High-value ingredients from marine biomass for health

KB-34-003-035

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Institute: WFBR, WLR, WMR and WFSR

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PO box 17, 6700 AA Wageningen, The Netherlands, T + 31 (0)317 48 00 84, E info.wfbr@wur.nl, www.wur.eu/wfbr.

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Summary

Low trophic marine biomass will play an important role in the development of circular and climate neutral food, feed, and non-food production systems. In the development of large offshore wind parks in the North Sea, one of the ideas is to 'multi-use' the space to produce low trophic marine biomass such as bivalves. The overall objective is to give more insight in the potential of low trophic marine biomass to produce high value, safe ingredients, to be applied in health promoting animal feed, and in food. Using biorefinery, three fractions from mussels can be produced: (1) a bioactive fraction, (2) a protein-rich fraction, and (3) shells. This project focusses on identifying applications for the bioactive and the protein-rich fractions within aquafeed and human applications, respectively.

The following activities were carried out:

- Biorefinery: (i) processing of 700 kg mussels in three fractions to obtain ample material for trials within the PPS project "*Development and implementation of the total use principle of bivalves"* (shrimp trials and food application tests), (ii) desalting the bioactive fraction to widen its applicability, and (iii) processing of ragworms into two fractions (bioactive and protein-rich) to determine whether the same biorefinery process can be applied for other low trophic marine organisms;
- Bioactive fraction: (i) analysing liver and spleen obtained from the in-vivo trial from 2022, using RNAseq, to understanding the underlying mechanisms that play a role in the alleviation of anaemia in sole fed ragworm, mussel and diets supplemented with mussel fractions, and (ii) supporting development of a bioassay based on these insights;
- Protein-rich fraction: (i) determining which kind of protein functionality is present to decide the applicability within food, and (ii) searching the Innova database to obtain an overview of possible food applications;
- Chemical safety: assessing chemical safety of the bioactive and protein-rich fractions by determining the microplastics and PFAS content;
- Outlook: (i) evaluating mussel production scenario's on the North Sea, and (ii) scanning the potential for the harvestability of *Ensis spp*., another low trophic marine resource.

Biorefinery

Mussels consisting of shells (50% starting material) and meat (16% dw) were processed. From 700 kg mussel, 13.2 kg freeze-dried pellet and 5.6 kg freeze-dried supernatant were obtained. This was ample amount to evaluate food applications and perform an in-vivo shrimp trail. Desalting the bioactive fraction using nanofiltration with a low MWCO membrane (600-800 Da) targeting 50% salt reduction is technically feasible. However, part of the bioactive compounds and nitrogen-containing components are lost (~20%). Higher yields (> 80%) may be obtained by using a membrane with a lower MWCO (300 Da). The economics eventually determine how much loss of nitrogen-containing and bioactive components is acceptable. The process developed for mussels was applied to ragworms; the various process conditions (blanching time and pH) influenced the moisture loss, and the colour of pellet and supernatant.

Understanding bioactive mechanism

The liver emerged as the more responsive organ to dietary interventions, compared to the spleen, showing significant alterations in gene expression related to iron homeostasis, lipid metabolism, and oxidative stress regulation. These findings emphasize the liver's critical role in alleviating anaemia and supporting metabolic adaptations. Gene Set Enrichment Analysis (GSEA) highlighted enriched pathways linked to enhanced metabolic performance, erythropoiesis, and reduced inflammation in fish fed with the diet supplemented with the bioactive mussel fraction compared to fish fed with the commercial pellet. The diet supplemented with the bioactive fraction consistently outperformed the commercial feed in improving hematopoietic responses and showed distinct but complementary effects compared to ragworms, which remains the most effective at addressing anaemia and supporting growth. These results underscore the potential of the bioactive mussel fraction as a functional feed ingredient to alleviate anaemia and enhance fish health and performance under anaemic conditions.

Development bioassay
One of the results of the RNAseq analysis is an upregulation of genes involved in oxidative stress response. As enzyme X was upregulated, the total X amount was measured. The total X content in mussel and ragworms shows promising results as it correlates to the in-vivo results with common sole. X analysis could potentially be used as a bioassay for indicating whether low marine resources can alleviate anaemia in common sole.

Applicability protein-rich fraction in foods
The protein-rich fraction is low in solubility, with limited emulsifying, foaming and gelling properties, probably because these functionalities rely mostly on native, soluble proteins. However, stable sheets could be formed, and a fibrous structure was observed during processing. Also, this insoluble (pellet) fraction can be used as a filler in foods and simultaneously increase the nutritional value. Furthermore, the colour and flavour are intense and umami-like, which may be an advantage in fish-like applications.

Chemical safety
The mussel pellet and supernatant were analysed on microplastics and PFAS. Polypropylene and other polymers were detected. The obtained results for microplastics are indicative, given the limited number of samples. At the moment, no legislation exists to establish the maximum levels of microplastics in food or feed. Measured concentrations of the four regulated PFAS and their sum in raw mussel, mussel pellet and supernatant did not exceed the legal limits set for crustaceans and bivalve mollusks within the EU. Despite the method's relatively low recovery, substantial traces of PFOSA were detected. No maximum limits exist for PFOSA in crustaceans and bivalve mollusks.

Outlook
Mussel production increase is considered to take place in the North Sea or Voordelta. An additional yearly potential of 960-1,900 tons of protein, 290-590 tons of total lipid (fat) and 190-390 tons of carbohydrate is estimated. Current policy and visions on food from the sea supports offshore production of mussels and the estimated production increase seems feasible within the carrying capacity of the system. However, technological improvements, economic perspectives, regulatory limitations, and social acceptance need to be developed and demonstrated. Razor clams (*Ensis spp*) were selected as a potential new low trophic marine resource. The razor clam stocks are 470 tons (ww) outside Natura2000, and 1,090 tons (ww) inside Natura2000 areas. Analysis demonstrate that a potential harvestable stock of razor clams is available. Whether these stocks have potential as a fisheries resource should be elucidated by an appropriate assessment, as part of an impact assessment or specific modelling or ecological studies.

1 Introduction

Low trophic marine biomass will play an important role in the development of circular and climate neutral food, feed and non-food production systems. In the development of large offshore wind parks in the North Sea, one of the ideas is to 'multi-use' the space to produce low trophic marine biomass such as seaweed and bivalves like mussels. The production of mussels has a much smaller ecological footprint than meat such as beef, pork and poultry, with mussels having 1.4 kg CO_2-eq emissions per kg meat, compared to 33-99 kg for beef, 12 kg for pork, and 10 kg for poultry [Poore & Nemecek, 2018; Gephart *et al.*, 2021]. This biomass can be cultivated for food, feed and non-food applications. In some types of biomass, aside from their nutritional value, health promoting properties may be present. Examples are seaweed biostimulants and animal health promoting properties found in mussels and polychaetes (e.g. ragworms).

The background knowledge for this project was created in the project KB34-3a-1 *"Diagnostics for biorefinery of low trophic marine resources to animal health application"* and project KB34-2c-4 *"Marine Resources"*. The influence of processing on the ability of mussel meat to increase haemoglobin levels in cultivated common sole (*Solea solea*) was studied. In addition, feed and food safety analyses and diagnostic analyses were developed. This project focuses on the valorisation of bivalves using the total use concept and possibilities to apply this concept to other low trophic marine resources. Using biorefinery, several fractions of the biomass are produced, each with its own application, hereby ensuring total use of the raw material and maximum valorisation: (1) a bioactive fraction, (2) a protein-rich fraction, and (3) shells. The project is linked to the PPS project "*Development and implementation of the total use principle of bivalves"* in which, together with partners, shrimp trials and food application tests will be carried out with the mussel fractions.

The bioactive fraction can be applied in aquafeed, in order to improve the health of cultured fish. The protein-rich fraction can be used in food applications. The specific properties of the material (high unsaturated fatty acid content [Grienke *et al.*, 2014]) and organoleptic properties supply a direction for a suitable food outlet, such as seafood- and fish-based products. Shells can find their way into concrete as filler or substitute for limestone. In this manner, all three fractions contribute to the overall valorisation of bivalves, following the total use principle. Naturally, food and feed safety will be taken into account for the fractions. Applications of shells as construction material are already investigated in the PPS project *"Mariene bouwstenen: Circulaire benutting van schelpen in betonproductie"* and in project KB34-3d-1 *"Negative GHG emissions & long-time sequestration through the development of new C-based products"*, and were therefore not be a focal point in this study.

The following research questions were addressed in this project:
- How to fractionate mussels into protein-rich fraction, bioactive fraction, and shells? (Chapter 2);
- Can the same process be applied to other low trophic marine organisms, like ragworms? (Chapter 2);
- Can we widen the applicability of the bioactive fraction by removing salt? (Chapter 2);
- What can we learn about the underlying mechanism by analysing the liver and spleen obtained from last year's successful in-vivo trial using RNAseq? (Chapter 3);
- What are specific properties of the protein-rich fraction? (Chapter 4);
- What hazardous components/contaminants for feed and food safety can be defined in the fractions, including their concentration and/or dilution during processing? (Chapter 5);
- What are important factors to take into account for the concepts put forward in this project, regarding raw material composition, productivity, cultivation, harvest? (Chapter 6);
- How may this research translate to other low trophic marine organisms? (Chapter 6).

1.1 Objective

The overall objective is to give more insight in the potential of total use of low trophic marine biomass to produce high value and safe ingredients, to be applied in health promoting animal feed, and in food. Of the

three main fractions resulting from processing mussels, the bioactive fraction and the protein-rich fraction are taken into account.

1.2 Approach

The results of the research activities are reported in this report:
- Process mussels into three fractions (bioactive fraction, protein-rich fraction, and shells) (Chapter 2);
- Process ragworms into two fractions (bioactive fraction and protein-rich fraction) (Chapter 2);
- Evaluate desalting bioactive fraction (Chapter 2);
- Analyse the liver and spleen obtained from the successful in-vivo trial of previous year using RNAseq to further unravel the underlying mechanisms into the working principle that play a crucial role in the alleviation of anaemia in sole fed mussels, mussel extract B and ragworm (Chapter 3);
- Evaluate specific properties of protein-rich fraction (Chapter 4);
- Assess safety of raw material and processed fractions for food and feed (Chapter 5);
- Evaluate potential productivity, seasonal variation in composition, and seasonal variation in quality (based on composition) for mussel production scenarios (Chapter 6);
- Scan of potential and boundaries for the productivity of a selected low trophic marine resource (e.g. *Ensis spp*) (Chapter 6).

The activities regarding stakeholders were:
- Submission of peer reviewed article: Kals J. Muizelaar W, Kootstra AMJ, Gerrits, van den Bosch HM, Geerdink P, Telleman Y. 2024. The effect of dietary supplementation of blue mussel fractions on haematology, plasma biochemical parameters, performance, and their ability to alleviate anaemia in common sole (*Solea solea* L.) *[Submitted to Animal Feed Science and Technology];*
- An interview in the Provinciale Zeeuwse Courant (5 juli 2023) published as 'De mossel is een medicijn (en vier andere feiten die je waarschijnlijk nog niet kende)'. https://www.pzc.nl/zeeuws-nieuws/de-mossel-is-een-medicijn-en-vier-andere-feiten-die-je-waarschijnlijk-nog-niet-kende~ae4c8bb2/;
- Position paper for a professional magazine for industry and policy makers: Poelman M, Kals J, Boon F. 2024. Wat kan je allemaal doen met schelpdieren. Visserijnieuws, 22 augustus 2024. https://www.visserijnieuws.nl/nieuws/algemeen/40172/wat-kan-je-allemaal-doen-met-schelpdieren;
- Video intended for both the general public as well as policy makers: Wageningen University & Research. The potential of mussels. https://www.youtube.com/watch?v=K4IEvi8d6kg.

