounder	Lemon sole	Megrim	Plaice	Thornback ray	
	n = 7	*n* = 5	*n* = 5	*n* = 10	*n* = 5	
Indispensable*	**Total-AA**					
	g 100 g^{-1}	g 100 g^{-1}	g 100 g^{-1}	g 100 g^{-1}	g 100 g^{-1}	*p*-value***
Histidine	0.27 ± 0.04^{b}	0.31 ± 0.04^{ab}	0.36 ± 0.08^{a}	0.28 ± 0.06^{ab}	0.26 ± 0.007^{b}	0.026
Isoleucine	0.57 ± 0.06^{b}	0.59 ± 0.01^{b}	0.71 ± 0.05^{a}	0.53 ± 0.06^{b}	0.59 ± 0.01^{b}	<0.001
Leucine	1.13 ± 0.12^{b}	1.06 ± 0.09^{b}	1.32 ± 0.09^{a}	1.03 ± 0.11^{b}	1.09 ± 0.02^{b}	<0.001
Lysine	1.40 ± 0.08^{b}	1.37 ± 0.14^{b}	1.63 ± 0.09^{a}	1.24 ± 0.17^{b}	1.26 ± 0.03^{b}	<0.001
Methionine	0.46 ± 0.05^{a}	0.20 ± 0.04^{b}	0.42 ± 0.12^{a}	0.37 ± 0.05^{a}	0.41 ± 0.04^{a}	<0.001
Phenylalanine	0.56 ± 0.06^{b}	0.54 ± 0.05^{b}	0.68 ± 0.05^{a}	0.51 ± 0.05^{b}	0.57 ± 0.01^{b}	<0.001
Threonine	0.69 ± 0.04^{b}	0.70 ± 0.1^{b}	0.85 ± 0.04^{a}	0.60 ± 0.06^{c}	0.69 ± 0.03^{bc}	<0.001
Valine	0.63 ± 0.07^{ab}	0.58 ± 0.07^{b}	0.72 ± 0.09^{a}	0.58 ± 0.06^{b}	0.58 ± 0.01^{b}	0.008
Σ IAA	6.14 ± 0.69^{b}	6.07 ± 0.78^{b}	7.24 ± 0.42^{a}	5.62 ± 0.65^{b}	5.88 ± 0.11^{b}	0.002
Non-indispensable						
Asparagine	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	<0.01 ± <0.01	0.833
Glutamine	0.03 ± 0.007^{b}	0.01 ± 0.004^{c}	0.05 ± 0.008^{a}	<0.01 ± <0.01^{c}	0.04 ± 0.01^{b}	<0.001
Arg/Gly**	0.82 ± 0.04^{ab}	0.83 ± 0.1^{ab}	0.90 ± 0.08^{a}	0.73 ± 0.07^{b}	0.77 ± 0.02^{ab}	0.004
Tyrosine	0.52 ± 0.06^{ab}	0.49 ± 0.04^{ab}	0.56 ± 0.1^{a}	0.45 ± 0.05^{b}	0.41 ± 0.04^{b}	0.005
Alanine	0.89 ± 0.1^{ab}	0.74 ± 0.16^{bc}	1.0 ± 0.15^{a}	0.69 ± 0.06^{c}	0.79 ± 0.02^{bc}	<0.001
Aspartate	1.39 ± 0.15^{ab}	1.35 ± 0.15^{ab}	1.61 ± 0.13^{a}	1.35 ± 0.17^{b}	1.32 ± 0.03^{b}	0.022
Glutamate	1.79 ± 0.19	1.83 ± 0.20	2.10 ± 0.15	1.95 ± 0.24	1.75 ± 0.05	0.049
Serine	0.74 ± 0.12^{ab}	0.85 ± 0.12^{a}	0.84 ± 0.14^{a}	0.60 ± 0.07^{b}	0.62 ± 0.01^{b}	<0.001
Σ Non-IAA	5.64 ± 0.62^{ab}	5.61 ± 0.52^{ab}	6.56 ± 0.52^{a}	5.34 ± 0.57^{b}	5.29 ± 0.11^{b}	0.005
Σ Total-AA	11.78 ± 1.3^{b}	11.68 ± 1.3^{b}	13.80 ± 0.9^{a}	10.96 ± 1.2^{b}	11.17 ± 0.2^{b}	0.002

Results presented as mean values ± SD. *Tryptophan is not detected due to acid hydrolysis. **Arginine/Glycine could not be separated. ***ANOVA was applied to detect differences in total-AA, where significant difference was detected ($\alpha < 0.05$), a Tukey HSD *post hoc* test was applied. Values with different superscript within a row are significantly different ($P < 0.05$).

TABLE 3 Average total digestible indispensable amino acid ratios and scores (DIAAS) for central fillets of flounder, lemon sole, megrim, plaice, and thornback ray based on recommendations for adults set by FAO et al. (28).

Amino acids	(28) Recommendations (mg/g protein)	Digestible indispensable amino acid ratios				
		Flounder	Lemon sole	Megrim	Plaice	Thornback ray
Histidine	15	1.3	1.5	1.5	1.4	1.3
Isoleucine	30	1.5	1.5	1.6	1.5	1.6
Leucine	59	1.5	1.4	1.5	1.5	1.5
Lysine	45	2.4	2.4	2.4	2.3	2.3
Methionine (+cys*)	22	1.6	0.7	1.3	1.4	1.6
Phenylalanine + tyrosine	38	2.0	2.4	2.0	1.1	2.0
Threonine	23	2.3	2.5	2.5	2.2	2.5
Tryptophan*	6	–	–	–	–	–
Valine	39	1.2	1.1	1.2	1.2	1.2
Total IAA	277	1.7	1.7	1.7	1.7	1.7
DIAAS (%)**		120%	70%	120%	110%	120%

*Cysteine/Tryptophan not measured in analysis. Scores above 1.0 indicate contents higher than recommendations (green cells), scores below 1.0 indicate content lower than recommendations (red cells). DIAAS (%) ** calculated by multiplying the lowest DIAA ratio by 100.

(120%), stressing the high protein quality of the studied fish. Lemon sole shows a poorer DIAAS (70%), which is possibly due to the lack of data from cysteine.

In addition to their nutritional importance, amino acids are associated with taste when occurring unbound in the form of free amino acids (FAA) in biological systems. Figure 2 shows the FAA distribution (mg/100 g sample ww) of the five investigated species. FAA are grouped according to their distinctive flavor as described by Fuke et al. (45), Kirimura et al. (46) and Sarower et al. (47). FAAs have been identified as essential taste contributors in seafood (45–47). Glutamic acid, in the form of glutamate, glycine, and alanine are commonly identified among the most important taste contributors. Glycine and alanine are linked to sweetness, while FAAs such as valine, arginine, and methionine are linked to bitter taste in seafood (47). Aspartate and glutamate both provide a sour taste. However, especially relevant in seafoods, these amino acids also give an umami taste in the presence of sodium salts, such as the familiar monosodium glutamate (MSG). Phenylalanine and tyrosine also have a bitter taste, but can enhance the umami flavor (47). Related to the present study, significant differences between species were found for all FAAs, but arginine/glycine ($p = 0.114$; Arg/Gly as combined values), threonine ($p = 0.866$) and methionine ($p = 0.872$). Lysine showed significant differences amongst the species ($p < 0.001$), with flounder and megrim having notably higher values. Flounder, lemon sole and plaice were identified to have significantly higher levels of histidine ($p < 0.001$), while megrim and thornback ray show comparatively low values of 0.51 and 0.84 mg/100 g sample, respectively. The most prevalent FAA are allocated in the group of "Sweet" amino acids with arginine/glycine having the highest content, followed by lysine and alanine.

Referring to the TAA and FAA distribution of the five investigated species, high variances within the species were observed, which is expressed by relatively large standard deviations as shown in Table 2 and Figure 2. External factors such as sex, maturity, and feeding behavior can influence the chemical composition of fish. Moreover, seasonality can play an important factor, as pointed out in a previous study on chemical composition of European plaice caught during three seasons by Kendler et al. (21).

3.3. Fatty acid composition

The following sections (section 3.3 and 3.4) highlight the potential of the studied species as sources for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in human nutrition. Flounder, plaice and thornback ray were found to have significantly higher total PUFA compositions than megrim with 50.7, 47.2, and 49.3%, respectively, as can be seen in Table 4. Significant differences were also found in the amount of present DHA. Thornback ray

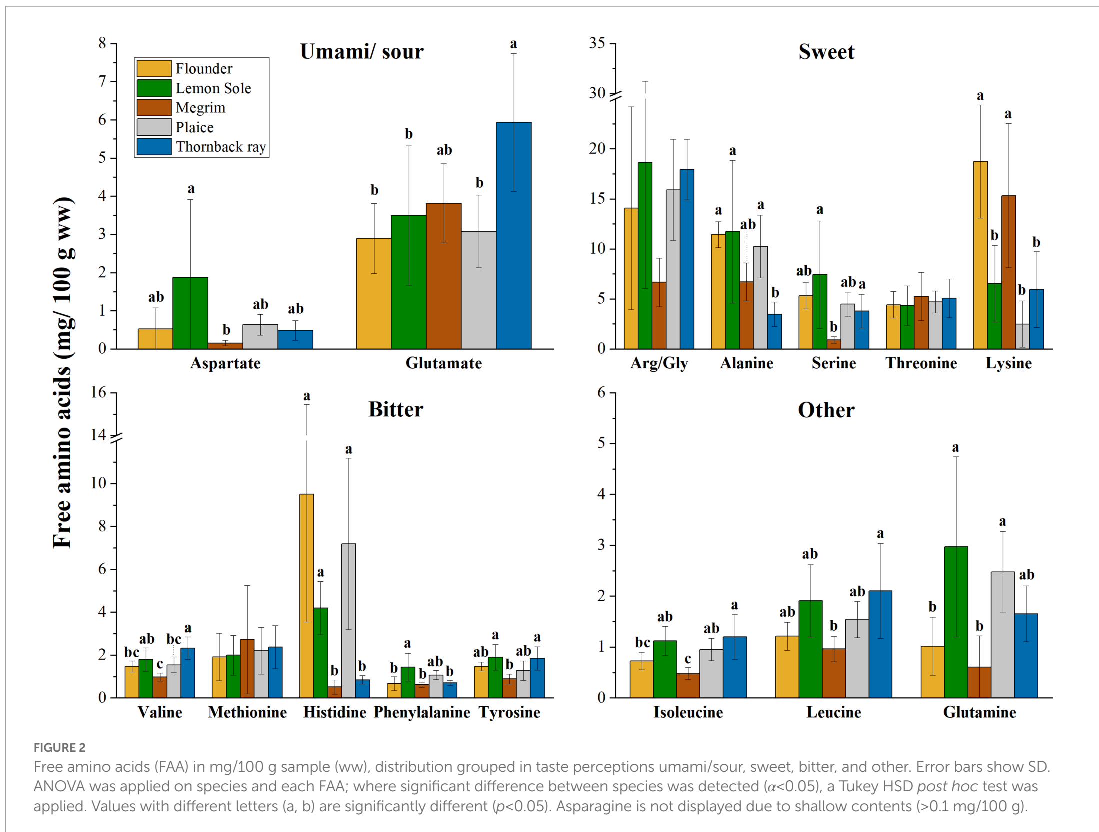

FIGURE 2
Free amino acids (FAA) in mg/100 g sample (ww), distribution grouped in taste perceptions umami/sour, sweet, bitter, and other. Error bars show SD. ANOVA was applied on species and each FAA; where significant difference between species was detected (α<0.05), a Tukey HSD *post hoc* test was applied. Values with different letters (a, b) are significantly different (p<0.05). Asparagine is not displayed due to shallow contents (>0.1 mg/100 g).

TABLE 4 Fatty acid composition (% of total fatty acids w w^{-1}) of flounder, lemon sole, megrim, plaice, and thornback ray.

	Species					
Fatty acids	Flounder	Lemon sole	Megrim	Plaice	Thornback ray	
	n = 3*	*n* = 3*	*n* = 4*	*n* = 10	*n* = 5*	
SFA	%	%	%	%	%	*P*-value**
C14:0	1.58 ± 0.03^{ab}	1.45 ± 0.3^{ab}	2.68 ± 0.6^{a}	2.71 ± 1.4^{a}	0.29 ± 0.3	0.003
C15:0	0.52 ± 0.3	0.37 ± 0.3	0.34 ± 0.2	0.17 ± 0.3	0.06 ± 0.1	0.110
C16:0	20.9 ± 1.9^{ab}	23.86 ± 6.2^{a}	20.37 ± 3.0^{ab}	17.1 ± 2.6^{b}	26.0 ± 1.6^{a}	<0.001
C17:0	0.66 ± 0.2^{ab}	1.05 ± 0.1.0^{a}	0.26 ± 0.2^{ab}	0.17 ± 0.3^{b}	0.29 ± 0.39^{ab}	0.034
C18:0	5.52 ± 0.7^{ab}	6.44 ± 1.1^{a}	4.64 ± 1.4^{ab}	3.50 ± 1.4^{b}	5.06 ± 0.3^{ab}	0.006
Σ SFA	29.19 ± 2.6^{ab}	33.17 ± 6.4^{a}	28.27 ± 3.3^{ab}	23.64 ± 2.8^{b}	31.66 ± 1.5^{a}	<0.001
MUFA						
C14:1	0.04 ± 0.06^{ab}	0.07 ± 0.1^{ab}	0.22 ± 0.2^{a}	0.03 ± 0.09^{b}	0.00 ± 0.0^{b}	0.041
C16:1 n7	2.88 ± 0.3^{bc}	1.81 ± 1.6^{bc}	4.78 ± 1.2^{ab}	6.93 ± 1.9^{a}	1.71 ± 0.09^{c}	<0.001
C17:1	0.13 ± 0.2	0.39 ± 0.4	0.31 ± 0.2	0.18 ± 0.3	0.00 ± 0.0	0.273
C18:1 n7	2.37 ± 0.3	1.80 ± 1.6	2.66 ± 0.7	4.16 ± 2.4	3.55 ± 0.6	0.228
C18:1 n9	10.08 ± 2.3	12.19 ± 4.9	13.68 ± 2.9	8.45 ± 3.4	8.36 ± 0.7	0.055
C20:1	1.97 ± 0.3^{ab}	0.77 ± 0.9^{b}	5.26 ± 1.2^{a}	4.89 ± 2.7^{a}	1.27 ± 0.7^{b}	0.003
C22:1	0.36 ± 0.6^{ab}	0.12 ± 0.2^{ab}	2.59 ± 2.0^{ab}	2.91 ± 1.9^{a}	0.00 ± 0.0^{b}	0.008
Σ MUFA	17.82 ± 2.3^{b}	17.16 ± 0.6^{b}	29.50 ± 5.8^{a}	27.53 ± 4.8^{a}	14.89 ± 1.8^{b}	<0.001
PUFA						
C16:2 n4	0.67 ± 0.2	0.73 ± 0.6	0.56 ± 0.4	0.40 ± 0.5	0.12 ± 0.3	0.351
C18:2 n6 (LA)	2.95 ± 1.8^{a}	1.17 ± 1.0^{ab}	0.88 ± 0.6^{b}	0.40 ± 0.5^{b}	1.49 ± 0.2^{ab}	0.002
C18:3 n3	0.77 ± 0.3	0.43 ± 0.4	0.72 ± 0.5	0.21 ± 0.3	0.10 ± 0.2	0.023
C18:4 n3	0.39 ± 0.3	0.17 ± 0.3	0.51 ± 0.6	1.48 ± 1.9	0.00 ± 0.0	0.249
C20:2 n6	0.11 ± 0.2^{ab}	0.39 ± 0.3^{a}	0.20 ± 0.1^{ab}	0.08 ± 0.1^{ab}	0.00 ± 0.0^{b}	0.037
C20:4 n6 (AA)	6.52 ± 1.6	8.94 ± 2.5	2.94 ± 1.8	4.11 ± 3.8	4.25 ± 0.6	0.07
C20:4 n3	0.16 ± 0.3	0.16 ± 0.3	0.62 ± 0.4	5.9 ± 7.0	0.14 ± 0.3	0.115
C20:5 n3 (EPA)	13.24 ± 2.6	14.93 ± 3.0	6.73 ± 2.3	10.07 ± 8.9	3.96 ± 0.7	0.119
C22:5 n3 (DPA)	2.09 ± 0.4	2.49 ± 2.2	2.31 ± 0.3	4.68 ± 5.6	3.11 ± 0.7	0.744
C22:6 n3 (DHA)	23.84 ± 4.4^{bc}	14.72 ± 2.7^{d}	24.80 ± 2.2^{b}	19.83 ± 2.8^{cd}	36.17 ± 1.4^{a}	<0.001
Σ PUFA	50.74 ± 3.3^{a}	44.12 ± 2.4^{ab}	40.26 ± 4.0^{b}	47.15 ± 4.0^{a}	49.34 ± 2.0^{a}	0.003
Σ n3	40.49 ± 5.2^{abc}	32.9 ± 2.55^{c}	35.67 ± 3.9^{bc}	41.72 ± 4.4^{ab}	43.48 ± 1.8^{a}	0.005
Σ n6	9.58 ± 2.2	10.50 ± 1.4	4.02 ± 1.1	4.92 ± 4.2	5.74 ± 0.6	0.022
n3/n6	4.5 ± 1.8^{bc}	3.2 ± 0.6^{c}	9.3 ± 2.4^{a}	6.1 ± 2.3^{abc}	7.6 ± 0.9^{ab}	0.003
Σ Others	2.24 ± 3.9	5.55 ± 5.3	1.97 ± 2.1	1.67 ± 2.9	4.12 ± 1.4	0.303