Activities regarding students:
- Bakker A, Ekelund H, Kemper J, Klein Holte K, Ruiter S, Vellekoop D. 2024. Exploring low trophic marine organisms as food, feed and biomass source. Report Case studies product quality. Wageningen University;
- Vourlas E, Nugroho F, Nguyen L, Ferretti F, Paardekooper L, Teske M, de Macedo F. 2024. Unveiling sustainable production systems: The North Sea's low trophic organisms as resource for food, feed and beyond. Report ACT group. Wageningen University.

2 Processing

2.1 Introduction

Three activities have been carried out:

- Pilot scale processing of 700 kg mussels into three fractions (bioactive fraction, protein-rich fraction, and shells) to obtain ample material for shrimp trials and food application tests;
- Desalting the bioactive fraction to widen the applicability;
- Lab scale processing of ragworms into two fractions (bioactive fraction and protein-rich fraction) to determine whether the same process for the mussels can also be applied for other low trophic marine organisms.

2.2 Results

2.2.1 Pilot scale processing of 700 kg mussels

700 kg mussel (Aqua-triton, provided by Lenger) was processed to obtain three fractions: shells, pellet and supernatant. The details are given in Annex 1. A fit-for-tasting protocol was followed to allow the pellet to be tested in food products. The composition of the fractions was analysed.

The mussels were blanched (30 s at 97ºC), cooled and peeled by hand. The mussel meat was milled with a colloid mill (VEA instruments), pH was adjusted to 6.0 with 6 M HCl. A pellet and supernatant were obtained using centrifugation (Sorval Lynx with 1 L buckets, 17.000xg, 30 min). Both fractions were freeze-dried, milled and packed in aluminium seal bags. The material was stored for further analyses at 4°C and for food application trials at -20°C. The total mass balance (wet weight, ww) is given in Table 2-1. 20% of the mass is lost during blanching and 50% of the initial mass consist of shells. After freeze-drying 13.2 kg pellet and 5.6 kg supernatant were obtained. The supernatant contained no microbial contamination, the pellet some small contamination. From a microbial point of view both fractions are fit-for-tasting.

Table 2-1 Mass balance (ww) processing 700 kg mussel (batch January 2024).

Process step		Fraction	kg	% starting material
Blanching	IN	Mussel	676	100
	OUT	Meat	211	31
		Shell	329	49
		Loss	136	20
Centrifugation	IN	Meat	211	31
	OUT	Pellet	129	19
		Supernatant	75	11
		Loss	7	1
Freeze-drying	IN	Pellet	129	19
		Supernatant	75	11
	OUT	Pellet	13.2	2
		Supernatant	5.6	0.8

The proximate composition of the source material, pellet and supernatant can be found in Table 2-2. The dry matter content of the raw mussel flesh is 16%. The pellet contains more protein and fat, the supernatant contains more ash and carbohydrates. This composition is the result of poor protein solubility at pH 6.0, as most of the protein is myo-fibrillar protein (muscle protein). The soluble proteins are the sarcoplasmic (blood) proteins, of which part is lost in the blanching step. Furthermore, blanching results in lowering the protein solubility due to denaturation. The ash (salt) is mostly in the aqueous phase, resulting in more ash in the supernatant. The fat was centrifuged out with the protein, as a light phase in the centrifuge tube. However, due to centrifugation at low temperature, the fat fraction stuck as a solid phase to the centrifuge

tube top and was recovered with the pellet. The insoluble fraction (pellet) was stored and later used as a food ingredient. The soluble fraction (supernatant) was stored for application as a feed ingredient for trials with shrimp and fish.

Table 2-2 Proximate composition of raw mussel, and pellet and supernatant after freeze-drying. Carbohydrates are assumed to be the mass that is left over after determining the protein, fat and ash content. For mussel (raw) in g/kg ww and for pellet and supernatant in g/kg dw

	Mussel (raw) (g/kg)	Mussel (raw) (%)	Pellet (g/kg)	Pellet (%)	Supernatant (g/kg)	Supernatant (%)
Dry matter	161	16	981	98	960	96
Crude protein	102	63	732	75	339	35
Crude fat	14	9	76	8	42	4
Ash	26	16	73	7	131	14
Carbohydrates	19	12	100	10	448	47

2.2.2 Desalting bioactive fraction

In the pilot scale processing of mussels (as described in paragraph 2.2.1), a supernatant was obtained as one of the final fractions. After freeze-drying, this fraction was analysed on bio-functionality and used in a fish trial yielding interesting and promising results. However, the high ash content of this supernatant fraction (16.2% dw) limits the inclusion level in aquafeed. Therefore, desalting the freeze-dried supernatant is necessary, aiming for 50% reduction of the ash content.

As a first attempt, dialysis with a 3.5 kDa tube was executed for desalting the supernatant fraction. Details can be found in Annex 1. Ash removal was above 90%; unfortunately, the protein yield was negatively affected under these process conditions. The membrane molecular weight cut-off (MWCO) of 3.5 kDa was apparently too high to retain most of the nitrogen-containing components (such as proteins, peptides and amino acids). Bioactivity (determined as antioxidant activity by DPPH assay) of the desalted fraction was unchanged indicating also partial removal of bioactive compounds during dialysis, together with other components.

As an alternative to the dialysis procedure, nanofiltration using a membrane with a lower MWCO[1] (600-800 Da) was experimentally tested, aiming for a reduced ash content of the supernatant fraction while preventing losses of nitrogen containing components and bioactive compounds in the process. Details can be found in Annex 1. The results indicate an ash removal of 40%, thereby lowering the ash content of the retentate fraction. Further reduction in ash content seems feasible by applying a diafiltration step with the selected membrane. Despite the lower MWCO of the membrane, the retention of nitrogen-containing components is far from complete (retention factor 0.7), resulting in an 80% yield. For a higher yield a membrane with a lower MWCO (300 Da) should be used. Bioactive compounds are partially retained by the membrane as evidenced by the bioactivity present in retentate as well as permeate fractions. It appears that the observed bioactivity can be attributed to multiple components with a wide range of molecular weights, thus a clear retention factor cannot be derived from the experimental data. However, using a membrane with a lower MWCO (300 Da) may retain more bioactive components in the retentate. In summary, it can be concluded that by desalting (50% salt removal) using nanofiltration, part of the bioactive compounds and nitrogen-containing components are lost with the permeate fraction (~20%). Higher yields (> 80%) may be obtained by using a membrane with a lower MWCO (300 Da). The economics eventually determine how much loss of nitrogen-containing and bioactive components is acceptable.

In addition to the desalting experiments, TGA was explored as alternative method for dry matter and ash determination in small sample sizes (< 1 mL), by testing various model solutions containing different mixtures of sucrose and NaCl. Details can be found in Annex 1. Accuracy of TGA for determination of the dry

[1] MWCO: molecular weight cut-off, defined as the lowest molecular weight (in Daltons) at which more than 90% of a solute with a known molecular weight is retained by the membrane.

matter content in model solutions is within 0.5%, with 0-30% deviation from the true value. TGA appears to be unsuitable for determining low ash or dry matter contents (<1% (w/w) in model solutions), although a lower limit was expected based on equipment specifications Applying the TGA measurement on freeze-dried samples of different NF fractions (with complex composition) did not yield results consistent with the current standard oven method. No clear explanations were found for these observed differences. For this type of samples with complex composition, it is recommended to investigate whether freeze-drying may have influenced the results obtained.

2.2.3 Lab scale processing ragworms

Ragworms were obtained from Topsy Baits (Wilhelmina polder, the Netherlands), transported by courier to Carus and kept in a water tank until use. The ragworms were in good condition before the trial. Approximately 12 kg of ragworms was divided in 4 batches for thermal treatment (no treatment, 30 s at 97ºC, 60 s at 97ºC and 300 s at 97ºC). The weight loss was significant during blanching. This weight loss is caused by the ragworms emptying their bowels during thermal treatment and the loss of moisture. The longer the treatment, the larger the weight loss (Table 2-3).

Table 2-3 Ragworm weight at the start (g) and after blanching (% starting material).

Blanching time at 97ºC (s)	Weight starting material ragworms (g)	Weight ragworms after blanching (% starting material)
30	3041	65,0
60	3699	61,2
300	3706	51,5

After blanching the ragworms were milled with a Thermomix cutting mill and the pH was adjusted to pH 4.5 (50%) and pH 6 (50%). The thermally treated ragworms were too viscous for separation after milling (Figure 2-1), therefore 500 mL demineralised water was added to aid mixing and centrifugation.

Figure 2-1 Ragworms before and after milling (left: fresh, right blanched).

The material was then centrifuged (Sorval Lynx with 1 L buckets, 17.000xg, 30 min) to create a pellet material and a supernatant (fractions listed in Table 2-4). These two fractions were collected separately and prepared for freeze-drying. The pellet and supernatant at pH 4.5 had to be adjusted to pH 6 because of freeze-dryer constraints for acids. After freeze-drying the material was milled or crushed depending on the hardness in order to create a homogeneous powder. This powder was used for further analyses.

Table 2-4 Fractions produced in the various treatments.

Treatment	pH (-)	Sample	Total (g)	Total (%)	pH (-)	Sample	Total (g)	Total (%)
Raw	4.5	pellet	472	34	6	pellet	318	22
	4.5	supernatant	918	66	6	supernatant	1,117	78
30 s	4.5	pellet	381	28	6	pellet	463	35
	4.5	supernatant	958	72	6	supernatant	868	65
60 s	4.5	pellet	448	30	6	pellet	699	43
	4.5	supernatant	1,069	70	6	supernatant	923	57
300 s	4.5	pellet	468	32	6	pellet	516	38
	4.5	supernatant	988	68	6	supernatant	842	62

The ragworm products after the various treatments are different in appearance. The supernatant becomes less coloured (more yellowish) with longer blanching times (Figure 2-2). The raw ragworms produce a supernatant that is very red.

Figure 2-2 Ragworms starting material (left), ragworms after blanching (centre), ragworm fractions after drying (right).

2.3 Conclusions

By processing 700 kg mussel, 13.2 kg freeze-dried pellet (2% of starting material) and 5.6 kg freeze-dried (0.8% of starting material) supernatant were obtained. These amounts were sufficient to evaluate food applications (pellet) and to perform an in-vivo shrimp trail in 2024 (supernatant), as part of the PPS project.

Desalting the supernatant using nanofiltration with a low MWCO membrane (600-800 Da) targeting 50% salt reduction is technically feasible. Both nitrogen-containing components and bioactive compounds were only partly retained. The retention factor for nitrogen-containing components was 0.7, resulting in an 80% yield. For the bioactive compounds, no retention factor could be derived from the experimental data, as the observed bioactivity can most likely be attributed to multiple components with a wide range of molecular weights. It can be concluded that by desalting (50% salt removal) using nanofiltration, part of the bioactive compounds and nitrogen-containing components are lost (~20%). Higher yields (> 80%) may be obtained by using a membrane with a lower MWCO (300 Da). The economics eventually determine how much loss of nitrogen-containing and bioactive components is acceptable.

TGA as alternative method for DM and ash measurement in small sample volumes (< 1 mL) appears to be unsuitable for determining low ash or dry matter contents (<1% (w/w) in model solutions). For higher contents, the accuracy of TGA for dry weight (dw) determination in model solutions is within 0.5%, with 0-30% deviation from the true value. Applying the TGA measurement on freeze-dried samples of different NF fractions (with complex composition) did not yield results consistent with the current standard oven method. Further method development is required for samples with complex composition, and it is recommended to investigate whether freeze-drying may have influenced the DM and ash measurement with the TGA.

The process developed for mussels was applied to ragworms, under various conditions (blanching time and pH). The conditions influenced the moisture loss, and the colour of pellet and supernatant. The fractions were assessed on bioactive properties (section 3.2.2).

3 Bioactive fraction

3.1 Introduction

The following research question was addressed by the activities described in this chapter:

- What can we learn about the underlying mechanism by analysing the liver and spleen obtained from last year's successful in-vivo trial using RNAseq?

3.2 Results

3.2.1 RNAseq

The liver and spleen obtained from the successful in-vivo trial in 2022 were used for RNAseq to further unravel the underlying mechanisms that could play a crucial role in the alleviation of anaemia in sole fed mussels, mussel extract B and ragworm. The results are shown in detail in Annex 2.

3.2.1.1 RNA extraction from liver and spleen tissue

Liver and spleen samples were flash frozen in liquid nitrogen immediately after collection and stored at -80°C until further processing. A small piece of tissue sample was cut and placed in an Eppendorf tube with Qiazol lysis reagent (Qiagen, Cat. No. / ID:79306) and a 5 mm stainless steel bead (Qiagen, 69989). The tissue samples were lysed using a tissue lyser (Qiagen) for 3 min at 20 Hz, repeated twice. The lysate was centrifuged at 12000xg and the supernatant was added to a tube with chloroform and gDNA eliminator. The sample was mixed by vortexing and then centrifuged for 15 min at 12000xg. Next, the aqueous phase was placed in a new tube, and the RNA was extracted according to the protocol of the RNeasy Plus Universal Mini kit (Qiagen, Cat. No./ID: 73404). Afterwards, the concentration was checked with a Qubit BR RNA assay kit (Thermo Fisher Scientific, Q10211) and RNA quality with the Bioanalyzer (Bioanalyzer kit: Agilent RNA 6000 Nano Kit Part Number 5067-1511).

3.2.1.2 RNA sequencing, processing and analysis

RNA sequencing was performed by Novogene Co., Ltd (Beijing, China), with an average of 20 M reads and approximately 6 Gb of data per sample. Libraries were prepared using poly-T oligo-attached magnetic beads and sequenced on an Illumina platform, generating 150-bp paired-end reads. Quality control included removal of low-quality reads, adapter sequences, and ambiguous bases, ensuring high data quality (Q30 > 92%). Processed data were delivered in FASTQ format and used for downstream analyses. Raw RNA-seq data were processed using FastQC and MultiQC to assess quality. Adapter sequences and poly-A tails were trimmed using TrimGalore, followed by a second round of quality checks. Reads were aligned to the reference *Solea solea* genome (GCA_958295425.1) using STAR with optimized alignment parameters. Gene expression was quantified with RSEM, generating expected counts, TPM, and FPKM values for each sample. For Gene Set Enrichment Analysis (GSEA) with the KEGG database, reads were also aligned to the Solea senegalensis genome (GCF_019176455.1).

The statistical analysis was performed in R (v4.2.0) with the following packages: DESeq2, ComplexHeatmap, vegan, tidyr, reshape2, ggplot2, edgeR, VennDiagram, org.Ss.eg.db, clusterProfiler, and AnnotationDbi. Data visualization included PCA plots, heatmaps of the top 1,000 variable genes. Differentially expressed genes (DEGs) were identified using DESeq2 with an FDR-adjusted p-value < 0.05 and |log2FC| > 1. Enrichment analysis was performed to explore pathways influenced by dietary treatments.

3.2.1.3 Sampling and study design

A total of 90 samples were collected from juvenile *Solea solea*, consisting of liver and spleen tissues from 15 fish per dietary treatment distributed across 3 tanks per treatment. The dietary treatments included ragworm (positive control), commercial pellet (CPEL, negative control), and mussel extract B (ME_B, experimental, supernatant, batch 2022). Liver and spleen tissues were selected for their respective roles in metabolism, erythropoiesis, and immune function.

3.2.1.4 Liver samples analysis

RNA sequencing generated approximately 23 million paired-end reads per sample mapped to the *S. solea* reference genome. Quality metrics, including duplication rates, quality scores, and adapter content, were within acceptable ranges for all samples. PCA was performed to visualize clustering patterns in the gene expression data. Two samples (S8 and S22) exhibited anomalous GC content and were removed based on PCA and heatmap clustering analyses. The PCA revealed treatment-based clustering, with a notable overlap between the ragworm and ME_B groups, suggesting shared gene expression profiles between these diets. Additionally, clustering by tank revealed a strong grouping of samples within individual tanks, indicating a significant tank effect on gene expression. After filtering, 14,609 of the original 36,761 genes (39.7%) were retained for downstream analysis. Genes were filtered to keep those with at least 10 counts in 14 or more samples, corresponding to the smallest treatment group size after outlier removal.

Differential expression analysis identified:

- ME_B vs ragworm: 0.68% of the filtered genes were differentially expressed, split evenly between upregulated and downregulated genes;
- ME_B vs CPEL: 1.76% of the filtered genes were differentially expressed, with 64% upregulated and 36% downregulated in ME_B compared to CPEL;
- Ragworm vs CPEL: 1.26% of the filtered genes were differentially expressed, with approximately equal proportions of upregulated and downregulated genes.

A Venn diagram of the significant DEGs revealed patterns of overlapping and unique gene expression profiles across treatments. The overlap between the ME_B vs ragworm and ME_B vs CPEL contrasts represented 34% and 13% of the total DEGs in these comparisons, respectively, likely reflecting pathways specifically influenced by the ME_B diet. The overlap between the ME_B vs ragworm and ragworm vs CPEL contrasts accounted for 15% and 8% of the total DEGs, respectively. Finally, the overlap between the ME_B vs CPEL and ragworm vs CPEL contrasts comprised 26% and 36% of the total DEGs, respectively.

Significant differences in gene expression were observed between the experimental diet ME_B and the commercial pellet CPEL. Genes associated with iron homeostasis and erythropoiesis showed altered expression patterns, suggesting distinct regulatory activities between the diets. Additionally, genes involved in lipid metabolism were upregulated in the ME_B group, while those linked to inflammatory processes were downregulated. Increased expression of genes associated with cell proliferation and better oxidative stress management was also observed in the ME_B-fed fish. The ME_B and ragworm diets exhibited distinct expression profiles in genes related to lipid and nitrogen metabolism, as well as stress response regulation. Variations in gene activity suggested differences in cholesterol metabolism and nitrogen processing. Expression patterns also indicated differences in muscle growth regulation and response to stress between the two groups. Finally, the gene expression patterns between the ragworm and CPEL diets highlighted the modulation of iron regulation and stress response pathways. The ragworm diet showed enhanced activity in iron-regulating pathways, contrasting with patterns observed in the CPEL group. Differences in metabolic and stress-response pathways further suggested diet-induced physiological adjustments.

3.2.1.5 Spleen samples analysis

The RNAseq spleen dataset comprehends 36,761 genes. After filtering to retain genes with at least 10 counts in a minimum of 15 samples (the smallest group size in the dataset), 16,539 genes (45%) were included for downstream analysis. Unlike the liver, no outliers were detected, and PCA revealed less distinct clustering with greater variability among individual samples.

Differential expression analysis showed fewer DEGs than the liver samples:

- ME_B vs ragworm: less than 0.1% of genes were differentially expressed, all upregulated in the ME_B;
- ME_B vs CPEL: 0.19% of genes were differentially expressed, with 19% upregulated and 81% downregulated in the ME_B group compared to CPEL;
- Ragworm vs CPEL: 0.18% of genes were differentially expressed, with 13% upregulated and 87% downregulated in the ragworm group compared to CPEL.

Key genes identified in the spleen were involved in modulating immune function, inflammation, coagulation, and nutrient absorption and supporting metabolic adjustments. These findings align with the spleen's critical role in immune surveillance and regulating erythrocyte and nutrient metabolism. However, the spleen samples exhibited a very small number of DEGs and overlapping clusters in the PCA plot, suggesting limited sensitivity of this organ to the dietary treatments included in this study compared to the liver. The liver plays a more prominent role in metabolic regulation and is likely a key organ in alleviating anaemia in *S. solea*. As a result, subsequent analyses focused primarily on the liver samples, as they provided more informative and reactive insights into the dietary impacts.

3.2.1.6 GSEA

Gene Set Enrichment Analysis (GSEA) was conducted for the liver samples to gain deeper insights into the biological pathways and processes influenced by dietary treatments. The analysis utilized both the *S. solea* (GCA_958295425.1) and *S. senegalensis* (GCF_019176455.1) genome annotations to ensure comprehensive pathway coverage, particularly to include KEGG pathway analysis. DEG re-analysis using the *S. senegalensis* genome produced highly consistent results with those from *S. solea*.

The GSEA revealed significant enrichment in pathways associated with iron metabolism, lipid metabolism, and oxidative stress regulation. The ME_B vs CPEL comparison highlighted pathways such as fatty acid transport, blood microparticle activity, and oxidative processes, suggesting a possible enhanced energy metabolism, alleviation of anaemia, and reduced inflammatory status in ME_B-fed fish. In the ME_B vs ragworm comparison, pathways related to cholesterol metabolism, nitrogen processing, and stress responses were prominent, indicating distinct metabolic adaptations between the two diets. Including KEGG pathway analysis further expanded these insights, revealing enrichment in pathways such as the MAPK signalling pathway, fatty acid biosynthesis, and proteasome activity for ME_B-fed fish. Lastly, the GSEA of ragworm vs CPEL showed enriched pathways, including iron ion binding, heme binding, fatty acid biosynthesis, and xenobiotic metabolic processes. These findings suggest enhanced iron metabolism and oxidative stress regulation in ragworm-fed fish compared to those on the CPEL diet.

Overall, the analyses revealed distinct pathway enrichment patterns associated with each dietary comparison, emphasizing differences in lipid metabolism, iron homeostasis, and stress-related processes among the three diets.