Results presented as mean values ± SD. *Samples were merged in order to provide enough lipid phase to analyze for fatty acid composition. **ANOVA was applied to detect differences in fatty acid composition; where significant difference was detected ($\alpha < 0.05$), a Tukey HSD *post hoc* test was applied. Values with different superscript (a, b) within a row are significantly different ($P < 0.05$).

shows considerable higher values (36.2%) comparing to the other species and profoundly lower values were observed in lemon sole (14.7%).

Although the studied fish are categorized as lean species, the fatty acid composition is of high importance. Health promoting effects due to prevention of cardiovascular diseases (CVD), tumor cell proliferation and inflammation processes as well as beneficial effects on brain, retina and neurodevelopment in children are primarily attributed to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (9).

3.4. Nutritional value and essential elements

The total EPA and DHA intake when consuming the five investigated species was calculated to highlight how the species contribute to providing these indispensable FAs to human diet. The contribution to the daily recommendations of 250 mg EPA and DHA by the EFSA Panel on Dietetic Products et al. (48) are shown in Figure 3. Moreover, the average contribution of all IAA given as DIAAS by FAO (24) including the daily requirements of IAA for an

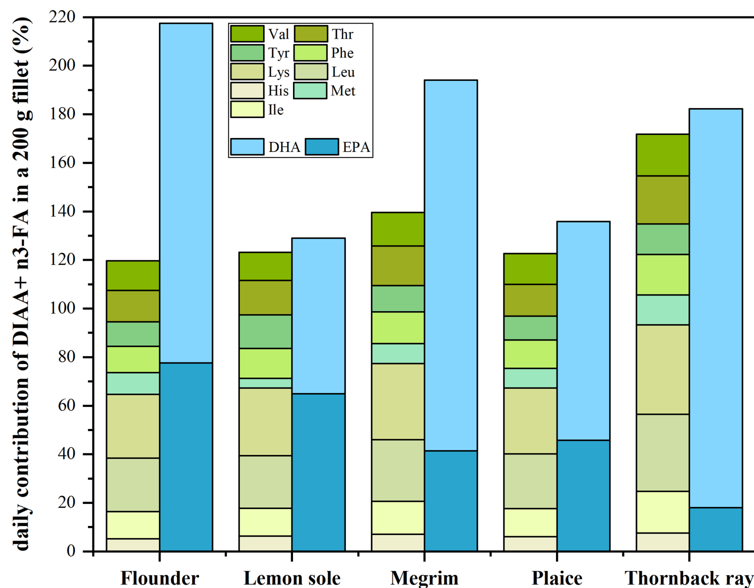

FIGURE 3
Given in green colors: average contribution of all DIAA to daily requirements for an 80 kg adult in a 200 g fillet portion by FAO, WHO (11); given in blue colors: average contribution of a 200 g fillet portion to cover the daily recommended intake of 250 mg DHA+EPA by EFSA (48).

80 g adult as proposed by FAO et al. (28) are shown in Figure 3. The values refer to a portion size of 200 g as recommended in a report on dinner serving sizes of foods by the Norwegian Food Authority (Mattilsynet) and were used for the calculations (49).

Figure 3 emphasizes on the nutritional value of the studied fish related to the content of DIAA as well as EPA and DHA in a 200 g fillet portion. The green bars show that the total required daily amount of DIAA for an 80 kg adult are met by all species. A fillet of 200 g of all species covers more than 120% of the total required DIAA, with thornback ray fulfilling the requirements to 172%. Nevertheless, to fully cover the daily intake of every individual indispensable amino acid (100%), a larger portion size than 200 g is needed for all investigated fish species. The daily recommended intake of, e.g., valine was restrictively covered in megrim (69%), hence a portion size of 371 g would be necessary to cover the daily demand of this particular amino acid (100%). Furthermore, valine is also the restrictive amino acid for thornback ray and flounder, where 54 and 61% of the daily demand is covered by a 200 g portion, respectively. In plaice, phenylalanine (53%) and in lemon sole, methionine (33%) are not fully covered with regards to a portion size of 200 g.

With regards to EPA and DHA, all investigated fish species contribute significantly to the daily suggested intake of 250 mg by EFSA (48). The relative contribution of DHA and EPA in 200 g fillets is shown in dark/light blue shading and was converted to mg/100 g edible portion using the conversion factors proposed by Weihrauch et al. (29) as described in section 2.6.2. Even tough being lean species, a 200 g portion of all of the five species contributes in average to more than 100% of the recommended average daily intake (AI) of EPA and DHA set by EFSA (48). The highest contribution was found in a 200 g portion of flounder (217%), followed by megrim (194%), thornback ray (182%), plaice (136%) and lemon sole (129%). With regards to the weekly recommended intake of 1.75 g EPA + DHA (250 mg × 7) this would mean a consumption of 3.2 portions of 200 g flounder, 3.6 of megrim, 3.8 of thornback ray, 5.2 of plaice and 5.4 portions of lemon sole, respectively. These results are highly relevant, as lean fish is usually not associated with providing sufficient levels of n3 fatty acids and the focus for covering n3 fatty acids was previously put on fatty fish such as salmon or trout in the past (50). All investigated species contain higher relative amounts of DHA, despite lemon sole, which has a 50:50 share of EPA and DHA.

Marine fish are good sources for both macro and trace elements, including minerals like calcium, magnesium or selenium, being vital for human health. All fish contain sufficient amounts of potassium (K) and magnesium as shown in Table 5. Significant differences between species were observed for the elements manganese ($p < 0.001$), magnesium ($p = 0.002$) and iron ($p = 0.047$). High values in selenium, ranging from 0.25 mg kg^{-1} in thornback ray to 0.49 mg kg^{-1} in lemon sole were found in this study. Compared to the study of Karl et al. (34) on different flatfish, selenium values ranging from 0.13 to 0.31 mg kg^{-1} were reported. When setting dietary recommendations, the dietary reference value (DRV) is used. The DRV in this study refers to either the average requirement (AR), the population reference intake (PRI) or the adequate intake (AI), depending on the available data from the expert panel on Dietetic Products, Nutrition and Allergies from EFSA (51–60). Even though no significant difference ($p = 0.213$) in selenium content between species was detected, an effect on the contribution to meet the DRV is visible, given as % of DRV. Hence, a 200 g fillet of lemon sole covers the selenium intake to 140%, whereas a 200 g fillet of thornback ray reaches only 74.3% of the daily selenium coverage. Moreover, all five species are a good source of potassium, covering around 20% of the DRV. Differences in species are visible for the

TABLE 5 Essential elements and nutritional contribution of a 200g portion of fillet of flounder, lemon sole, megrim, plaice, and thornback ray.

		Species															
		Flounder (n=2)			Lemon sole (n=2)			Megrim (n=2)			Plaice (n=6)			Thornback ray (n=2)			
Element	DRV (mg/day)	μg kg^{-1}	EDI	% DRV	μg kg^{-1}	EDI	% DRV	μg kg^{-1}	EDI	% DRV	μg kg^{-1}	EDI	% DRV	μg kg^{-1}	EDI	% DRV	P-value*
Mn	3.0 (51)	70.5 ± 5.5^{bc}	0.014	0.47	94.8 ± 2.2^{ab}	0.019	0.63	107 ± 16.6^{ab}	0.021	0.71	39.8 ± 14.5□^{c}	0.008	0.27	145.8 ± 27.1^{a}	0.029	0.97	<0.001
Mo	0.065 (52)	0.7 ± 0.06	1×10^{-4}	0.22	1.2 ± 0.38	3×10^{-4}	0.38	0.06 ± 0.08	1×10^{-5}	0.012	0.87 ± 0.4□	2×10^{-4}	0.28	1.82 ± 1.5	4×10^{-4}	0.6	0.114
Co	/	2.5 ± 0.15	5×10^{-4}		2.4 ± 1.12	5×10^{-4}		0.52 ± 0.12	1×10^{-4}		2.52 ± 1.1□	5×10^{-4}		1.08 ± 0.23	2×10^{-4}		0.098
		mg kg^{-1}			mg kg^{-1}			mg kg^{-1}			mg kg^{-1}			mg kg^{-1}			
K	3,500 (53)	$3,622 \pm 315$	724.4	20.7	$3,411 \pm 409$	682.2	19.5	$3,689 \pm 4.3$	737.8	21.1	$3,493 \pm 507$	698.6	20.0	$3,495 \pm 154$	699	20.0	0.847
Na	2000 (54)	919 ± 71.4	183.8	9.2	730 ± 99.9	145.9	7.3	644 ± 201	128.9	6.4	$1,085 \pm 248$	217	10.9	774.5 ± 86.0	154.9	7.7	0.191
Mg	300–350 (55)	244 ± 28.2^{ab}	48.7	13.9	218 ± 34.6^{bc}	43.5	12.4	280 ± 6.7^{a}	56.0	16.0	188.7 ± 7.8^{c}	37.74	10.8	232.1 ± 2^{abc}	46.4	13.3	0.002
Ca	950–1,000 (56)	151 ± 30.2	30.24	3.0	149 ± 35.7	29.8	3.0	146 ± 28.6	29.12	2.9	153.7 ± 59.1	30.74	3.1	172.5 ± 107	34.5	3.5	0.993
Fe	11–16 (57)	0.8 ± 0.24	0.16	1.0	0.83 ± 0.25	0.17	1.0	0.74 ± 0.23	0.15	0.9	0.94 ± 0.25□	0.188	1.2	1.65 ± 0.5	0.33	2.1	0.047
Zn	12.7–16.3 (58)	3.7 ± 0.2	0.74	4.5	3.18 ± 0.16	0.64	3.9	3.75 ± 0.08	0.75	4.6	3.90 ± 0.48□	0.78	4.8	3.23 ± 0.3	0.65	4.0	0.172
Se	0.070 (59)	0.4 ± 0.02	0.07	100	0.49 ± 0.01	0.098	140	0.40 ± 0.02	0.08	114.3	0.40 ± 0.12□	0.08	114	0.26 ± 0.0	0.052	74.3	0.213
Cu	1.3–1.6 (60)	0.2 ± 0.06^{ab}	0.034	2.1	0.16 ± 0.01^{ab}	0.032	2	0.2 ± 0.002^{a}	0.038	2.4	0.11 ± 0.02□^{b}	0.022	1.4	0.2 ± 0.03^{ab}	0.032	2	0.031

n = pooled samples constituting of multiple individuals per sample. Results presented as mean values ± SD; DRV, dietary reference values; EDI, estimated daily intake, mg/200 g fillet. □Results extracted from a previous study by Kendler et al. (21). *ANOVA was applied to detect differences in trace elements, where significant difference was detected ($\alpha < 0.05$), a Tukey HSD post hoc test was applied. Values with different superscript (^{a,b}) within a row are significantly different ($p < 0.05$). DRV is expressed as PRI (population reference intake) or AI (adequate intake), dependent on the relative scientific opinion from EFSA as given in the references (51–60). For EDI calculation, average DRV values of Mg, Fe, Zn, and Cu were used.

TABLE 6 Toxic trace elements of flounder, lemon sole, megrim, plaice, and thornback ray.

	Season					
Toxic trace elements	Flounder	Lemon sole	Megrim	Plaice☐	Thornback ray	
	$n = 2$	$n = 2$	$n = 2$	$n = 6$	$n = 2$	P-value*
	µg kg^{-1}	µg kg^{-1}	µg kg^{-1}	µg kg^{-1}	µg kg^{-1}	
V	5.54 ± 4.41	10.20 ± 10.57	2.54 ± 1.69	12.4 ± 16.3	0.67 ± 0.05	0.756
Cr	14.71 ± 2.99^{bc}	37.89 ± 6.28^{ab}	12.56 ± 2.63^{bc}	7.6 ± 2.1^{c}	56.36 ± 29.12^{a}	0.002
Ni	16.52 ± 2.4^{a}	9.29 ± 0.06^{b}	8.92 ± 0.8^{b}	2.01 ± 0.6^{c}	10.75 ± 1.07^{b}	<0.001
Ag	0.24 ± 0.1	0.35 ± 0.49	0.13 ± 0.03	0.19 ± 0.1	0.51 ± 0.02	0.316
Cd	0.19 ± 0.01^{b}	0.13 ± 0.03^{b}	0.12 ± 0.05^{b}	0.17 ± 0.09^{b}	0.58 ± 0.05^{a}	<0.001
Pb	2.42 ± 0.67^{ab}	2.98 ± 0.17^{a}	3.50 ± 0.37^{a}	0.81 ± 0.5^{c}	1.20 ± 0.2^{bc}	<0.001
Hg	39.78 ± 1.74	58.05 ± 2.29	70.77 ± 10.69	112.9 ± 54.1	174.37 ± 169	0.350
	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	
As	19.87 ± 7.05	105.3 ± 65.6	3.51 ± 0.63	58.9 ± 28.3	29.94 ± 9.33	0.051
Σ toxic elements	19.95 ± 7.1	105.4 ± 65.6	3.60 ± 0.6	59.05 ± 28.4	30.18 ± 9.5	

n = pooled samples constituting of multiple individuals per sample. Results presented as mean values ± SD. ☐Results extracted from a previous study by Kendler et al. (21). *ANOVA was applied to detect differences in trace elements and PCBs; where significant difference was detected ($\alpha < 0.05$), a Tukey HSD *post hoc* test was applied. Values with different superscript ($^{a, b}$) within a row are significantly different ($P < 0.05$).

magnesium contribution, where a 200 g portion of plaice covers 10% of the DRV and megrim contributes up to 16% of the daily recommended intake.