3.2.2 Bioassay

Several studies have shown that antioxidants can have a beneficial effect on oxidative stress-related haemolytic anaemia [Saputra *et al.*, 2020; Mas'adah & Djaelani 2019; Buka *et al.*, 2018; Sènou *et al.*, 2017]. This finding, and the anti-oxidative properties of particular small peptides [Lorenzo *et al.*, 2018; Chai *et al.*, 2017; Chi *et al.*, 2015], serve as the basis for the processing of low trophic marine resources to concentrate such peptides, and their cumulative antioxidant activity, in the supernatant. Therefore, in the first part of this project, the antioxidant capacity of mussel fractions (supernatant and pellet) and ragworm (whole) was measured using the DPPH assay. The EC50 of the supernatant showed a higher numerical value than the pellet (2.2 mg/mL compared to 1.1 mg/mL) and the ragworms did not show any DPPH antioxidant capacity. These values did not correlate with the results obtained in the in-vivo trail [Kals *et al.*, submitted].

The RNAseq results have been used to look for other possible pathways or key-enzymes that might be involved in the improved health status of common soles fed with mussels or ragworms (alleviate anaemia). One of the results of the RNAseq analysis is an upregulation of genes that are involved in oxidative stress response. As enzyme X was upregulated, we have measured the X amount in the mussel supernatant

fraction, the mussel pellet fraction and all ragworm fractions from section 2.2.3 using a commercial kit. The X content was expressed as µM X per mg dry matter.

All total X results can be found in Figure 3-1 for ragworm and in Table 3-1 for mussel. In the ragworm fractions a higher X content was found than in the mussel fractions. The X content appeared to be higher in the ragworm supernatant than in the pellet, the same result was found for the mussel. These results are promising as they correlate with the in-vivo results showing an alleviation of anaemia by both mussel and ragworms. The improvement was better with the mussel supernatant fraction than the pellet and with ragworms the improvement was better than with mussel [Kals *et al.*, submitted].

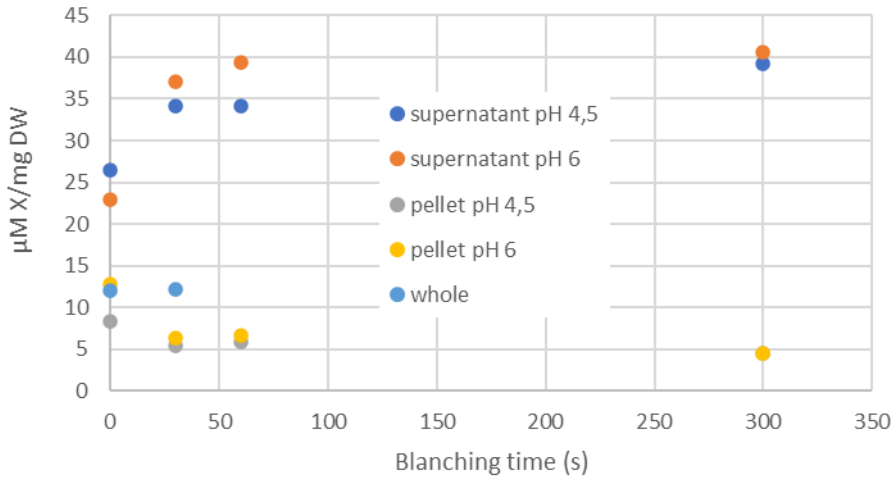

Figure 3-1 X content in ragworm and ragworm fractions (pellet and supernantant) as function of blanching time and pH.

Table 3-1 X content in mussel fractions.

Mussel samples	µM total X per mg dry matter	
	Supernatant	Pellet
Mussel (batch 2022)	7.6	4.6
Mussel (batch January 2024)	17.5	9.9

In the assay the total X amount is measured, including the oxidized X. Since the enzyme X is upregulated, the oxidized form is not used by the enzyme. It is therefore recommended to exclude oxidized X from the bioassay, which requires a relatively simple adaptation of the assay.

3.3 Conclusions

The liver emerged as the more responsive organ to dietary interventions, compared to the spleen, showing significant alterations in gene expression related to iron homeostasis, lipid metabolism, and oxidative stress regulation. These findings emphasize the liver's critical role in alleviating anaemia and supporting metabolic adaptations. Gene Set Enrichment Analysis (GSEA) highlighted enriched pathways linked to enhanced metabolic performance, erythropoiesis, and reduced inflammation in fish fed with the diet supplemented with the bioactive mussel fraction compared to fish fed with the commercial pellet. The diet supplemented with the bioactive mussel fraction consistently outperformed the commercial feed in improving hematopoietic responses and showed distinct but complementary effects compared to ragworm, which remains the most effective at addressing anaemia and supporting growth. These results underscore the potential of the bioactive mussel fraction as a functional feed ingredient to enhance fish health and performance under anaemia conditions. Future studies should explore the synergistic effects of combining the bioactive mussel fraction with other diets and conducting bioassays to validate the molecular mechanisms underlying these findings.

The total X content in mussel and ragworms shows promising results as it correlates to the in-vivo results with common sole. X analysis could potentially be used as a bioassay for indicating whether low marine resources can alleviate anaemia in common soles. It is recommended to measure the X levels in other shellfish and compare these with literature data on alleviation of anaemia. Furthermore, it is recommended to setup an in-vivo trial with common sole, including added X in the commercial feed, to show whether X is (one of) the compound(s) in mussel and ragworm that alleviates anaemia in sole.

4 Protein-rich fraction

4.1 Introduction

To assess the potential of the protein-rich pellet fraction from mussel within food applications, two activities were carried out:
- Experiments were performed to determined which kind of protein functionality was present;
- A search in the Innova database was carried out to obtain an overview of possible applications.

4.2 Results

The results are shown in detail in Annex 3.

4.2.1 Assessment of protein functionality

For the assessment of the protein functionality of the protein-rich fraction, several tests were carried out: determining the emulsifying and foaming capacity, heating to obtain an indication of gelling capacity, making structured products by applying shear by hand, kneading and the use of a pasta machine, freeze texturizing to create fibre-like structures, and some tests adding transglutaminase as a crosslink agent for gelling. In addition, Differential Scanning Calorimetry (DSC) was used to determine the enthalpy change (thermal denaturation), which is a measure for protein denaturation.

The emulsifying capacity for both oil-in-water and water-in-oil mixtures, tested at different pH's, was limited. The emulsions were unstable, and it was decided not to pursue this further. The same was true for gelling and foaming. The application of shear caused some structure formation in the farinograph (kneading): it was possible to produce "lasagna" sheets using the pasta machine, that could be boiled without falling apart, and "spaghetti" by using shear by hand that did not fall apart in water. It was not possible to create a fibre-like structure using freeze texturizing and the addition of transglutaminase had no noticeable effect. The results of the DSC showed almost no peak, indicating a very low amount of native protein.

It can be concluded that the protein-rich fraction (pellet) is low in solubility, with limited emulsifying, foaming and gelling properties, possibly because the latter three functionalities require soluble protein (which is absent in the insoluble pellet fraction). Still, the fraction has high water holding capacity, and stable sheets could be made using this fraction. It could also serve as a filler in foods. Furthermore, it can be used to increase the nutritional value. The colour and flavour are intense and umami-like, which may be an advantage in fish-like applications.

During centrifugation as part of mussel processing, a fibre-like structure was observed to form in the pellet looking similar to the adductor muscles of the mussel. Since this functionality was not observed in the freeze-dried fraction, either the freezing, the batch or age of the sample (pellet produced in 2022 was used for these tests) has an effect on its functionality. However, it does show possibilities to further broaden the application of the protein-rich fraction.

4.2.2 Possible food applications

Based on the Innova database, various application routes for mussel extract have been identified:
- As a filler: pasta, cookies, kroepoek, crackers, bread, meat replacer, coating (breadcrumbs, batter) and in extruded snacks;
- As a flavour: flavouring in sauce, soup, crisps, as savoury drink and in spreads;
- Other: pet food, jerky-type products/biltong and in supplements.

If the gelling properties could be increased (possibly by using the frozen instead of freeze-dried extract), a surimi-type product may also be of interest. The number of product introductions with mussel as an ingredient is increasing, with most introductions in the United States, Spain and Germany. This could be due to the rising awareness of the health benefits of mussels.

4.3 Conclusions

The protein-rich fraction is low in solubility, with limited emulsifying, foaming and gelling properties, probably because these functionalities rely mostly on native, soluble proteins. However, stable sheets could be formed, and a fibrous structure was observed during processing. Also, this insoluble (pellet) fraction can be used as a filler in foods and simultaneously increase the nutritional value. Furthermore, the colour and flavour are intense and umami-like, which may be an advantage in fish-like applications.

5 Chemical safety

5.1 Introduction

Microplastics are widely present in the marine environment and can end-up in fishery products such as bivalve mollusks [Ajith *et al.*, 2020; Alberghini *et al.*, 2022]. Microplastics, also referred to as synthetic polymers, can be found in many different shapes, colours and sizes. In marine life, it can be expected to find polyethylene terephthalate (PET), polypropylene (PP), polyethylene (PE), polyamide (PA) and polystyrene (PS) from the use of fishing nets [Andrady, 2011].

Other highly persistent contaminants in the environment are PFAS (per- and polyfluoroalkyl substances). PFAS are chemical compounds generally applied as water and grease repellents such as coatings for furniture and clothes, but also in flame retardants. The production and usage of PFAS accounts for their spread in the environment. Short-chain PFAS are more water soluble and are therefore expected in water, whereas long-chain PFAS are expected in (fatty) fishery products, meat and eggs. Maximum levels of four legacy PFAS and their sum in food, namely perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA) and perfluorohexane sulfonic acid (PFHxS), are set out in Commission Regulation (EU) 2023/915 [EC, 2023].

5.2 Results

5.2.1 Microplastic analyses using µFTIR of the processed fractions

The methods fluorescence microscopy (µFL), micro fourier transform interferometry (µFTIR) and pyrolysis–gas chromatography–mass spectrometry (Py-GC/MS) were explored for the analyses of microplastics in mussel pellet and supernatant. Unfortunately, microplastic analyses could not be performed in raw mussel due to the unavailability of raw mussel. Target limitations with µFL (no identification of detected polymers) and technical limitations with Py-GC/MS led to the decision to use µFTIR. With µFTIR, microplastics with a size between 50-500 µm could be detected and identified using a combination of optical imaging (microscopy) and chemical imaging (µFTIR).

For the analyses using µFTIR, 2 g of each sample was used. Sample preparation consisted of alkaline digestion using potassium hydroxide (KOH, 10%) at 50°C overnight with mild shaking. This was followed by wet peroxide oxidation (H_2O_2, 30%) with Fenton's reagent ($FeSO_4$) at pH 2.5. After digestion, the samples were filtered using a stainless steel filter (10 µm pore size) and a final filtration step using an aluminium oxide filter (Anodisc, 0.2 µm pore size). Particle count and particle size distributions were determined by individually evaluating the microscopy and FTIR images. It was decided to focus on the presence of the polymers PET, PP, PE, PA and PS. The negative control consisted of the chemicals used during the digestion. Due to the laborious method and long acquisition times, the positive controls were not measured together with the samples. Positive controls (PE, 125-150 µm) were measured during the method development.

KOH successfully digested most of the sample organic material, and the consecutive wet peroxide oxidation step was necessary to prevent clogging of the Anodisc filter and significantly improve filterability. The optical images of the mussel pellet and supernatant (Figure 5-1 and Figure 5-2, respectively) showed the presence of mainly polymeric fibres, but also minerals and sand. In the mussel pellet, 3 PP, 1 PE, 2 PS and 1 PA fragments were found. In the mussel supernatant, 11 PP, 2 PS and 1 PA fragments were found. The size of particles indicatively ranged from 75-500 µm. The negative control (Figure 5-3) showed background contamination of PP (2 particles). More details on the results are shown in Annex 4.

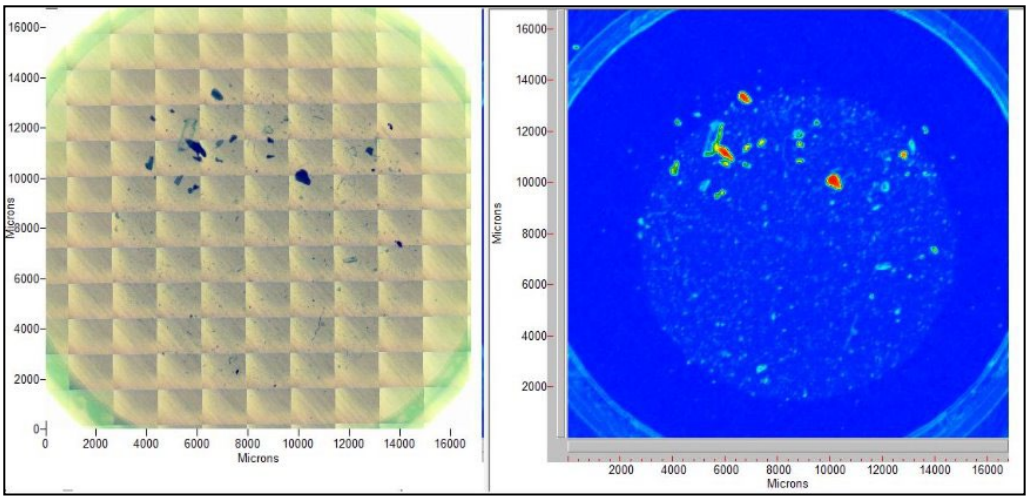

Figure 5-1 Optical image (left) and chemical image (right) of digested mussel pellet.

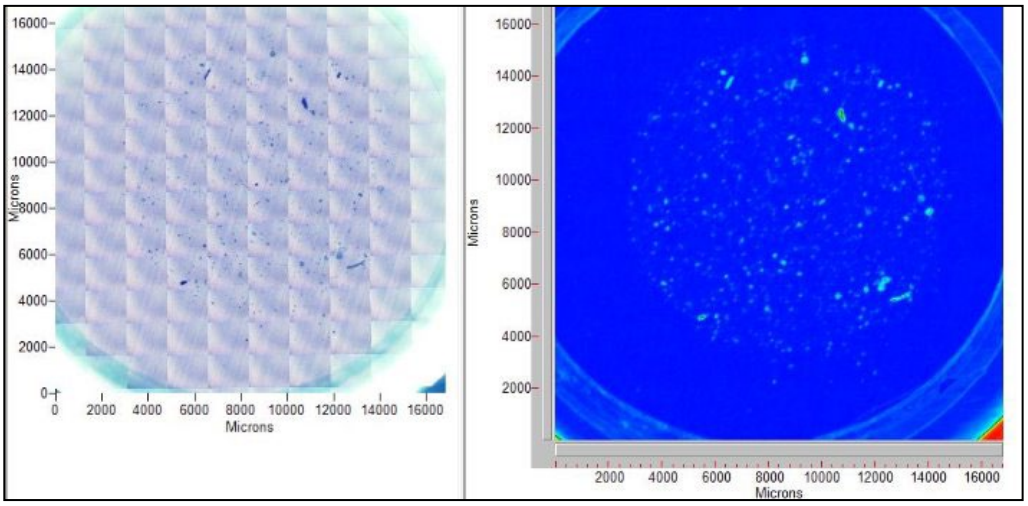

Figure 5-2 Optical image (left) and chemical image (right) of digested mussel supernatant.

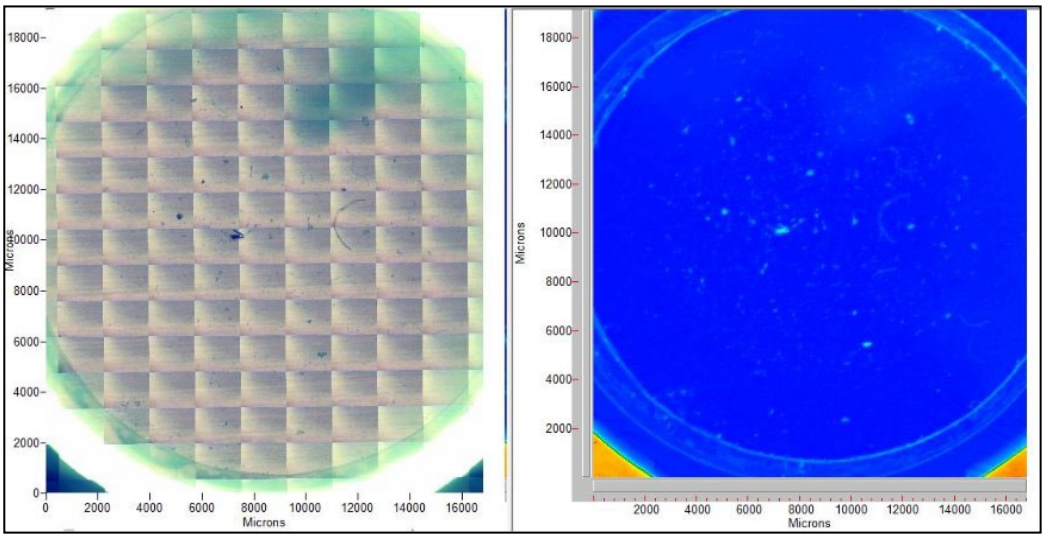

Figure 5-3 Optical image (left) and chemical image (right) of chemical blank (negative control).

5.2.2 PFAS analyses of raw material and processed fractions

The raw mussel was first cryogenically grinded to obtain a homogenous sample. The sample was processed and analysed according to the monitoring method of PFAS in fish and shellfish products. 1 g of sample was weighed in a 50 mL Greiner tube and 25 µL internal standard (100 ng/mL) was added for quality control and quantification. The sample was extracted with 10 mL methanol and vortexed for 1 min, followed by shaking

for 30 min and centrifugation. The clear supernatant was transferred to a SPE column (Strata-X-AW Polymeric WAX (200 mg/6 mL, 33 µm, Phenomenex)) and underwent solid phase extraction (SPE) using 5 mL 25 mM sodium acetate buffer and 3 mL 0.04 M HCl in MeOH. Next, the extract was dried and reconstituted in a buffer solution. An injection standard solution was added for quality control, and the samples were then analysed using LC-MS/MS. Quantification was performed using a solvent-based calibration curve.

For the mussel pellet and supernatant a not formally validated method for PFAS detection in animal feed was applied. Approximately 1 g of dry sample was weighed into a 50 mL centrifuge tube, and 5 mL of LC-MS water was added. An internal standard mix was introduced for quality control and quantification. The samples were extracted with 10 mL of acetonitrile, vortexed for 1 min, and then ultrasonicated for 15 min. Following this, the tube was shaken for 30 min and centrifuged. The clear supernatant was transferred to a 50 mL Greiner tube, and the extraction process was repeated with an additional 10 mL of acetonitrile. The second extract was combined with the first in the same 50 mL Greiner tube. To prepare for further clean-up, 8 mL of LC-MS water was added to a clean 15 mL centrifuge tube, and 5 mL of the sample extract was transferred into this tube. The final solution underwent SPE using weak anion exchange and graphitized carbon black for clean-up. After the clean-up process, the extracts were dried and reconstituted in a buffer solution. An injection standard solution was added for quality control, and the samples were then analysed using LC-MS/MS. Quantification was performed using a solvent-based calibration curve.

A slightly higher concentration of PFOSA was found in mussel pellet compared to the supernatant (Table 5-1), which could be attributed to the hydrophobic character of PFOSA [Wang *et al.*, 2011]. Overall, the concentrations and sum of all four legacy PFAS did not exceed the maximum levels set for crustaceans and bivalve mollusks (5 µg/kg ww) in all three matrices (Table 5-1). For the sum of the four legacy PFAS, maximum levels refer to lower bound concentrations. With this, concentrations are calculated following the assumption that all the values below the limit of quantification are zero.

Table 5-1 Concentration of PFOSA and the four EFSA legacy PFAS, PFOS, PFOA and PFNA and PFHxS in fresh mussel, mussel pellet and supernatant (µg/kg ww).

	PFOSA	PFOS	PFOA	PFNA	PFHxS	Sum of PFOS, PFOA, PFNA and PFHxS
Raw mussel	-	0.22	<0.1	<0.1	<0.03	0.22
Mussel pellet	1.87	0.93	0.06	0.076	0.03	1.10
Mussel supernatant	1.44	0.38	0.05	<0.08	<0.03	0.43
Maximum limit in crustaceans and bivalve mollusks	-	3.0	0.7	1.0	1.5	5.0

As PFOSA is not a regulated contaminant [EC, 2023], it was not analysed in the raw mussel sample using the monitoring method of PFASs in fish and shellfish products. Therefore, no mass balance calculations could be made. For PFOA, PFNA and PFHxS also no mass balance calculations could be performed as they were measured below the limit of quantification in the raw mussel sample. Hence, Table 5-2 shows a nearly complete mass balance for PFOS only.

Table 5-2 PFAS mass balance of the three mussel fractions (dw).

	Raw mussel (µg/kg dw)	Mussel pellet (µg/kg dw)	% starting material	Mussel supernatant (µg/kg dw)	% starting material	SUM%
PFOS	1.37	0.95	69.2	0.40	29.1	98.3

5.3 Conclusions

The mussel pellet and supernatant were successfully analysed on microplastics using the µFTIR method, confirming the presence of polypropylene and detecting other polymers as well. The method's LOD size does not allow for the detection of polymeric structures smaller than 50 µm and one should consider the presence of 'background' microplastics. The obtained results for microplastics are indicative, given the limited number of samples. To ensure more robust results, a wide variety of samples should be analysed, including negative and, if possible, positive controls. However, positive controls may pose challenges due to the environmental polymeric contamination of the bivalve mollusks, which includes a broad number of polymers and sizes. Additionally, Py-GCMS is recommended to determine the polymeric fraction < 50 µm. At the moment, no legislation exists to establish the maximum levels of microplastics in food or feed.

Measured concentrations of the four regulated PFAS and their sum in raw mussel, mussel pellet and supernatant did not exceed the legal limits set for crustaceans and bivalve mollusks within the EU. Although the method applied on the mussel pellet and supernatant was not formally validated, rigorous quality control measures were implemented to ensure the accuracy of the results. The data showed consistent and reliable outcomes, with no significant deviations. Despite the method's relatively low recovery, substantial traces of PFOSA were detected. No maximum limits exist for PFOSA in crustaceans and bivalve mollusks. However, it is interesting to measure PFOSA levels as it is a precursor for PFOS.