3.5. Contaminants

Being demersal fish species, *Pleuronoectiformes* and *Rajiformes* are more likely to accumulate PCBs and hazardous trace elements than other fish species (13). Therefore, when frequently eating flatfish or ray, the matter of food safety must be considered. However, both intrinsic and environmental variables have a role in the bioaccumulation of hazardous as well as beneficial compounds (31). Individuals different in sizes and sexes, exhibit different concentrations in trace elements and contaminants, due to a variety in habitat and migration behaviors. In this study several toxic trace elements were determined (Table 6). The analysis on PCBs, including both non-dioxin and dioxin-like PCB congeners, show values lower than the detection limit (LOD) for flounder, lemon sole, megrim, and thornback ray. Hence no significant accumulation of any of the investigated PCB congeners were detected in those four species. Concerning plaice, traces of PCB 3, 52, 101, 118, 138, 153, and 180 were detected, as previously reported in the study of Kendler et al. (21) that looked into seasonal differences. A reason for the lower PCB values compared to plaice could be the lower fat content as well as overall lower fish size in the three other flatfish and the thornback ray. With regards to toxic trace elements, significantly different values between the species were detected for the elements chromium ($p = 0.002$), nickel ($p < 0.001$), cadmium ($p < 0.001$) and lead ($p < 0.001$). The highest accumulation of chromium and cadmium were found in thornback ray with 56.36 ± 29.12 µg kg^{-1} and 0.58 ± 0.05 µg kg^{-1} respectively. Large varieties within species can be seen for all elements, with particular great SD in the mercury content of plaice (112.9 ± 54.1 µg kg^{-1}) and thornback ray (174.37 ± 169 µg kg^{-1}). Despite differences between species as well as individuals, the maximum levels of cadmium (0.1 mg kg^{-1}), lead (0.3 mg kg^{-1}) and mercury (0.5 mg kg^{-1}) set by the EC (61) are not exceeded. When considering arsenic as potential hazardous component, organic and inorganic arsenic must be differentiated. Inorganic arsenic is the toxic form and according to Sloth et al. (62) a maximum of 1% of total arsenic in marine species is found in the form of hazardous arsenite and arsenate. The calculations in the previous study of Kendler et al. (21) on arsenic content in European plaice were followed for the other four species in this study. Considering the suggestion of 1% inorganic arsenic (58), it is safe to consume the recommended portion size by the Norwegian Food Authority (49) of 200 g of each of the five investigated species.

4. Conclusion

This study highlighted the nutritional composition of flounder, lemon sole, megrim, plaice, and thornback ray. The distribution and contribution of DIAA and the two main n3 fatty acids EPA and DHA show remarkable nutritional quality in all five species. A 200 g fillet portion of each of the five species covers the total DIAA and the recommended average daily intake of n3 fatty acids for an adult person. The nutritional score, emphasizing on DIAA and n3-fatty acids, can be regarded as profitable with good overall quality of all five fish. This study emphasized on the benefits of consuming these five species, mainly in the form of n3-fatty acids, DIAA, and essential minerals, but also investigated potential hazardous components. Potential risk factors in the form of PCBs and toxic trace elements were analyzed and have shown only minor bioaccumulation of single elements below the suggested upper intake limits. In conclusion, our study provides important insights into the nutritional profile of five underutilized fish species in Norway. However, it is important to note that there were some limitations in our study, including an incomplete TAA profile analysis, by not covering tryptophan and cysteine, as well as a potential overestimation of the protein content due to the chosen conversion factor. To further improve the understanding of the amino acid composition and total protein content, future studies should include cysteine analysis and focus on evaluating species-specific

conversion factors Future work should also put a stronger focus on assessing the risks and benefits of these fish that come with increased consumption. This is necessary to promote a safe consumption and integrate these fish, which have not yet been considered commercially in Norway, into the diet.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SK: conceptualization, methodology, formal analysis, investigation, and writing – original draft. FT: investigation and writing – original draft. AJ: conceptualization, methodology, and supervision. JL: conceptualization, methodology, supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Paper IV

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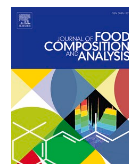

Risk-benefit assessment of five underutilized fish species in Norway

Sophie Kendler[a,*], Sara Monteiro Pires[b], Anita Nordeng Jakobsen[a], Jørgen Lerfall[a]

[a] Department of Biotechnology and Food Science, Norwegian University of Science and Technology (NTNU), NO-7491 Trondheim, Norway
[b] National Food Institute, Technical University of Denmark (DTU), Lyngby, Denmark

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ABSTRACT

Risks and benefits of increasing the consumption of five underutilized demersal fish species in Norway by applying the Benefit-Risk Analysis for Foods (BRAFO)-tiered approach were assessed. A reference scenario with zero intake was opposed to two different alternative scenarios of 250 (AS1) and 450 g (AS2) per week. Health-benefit vs. health-risk calculations were computed. Moreover, tolerable weekly intake and recommended weekly intake were considered for the general public and women of childbearing age. In addition, the molar ratio of Selenium and Mercury (Se:Hg) and the Health Benefit Value of Selenium (HBV_{Se}) were calculated and considered. Results suggest that a consumption of 250 g, when combined with a weekly portion of fatty fish, is the optimal intake scenario for adequate polyunsaturated fatty acids. Flounder and megrim feature the significantly highest eicosapentaenoic+doxosahexaenoic acid values with 678 and 606 mg in AS1. A surplus of selenium was detected in all five species, with flounder and lemon sole showing significantly highest Se:Hg (21; 22). Moreover, no detrimental effects were found due to an increased contaminant intake among those eating fish. Consequently, results revealed a net beneficial health effect by increasing the consumption of the five underutilized fish species. Thus, their consumption can be recommended.

1. Introduction

The health effects of fish and seafood are widely known and appreciated, and the main attributed beneficial effects are due to high amounts of proteins and long-chain polyunsaturated fatty acids (LC-PUFAs). In fact, fish and seafood contain satisfying amounts of important LC-PUFAs that promote physiological, molecular as well as cellular processes (Calder, 2014). Nevertheless, when consuming fish, possible exposure to environmental and chemical contaminants must be considered. Methylmercury (MeHg) is the primary form of mercury found in foodstuffs and due to its high toxicity, regulations for the maximum concentration have been established. The European Commission set a maximum level for fish and fishery products of 0.5 mg/ kg (wet weight) (EC, 2006). Moreover, the European Food Safety Authority (EFSA) Scientific Panel on Contaminants in the Food Chain (CONTAM) prompted a tolerable weekly intake (TWI) for MeHg of 1.3 µg/ kg body weight (b.w.) (EFSA, 2012a). Seafood is the primary source of human MeHg intake and is known to be neurotoxic and to cause oxidative stress, due to its interactions with sidechains of proteins and non-proteins (Farina et al., 2011). The antagonistic relationship between MeHg and Selenium (Se) has been recognized previously, but it is not yet completely certain which metabolic pathways are predominantly responsible for the high toxicity of MeHg (Khan and Wang, 2009). On the other hand, a constant supply of Se is required to synthesize vital selenoenzymes, which are essential for shielding the brain tissues from oxidative stress. Therefore, an adequate intake (AI) of 70 µg/ day was established by EFSA (2014) Moreover, fish can also be a source of dioxins and polychlorinated biphenyls and need to be considered when consuming fish, as contaminants can lower the beneficial effects from LC-PUFAs amongst others (Sofoulaki et al., 2019) Therefore, risk-benefits assessments (RBA) can be applied to assess the net beneficial vs. detrimental health effects of consuming foodstuffs. RBAs are valuable tools for authorities and governments to give guidelines on safe consumption of certain foodstuffs. As reviewed by Thomsen et al. (2021) RBA is a useful tool to assess the health impact of consumption patterns of fish and can help promoting the consumption of certain fish species. RBAs are heterogeneous in its nature, depending on if they are concluded for specific fish, consumer groups, in between different species or for specific countries. However, what they all have in common is the identification and comparison of beneficial and detrimental components and their consequences on human health. According to Thomsen et al. (2021) the minority of RBAs is conducted on fish consumption

* Corresponding author.
E-mail address: Sophie.kendler@ntnu.no (S. Kendler).

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of a specific country whereas a majority of RBAs compares the risk-benefit of consuming different fish species or seafood products. Moreover, a majority of RBAs on fish and seafood focusses on eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and Se as beneficial components opposing them to MeHg, dioxins and dioxin-like polychlorinated biphenyls (dl-PCBs) (Afonso et al., 2013; Prato et al., 2019; Reyes et al., 2017; Sofoulaki et al., 2019; Strandberg et al., 2016).

Fish are an important food source for the Norwegian population, marked by a yearly per capita consumption of 31.5 kg of whole fish (round weight) and 13.3 kg of fillets (Norwegian Directorate of Health, 2022). However, Norwegian waters, being the second longest coastline worldwide, inhabit over 220 fish species, including species being so far underutilized and of minor commercial interest (Directorate of Fisheries, 2022). In particular, the five species considered in this study, European plaice (*Pleuronectes platessa*), megrim (*Lepidorhombus whiffiagonis*), European flounder (*Platichthys flesus*), lemon sole (*Microstomus kitt*), and thornback ray (*Raja clavata*), can be categorized as underutilized in Norway as partly described by Bjørklund, Henriksen. (2011). The European Commission (2020) announced that stocks of e.g. European plaice are moving further north and will possibly decline in the Northern sea, suggesting increasing stocks in the Norwegian sea. Since all five species are bottom-living fish and inhabit the same regions, these migration trends will be relevant for local fishermen in Norway. The previous study by Kendler et al. (2023) featured the favorable chemical and nutritional composition of the five demersal fish species, and also elaborates on micronutrient and contaminant levels. Moreover, Kendler et al. (2023) studied the seasonal effect on the chemical composition and contaminants of European plaice and Tsoukalas et al. (2022) looked into different packaging and storage conditions on the microbial and physiochemical quality of European plaice. However, no direct comparison between beneficial and health risk factors have been conducted up to date. To promote the consumption of these five underutilized fish species, it is important to consider both the potential health effects and risks that come with increased consumption. To our knowledge, no risk-benefit assessment on the consumption of those five species has been carried out. A recent report conducted by the Norwegian Scientific Committee for Food and Environment (VKM) as well as previous studies from VKM, focus on the risk assessment of the main consumed fish species in Norway, with limited and more general data on, e.g. European flounder and European plaice as no consumer data of these fish is available (Norwegian Scientific Committee for Food and Environment et al., 2022).

To our knowledge, no data on the specific consumption of the five species of interest has been collected in Norway up to date. Therefore, this study aimed to assess the net health effect of increased consumption of European plaice, European flounder, megrim, lemon sole and thornback ray originating from the west-coast of Norway. The objective was to establish recommendations for consuming these five underutilized fish species following the Benefit-Risk Analysis for Foods (BRAFO)-tiered approach.

2. Materials & methods

2.1. Scope of RBA study

The scope of this risk-benefit assessment lies purely on the five mentioned underutilized species, originating from the Norwegian west coast: European plaice, European flounder, megrim, lemon sole and thornback ray. The safety of different possible patterns of consumption has been evaluated, which can be reflected in recommendations for consumption for the Norwegian population of these fish. As this RBA focuses on fish originating from coastal waters on the west coast of Norway as the fishing region and the Norwegian adult population as the target group, existing data on species originating from, e.g. the Mediterranean sea or Northern sea were not considered in the assessment.

The focus of the study was on the general public and women of childbearing age for all intake calculations and the assessment of risks and benefits. Pregnant women were considered for calculations on possible IQ changes of infants in AS1 and AS2, but children below 18 years or elderly people above the age of 70 were not considered in this risk-benefit assessment.

2.2. Fish species and sampling

The fish species that were included in this RBA are five underutilized fish species in Norway, four flatfish (*Pleuronoectiformes*) and one belonging to the family of rays (*Rajiformes*). More precisely, individuals of European plaice (*Pleuronectes platessa*), flounder (*Platichthys flesus*), lemon sole (*Microstomus kitt*), megrim (*Lepidorhombus whiffiagonis*), and thornback ray (*Raja clavata*), were investigated. The sampling of the fish from two previous studies by Kendler et al. (2023) and Kendler et al. (2023) took place in autumn and winter 2020 as well as spring 2021 in the fishing area 2.a.2 as defined by (FAO, 1990–2021) along the west-coast of Norway. The sample size for each species was as following: n(flounder)= 7, n(lemon sole)= 5, n(megrim)= 5, n(plaice)= 10, n (thornback ray)= 5. During handling and processing of samples, the fish was constantly kept on ice and subsequently frozen at − 80 °C until further analysis. Due to its morphology, two sampling points for the four flatfish species (n= 2 ×2) and one sampling point for thornback ray were chosen, as visualized in the study by Kendler et al. (2023).

2.3. Sample analysis: consideration of results from previous studies

Kendler and co-authors have previously published results on nutritional composition, chemical and environmental contaminants as well as storage stability under different conditions on the five species of interest. Detailed information on the analyses of fatty acid distribution as well as essential trace elements and chemical and microbiological contaminants, can be found in the respective studies of Kendler et al. (2023); Kendler et al. (2023) and Tsoukalas et al. (2022).

For this study, values from e.g. fatty acids, selenium or mercury were re-calculated to be suitable for assessing benefits and risks (Section 2.5). Moreover, a literature review on contaminant and nutritional data of the five species of interest was carried out. The Marine Research Institute, Norway, obtained values of multiple contaminants in European plaice in a report on “Contaminants in plaice, anglerfish and pollack” (Frantzen et al., 2020). Those values were considered in the discussion part of the RBA.

2.4. Risk-benefit assessment methodology and approach

The BRAFO-tiered approach evaluates risks and benefits in a five-step process as previously presented by Boobis et al. (2013) and Hoekstra et al. (2012), and summarized by Nauta et al. (2020). This study attempted to follow this five-step assessment approach in a consecutive matter on the five species of interest, starting with the pre-assessment and problem formulation (step 1), which defines suitable intakes as a reference and alternative scenarios. Followed by tiers 1 and 2, containing the evaluation of the risk-benefit question (RBQ), including the individual assessment of risks and benefits (tier 1, step 2). If no benefits are detected, the consumption of the reference scenario can be advised. In contrast, the alternative intake scenario can be suggested if no additional risks go along with the respective alternative scenario. In both cases, the RBA can be stopped, and no further evaluation is necessary. In step 3 (tier 2), a quantitative integration of risks and benefits is carried out. In this step, the reference scenario is opposed to the newly proposed alternative intake scenarios. Here, either the reference or alternative scenario can be suggested if the benefits/ risks prevail over each other. In tier 3 (step 4), a quantitative comparison of risks and benefits is carried out by applying a deterministic computation with a common health metric (Hoekstra et al., 2012). This usually results in calculating Disability-adjusted life years values (DALYs) or Quality-adjusted life

years (QALYs). The computation of DALYs or QALYs is only possible if sufficient consumption data or epidemiological data on the consuming population is available on the species of interest. As previously stated in the aims of the study, to our knowledge there is no intake data of the five species of interest available in Norway. That is why no exposure/ intake data of identified beneficial and detrimental components could be obtained from public studies. Consequently, this RBA was conducted with a qualitative approach, using analytical results from previous studies as a base on the intake of the specific nutrients/ contaminants. Furthermore, with no available consumption data of the five underutilized fish species, the study will follow the BRAFO-tiered approach from the pre-assessment and problem formulation, tier 1 until tier 2.