6 Outlook

6.1 Introduction

In this chapter the potential productivity, seasonal variation in composition, and seasonal variation in quality (based on composition) is evaluated for mussel production scenarios. Secondly, a scan is made of the potential and boundaries for the productivity of a selected low trophic marine resource (e.g. *Ensis sp*p).

6.2 Results

6.2.1 Case study outlook upscaling mussel production for total use

The Dutch shellfish industry, particularly the mussel sector, plays a significant role in national aquaculture. Recent years have seen a decline in mussel production, with an average annual yield of 43,000 tons between 2016 and 2020 [Agrimatie, 2022]. The blue mussel (*Mytilus edulis*) remains the predominant aquaculture product, cultivated primarily in the Wadden Sea and Oosterschelde. In 2019/2020, approximately 33,000 tons of mussels were produced using bottom cultivation methods, with two-thirds originating from the Oosterschelde and one-third from the Wadden Sea. In 2021/2022, production reached 33,000 tons, generating 66 M€ in revenue. However, fluctuations in production and market prices due to factors like mortality, nutrient availability, and plot suitability have led to variability in sector performance.

In the current Shellfish policy [LVVN, 2023] and the ambitions for Food from the Sea (Visie Voedsel uit Zee [LVVN, 2024]), both the industry and government have set ambitions to sustain or increase the production of low trophic species (primarily mussel/oyster and seaweed). Production in current production areas is seen as limiting due to the complexity in the production scale-up, therefore new production areas are being explored. The mussel sector has an agreement with governments and NGOs on the transition of mussel cultivation. This agreement focuses on improving productivity, sustainability and production perspective, with an emphasis on new methods and new production areas.

In the Netherlands, mussel seed is usually collected in important nature reserves, where for example (breeding) birds also depend on the mussel supply. The number of required/usable cultivation plots depends on four factors [Smaal *et al.*, 2010]:

- The supply of mussel seed;
- Growth and survival on the breeding plots;
- The need for mussel seed for cultivation in other areas;
- Harvest for market supply.

The size of the stocks present (the biomass) is variable in time and space, which influences the density and distribution of the stock, which in turn influences the quota of mussel brood (mussel seed). The amount of available seeds is important for the mussel sector. The government sets quotas based on an annual assessment of the amount of mussel seed present. Seed mussel collection systems (SMCs) are used to reduce the dependency on wild mussel seed [Smaal *et al.*, 2019]. In the Netherlands, mussels are mainly cultivated on bottom plots. Suspended cultures only form a limited percentage of the production volume. Mussels are cultivated in the Wadden Sea and Oosterschelde, after which the production is transferred to the processing companies, the majority of which are concentrated in the southwestern region around Yerseke.

The biggest challenge for the mussel sector to realize an increase in production efficiency and production potential is the lack of available mussel seed and sufficient qualitative production grounds. Part of the production has been optimized in the past decade by research into the development of mussel seed capture systems. In addition, it is being explored whether there are also opportunities outside the existing production areas, which may become available due to the change in spatial organisation of the North Sea.

The Dutch government has set itself the goal of transforming the North Sea into an area where food production, renewable energy, nature conservation and CO_2 storage can be combined by 2050 [Strietman *et al.*, 2019]. Wind farms are being built in the Dutch economic exclusive zone (EEZ) for sustainable energy. The goal is to increase energy production from wind farms from 4.5 GW to 11.5 GW between 2024 and 2030. This means that increasingly larger areas of the Dutch EEZ are reserved for these wind farms [Rijksoverheid, 2018]. There is a North Sea Agreement in which agreements are made about developments in the field of food from the sea. The government is exploring examples such as growing seaweed in the North Sea and the use of seed mussel collectors. Therefore, the possibilities of multifunctional use of wind farms are being examined [Rijksoverheid, 2022]. Developments such as offshore wind farms can offer opportunities to implement cultivation steps outside the current cultivation plots or to produce feasible smaller mussels offshore. This report takes into account the fact that the current mussel sector can change to a more offshore-oriented industry. At the moment there are uncertainties about the economic feasibility and the future market absorption. Different trajectories around square value of mussels are explored in order to generate an improved market perspective, fitting the challenges of the future (changing consumer, market and production).

At the moment there are many scenarios to bring about an improvement and maximization in mussel production. However, a scalable scenario over a period of 10-20 years is chosen. In this, a doubling of the current sector is seen as feasible despite uncertainties. Scaling up mussel production will be accompanied by a change in the production chain and the production/processing strategy. The market for fresh mussels may change (but probably not grow significantly), which means that changing processing scenarios must certainly be taken into account for future developments. Since there is no specific consensus on the future of the current market, various scenarios have been developed. In this study, maximum focus is placed on processes that contribute to the separation of product and the concentration of the by-product stream (specifically the shell).

In this study we assume 37,500 tons as a reference production volume for the current supply chain. In addition, there is a supply of 37,500 tons of production from import (supply from Germany, Denmark, Ireland and the United Kingdom). Together, the reference volume for production and processing in the Netherlands is 75,000 tons. In a study exploring the potential for shell utilization [Foekema *et al.*, 2024] several scenarios were developed to assess the feasibility of processing increase for mussels (Table 6-1):

- No increase in production (current status: 37,500 tons Dutch production plus 37,500 tons supply from import);
- No increase in production, but a change in the percentage of processed product for total use principles (50%);
- Production increase 50% (increase 37,500 tons), processed product for total use principles (100%);
- Production increase 100% (increase 75,000 tons), processed product for total use principles (100%).

The production increase should take place in the North Sea or Voordelta (as the most suitable location). The uncertainties for mussel production increase are high and currently considered mainly in economic, legal and social barriers. In the case studies we assume a processing potential for total use purposes of 9,750 tons of mussel flesh. According to the Nutritional standard table, flesh contains 83.4 % water, 9.9% protein, 3% total lipid (fat), and 2% carbohydrates [Voedingswaarde table, 2024]. This indicates an availability of 956 tons protein, 293 tons total lipid (fat) and 195 tons carbohydrate in two scenarios (current production with redistribution of processing, and 50% production increase; Table 6-2). In the scenario for upscaled mussel production with 75,000 tons increase and 100% total use application, 1,911 tons protein, 585 tons total lipid (fat) and 390 tons carbohydrate is available. These indications will be used to further elucidate the economic, technical and production potential for mussel total use upscaling.

End products can consist of various processed products such as:

- Mussel products based on mussel meat (cooked or uncooked mussel material);
- Mussel extracts (e.g. protein, oil, specific extracts);
- Mussel oil and mussel fat purified;
- Protein concentrates;
- Traditionally processed products (cooked, frozen).

Table 6-1 Scenarios for mussel production in the Netherlands. The reference scenario is considered as 37,500 tons Dutch shellfish production, and 37,500 tons imported shellfish for processing (raw product). An average of 26% flesh content is assumed for the calculation of whole mussel to flesh (ww).

	Reference production volume (tons whole mussel)	Additional production volume (tons whole mussel)	Additional production volume for fresh market (tons whole mussel)	Additional production for processing (tons whole mussel)	Additional production for processing (tons flesh ww)
No increase in production (37,500 tons Dutch production, 37,500 tons supply from import)	75,000	0	0	0	0
No increase in production, but changed percentage of processed product (50%)	75,000	0	-37,500	37,500	9,750
Production increase 50%	75,000	37,500	0	37,500	9,750
Production increase 100%	75,000	75,000	0	75,000	19,500

Table 6-2 Scenarios for mussel production in the Netherlands. The reference scenario is considered as 37,500 tons Dutch shellfish production, and 37,500 tons imported shellfish for processing (raw product). An average of 26% flesh content is assumed for the calculation of whole mussel to flesh (ww). Composition assumed: water 84.2%, protein 9.8%, lipid 3%, and carbohydrates 2% [Voedingswaarde tabel].

	Reference production volume (tons whole mussel)	Additional production for processing flesh (tons ww)	Additional production for processing flesh (tons dw)	Additional production for processing protein (tons)	Additional production for processing lipid (tons)	Additional production for processing carbohydrates (tons)
No increase in production (37,500 tons Dutch production, 37,500 tons supply from import)	75,000	0	0	0	0	0
No increase in production, but changed percentage of processed product (50%)	75,000	9,750	1,541	956	293	195
Production increase 50%	75,000	9,750	1,541	956	293	195
Production increase 100%	75,000	19,500	3,081	1,911	585	390

6.2.2 Case study outlook razor clam production loss

To assess the potential and boundaries for the production of low trophic marine resources, a case study on razor clams (*Ensis leei*) was conducted. Historically, fisheries management is mainly focussing on market sized fisheries products. Fisheries is regulated for species and targeted size classes, which are fished and processed for direct raw or cooked consumption. These are traditionally fished based on their market value and consumer preferences for raw products. Age classes of shellfish are typically multiple years, and size classes are in accordance. When shellfish stocks are also utilised for total use principles, other considerations for material sourcing may be explored. They may be derived from different size classes if this is feasible within sustainable management practices.

Shellfish, in the natural (or fished) environment, recruit and grow under specific conditions and in specific habitats. During their life cycle, mortality of stocks occurs, which can be related to, among other things, storm losses, density dependent mortalities, predation, and fisheries mortality (harvest). Ideally, fisheries

management takes these mortalities into account for sustainable exploration of shellfish stocks. Currently, due to the lack of market potential, juvenile stocks are not directly considered in the management of fisheries or in fisheries plans. Juvenile stocks are the feed source for many birds and marine organisms, and thus need thoughtful considerations, when exploiting them. However, there can be specific interest in juvenile stocks, which are known for specific mortalities during the first year of the life cycle, like *Ensis leei*. These stocks are assessed, or more specifically the part which is currently lost due to density mortality or storm losses, for their potential to be harvested. Therefore, stocks which would not survive naturally, could be considered for fisheries. The combination of juvenile resourcing, and processing of the flesh could result in a potential new product source, while maintaining sustainable boundaries.

The life cycle of most shellfish begins when adult individuals release eggs and sperm into the water [Kamermans 2005]. Fertilized eggs rapidly develop into larvae, which later undergo metamorphosis into small shells and settle. The timing of spawning depends on the water temperature, which is 15°C for razor clams. The number of days until metamorphosis is influenced by factors such as water temperature and food availability. For razor clams, this process takes approximately 10 days at 24°C but can extend to 27 days [Gollasch *et al.*, 2015]. Survival afterward depends on predation, habitat suitability, and the temperature during the following winter.

Annual monitoring of shellfish stocks in Dutch coastal waters occurs within the framework of statutory research tasks in fisheries (the WOT Fisheries program) [Troost *et al.*, 2023]. This has been conducted since 1995 each spring, focusing on the banded wedge shell (*Spisula subtruncata*) and the American razor clam (*Ensis leei*). Data is collected at more than 800 locations, categorizing razor clams into large and small individuals. The threshold between the two is a shell width of 16 mm (measured 5 mm below the tip), equivalent to a shell length of 106 mm. Since 2010, efforts have been made to measure as many shellfish as possible, providing data on length-frequency distributions (Table 6-3).

Table 6-3 Stock of one-year-old individuals in millions of individuals (N stock) and millions of kg fresh weight (B stock).

Year	N stock (millions)	B stock (tons)
2010	48,520	50,360
2011	48,900	50,760
2012	48,500	50,350
2013	39,700	41,200
2014	52,000	53,970
2015	0	0
2016	12,100	12,560
2017	87,180	90,500
2018	21,780	22,620
2019	133,400	138,480
2020	32,400	33,640
2021	9,600	9,960

A recent cohort analysis based on these data distinguished between one-year-old and older individuals [Craeymeersch *et al.*, in prep]. The average length of one-year-old individuals ranges from 36 to 47 mm, allowing for an estimate of the population of one-year-old individuals. This was converted into biomass in fresh weight using the following parameters:

- An average length of one-year-olds of 41.5 mm;
- The relationship between length (L, mm) and width (W, mm): $L = 6.4624 \cdot W - 3.5124$ [Craeymeersch & Van der Land 1998];
- The relationship between width (W, mm) and biomass (B, g): $B = 0.0015 \cdot W^{3.3693}$ [Troost *et al.*, 2024].

Biomass varies annually: in 2015, no one-year-old individuals were observed. In other years, biomass ranged from approximately 10 million kg fresh weight (2021) to nearly 140 million kg (2019).

A strong relationship between the number of older individuals and one-year-individuals was identified. In many shellfish species, recruitment is highest near adults. This has also been observed for razor clams in Belgian waters [Houziaux *et al.*, 2011]. In the Voordelta, where fall monitoring was conducted as part of nature compensation monitoring [Prins *et al.*, 2020], young individuals (0-year-olds) were found almost everywhere [Craeymeersch *et al.*, 2015]. Spring surveys under the WOT framework revealed that one-year-old individuals and older individuals co-occur predominantly. In less suitable areas, mortality plays a significant role. However, data on the rest of the Dutch coastal zone is lacking.

This indicates there is a potential stock of razor clams, which does not survive the first year. These individuals are not allowed to harvest through regular fisheries due to their undersized status, for lack of permit for this size class. However, harvesting them for purposes other than consumption may be feasible in areas where survival is unlikely. This suggestion is analogue to sublittoral mussel seed fisheries in the Wadden Sea, where unstable stocks can be fished in the fall. The assessment of the available stocks, which may be subjective to this type of fisheries regime cannot be quantified. Data on survival is only available for a limited regions [Craeymeersch *et al.*, in prep], and is not present for the entire coastal zone. Assessment of mortality calculations require data of monitoring in fall, where typically programs focus on spring monitoring for stock assessment purposes.

In order to obtain insight into the stocks of individuals from a small size class, data from the annual monitoring of shellfish stocks in Dutch coastal waters within the framework of statutory research tasks in fisheries (the WOT Fisheries program) is used [Troost *et al.*, 2024]. The stocks are reported for shell width of 16 mm (measured 5 mm below the tip), equivalent to a shell length of 106 mm, and for larger individuals. Since stocks of larger individuals are already part of the fisheries management and regulation, our focus is to estimate indications for maximum stock availability of the small size class. In Table 6-4 shellfish stocks outside the Natura 2000 areas are used as a reference, where stocks in Natura 2000 areas are reported for potential assessment. For both, stocks in- and outside Natura 2000 areas, an indication of mortality due to fisheries (harvest) of 1.6% based on the current fisheries percentage [Keus, 2023] and 4% based on the licensed harvestable biomass for large individuals is used to estimate current biomass losses.

Table 6-4 Overview of shellfish stocks based on the Shellfish monitoring network in the Netherlands [Troost et al., 2024]. Potential biomass loss due to mortality (based on current fisheries mortality numbers) are indicative. Biomass reported in wet weight (ww) for shell and flesh. Large is a width >16 mm, small <16 mm. Current fisheries are approximately 7,000 tons/year (1.6% of available stock), the licensed harvestable biomass for large individuals is 17,500 tons/year (4% of available stock), which is extrapolated to small individuals as a reference.

	Biomass available (tons ww)	**Potential biomass loss (based on 1.6% mortality) (tons ww)**	**Potential biomass loss (based on 4% mortality) (tons ww)**
Outside Natura 2000 biomass large individuals	199,000	n.a.	n.a.
Outside Natura 2000 biomass small individuals	30,000	480	1,200
Natura 2000 biomass large individuals	142,000	n.a.	n.a.
Natura 2000 biomass small individuals	68,000	1,090	2,720
Total biomass small individuals	98,000	1,570	3,920
Total biomass large individuals	341,000	n.a.	n.a.
Total biomass	439,000	1,570	3920

The razor clam stocks, which may result in losses for production, are 470 tons (ww) outside Natura2000, and 1,090 tons (ww) in Natura2000 areas. Whether these stocks have potential as a fisheries resource should be elucidated by an appropriate assessment, or as part of an impact assessment. Including the effect and consequences for fisheries management, sustainable practices, and regulatory limitations. This overview, however, may provide a basis for discussion on new management approaches.

6.3 Conclusions

Mussel production increase is considered to take place in the North Sea or Voordelta. An additional yearly potential of 960-1,900 tons of protein, 290-590 tons of total lipid (fat) and 190-390 tons of carbohydrate is estimated, based on production scenarios as derived from earlier studies. Current policy and visions on food from the sea supports exposed and offshore production of mussels; the barriers are currently found in technical, economic, legal and social feasibility. Production within the carrying capacity of the system seems feasible. However, technological improvements, economic perspective, regulatory limitations, and social acceptance need to be developed and demonstrated.

Razor clams (*Ensis spp*) were selected as a potential new low trophic marine resource, because of stock data availability. The razor clam stocks are 470 tons (ww) outside Natura2000, and 1,090 tons (ww) inside Natura2000 areas. Analysis demonstrate that a potential harvestable stock of razor clams is available, particularly the part which does not survive the first year of their life. These individuals are currently not allowed to be harvested through regular fisheries due to their undersized status, for lack of permit for this size class. However, harvesting them for purposes other than consumption "whole shell fresh" may be feasible in areas where survival is unlikely. This is in line with the policy for the Dutch mussel industry in which unstable mussel beds may be fished as a seed source. Whether these stocks have potential as a fisheries resource should be elucidated by an appropriate assessment, as part of an impact assessment or specific modelling or ecological studies. This study explores primarily the harvesting potential based on natural production losses.

7 Literature

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Annex 1 Processing

Pilot scale processing of 700 kg mussels

Processing 700 kg mussel to obtain pellet and supernatant

Peter Geerdink

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Processing 700 kg mussels - Aim

- Reproduce previous process (300 kg mussels)
- Produce bioactive fraction – feed ingredient
- Produce protein-rich fraction – food ingredient
- ~~Study the effect of scale-up~~
- Monitor food-related pathogens while processing

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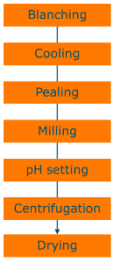
2

Processing 700 kg mussels - Method

- **Daily routine – 170 – 180 kg mussels**
- Blanching of mussels – 30 sec. 97°C
- Cooling after blanching – 3 min. running tap water
- Peeling of mussels by hand
- Milling of the mussel meat
- Setting of pH and stirring
- Separating solids and liquid with lab-centrifuge
- Freeze drying of pellet and supernatant



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Processing 700 kg mussels - Results

- Box of 10 kg = 9.9 kg mussels
- Meat yield 31% (closed & broken discarded)
- Loss is also moisture during blanching
- Processing in 1 day, 4 times
  - Shorter processing times
  - Better oversight
  - Options to alter processing when problems occur

	kg	%
Mussels	676	100
Shells	329	49
Meat	211	31
Loss	136	20

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Processing 700 kg mussels - Results

- Milling of material very easy with colloid mill
  - Smooth process. No problems
  - No temperature increase during milling
- Setting of pH and centrifugation directly after milling
  - Short processing time
  - Less time for microbial growth
  - Material frozen as quickly as possible

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Processing 700 kg mussels - Results

- Material centrifuged in 1 L buckets, 3 phases
  - Large sticky solid phase
  - Brown turbid heavy liquid phase
  - Small amount of light liquid (fat) phase
- Solid phase collected separate from liquid phase
- Both frozen in "bami-trays" for freeze drying

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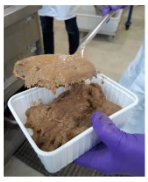
Processing 700 kg mussels - Results

- Separation
  - Meat 211 kg
    - Liquid => 75 kg (37%)
    - Solid => 129 kg (63%)
- After freeze drying
  - Total:
    - Liquid: 5.6 kg
    - Solid: 13.2 kg



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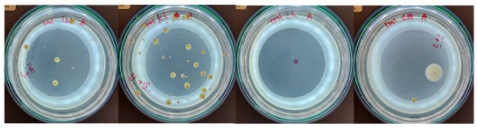
Processing 700 kg mussels - Results

- Cleaning procedures upfront assessed with PCA
  - Equipment (blancher) is clean
  - Cooling water is clean
  - Water after cooling mussels is just above drinking water threshold (log 2.3)



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Processing 700 kg mussels - Results

- Material after freeze drying assessed on microbial contamination
- Liquid phase (supernatant)
  - No contamination (below detection limit)
  - Fit for tasting
- Solid phase (pellet)
  - Small contamination
    - aerobic mesophilic flora 200 cfu/g
    - Rest below detection limit
  - Fit for tasting

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9

Processing 700 kg mussels – Conclusions

- New trial to produce material and apply learnings of trial 2023
  - Better disinfection
  - Other source of ice => running water
  - Colder processing => less heat production
  - Shorter processing time (same day, smaller batches)
  - Batch centrifugation (fixed-angle centrifuge) => works
    - What about decanter centrifuge?

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Processing 700 kg mussel to obtain pellet and supernatant – Part 2

PPS Bivalves consortium meeting, 30th May 2024

Jeroen Kals et al.

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Desalting bioactive fraction – Dialysis

KB/PPS
Dialysis experiments, Trail 2

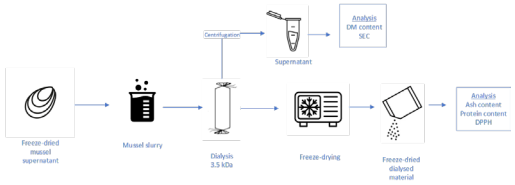
November 2023

Goal

- This freeze-dried fraction after the large-scale mussel experiment showed interesting and promising results. However, the high ash content limits the inclusion level in aquafeed. For this reason, desalting the freeze-dried supernatant is necessary. The aim is to lower the ash content from 16% to 7%, while retaining the bioactive properties of the material

Material & Method

- Freeze-dried mussel supernatant (code: Extract BS/ 23-08-2022)



- Dialysis is done with 30 g FD supernatant + 250 g demineralised water.
- Dialysis tubes of 3.5 kDa
- 24 hours in 50 L water. Water was refreshed once



Figure 1. Left: Starting material before dialysis (freeze-dried supernatant)
Middle: Slurry made of freeze-dried supernatant and water
Right: Slurry inside the dialysis tube

Results

Table 1: Results after dialysis of the freeze-dried slurry in the 3.5 kDa tube

Sample	DM content (%)	Ash content (%)	Protein content (%)	DM (g)	Ash (g)	Protein (g)	IC50* (mg/mL)
Starting material	96	16.2	42.1	28.8	4.9	12.6	1.6
3.5 kDa	3.9	2.6	42.6	13.8	0.4	5.9	1.6
Yield				48%	8%	49%	

* Freeze-dried samples were used for IC50 measurement.