2.5. Pre-assessment and problem formulation

The Norwegian Directorate of Health (2011) recommends adult fish consumption of 300–450 g per week, of which 200 g should be obtained from fatty fish. In a recently published comprehensive general report on the assessment of benefits and risks of fish in the Norwegian diet from the Norwegian Scientific Committee for Food and Environment et al. (2022), alternative scenarios with 300 as well as 450 g fish intake were chosen. The study of the Norwegian Scientific Committee for Food and Environment included the general intake of fatty and lean fish, rather than recommendations for specific fish species, for the Norwegian public.

Therefore, a theoretical reference scenario (RS) with zero intake of the five species was selected and followed in the assessment. As alternative scenario 1 (AS1), a weekly intake of 250 g of the five fish species of interest was chosen. Together with the suggested intake of 200 g fatty fish by the Norwegian Directorate of Health (2011), AS1 would lead to a total of 450 g fish per week, suggesting that the 250 g is covered by one of the five species as a source for lean fish. As alternative scenario 2 (AS2), a weekly intake of 450 g for the five lean fish species was chosen to assess whether the consumption of 450 g consisting of at least one of the five fish species is reasonable in terms of benefits and risks connected to its consumption, or not. Moreover, whether AS2 is sufficient in providing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), usually associated with the intake of fatty fish. In this study, the intake of fatty fish was not assessed, but it was expected that 200 g fatty fish are consumed additionally to the 250 g as proposed in AS1. Moreover, the focus of the assessment lies purely on the possible risks and benefits of an increased consumption (AS1 vs. AS2) of the five lean fish and does not include any potential risks/ benefits through additional fish consumption.

This leads to the following three scenarios that were compared in this study:

1. Reference scenario (RS): no consumption of the five species, which refers to the current intake, assuming no frequent consumption of these fish. The current weekly intake data assessed from the Norwegian Scientific Committee for Food and Environment et al. (2022) was used.
2. Scenario 1 (AS1): consumption of 250 g fish/ per week, assuming that the suggested consumption of 250 g lean fish per week by the Norwegian Directorate of Health (2011) is fulfilled by consuming the five species in the study. Additionally, the Norwegian Directorate of Health (2011) suggests the intake of 200 g fatty fish. The calculations for AS1 are conducted for the 250 g portion of lean fish.
3. Scenario 2 (AS2): consumption of 450 g fish/ per week, assuming the total recommended weekly fish intake from the Norwegian Directorate of Health (2011) is fulfilled by consumption of the five species in the study.

In the current risk-benefit assessment, the positive health impacts of fish consumption are compared to the adverse health effects of consumption of the five species. Hereby, benefit is defined as a decreased likelihood of adverse health effects associated with eating the fish or ingesting fish-related substances like nutrients. Whereas risk is defined as an increased likelihood of adverse health effects associated with fish consumption or ingesting fish-related substances like contaminants (Norwegian Scientific Committee for Food and Environment et al., 2022, p. p. 856).

2.6. Calculations related to potential risks and benefits

2.6.1. Selenium:Methylmercury ratio (Se:Hg) and Health Benefit Value of Selenium (HBV_{Se})

Previous studies have shown that approximately 90% of the total mercury (Hg) present in seafood occurs in the form of methylmercury (MeHg) (Afonso et al., 2019; Barone et al., 2021; EC, 2006). Based on this and the risk-benefit comparison approach of the Food and Agricultural Organization of the United Nations and World Health Organization (FAO/WHO, 2011) on fish, TWI calculations were based on the total content of Hg assuming 100% of Hg to be in the form of MeHg, to be certain of not exceeding the TWI of MeHg.

The molar ratio (Se:Hg in μmol/ g) was computed by dividing the concentrations of Selenium (Se) and Hg by their corresponding molecular weights, being 78.96 for Se and 200.59 for Hg (Barone et al., 2021; Ralston et al., 2016). Moreover, the HBV_{Se} was calculated by applying the established equation of Ralston et al. (2016) as shown in Eq. (1):

$$HBV_{Se} = \left(\frac{[Se - Hg]}{Se}\right) \times (Se + Hg) \tag{1}$$

The HBV_{Se} demonstrates whether consuming the fish will elevate (positive values) or degrade (negative values) the existing Se level. Moreover, it depends on how high the HBV_{Se} is, as this reflects the relative Se surplus or deficit brought about by consuming the fish (Ralston et al., 2016). The HBV_{Se} helps understanding the net benefit of Se coming with consumption of foodstuffs that also contain Hg, and that, moreover, possibly mitigate the toxic impact of MeHg in the body (Farina et al., 2011).

2.6.2. Health-benefit vs. health-risk related factors

In the Joint FAO/ WHO Expert Committee report about the risks and benefits of fish consumption, a framework for assessing the net health benefits/ risks of fish consumption was established, and in context, health-benefit vs. health-risk calculations were determined (FAO/WHO, 2011). Following the guidance of the FAO/ WHO, the data this RBA refers to, was used to assess the deaths prevented per million people (Eq. (2)) due to sufficient EPA+DHA intake, cancer deaths caused per million people (Eq. (3)) due to dioxin/DL-PCB intake as well as the IQ gain (Eq. (4)) and IQ loss (Eq. (5)), due to elevated intake of MeHg vs. the intake of DHA, effecting the neurodevelopment of infants. AS1 (250 g) and AS2 (450 g) were considered in the calculations.

$$\frac{Deaths \quad prevented}{million \quad people} = \frac{[EPA + DHA] \times 100 \times \frac{x}{7}}{250} \times 0.36 \times D \tag{2}$$

Where;.

- [EPA + DHA] is the total concentration of EPA plus DHA in fish (mg/ g); applies also to Eq. (3)
- 100 is the estimated fish serving size (g); applies also to Eqs. (2)–(5)
- x is the number of servings of fish per week (7 days); applies also to Eqs. (2)–(5)
- 0.36 is the proportional reduction in coronary heart disease deaths, with reduction in deaths assumed to be linearly related to DHA intake up to 250 mg/ day;
- D is the estimated number of coronary heart disease deaths per million people (1580 deaths per year per million people, calculated over 70 years).

$$\frac{Cancer \quad deaths \quad caused}{million \quad people} = \frac{[Dioxins] \times 100 \times \frac{x}{7}}{60} \times 1 \times 10^{-3} \times 10^{6} \tag{3}$$

Where;.

- [Dioxins] is the concentration of dioxins in fish (pg TEQ/ g); toxic equivalence (TEQ). The TEQ is calculated by multiplying the actual concentration with the toxic equivalence factor (TEF) of 3 * 10^{-5} as previously proposed by the World Health Organization (WHO) and reviewed by Van den Berg et al. (2006).
- 60 is the estimated body weight (kg) of a female person

$$IQ \quad points \quad gained = [EPA + DHA] \times 100 \times X \times \frac{x}{7} \times 0.04 \tag{4}$$

Where;.

- X: FAO/WHO (2011) used 0.67 as a factor to estimate the DHA concentration from [EPA + DHA]; here, specific factors were calculated for each fish species relatively, being 0.64 (Flounder), 0.50 (Lemon sole), 0.79 (Megrim), 0.90 (Thornback ray), 0.66 (Plaice)
- x is the number of servings of fish per week
- 0.04 is the coefficient relating IQ points gained to milligrams of DHA intake per day.

$$IQ \quad points \quad lossed = \frac{[MeHg] \times 100 \times \frac{x}{7}}{60} \times 9.3 \times (-0.18 or - 0.7) \tag{5}$$

Where;.

- [MeHg] is the concentration of methylmercury in fish (μg/ g); calculations were based on the total content of Hg assuming 100% of Hg to be in the form of MeHg
- 60 is the estimated maternal body weight (kg);
- 9.3 is the correlation between maternal MeHg intake and maternal hair mercury level;
- 0.18 is the central estimate of IQ points gained per microgram per gram hair mercury gained; and − 0.7 is the upper-bound estimate of IQ points gained per microgram per gram hair mercury gained. In the RBA, the upper-bound estimate of− 0.7 was applied in the calculations.

2.6.3. Fatty acids content

Eq. (6) was used to re-calculate the weight% of fatty acid methyl esters (FAME) of the studied species to obtain values in g FA/ g fillet wet weight (ww). Weihrauch et al. (1977) has previously conducted detailed research on establishing lipid conversion factors in different fish species, given as fatty acid conversion factor (FACF) in Eq. (7).

$$\frac{g \quad fatty \quad acid}{100g \quad fillet} = weight\%FAME \times FACF \times TLC \tag{6}$$

Where;.

- %FAME: results obtained from FAME analysis, presuming the same as weight%-FA since marine lipids primarily entail PUFA. The results from the established FAME procedure (Lerfall et al., 2016; Metcalfe et al., 1966) carried out by Kendler, Thornes, et al. (2023) and Kendler et al. (2023) were used as basis for calculation.
- FACF: fatty acid conversion factor expressed in g FA/ g lipid, applying the FACF (Saavedra et al., 2017; Weihrauch et al., 1977) and shown in Eq. 7.

$$FACF = \frac{0.933 - 0.143}{TLC} \tag{7}$$

Where;.

- TLC: total lipid content in g lipid/ g fillet ww (Kendler et al., 2023; Kendler et al., 2023)

2.7. Characterization of nutrients and contaminants

To define the intake of nutrients and contaminants, dietary values and health-based guidance values are employed in this study. These values are the average requirement (AR), upper limit (UL) or adequate intake (AI) for nutrients and tolerable weekly intake (TWI) for contaminants, as previously employed by the Norwegian Scientific Committee for Food and Environment et al. (2022) in a comprehensive report on fish consumption. Hereby, the scientific opinions from the European Food Safety Authority (EFSA) on specific intake values are considered for calculations of identified risks and benefits.

2.8. Statistics

For statistical analyses, the software IBM SPSS (release 28, IBM Corporation, USA) was applied. Analysis of variance (ANOVA) was carried out between the five different species, and when significant difference detected ($p < 0.05$), a Tukey HSD post hoc test was performed to investigate the differences between groups. An α-level of 0.05 was used.

3. Results

3.1. RBA assessment: BRAFO-tiered approach

3.1.1. Individual assessment of risks and benefits (Tier 1): Identification of positive health effects and hazards

Previous RBAs focusing on seafood intake identified eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), methylmercury (MeHg) as well as dioxins and dioxin-like polychlorinated bisphenyls (PCBs) as main benefit-risk components as reviewed by Thomsen et al. (2021). Based on this and the previously obtained results by Kendler et al. (2023) and Kendler et al. (2023) on contaminant levels and nutritional composition of the five fish species of interest, the following risks and benefits, as illustrated in Fig. 1, were identified for the five species.

More precisely, the concentrations of relevant components were used as a basis to assess the benefits and risks that come along with the consumption of the five fish and can be seen given per 250 g (AS1) and 450 g (AS2) in Table 2. For this, health-benefit-related calculations were executed and used as a basis for the assessment. Table 1 shows the chosen beneficial and hazardous components for this RBA, including their potential positive or negative health effects as well as suggested intakes.

3.1.2. Qualitative integration of risks and benefits (Tier 2)

The two alternative intake scenarios were opposed to the RS of zero intake and health-benefit/-risk-related factors were considered for the assessment, as previously established by FAO/WHO (2011) and Ralston et al. (2016) and can be seen in Table 2. Moreover, Table 2 shows the increase of EPA+DHA from AS1 to AS2. In AS1, the EPA+DHA contribution of the individual species ranged from 23.0% (Lemon sole) to 38.8% (Flounder) of the total RWI of EPA+DHA. Since the RS is at zero intake of the fish, health-benefit/-risk calculations on the RS are not compelling and not included in the tables. Moreover, it should be mentioned that all fish contain other LC-PUFA such as docosapentaenoic acid (DPA), being not included in RWI calculations, as no RWI suggestions are available up to date.

The increased intake of EPA+DHA and its effect on neurodevelopment in unborn infants (due to intake of the mother) is expressed as IQ points gained in Table 3 and opposed to IQ points lost due to MeHg intake. The net IQ points gained could be significantly increased from AS1 to AS2, being on average two points higher in AS2. The most significant effect on net IQ points gained was observed in megrim,

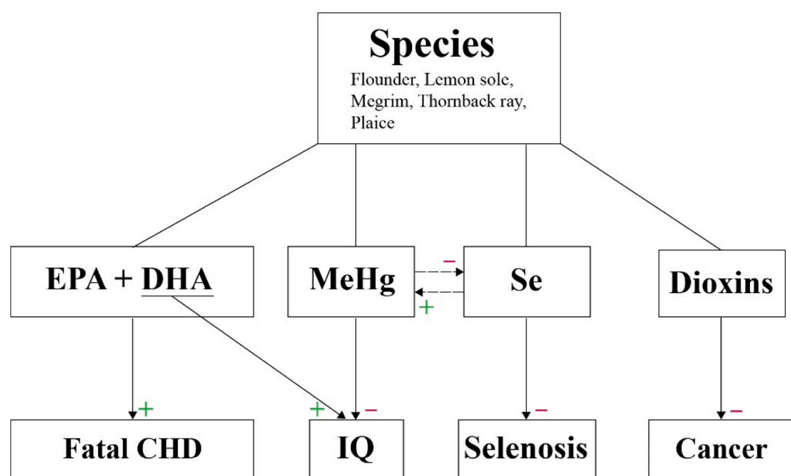

Fig. 1. Identified beneficial and hazardous components in the fish species of interest, as well as their respective health effects (+ signalling positive effects; - signalling adverse health effects); eicosapentaenoic acid (EPA); docosahexaenoic acid (DHA); methylmercury (MeHg); selenium (Se); coronary heart disease (CHD); intelligence quotient (IQ).

Table 2
concentrations of EPA+DHA (mg/ AS1; mg/ AS2) including average% of recommended weekly intake (RWI); concentrations for DPA (mg/ AS1; mg/ AS2); concentrations of Se (µg/ AS1; µg/ AS2) and% of TWI; concentrations of MeHg (µg/ AS1; µg/ AS2) as well as% of tolerable weekly intake (TWI) shown for the fillets of flounder, lemon sole, megrim, thornback ray and plaice.

Compound	Flounder	Lemon sole	Megrim	Thornback ray	Plaice	p-value[1]
EPA+DHA						
mg/ 250 g (AS1)	679.6 ± 56.8[a]	403.3 ± 45.0[c]	606.4 ± 30.2[a, b]	569.7 ± 13.1[b]	424.5 ± 56.4[c]	< 0.001
% of RWI	38.8 ± 3.3	23.0 ± 2.6	34.7 ± 1.7	32.6 ± 0.8	24.3 ± 3.2	
mg/ 450 g (AS2)	1223.2 ± 102.3[a]	725.9 ± 81.[c]	1091.5 ± 54.4[a, b]	1025.5 ± 23.6[b]	764.2 ± 101.5[c]	< 0.001
% of RWI	69.9 ± 5.8	41.5 ± 4.6	62.4 ± 3.1	58.6 ± 1.3	43.7 ± 5.8	
DPA						
mg/ 250 g (AS1)	38.37 ± 7.6	33.85 ± 45.0	44.49 ± 5.1	44.21 ± 14.7	66.47 ± 108.5	p = 0.942
mg/ 450 g (AS2)	69.06 ± 13.7	60.93 ± 81.0	80.08 ± 9.2	79.57 ± 108.5	119.6 ± 195.3	
Se						
µg/ 250 g (AS1)	88.41 ± 4.1[a, b]	121.45 ± 1.7[a]	99.61 ± 4.7[a, b]	64.98 ± 0.3[b]	99.99 ± 29.2[a]	< 0.009
% of RWI	18.0 ± 0.8	24.8 ± 0.4	20.3 ± 1.0	13.3 ± 0.1	20.4 ± 6.0	
µg/ 450 g (AS2)	159.14 ± 7.3[a, b]	218.61 ± 3.1[a]	179.30 ± 8.5[a, b]	116.96 ± 0.6[b]	179.99 ± 52.6[b]	< 0.009
% of RWI	32.5 ± 1.5	44.6 ± 0.6	36.6 ± 1.7	23.9 ± 0.1	36.7 ± 10.7	
MeHg*						
µg/ 250 g (AS1)	9.43 ± 0.4	14.5 ± 0.6	17.7 ± 2.7	43.6 ± 42.3	28.24 ± 13.5	0.178
% of TWI	12.7 ± <0.01	18.6 ± <0.01	22.7 ± <0.01	55.9 ± 0.05	36.2 ± 0.02	
µg/ 450 g (AS2)	17.9 ± 0.8	26.1 ± 1.0	31.8 ± 4.8	78.5 ± 76.1	50.83 ± 24.3	0.178
% of TWI	22.9 ± <0.01	33.5 ± <0.01	40.8 ± 0.01	100.6 ± 0.10	65.2 ± 0.03	

*MeHg is expressed as Hg, calculations made by presuming 100% of Hg is MeHg
RWI's were calculated as AI x 7
[1] ANOVA was applied to detect differences in EPA+DHA, EPA, Se and MeHg concentrations between species respectively; where significant difference was detected ($\alpha < 0.05$), a Tukey post hoc test was applied. Values with different superscript letters within a row are significantly different ($p < 0.05$)

increasing from 2.45 in AS1 to 4.41 in AS2The EFSA Scientific Panel on Contaminants in the Food Chain (CONTAM) sets a TWI of MeHg of 1.3 µg/kg body weight (b.w.) (EFSA, 2012a). Considering this suggested TWI, values respective to the alternative intake scenarios, for a person with 60 kg b.w. were calculated (Table 2). Moreover, AI's for Se intake of 70 µg/day was applied, as suggested by the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) and thereof TWI's (AI x 7) were computed (EFSA, 2014). The TWI of MeHg is not exceeded in AS1, showing acceptable values for flounder, lemon sole, megrim and plaice. In contrast, a 250 g fillet of thornback ray contributes to approximately 55.9% of the suggested TWI of MeHg. Considering the intake increase in AS2, values of thornback ray exceed the TWI (100.6%) but are within the TWI for the four other fish.

The Se:Hg molar ratio and HBV_{Se} can be used to assess whether the available Se concentration exceeds the Hg concentration and if Se moderates and even counteracts the toxicity of Hg (hence MeHg) (Barone et al., 2021). Fig. 2 visualizes the molar ratio of Se:Hg as well as the HBV_{Se} of the five fish of interest. The highest HBV_{Se} was computed for lemon sole, and showed the second highest Se:Hg after flounder. In addition, positive HBV_{Se} were calculated for all five species and the net Se concentration predominates in all species. However, no significant difference ($p = 0.167$) of the HBV_{Se} between the five species was found. All five fish show positive Se:Hg, indicating a surplus of Se over Hg. Significant differences ($p < 0.001$) were found between the five species, with flounder and lemon sole, showing significantly higher values compared to megrim, thornback ray and plaice.

The collected data on 12 different PCB and dioxin-like (DL-) PCB congeners in the studies of Kendler et al. (2023) and Kendler et al.

Table 1
Identified beneficial and hazardous components, their health effect and suggested intake.

Component	Health effect	Suggested intake/ calculation
EPA+DHA	Health-benefiting effects of LC-PUFAs: IQ point gain Decreased mortality caused by cancer and CVD	RWI[1] of 1.75 g (250 mg per day) as proposed by EFSA (2012b)
Selenium	Antagonistic effect to methylmercury; important trace element	RWI of 490 μg (based on AI x 7); UL of 255 μg/ day are considered (EFSA, 2014; EFSA Panel on Nutrition et al., 2023) HBV_{Se} and molar ratio Se:Hg
Methylmercury	Adverse-health effects of MeHg: IQ point loss due to neurotoxicity	TWI of 1.3 μg/ kg body weight (EFSA, 2012a) Molar ratio Se:Hg
Dioxins+DL-PCBs	Adverse-health effects of dioxins + DL-PCBs Increased mortality	TEQ of specific congeners

[1] Abbreviations: recommended weekly intake (RWI); tolerable weekly intake (TWI); cardiovascular diseases (CVD); health benefit value of selenium (HBV_{Se}); toxic equivalent (TEQ); intelligence quotient (IQ)

(2023) is insufficient to be included in an RBA, with only determining DL-PCB 118. DL-PCBs and dioxins are causing main health issues in humans, when compared to non-dioxin like PCBs. DL-PCB 118 was detected in European plaice in the study of Kendler et al. (2023) but below the LOD in the other four species (Kendler et al., 2023), making further calculations not feasible for all five species. Therefore, the cancer deaths caused per million people were only computed for European plaice, considering the TEQ of DL-PCB 118 for the calculation. The results given in Table 4 visualize that an increasing intake of EPA+DHA in AS1 and AS2 leads to net prevention of deaths. The concentration of PCB-118 in plaice was relatively low, showing a minor impact on mortality, which can also be expected for the four other species due to values below LOD. Nevertheless, it must be mentioned, that other dioxins or DL-PCBs might be present in the five fish species, which were not considered in the previous studies conducted by Kendler et al. (2023) and Kendler et al. (2023). Hence, values on mortality must be regarded with caution, showing rather a trend than an absolute directive.

3.1.3. *Deterministic computation of common health metric (Tier 3)*

The health-benefit vs. health-risk calculations in Tables 2, 3 and 4 indicate that the net benefits from both alternative scenarios predominate compared to the net risks, considering the mortality and child IQ point gain as indicators for assessing the public and women of childbearing age in Norway. Furthermore, the HBV_{Se} and Se:Hg ratio show a surplus of Se as shown in Fig. 2, possibly alleviating the adverse effects of an increased MeHg intake due to an increase in AS1 and AS2. Results in the current study revealed that the net benefits of increasing the intake of all five fish species are higher than the net risks for the general population and women of childbearing age with regards to IQ calculations of children. Both alternative scenarios can be considered safe, with AS2 significantly improving the intake of EPA+DHA, contributing up to 69.9% to the RWI of LC-PUFAs. Considering the estimated health benefiting and adverse factors, the assessment can be stopped at Tier 2, making further computations in Tier 3 and 4 obsolete.

4. Discussion

The present study evaluated the risks and benefits of increasing the consumption of five underutilized demersal fish species in Norway using the BRAFO-tiered approach. Moreover, RBAs carried out by the Norwegian Scientific Committee for Food and Environment highlight the importance of a sufficient intake of fish for providing essential nutrients as EPA and DHA for the Norwegian population (Norwegian Scientific Committee for Food and Environment, 2014). The previous study of Kendler et al. (2023) on the nutritional profile of the five fish species investigated in the present RBA pointed out the beneficial nutritional composition of those species, describing them as important suppliers of LC-PUFAs. Afonso et al. (2013) reports that already one portion (160 g) of megrim (*Lepidorhombus whiffiagonis*) contributes significantly to reach the EPA+DHA recommendations, coming along with low risks due to minimal concentrations of toxic trace elements. Moreover, the present RBA identified that, although all five species are considered lean fish with low fat contents in the range of 0.75–1.55% (Kendler et al., 2023; Kendler, Tsoukalas, et al., 2023), their fatty acid composition should not be underestimated, as they significantly contribute to the daily requirements set by the European Food Safety Authority (EFSA, 2012b). We estimated that the increase of portion size from 250 g (AS1) to 450 g (AS2) would lead to an average < 20% increase of n3 (omega-3)

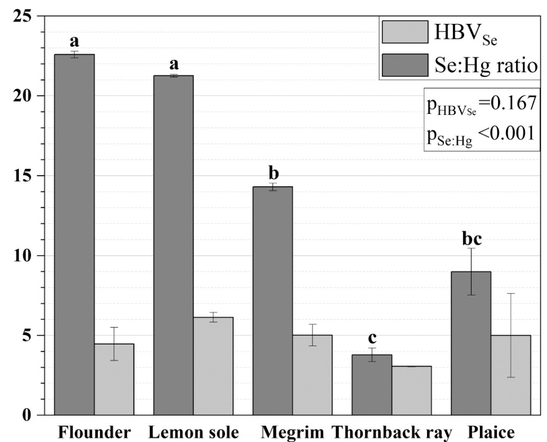

Fig. 2. Molar ratio of Se:Hg and Health Benefit Value (HBV_{Se}) of Se for the five species of interest. Error bars show SD. ANOVA was applied on species and HBV_{Se} and species and Se:Hg; where significant difference was detected ($\alpha < 0.05$), a Tukey HSD post hoc test was applied. Values with different letters (a, b) are significantly different ($p < 0.05$).

Table 3
Change of IQ Points in infants calculated from EPA+DHA intake vs. Methylmercury intake; Calculations made for the intake of for AS1 (250 g intake) and AS2 (450 g intake).

			EPA + DHA vs. Methylmercury				
			Flounder	Lemon sole	Megrim	Thornback ray	Plaice
Change of IQ Points	250 g (AS1)	IQ points gain (+)	+ 2.50	+ 1.14	+ 2.73	+ 2.93	+ 1.61
		IQ points loss (–)	– 0.15	– 0.22	– 0.27	– 0.68	– 0.22
		Net IQ gain	+ 2.34	+ 0.92	+ 2.45	+ 2.26	+ 1.38
	450 g (AS2)	IQ points gain (+)	+ 4.49	+ 2.06	+ 4.91	+ 5.28	+ 2.90
		IQ points loss (–)	– 0.28	– 0.40	– 0.49	– 1.22	– 0.79
		Net IQ gain	+ 4.22	+ 1.65	+ 4.41	+ 4.07	+ 2.11

Table 4
Change of mortality (deaths/million people) calculated from DL-PCB 118 intake. Calculations made for the intake for AS1 (250 g intake) and AS2 (450 g intake).

			EPA + DHA vs. Dioxins (PCB-118)*				
			Flounder	Lemon sole	Megrim	Thornback ray	Plaice
Change of mortality	250 g (AS1)	Prevented deaths (+)	+ 15461	+ 9176	+ 13797	+ 12963	+ 9659
		Caused deaths (–)	/	/	/	/	– 1.8
		Net prevented deaths	/	/	/	/	9657
	450 g (AS2)	Prevented deaths (+)	+ 27830	+ 16517	+ 24835	+ 23333	+ 17386
		Caused deaths (–)	/	/	/	/	– 3.3
		Net prevented deaths	/	/	/	/	17383

*values of PCB-118 are below the limit of detection (LOD) and hence not included in this RBA (Kendler et al., 2023).

long-chain polyunsaturated fatty acids. For example, when comparing these two scenarios for flounder, an increase in the contribution of EPA+DHA from 38.8% to 69.9% was identified. Nevertheless, AS2 is not sufficient in providing 100% of the daily required EPA+DHA concentrations, which is why substituting the intake of fatty fish with 250 g of any of the five investigated lean fish as suggested in AS1 should be considered. In spite of the lack of an RWI for DPA, research indicates that the evidence on health benefiting characteristics of DPA is increasing (Calder, 2014). DPA is the intermediate product of EPA and DHA, and is suggested to mediate similar functions in human metabolic processes (Kaur et al., 2011). This contributes to the health benefits of an increased intake of the five species through LC-PUFAs.

The MeHg values for the four flatfish species were found to be considerably below the TWI. However, when consuming thornback ray, higher exposure to MeHg must be considered in intake suggestions, with AS1 contributing to 55.9% and AS2 exceeding the TWI (100.6%) of MeHg. Elasmobranchs, such as thornback ray, are potentially more exposed to accumulation of pollutants and toxic trace elements due to their higher trophic level in the food chain as well as general slow reproductivity and maturity, similar to large mammals (Tiktak et al., 2020). However, health-benefiting properties can be linked to the consumption of all fish species with regards to Se. The present study estimated that the five fish's Se concentrations contribute on average with 20% to the RWI in AS1 and up to 44.6% in AS2. The upper limit for Se for adult people of 255 µg/day (EFSA Panel on Nutrition et al., 2023) was not exceeded in any scenario for any species. The beneficial Se concentrations can be opposed to the concentrations of toxic MeHg. The Se:Hg as well as HBV_{Se} were considered for the five fish species (Fig. 2). Se:Hg exceeding 1 indicate a protective effect of Se against the toxicity of Hg (Peterson et al., 2009). All five investigated fish have higher total Se concentrations than Hg, hence exceed a molar ratio of 1, with flounder showing the highest (Se:Hg > 22). Moreover, HBV_{Se}'s of three to six were found in the current study, indicating a surplus of Se and preventive effects opposing the MeHg exposure. In a comparable RBA considering Hg, MeHg and Se concentrations in elasmobranch meat, thornback ray showed the highest HBV_{Se} compared to other rays, skates and sharks with a value of about 6 (Storelli et al., 2022). This is in accordance to a positive HBV_{Se} found in the present study and indicates a positive antagonistic effect of Se on the MeHg toxicity. Azad et al. (2019) found a molar ratio (Se:Hg) of 23.2 and HBV_{Se} of 4.76 for plaice in a study from the Northeast Atlantic. Barone et al. (2021) found Se:Hg's of approximately three to four for three different rays as well as for turbot and Common sole. Moreover, HBV_{Se}'s of around 2–6.5 were found by Barone et al. (2021). In addition, child IQ gain/ loss as the common health metrics considering MeHg and EPA+DHA intake was considered. The MeHg intake showed almost no influence on IQ points due to low concentrations in all five fish species, suggesting low exposure when consuming these species. Moreover, a net gain in IQ points was identified in both AS1 and AS2 for all fish, due to a satisfying intake of EPA+DHA.

The European Commission (EC) has set maximum levels of 6.5 ng TEQ/ kg muscle meat of fish and fishery products to address the risks of unwanted intake of dioxins and DL-PCBs (EC, 2011). Frantzen et al. (2020) assessed multiple dioxins and DL-PCBs for European plaice in 54 pooled samples, containing 448 individuals and got an average concentration of 0.52 and median of 0.50 ng TEQ/ kg fillet ww of for all samples. Both the obtained values in the comprehensive report of Frantzen et al. (2020) and the values used in this RBA are significantly lower than the recommended values of the EC. Moreover, calculations on the mortality due to PCB and dioxin intake vs. EPA+DHA intake concluded a net prevention of death when consuming plaice, promoting the overall low values of contaminants. It can be suggested that an increased consumption of plaice as suggested in AS1 and AS2 can be regarded as safe with respect to the relative dioxin and DL-PCB concentrations. Furthermore, it can be argued that the consumption of the four other fish species can be assumed as safe, as they inhabit the same environments as European plaice, making a proximate evaluation of their expected contaminant concentrations and health effects possible. Due to a high lipophilicity PCBs and DL-PCBs tend to accumulate in the adipose tissue of fish (Zhang et al., 2012), whereas Hg and MeHg are supposed to accumulate in the muscle tissue, due to closely binding to thiol groups in (seleno)proteins (Bosch et al., 2016). The five fish species in the present study are regarded lean fish with fat contents below 2%, which speaks against the general likelihood of elevations of PCBs or dl-PCBs (Kendler, Thornes, et al., 2023; Kendler, Tsoukalas, et al., 2023). Zhang et al. (2012) reported higher PCB accumulation in tails, compared to dorsal and ventral muscle samples. In addition, Barbosa et al. (2018) found differences of toxic element accumulation between different muscle parts, reporting higher accumulation of Hg (among others) in the central muscle compared to edge parts of megrim (*Lepidorhombus whiffiagonis*), but being within the defined acceptable limit of 1 mg/ kg for *Lepidorhombus* species (EC, 2006). This is in accordance with results obtained for megrim (0.071 mg/ kg or 17.7 µg/ 250 g) in the present study.

The BRAFO-tiered approach is a well-established method to assess risks and benefits of foods and has been followed in multiple assessments (e.g. Gao et al. (2015); Hoekstra et al. (2013); Schütte et al. (2012); Watzl et al. (2012)). A RBA study on the consumption of marine species in the Chinese population carried out by Gao et al. (2015) applied similar health metrics established by FAO/WHO (2011), as the present study. The qualitative RBA of Gao et al. (2015) lead to similar results, where the alternative scenario led to clear net beneficial effects on the prevention of deaths and child IQ gain outweighed the exposure of dioxins and MeHg for fish consumed in China. In addition, the results in the present RBA stress that the BRAFO-tiered approach is a useful methodology to clearly weigh out risks and benefits of marine fish.

It is important to note that there were some limitations in our RBA study, including a fragmentary screening of toxins, excluding major toxic dioxins as well as the limited availability of consumer intake data which only allowed for a qualitative RBA approach. To conduct a quantitative RBA, a comprehensive data set including sufficient information on intake frequency and amount of the investigated species is required. Quantitative RBAs on fish as carried out by Carvalho et al. (2022) can give comprehensive information on the prevention of DALYs due to sufficient and regular fish intake, especially important for policy makers and authorities. Nevertheless, a qualitative RBA, such as carried

out in this study, generates important knowledge, and gives information to relevant policy makers and authorities in Norway. Our RBA created valuable knowledge for five species that have so far not been of large commercial interest in Norway. This is the first RBA carried out on these five fish species in the country, and it can be used as a directive for safe consumption of these underutilized fish species.

5. Conclusions

The present study not only emphasized the beneficial outcomes of consuming the five investigated underutilized fish species, but also highlighted that these benefits outweigh potential risks. Consequently, the findings strongly support an increased intake of these five species originating from Norway for the Norwegian population, effectively highlighting their positive health benefits. An optimal weekly fish intake of 300–450 g of which 200 g should be fatty fish can be regarded as feasible intake scenario. The substitution of 250 g of any of the five investigated lean fish should be preferred, as suggested in AS1. To get information on DALY and QALY connected to the consumption of the five studied fish, more data on the population is required, which can be done in future works in this field. Even though not all possible contaminants (e.g. multiple dioxin, DL-PCB congeners) were considered in this study, our results support a recommendation for increasing the consumption of these five species. We believe that this RBA can help promote the consumption and commercialization of flatfish and rays in the Norwegian market.

CRediT authorship contribution statement

Sophie Kendler: Conceptualization, Methodology, Investigation, Writing – original draft. **Sara Monteiro Pires**: Validation, Methodology, Writing – review & editing. **Anita Nordeng Jakobsen**: Conceptualization, Supervision, Validation, Writing – review & editing, Funding acquisition. **Jørgen Lerfall**: Conceptualization, Supervision, Validation, Writing – review & editing, Project administration, Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Paper V

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EDITED BY
Dandan Ren,
Dalian Ocean University, China

REVIEWED BY
Abdo Hassoun,
Sustainable AgriFoodtech Innovation & Research (Safir), France
Zhijian Tan,
Chinese Academy of Agricultural Sciences, China

*CORRESPONDENCE
Sophie Kendler
✉ sophie.kendler@ntnu.no

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The application of microwave and ultrasound technologies for extracting collagen from European plaice by-products

Sophie Kendler*, Sine Marie Moen Kobbenes, Anita Nordeng Jakobsen, Kirill Mukhatov and Jørgen Lerfall

Department of Biotechnology and Food Science, Norwegian University of Science and Technology, Trondheim, Norway

This study's main aim was to utilize green extraction protocols to recover collagen from by-products originating from European plaice. Moreover, the objective was to evaluate pre-treatments, the composition of the up-cycled product as well as to identify the most promising by-product fraction. Microwave (MAE) and ultrasound-assisted extractions (UAE) were performed on untreated, pre-treated (salt-washed or enzymatic hydrolysis) fractions of backbones, skins, and heads. Both MAE and UAE were performed for 15 and 35 min. After pre-treatment and extraction, the quality and yield of products were evaluated. Protein and collagen concentration, as well as amino acid profiles, were measured. Skins deliver significantly higher yields of collagen and protein than heads and backbones ($p < 0.05$). Enzymatic hydrolysis resulted in the highest collagen yields for skins (77%), while salt-washing gave the highest results for backbones (43%) and heads (41%) regardless of extraction method and time. Total and free amino acid profiles differed between the three fractions, with backbones and heads showing overall more similarity in composition compared to skins. The study showed that MAE and UAE technologies are suitable for generating collagen from marine by-products. Additional research is recommended to optimize pre-treatment and extraction for skin, as most promising collagen supplier.

KEYWORDS

green extraction methods, marine collagen, by-product valorization, upcycling, side-streams utilization, innovative collagen extraction

1. Introduction

Global fish production reached a substantial 176 million tonnes in 2020, of which 157 million tonnes were used for direct human consumption and an increasing share of fish getting further processed and not sold as whole fish (FAO, 2022). This leads to a high amount of by-products, which can reach up to 70% of the total mass of the fish. By-products refer to the portions of the catch that are not considered the main saleable product, including skins, scales, heads, viscera, backbones, and fins (Ozogul et al., 2021). Europe has increased its by-product utilization significantly in the past year, however, the main share is used for low-value applications like fishmeal, fish oil, biogas or fertilizers (FAO, 2020). However, over the past few decades, extensive research has revealed that these by-product fractions contain valuable components that can be further up-cycled into products for human consumption (Al Khawli et al., 2019; Ozogul et al., 2021). Especially collagen has versatile fields of applications, such as a functional food ingredient or stabilizer in food, as biomaterial to regenerate skin and

bones as well as applications in cosmetics as skincare products, proven to exhibit bioactive properties (Avila Rodríguez et al., 2018; Lu et al., 2023). Moreover, collagen, in its application as a novel functional food ingredient, contains valuable essential amino acids which positively impact the nutritional quality of the product and moreover exhibit bioactive properties suitable for application as natural antioxidants (Pal and Suresh, 2016).

The demand for a more sustainable global food production system prompted the European Union (EU) Commission to launch the Circular Economy Action Plan in 2020, building upon strategies outlined in the European Green Deal of 2015. This plan aims to establish a framework that promotes the norm of sustainable products and contributes to achieving the 2030 Sustainable Development Goals (Commission and Communication, 2020). Especially novel technologies, such as green extraction procedures for obtaining bioactive components from marine by-products have attracted considerable interest in recent years, due to their reduced impact on the environment and decreased economic waste (Ozogul et al., 2021) Pal and Suresh (2016) list ultrasonic (ultrasound), microwave, super critical fluids and high pressure as emerging green extraction methods for marine collagen, but also states that these extractions require heating which potentially affect the structure of marine collagen. Previously, ultrasound-assisted extraction has been used to extract collagen from marine by-products (Ali et al., 2018; Shaik et al., 2021; Lu et al., 2023). Moreover, chitin and chitosan from shrimp shells have formerly been generated by microwave-assisted extraction (El Knidri et al., 2019; Santos et al., 2019). Ozogul et al. (2021) state that the extraction of marine collagen is most efficiently performed with organic acids or with enzymatic hydrolysis. However, to our knowledge, the research and knowledge on the utilization of ultrasound or microwave-assisted extraction on generating collagen from fish by-products has not been followed-up to date.

Thus, the present study aimed to investigate the utilization of green extraction technologies for valorizing three by-product fractions derived from European plaice (*Pleuronectes platessa*). Specifically, the focus was on extracting collagen from backbones, skins and heads using microwave or ultrasound-assisted extraction. Next to collagen, the total protein content and amino acid profiles were investigated. Additionally, the study examined the effects of enzymatic hydrolysis or salt-washing prior to extraction as suitable pre-treatments. By exploring these objectives, the study aimed to provide insights into optimal green extraction procedures and conditions for obtaining collagen from different by-product fractions of European plaice.

2. Materials and methods

2.1. Raw material and experimental design

Skins, heads and backbones from European plaice (*Pleuronectes platessa*), previously collected during the study of Kendler et al. (2023b), were separated, homogenized and kept frozen at −80°C until further analyses. Samples underwent pre-treatment by salt-washing or enzymatic hydrolysis or were used untreated for further extractions by microwave or ultrasound technologies, each for 15 and 35 min (MAE15, MAE35, UAE15, and UAE35). After extraction, samples got lyophilized for 24 h at −50°C and 13.3 Pa and frozen prior to further analyses.

2.2. Pre-treatments

2.2.1. Salt pre-treatment

The salt pre-treatment followed the method of Kołodziejska et al. (2008) with minor modifications. Minced raw material was thawed at 4°C. A 0.45 M NaCl-solution (6:1 v/w) was added and shaken with the material for 10 min at 4°C. The solution was filtered, and the salt-wash was discarded. Steps one and two were repeated two more times. The material was then washed in cold tap water. Next, the material was shaken in a 10% ethanol solution (6:1 v/w) for 30 min at 4°C, filtered again, and subsequently washed in cold tap water. The salt pre-treated material was stored at 4°C until extraction with microwave or ultrasound.

2.2.2. Enzymatic hydrolysis

Enzymatic hydrolysis was performed by following an in-house protocol, as described by Hjellnes et al. (2021, p. 26), using the peptidase Alcalase 2.4 L (provided by Novozymes AS, Denmark) as the catalyst. The hydrolysis was carried out in a laboratory-scale bioreactor (Model No. 2101000; Syrris Atlas) equipped with a stirring unit, thermostat, and pH meter.

Pre-heated water (50°C) and raw material (1:1% ww) were added and stirred in the reactor. Once the mixture reached 50°C, a portion was taken out as a 0-sample (prior to enzyme addition) and deactivated in a 90°C water bath for 5 min with stirring. Alcalase 2.4 L was then added to the remaining sample (0.1% of weight), and the stirring was initiated. Samples were taken out at 60 min and deactivated as described earlier. The pH was maintained at 7.0 throughout the hydrolysis, with NaOH added if necessary. After 60 min, the hydrolysis was stopped.

All samples were centrifuged and frozen overnight at −40°C. The following day, the frozen samples were divided into three fractions: fat, protein hydrolysate, and sediment. The weight of each fraction was recorded for yield calculation, and the sediment and protein hydrolysate fractions were further analyzed. To determine which fraction contained more collagen for subsequent extractions, both fractions were lyophilized and assessed for collagen content.

2.3. Green extraction technologies

Both untreated and pre-treated raw material was subjected to microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE).

2.3.1. Microwave-assisted extraction

Microwave-assisted extraction (MAE) was conducted with a MARS 6 microwave digestion system (CEM Corporation, NC), equipped with 30 digestion vessels. About 30–40 g (ww) raw material was homogenized (Polytron PT3100; Kinematica) at 10000 rpm for 1 min and subsequently mixed with deionized water (6:1 v/w). For hydrolysates, 2–3 g lyophilized samples were used and deionized water was added (6:1 v/w according to wet weight of hydrolysates). The water-sample mixture was shaken vigorously to mix water and hydrolysate. Finally, the material was added to the digestion vessels and following parameters were chosen for each extraction round: ramp time (5 min), hold time (15 or 35 min), temperature 40°C, power (350 W), stirring (medium speed).

2.3.2. Ultrasound-assisted extraction

For the Ultrasound-assisted extraction (UAE), an ultrasonic cleaning bath, of type USC-T (VWR) was used. The material was prepared, and same sample amount used as described in section 2.31. Basic 50 mL centrifuge tubes were used for extraction. The ultrasound water bath was pre-heated to 40°C and the centrifuge tubes were placed in the water bath when the temperature reached 35°C to obtain a ramp time of 5 min, like MAE.

After water, MAE or UAE extraction, the samples were centrifuged (34,000 g, 10 min, 20°C) and the two obtained phases, being the sediment and soluble protein phase were separated and weighted. The soluble protein phase was then lyophilized for further analyses.

2.4. Chemical analyses

2.4.1. Total protein and amino acid profile

The determination of total crude protein (%) was carried out using the Kjeldahl method (AOAC, 1990), using a Kjeldahl apparatus equipped with a digestion and auto titration unit (K-449 and K-375, Büchi Labortechnik, Schwitzerland). The procedure was carried out as previously described in Kendler et al. (2023a,b). A conversion factor of 6.25 x nitrogen (%) was applied to determine the protein content of samples (NMKL, 2003).

The determination of the total amino acid (TAA) distribution (%) followed the method of Blackburn (1978) utilizing acid hydrolysis. The procedure was previously described in detail by, Kendler et al. (2023a,b).

The method from Osnes and Mohr (1985) was followed to determine the free amino acid (FAA) distribution (%). The sample preparation was previously described in Kendler et al. (2023a,b).

The HPLC analysis for TAA and FAA distributions (%) was conducted by a ultra-high-performance liquid chromatography system (HPLC, UltiMate 300, Thermo Fisher Scientific, United States). The same HPLC system setup as previously described in detail by Kendler et al. (2023a,b) was used.

2.5. Collagen content

Collagen is a protein composed of repetitive triple helixes with proline and hydroxyprline as composites Zanaboni et al. (2000). Which is why the determination of hydroxyproline in section 2.5.1 gives information of the total collagen content.

2.5.1. Hydroxyproline

The determination of hydroxyproline content is based on the method of Leach (1960). An L-hydroxyproline stock solution was used (0.05 g hydroxyproline in 400 mL distilled water, 20 mL concentrated HCl, adding distilled water until volume reached 500 mL). From the stock solution, four standards of 2.5, 5.0, 10.0 and 15.0 µg hydroxyproline/mL were prepared.

The absorbance of the standard and sample solutions were measured against a blank using a GENESYS 10S UV–VIS Spectrophotometer (Thermo Fisher Scientific Inc., Unite States) at a wavelength of 555 nm.

A standard curve based on the absorbance of the standards was used to calculate the concentration of hydroxyproline in samples. The collagen content was then determined by using the equations 1–3 with the species specific collagen conversion factor of 9.6 for flounder (Sikorski et al., 1984).

$$Hyp_{content}\left(\frac{mg\ collagen}{ml\ hydrolysed\ sample}\right) = \frac{(OD - b)\cdot DF}{a} \quad (1)$$

$$Hyp\%of\ freeze\ dried\ sample = \frac{Hyp_{content}\cdot V}{m\cdot 10^6}\cdot 100\% \quad (2)$$

$$Collagen\%of\ freeze\ dried\ sample = Hyp\%\frac{100}{CF} \quad (3)$$

Where:

OD, Optical density (absorbance from spectrophotometer); DF, Dilution factor; a, A-value from standard curve ($y = ax + b$) (y = absorbance, x = hyp-content); b, B-value from standard curve; V, mL sample after acid hydrolysis; m, Mass of lyophilized sample for hydrolysis; *CF*, Conversion factor Statistics.

2.6. Statistics

All analyses were performed in triplicates, if not other stated ($n = 3$). The data from the analyses was tested by a General Linear Model (GLM) using the software IBM SPSS (release 28, IBM Corporation, United States). Where applicable, analysis of variance (ANOVA) was carried out, and when significance detected in the GLM or ANOVA, a Tukey HSD *post hoc* test was carried out to investigate the differences between groups. The α-level was set to 0.05.

3. Results and discussion

3.1. Pre-assessment of raw material quality and fractions

Analyses of the three raw material fractions prior to pre-treatment and extraction showed significant differences in their initial proximate composition. The skins protein content of 17.37 ± 1.4 g/100 g ww (wet weight) is significantly higher ($p < 0.001$) compared to backbones (11.88 ± 0.4 g/100 g ww) and heads (12.57 ± 0.8 g/100 g ww). Additionally, the skins ash content (1.31 ± 0.03 g/100 g ww) is significantly lower ($p < 0.001$) than the content of the backbones (5.44 ± 1.06 g/100 g ww) and heads (5.41 ± 1.5 g/100 g ww), which can be explained by the higher content of inorganic matter in backbones and heads. No significant differences in lipid and moisture content were observed. Moreover, the initial collagen content of skins (32.54 ± 0.4 g/100 g dw; dry weight) is significantly higher (p < 0.001) compared to backbones (18.71 ± 1.1 dw) and heads (12.73 ± 0.2 g/100 g dw) which is of special relevance for the extraction of collagen.

Analyses on the collagen content of sediment and protein hydrolysates generated through enzymatic hydrolysis showed that skin protein hydrolysates contain collagen of 52.07 ± 13.0% dw followed by heads protein hydrolysates with 34.63 ± 12.1% dw. Moreover, the collagen content in the sediment fractions of skins (28.3 ± 3.0% dw) and heads (13.2 ± 4.8%) were significantly lower ($p = 0.043$) than for

their respective protein fraction. The lowest collagen content was found in the sediment fraction of backbones (12.03 ± 1.7% dw). Following these results, merely the protein hydrolysate fractions were considered for further MAE and UAE, to guarantee the highest possible yield.

3.2. Total protein and collagen content after extraction

Analyzing the main effects of raw material, pre-treatments and extraction methods and times, it was found that regardless of pre-treatment, extraction and time, skins have the significantly ($p < 0.001$) highest collagen (%) and highest protein (%) concentrations of all three fractions. Moreover, pre-treatment could significantly increase collagen content ($p < 0.001$). In addition, the GLM demonstrated that both MAE and UAE, including both times, lead to a significant ($p < 0.001$) increase in the collagen (%) and protein content (%) compared to untreated raw material and without extraction.

Figure 1 shows the collagen and protein concentrations depending on pre-treatment (a, c) as well as after extractions with microwave or ultrasound for two different times (b, d). It is visible (Figure 1A) that skin yields the significantly highest collagen, regardless of pre-treatment ($p < 0.05$). Moreover, salt-washing is most effective in generating collagen for heads and backbones, whereas enzymatic hydrolysis yielded the most collagen for skins regardless of extraction method and time ($p < 0.05$). When looking at the different pre-treatments (Figure 1C) and their effect on the yield of protein of each raw material, it is visible that skins show significantly highest protein concentration for each pre-treatment. Thus, skins being the fraction with highest collagen and total protein yield regardless of pre-treatment. For both collagen and protein yield of skins, enzymatic hydrolysis was the most effective method. In addition, Figure 1B highlights that skins, regardless of extraction method and time, produce the highest collagen content ($p < 0.05$). Among the different extraction methods applied to backbones, MAE 35 has a significantly ($p < 0.05$) lower collagen content (27.96 ± 5.6%) compared to MAE 15, UAE 15, and UAE 35. No significant differences were observed among MAE 15, UAE 15, and UAE 35 for backbone as raw material. A different trend was observed for skins, where MAE 35 led to significantly higher collagen (75.58 ± 4.2%) compared to the lowest concentrations observed for UAE 15. Generally, the same trend for a suitable pre-treatment or extraction method and time was observed for backbones and heads, with enzymatic hydrolysis and UAE 35 yielding the highest protein concentrations for both fractions.

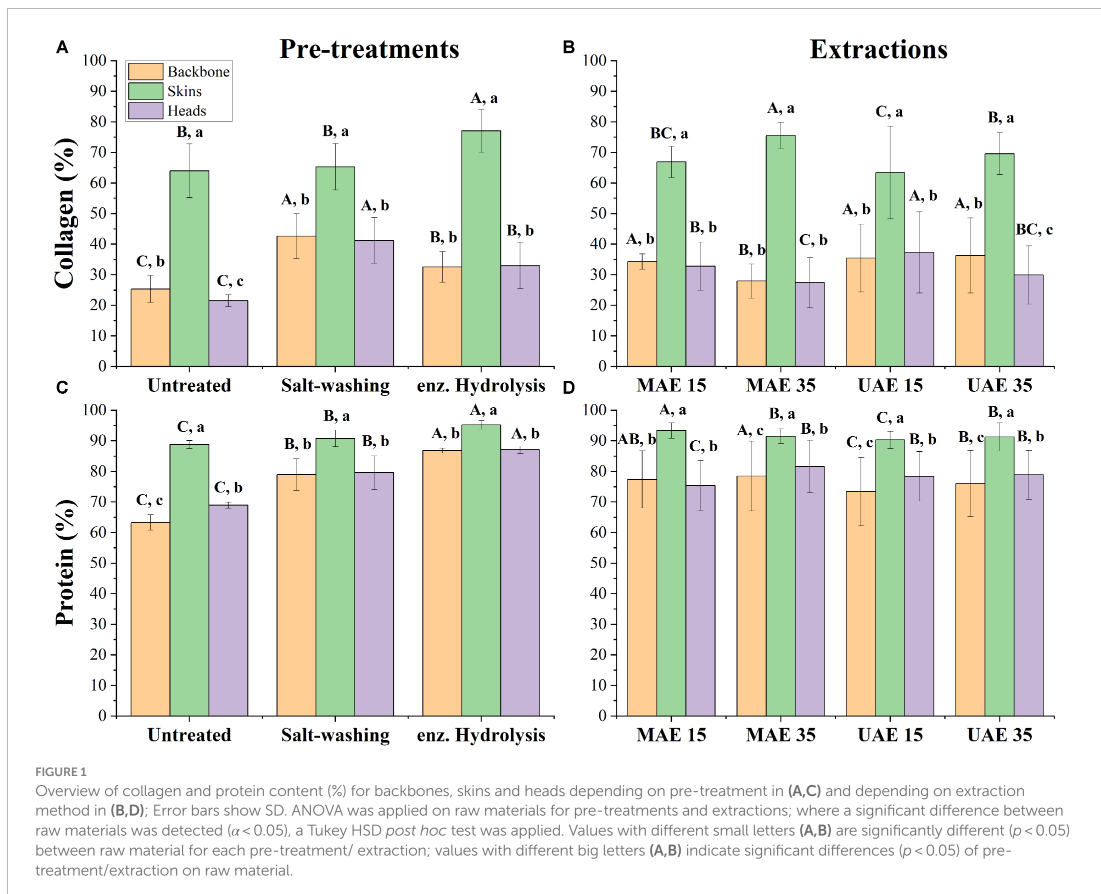

FIGURE 1
Overview of collagen and protein content (%) for backbones, skins and heads depending on pre-treatment in **(A,C)** and depending on extraction method in **(B,D)**; Error bars show SD. ANOVA was applied on raw materials for pre-treatments and extractions; where a significant difference between raw materials was detected ($\alpha < 0.05$), a Tukey HSD *post hoc* test was applied. Values with different small letters **(A,B)** are significantly different ($p < 0.05$) between raw material for each pre-treatment/ extraction; values with different big letters **(A,B)** indicate significant differences ($p < 0.05$) of pre-treatment/extraction on raw material.

3.3. Amino acid profile

Table 1 visualizes the TAA distribution of the three by-product fractions after extraction with MAE15, MAE35, UAE15, and UAE35. Pre-treatment by salt-washing or enzymatic hydrolysis both led to an increase in the TAA content of backbones ($p = 0.009$) and heads ($p < 0.001$) compared to no pre-treatment, while pre-treatments showed no difference in the composition for skins ($p = 0.418$). No between-subjects effects of extraction+time and pre-treatment for any of the three raw materials ($p > 0.05$) was observed. Moreover, the extraction method

TABLE 1 Total amino acid composition (mg/g) of backbones, skins and heads depending on extraction methods, regardless of pre-treatment; values presented as mean ± SD.

Raw material	Amino acids	MAE15	MAE35	UAE15	UAE35
	Essential AA	mg/g	mg/g	mg/g	mg/g
Backbones	Histidine	9.6 ± 2.2^{A}	9.6 ± 2.3^{A}	8.6 ± 2.0^{B}	9.1 ± 2.1^{AB}
Skins		$6.5 \pm 1.8^{b,B}$	$7.6 \pm 2.4^{ab,B}$	$7.8 \pm 1.3^{a,B}$	$8.2 \pm 2.6^{a,B}$
Heads		9.4 ± 1.5^{A}	9.9 ± 1.9^{A}	10.0 ± 1.2^{A}	10.5 ± 2.0^{A}
Backbones	Isoleucine	17.7 ± 5.0^{A}	17.5 ± 4.5^{A}	16.7 ± 4.0^{A}	15.3 ± 4.0^{A}
Skins		$10.4 \pm 4.4^{b,B}$	$11.3 \pm 3.3^{ab,B}$	$12.1 \pm 3.2^{ab,B}$	$12.8 \pm 3.1^{a,B}$
Heads		17.7 ± 4.5^{A}	17.2 ± 4.1^{A}	16.7 ± 4.2^{A}	17.7 ± 3.7^{A}
Backbones	Leucine	35.6 ± 7.6^{A}	34.4 ± 7.4^{A}	35.3 ± 7.3^{A}	33.8 ± 10.5^{A}
Skins		$23.3 \pm 8.1^{b,B}$	$24.9 \pm 9.9^{ab,B}$	$25.1 \pm 6.6^{ab,B}$	$28.7 \pm 8.1^{a,B}$
Heads		35.9 ± 5.8^{A}	33.3 ± 8.0^{A}	34.0 ± 9.2^{A}	35.6 ± 8.1^{A}
Backbones	Lysine	43.6 ± 16.0^{A}	40.4 ± 10.1^{A}	37.5 ± 8.5^{A}	35.8 ± 10.5
Skins		$24.3 \pm 11.1^{b,B}$	$32.2 \pm 8.6^{ab,B}$	$30.8 \pm 4.5^{ab,B}$	36.4 ± 9.2^{a}
Heads		38.9 ± 8.0^{A}	39.2 ± 10.5^{A}	38.3 ± 8.7^{A}	40.3 ± 10.4
Backbones	Methionine	$13.5 \pm 2.9^{A,B}$	13.2 ± 2.6	14.1 ± 2.8	$13.2 \pm 4.7^{A,B}$
Skins		12.2 ± 4.1^{B}	12.3 ± 2.9	12.5 ± 2.8	14.7 ± 3.1^{A}
Heads		$15.7 \pm 2.8^{a,A}$	13.5 ± 3.2^{ab}	14.6 ± 4.7^{a}	$10.5 \pm 6.5^{b,B}$
Backbones	Phenylalanine	20.0 ± 3.7	20.1 ± 4.0	18.7 ± 2.3	18.2 ± 3.5
Skins		17.2 ± 2.8^{b}	18.0 ± 3.3^{ab}	18.0 ± 2.6^{ab}	20.1 ± 4.3^{a}
Heads		20.4 ± 3.0	20.5 ± 3.5	19.9 ± 4.0	20.4 ± 3.0
Backbones	Threonine	25.3 ± 5.9	25.7 ± 6.7	25.3 ± 5.8	24.5 ± 7.2^{B}
Skins		23.4 ± 5.3^{b}	26.6 ± 7.0^{ab}	25.9 ± 4.4^{ab}	$29.8 \pm 8.1^{a,A}$
Heads		26.2 ± 3.3	25.5 ± 6.2	26.6 ± 8.3	$26.9 \pm 6.5^{A,B}$
Backbones	Valine	20.5 ± 4.6^{A}	19.5 ± 4.6^{A}	19.7 ± 3.5^{A}	21.4 ± 5.2
Skins		$15.1 \pm 4.2^{b,B}$	$17.2 \pm 4.4^{ab,B}$	$16.7 \pm 2.3^{ab,B}$	19.1 ± 4.5^{a}
Heads		20.4 ± 3.8^{A}	21.3 ± 4.7^{A}	20.5 ± 3.5^{A}	21.3 ± 5.1
	Non-essential AA				
Backbones	Asparagine	$0.01 \pm 0.01^{b,AB}$	$<0.01 \pm <0.01^{b}$	$0.01 \pm <0.01^{b}$	0.07 ± 0.1^{a}
Skins		0.02 ± 0.01^{A}	0.02 ± 0.02	$<0.01 \pm <0.01$	0.01 ± 0.01
Heads		$<0.01 \pm <0.01^{B}$	0.02 ± 0.04	0.01 ± 0.01	0.05 ± 0.1
Backbones	Glutamine	15.6 ± 25.8^{a}	$0.12 \pm 0.2^{b,B}$	$21.9 \pm 32.7^{a,AB}$	0.67 ± 0.7^{b}
Skins		11.1 ± 17.8^{b}	$0.87 \pm 07^{c,A}$	$31.6 \pm 29.3^{a,A}$	0.59 ± 0.6^{c}
Heads		16.4 ± 25.4^{a}	$<0.01 \pm <0.01^{b,B}$	$10.5 \pm 15.4^{a,B}$	0.29 ± 0.5^{b}
Backbones	Arg/Gly	173.4 ± 55.2^{B}	167.3 ± 72.0^{B}	160.2 ± 48.5^{B}	152.6 ± 51.4^{B}
Skins		$260.5 \pm 27.8^{b,A}$	$266.1 \pm 48.8^{ab,A}$	$257.2 \pm 26.9^{b,A}$	$298.9 \pm 72.1^{a,A}$
Heads		166.5 ± 36.2^{B}	169.9 ± 54.2^{B}	140.2 ± 45.2^{C}	161.4 ± 52.4^{B}
Backbones	Tyrosine	12.0 ± 2.0^{A}	11.3 ± 1.9^{A}	11.7 ± 2.4^{A}	10.5 ± 2.9^{A}
Skins		$6.1 \pm 2.1^{b,B}$	$6.0 \pm 1.3^{b,B}$	$6.9 \pm 1.6^{ab,B}$	$7.3 \pm 1.7^{a,B}$
Heads		12.7 ± 1.7^{A}	10.8 ± 2.4^{A}	13.0 ± 4.2^{A}	12.1 ± 2.6^{A}

(Continued)

TABLE 1 (Continued)

Raw material	Amino acids	MAE15	MAE35	UAE15	UAE35
Backbones	Alanine	46.8 ± 11.2[B]	46.4 ± 15.4[B]	44.5 ± 9.8[B]	43.5 ± 12.4[B]
Skins		59.9 ± 7.2[ab,A]	59.5 ± 11.5[ab,A]	57.9 ± 7.5[b,A]	67.5 ± 14.8[a,A]
Heads		46.1 ± 7.9[B]	46.6 ± 7.9[B]	42.1 ± 10.5[B]	46.8 ± 12.9[B]
Backbones	Aspartate	54.7 ± 11.3	53.2 ± 12.5	51.6 ± 9.6	50.0 ± 13.8
Skins		48.3 ± 8.8	50.3 ± 11.7	49.0 ± 6.5	55.1 ± 12.6
Heads		54.7 ± 7.7	52.8 ± 12.6	52.4 ± 12.3	54.5 ± 12.8
Backbones	Glutamate	72.9 ± 16.9	73.4 ± 19.0	68.7 ± 14.4[AB]	72.8 ± 26.2
Skins		64.2 ± 9.5[b]	71.0 ± 20.7[ab]	61.8 ± 6.8[b,B]	78.0 ± 20.9[a]
Heads		72.6 ± 11.2	72.0 ± 20.6	70.9 ± 22.8[A]	74.6 ± 21.2
Backbones	Serine	34.5 ± 9.2	34.1 ± 10.8	32.8 ± 6.5[B]	30.3 ± 8.2[B]
Skins		38.3 ± 5.8[b]	39.6 ± 7.6[ab]	39.7 ± 5.1[ab,A]	44.6 ± 10.5[a,A]
Heads		34.6 ± 5.2	34.6 ± 8.8	31.5 ± 7.0[B]	35.49.2 ± 8.1[B]
Backbones	∑ Total-AA	463.3 ± 291	568.1 ± 167	567.3 ± 132	471.1 ± 228[B]
Skins		551.8 ± 226	643.5 ± 140	580.3 ± 233	721.8 ± 169[A]
Heads		588.2 ± 108	567.2 ± 149	541.0 ± 143	568.4 ± 146[AB]

ANOVA was applied to detect differences between raw materials and between extraction methods; where a significant difference was detected ($\alpha < 0.05$), a Tukey HSD *post hoc* test was applied. Values with different superscript (a, b within a row; A, B within a column) are significantly different ($p<0.05$).

and time did not significantly influence the total amino acid contents ($p > 0.05$). Moreover, backbones and heads show significantly higher ($p < 0.05$) values for, e.g., histidine, isoleucine, leucine and valine, all essential amino acids, after extraction with MAE15 compared to skins. This pattern can also be observed for MAE35, UAE15, and UAE35. Nevertheless, when looking closer at the sum of all the TAA, a significant difference ($p < 0.05$) between the three by-products was only observed for UAE35, with skin having a significantly higher TAA content. This leads to the assumption that skins have a significantly different amino acid profile than the two other fractions, but all in all same total content. Skins showed significantly ($p < 0.05$) higher values in serine for UAE15 and UAE35. Moreover, the alanine concentrations in skins are higher in all four extraction methods compared to backbones and heads. In addition, Table 1 points out that some amino acids are more influenced by extraction method and time than other amino acids, which show no significant different concentrations. Moreover, arginine/glycine were identified as the highest concentrations in all three fractions, which was also observed (for glycine) by Ali et al. (2018); Tamilmozhi et al. (2013). This is reasonable because the strict repeating of Gly-X-Y creates the α-triple-helix of collagen (Zanaboni et al., 2000).

Table 2 shows the FAA profile of untreated by-products after extraction with MAE15, MAE35, UAE15, and UAE35. Overall, similar FAA profiles between backbones and heads were found, with significantly higher ($p < 0.05$) values of leucine and glutamine, regardless of extraction method. Moreover, the values of lysine are significantly ($p < 0.05$) lower in skins when compared to the two other fractions. The total FAA content differs significantly between the three by-products as well as between the four extractions. For backbones, no significant differences between the four extractions were found, whereas UAE15 was identified to deliver most FAA in skins, and UAE15 as well as UAE35 for heads. Similar to TAA, glycine (combined Arg/Gly) was also identified as the main FAA in all three fractions. Higher free glycine can be linked to a disruption of the α-triple-helix and potentially increase the interfacial properties, as previously described in the study of Feng et al. (2021) on microwave extraction of fish skin gelatine.

The study highlights the potential value of the three studied fractions for collagen extraction. The results support the findings of Mohamad Razali et al. (2023), stating that green extraction methods such as UAE and MAE increase the extraction efficiency of collagen. Moreover, Mohamad Razali et al. (2023) review the current progress of green extraction methods for collagen and state that MAE is a versatile technology, enabling the transfer of technology from small (lab) to big (industry) scale (Destandau et al., 2013). Furthermore, Mohamad Razali et al. (2023) highlights that UAE and MAE have been successfully applied in multiple studies to increase the extraction efficiency of collagen, hereby stressing the significance of these green extraction methods. The study carried out by Shaik et al. (2021) on UAE of stingray skin collagen found UAE to increase the collagen yield, which agrees with the present study, underlining skin as a high collagen and high protein fraction with great potential for further utilization.

4. Conclusion

The findings of the present study contribute to the development of sustainable extraction processes of marine collagen from plaice by-products. Furthermore, the results feature how pre-treatment and extraction techniques affect the final protein and collagen concentration of the three studied by-products, with skins being the most promising collagen supplier. However, to fully understand and interpret the impact of the results, the knowledge of collagen extraction from skin, should be expanded. In conclusion, the current study highlights the potential of the applied green extraction procedures suitable for application in food, ingredient and cosmetic industries and suggests further research on the characteristics of the collagen structure as the next step to understand the functionality and applicability of the gained product.

TABLE 2 Free amino acid composition (mg/g) of backbones, skins and heads depending on extraction methods, excluding effect of pre-treatment; values presented as mean $\pm$ SD.

Raw material	Amino acids	MAE15	MAE35	UAE15	UAE35
	Essential AA	mg/g	mg/g	mg/g	mg/g
Backbones	Histidine	$0.51 \pm 0.02^{a,A}$	$0.31 \pm 0.06^{b,B}$	$0.52 \pm 0.02^{a,B}$	$0.53 \pm 0.01^{a,B}$
Skins		$0.25 \pm 0.04^{a,B}$	$0.09 \pm 0.08^{b,C}$	$0.24 \pm 0.02^{a,C}$	$0.23 \pm 0.03^{a,C}$
Heads		$0.53 \pm 0.02^{b,A}$	$0.55 \pm 0.05^{b,A}$	$0.67 \pm 0.07^{a,A}$	$0.76 \pm 0.02^{a,A}$
Backbones	Isoleucine	0.42 ± 0.14^{A}	0.15 ± 0.01^{AB}	0.50 ± 0.24	0.30 ± 0.01^{A}
Skins		0.04 ± 0.01^{B}	$0.03 \pm <0.001^{B}$	0.13 ± 0.12	0.10 ± 0.09^{B}
Heads		0.33 ± 0.2^{AB}	0.39 ± 0.21^{A}	0.47 ± 0.27	0.17 ± 0.01^{B}
Backbones	Leucine	$0.53 \pm 0.17^{ab,A}$	$0.33 \pm 0.01^{b,A}$	$0.60 \pm 0.04^{a,A}$	$0.50 \pm 0.02^{ab,A}$
Skins		$0.06 \pm 0.02^{bc,B}$	$0.05 \pm 0.01^{c,B}$	$0.12 \pm 0.02^{a,B}$	$0.09 \pm 0.01^{ab,C}$
Heads		0.40 ± 0.09^{A}	0.44 ± 0.11^{A}	0.49 ± 0.10^{A}	0.28 ± 0.03^{B}
Backbones	Lysine	0.31 ± 0.34	0.21 ± 0.01^{B}	0.42 ± 0.03^{AB}	0.27 ± 0.01^{A}
Skins		0.03 ± 0.01	0.01 ± 0.01^{C}	0.04 ± 0.03^{B}	0.04 ± 0.02^{B}
Heads		0.37 ± 0.12^{ab}	$0.37 \pm 0.06^{ab,A}$	$0.67 \pm 0.27^{a,A}$	$0.25 \pm 0.03^{b,A}$
Backbones	Methionine	0.77 ± 0.2	0.54 ± 0.02	0.62 ± 0.14	0.59 ± 0.03
Skins		0.43 ± 0.03^{a}	0.34 ± 0.03^{b}	0.43 ± 0.02^{a}	0.34 ± 0.04^{b}
Heads		0.63 ± 0.4	0.63 ± 0.29	0.69 ± 0.34	0.80 ± 0.03
Backbones	Phenylalanine	$0.82 \pm 0.08^{a,A}$	$0.50 \pm 0.02^{b,AB}$	0.76 ± 0.02^{a}	$0.71 \pm 0.01^{a,A}$
Skins		0.13 ± 0.02^{B}	0.11 ± 0.01^{B}	0.28 ± 0.18	0.21 ± 0.16^{B}
Heads		0.57 ± 0.33^{AB}	0.57 ± 0.29^{A}	0.65 ± 0.32	0.33 ± 0.05^{B}
Backbones	Threonine	3.28 ± 3.0	1.37 ± 0.04^{A}	1.36 ± 0.02^{B}	1.71 ± 0.12^{B}
Skins		0.83 ± 0.01	0.40 ± 0.34^{B}	0.77 ± 0.04^{C}	0.73 ± 0.02^{C}
Heads		1.58 ± 0.12^{c}	$1.85 \pm 0.04^{b,A}$	$1.98 \pm 0.14^{b,A}$	$2.54 \pm 0.07^{a,A}$
Backbones	Valine	$0.58 \pm 0.07^{a,AB}$	$0.40 \pm 0.03^{b,AB}$	$0.66 \pm 0.07^{a,A}$	$0.61 \pm 0.03^{a,A}$
Skins		$0.12 \pm 0.01^{ab,B}$	$0.11 \pm 0.02^{b,B}$	$0.27 \pm 0.08^{a,B}$	$0.20 \pm 0.08^{ab,C}$
Heads		0.67 ± 0.3^{A}	$0.75 \pm 0.34^{A,}$	0.81 ± 0.24^{A}	$0.44 \pm 0.03^{a,B}$
	Non-essential AA				
Backbones	Asparagine				
Skins		<0.001	<0.001	<0.001	<0.001
Heads					
Backbones	Glutamine	$0.56 \pm 0.03^{a,A}$	$0.36 \pm 0.10^{b,B}$	$0.56 \pm 0.03^{a,B}$	$0.42 \pm 0.03^{ab,B}$
Skins		$0.36 \pm 0.02^{a,B}$	$0.23 \pm 0.02^{c,B}$	$0.34 \pm 0.01^{ab,V}$	$0.31 \pm 0.02^{b,C}$
Heads		$0.56 \pm 0.04^{b,A}$	$0.57 \pm 0.03^{b,A}$	$0.70 \pm 0.04^{a,A}$	$0.52 \pm 0.01^{b,A}$
Backbones	Arg/Gly	$6.60 \pm 0.09^{b,A}$	$5.9 \pm 0.24^{c,A}$	$6.67 \pm 0.22^{b,A}$	$7.71 \pm 0.20^{a,A}$
Skins		$4.55 \pm 0.03^{b,C}$	$3.13 \pm 0.14^{c,C}$	$4.78 \pm 0.08^{a,C}$	$4.45 \pm 0.06^{b,C}$
Heads		$5.01 \pm 0.07^{c,B}$	$4.51 \pm 0.12^{d,B}$	$5.66 \pm 0.14^{b,B}$	$6.75 \pm 0.16^{a,B}$
Backbones	Tyrosine	2.39 ± 3.1	0.86 ± 0.03^{A}	0.58 ± 0.01^{A}	1.00 ± 0.04^{A}
Skins		0.29 ± 0.08^{b}	$0.25 \pm 0.01^{b,C}$	$0.54 \pm 0.01^{a,B}$	$0.46 \pm 0.01^{a,B}$
Heads		0.26 ± 0.03	0.36 ± 0.02^{B}	0.39 ± 0.02^{C}	0.54 ± 0.26^{B}
Backbones	Alanine	$2.15 \pm 0.97^{ab,A}$	$1.10 \pm 0.04^{b,B}$	$2.39 \pm 0.03^{a,B}$	$2.60 \pm 0.01^{a,A}$
Skins		$0.74 \pm 0.03^{c,B}$	$0.79 \pm 0.04^{bc,C}$	$1.03 \pm 0.02^{a,C}$	$0.82 \pm 0.01^{b,C}$
Heads		$1.95 \pm 0.02^{b,AB}$	$2.66 \pm 0.08^{a,A}$	$2.75 \pm 0.03^{a,A}$	$1.98 \pm 0.21^{b,B}$
Backbones	Aspartate	0.41 ± 0.06^{B}	0.34 ± 0.05^{B}	0.50 ± 0.13^{B}	0.49 ± 0.05^{A}
Skins		$0.18 \pm 0.02^{b,C}$	$0.18 \pm 0.02^{b,C}$	$0.29 \pm 0.03^{a,C}$	$0.20 \pm 0.02^{b,B}$
Heads		$0.53 \pm 0.01^{bc,A}$	$0.54 \pm 0.05^{b,A}$	$0.73 \pm 0.04^{a,A}$	$0.45 \pm 0.01^{c,A}$

(Continued)

TABLE 2 (Continued)

Raw material	Amino acids	MAE15	MAE35	UAE15	UAE35
Backbones	Glutamate	$0.33 \pm 0.02^{b,A}$	$0.50 \pm 0.05^{a,A}$	$0.22 \pm 0.04^{c,B}$	$0.45 \pm 0.02^{a,B}$
Skins		$0.11 \pm 0.02^{a,B}$	$0.07 \pm 0.01^{b,C}$	$0.11 \pm 0.01^{a,C}$	$0.11 \pm 0.01^{a,C}$
Heads		$0.35 \pm 0.01^{d,A}$	$0.39 \pm 0.02^{c,B}$	$0.44 \pm 0.01^{b,A}$	$0.78 \pm 00.01^{a,A}$
Backbones	Serine	$1.26 \pm 0.04^{ab,B}$	$1.22 \pm 0.15^{b,B}$	$1.38 \pm 0.06^{ab,B}$	$1.46 \pm 0.05^{a,B}$
Skins		1.24 ± 0.04^{B}	1.01 ± 0.28^{B}	1.33 ± 0.03^{C}	1.22 ± 0.02^{C}
Heads		$1.86 \pm 0.02^{d,A}$	$2.16 \pm 0.08^{c,A}$	$2.71 \pm 0.08^{b,A}$	$3.27 \pm 0.10^{a,A}$
Backbones	∑ Free-AA	20.9 ± 5.7^{A}	14.1 ± 0.7^{B}	17.7 ± 0.5^{B}	19.3 ± 0.2^{A}
Skins		$9.3 \pm 0.3^{b,B}$	$6.81 \pm 0.2^{c,C}$	$10.7 \pm 0.5^{a,C}$	$9.5 \pm 0.4^{b,B}$
Heads		$15.6 \pm 0.2^{b,AB}$	$16.7 \pm 0.5^{b,A}$	$19.8 \pm 0.4^{a,A}$	$19.9 \pm 0.9^{a,A}$

ANOVA was applied to detect differences between raw materials and between extraction methods; where a significant difference was detected ($\alpha < 0.05$), a Tukey HSD *post hoc* test was applied. Values with different superscript (a, b within a row; A, B within a column) are significantly different ($p < 0.05$).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SKe: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. SKo: Conceptualization, Investigation, Formal analysis. KM: Validation, Methodology, Formal analysis. AJ: Conceptualization, Supervision, Validation, Writing – review & editing, Funding acquisition. JL: Conceptualization, Supervision, Validation, Writing – review & editing, Project administration, Resources, Funding acquisition.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Paper VI

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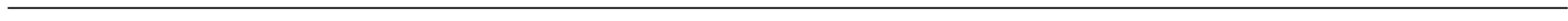

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