Material IN: 277 g. Material OUT: 354 g

Conductivity of slurry before dialysis: 21 mS/cm. After dialysis: 787 µS/cm

Desalting bioactive fraction – Nanofiltration

Nanofiltration (NF) of mussel extract

February 2024 - Yvette Telleman & Ronald Vroon

WAGENINGEN UNIVERSITY & RESEARCH

Materials & Methods

- Freeze-dried mussel extract (supernatant)
  - Code: Mossel sup 9-2-24
  - Ash content: 14.7% (Sample 2023: 16.2%)
- Slurry of mussel extract centrifuged, supernatant 1:1 diluted with water and used as feed for NF trial
- Sterlitech pressure vessel used for NF trial
  - Membrane: Synder NFG (600-800 Da)
  - TMP: 35 bar

Fig 1. Sterlitech pressure vessel 2

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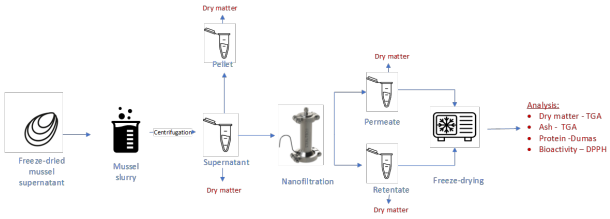
Process



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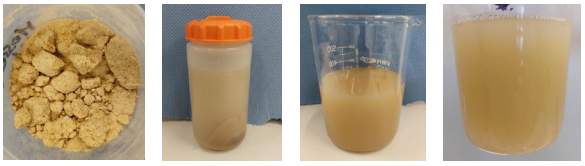
3

Results



Mussel extract (freeze-dried supernatant) | Centrifuged slurry | Supernatant | Feed (1:1 diluted supernatant)

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4

Results

Permeate
Before/after freeze-drying

Retentate
Before/after freeze-drying

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5

Results

Table 1. Results of the NF feed, permeate and retentate

	NF Feed	Permeate	Retentate
Mass wet (g)	718	297	373
Mass freeze-dried (g)	-	1.8	13.0
DM (%)	12.8*	0.6	3.2
Conductivity (mS/cm)	5.7	5.2	6.5
Protein (%)**	32.1	37.2	30.8
Protein (yields) (g)	4.6	0.7 (15%)	3.7 (78%)
Ash (%)	14.7*	TGA	TGA

** Measured in freeze -dried sample ** Kf: 6.25*

	IC50 (mg/mL)
Pellet	0.7
Supernatant	0.9
Retentate	0.8
Permeate	0.7
Uric acid (ref.)	0.5

IC50 depends on the DM content

- Bioactive components are partially retained by the membrane as evidenced by the bioactivity present in retentate as well as permeate fractions → observed bioactivity most likely attributed to multiple components with different molecular weights

6

Results NF process performance

	Amount* (g)	EC (mS/cm)	DM (g/L)	Kjeldahl (g/L)	*Salt*** *(g/L)*	Kjeldahl (% d.b.)	Salt (% d.b.)
NF feed	699	5.69	20.2	6.5	*3.17*	32.1	15.7
NF retentate	373	6.45	31.8	9.8	*3.59*	30.8	11.3
NF permeate	326	5.18	6.3	2.3	*2.89*	37.2	45.8
Mass balance (OUT/IN) (-)	1.00		0.99	0.98	1.03		
Concentration factor (CF) (-)	1.87						
Retentate yield (Kjeldahl, salt) (-)				0.81	0.61		
Retention factor (-)				0.66	0.20		

* Mass balance corrected for NF losses (feed = ret + perm)
** EC expressed as salt concentration (approximate value, assuming NaCl as major ash component)

- EC data converted to salt concentration indicate around **40% of salt removal**, resulting in decreased salt content of retentate fraction (15.7 → 11.3%)
- Further reduction in retentate salt content feasible by applying diafiltration → retention factor of ~0.2 for salt components (mainly monovalent)
- Nitrogen-containing components are partially retained by the membrane (80% yield), with a retention factor of 0.66, indicating presence of low molecular weight N-components

7

Desalting bioactive fraction – TGA method development

TGA measurement of dry matter and ash content - Method development

October 2024 - Yvette Telleman & Ronald Vroon

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Materials & Methods

- Trials and method development to test applicability of TGA fordetermination of dry matter and ash content in liquid and freezedried samples
- Equipment used: PerkinElmer STA 6000
- Method (temperature-time profile) used
  - [1] 30→105°C (10°C/min); [2] 4 h @ 105°C; [3] 105→550°C (10°C/min); [4] 4 h @ 550°C
- Test samples used
  - Model solutions of sucrose and NaCl, with composition as indicated
  - Freeze-dried fractions of NF experiment with musselextract

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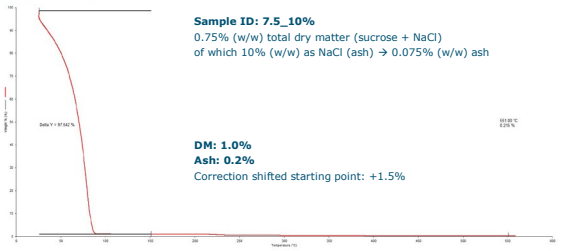
TGA results model solutions



Sample ID: 7.5_10%
0.75% (w/w) total dry matter (sucrose + NaCl)
of which 10% (w/w) as NaCl (ash) → 0.075% (w/w) ash

DM: 1.0%
Ash: 0.2%
Correction shifted starting point: +1.5%

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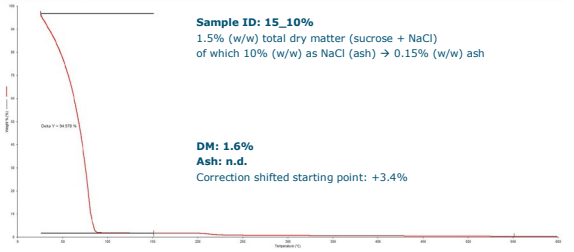
TGA results model solutions



Sample ID: 15_10%
1.5% (w/w) total dry matter (sucrose + NaCl)
of which 10% (w/w) as NaCl (ash) → 0.15% (w/w) ash

DM: 1.6%
Ash: n.d.
Correction shifted starting point: +3.4%

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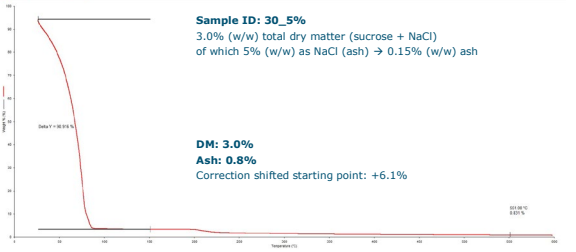
TGA results model solutions



Sample ID: 30_5%
3.0% (w/w) total dry matter (sucrose + NaCl)
of which 5% (w/w) as NaCl (ash) → 0.15% (w/w) ash

DM: 3.0%
Ash: 0.8%
Correction shifted starting point: +6.1%

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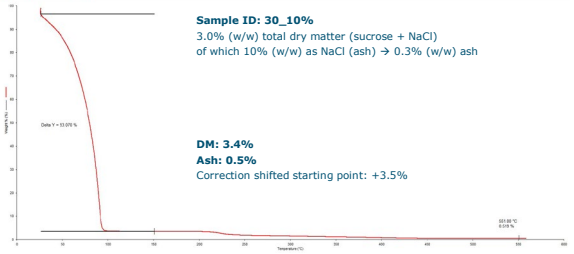
TGA results model solutions



Sample ID: 30_10%
3.0% (w/w) total dry matter (sucrose + NaCl)
of which 10% (w/w) as NaCl (ash) → 0.3% (w/w) ash

DM: 3.4%
Ash: 0.5%
Correction shifted starting point: +3.5%

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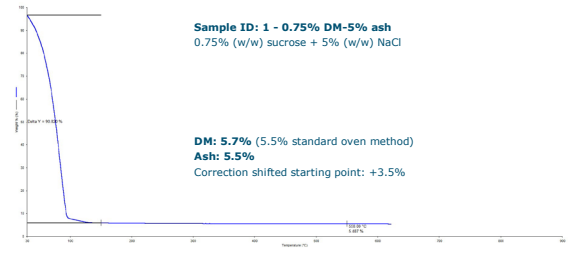
Previous TGA results model solutions



Sample ID: 1 - 0.75% DM-5% ash
0.75% (w/w) sucrose + 5% (w/w) NaCl

DM: 5.7% (5.5% standard oven method)
Ash: 5.5%
Correction shifted starting point: +3.5%

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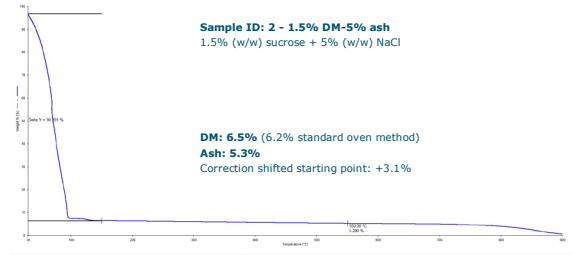
Previous TGA results model solutions



Sample ID: 2 - 1.5% DM-5% ash
1.5% (w/w) sucrose + 5% (w/w) NaCl

DM: 6.5% (6.2% standard oven method)
Ash: 5.3%
Correction shifted starting point: +3.1%

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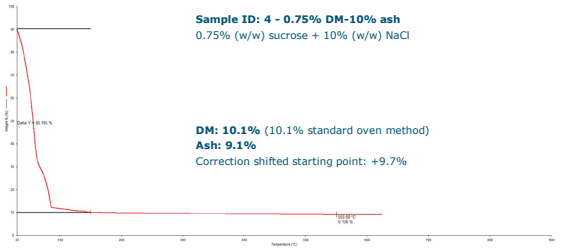
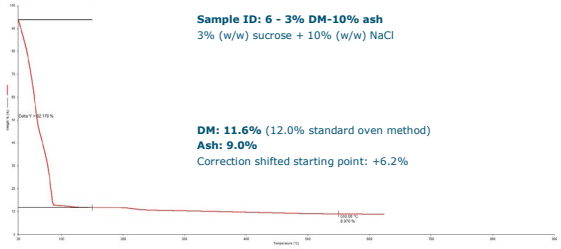
Previous TGA results model solutions



Sample ID: 4 - 0.75% DM-10% ash
0.75% (w/w) sucrose + 10% (w/w) NaCl

DM: 10.1% (10.1% standard oven method)
Ash: 9.1%
Correction shifted starting point: +9.7%

Previous TGA results model solutions



Sample ID: 6 - 3% DM-10% ash
3% (w/w) sucrose + 10% (w/w) NaCl

DM: 11.6% (12.0% standard oven method)
Ash: 9.0%
Correction shifted starting point: +6.2%

Conclusions model solutions

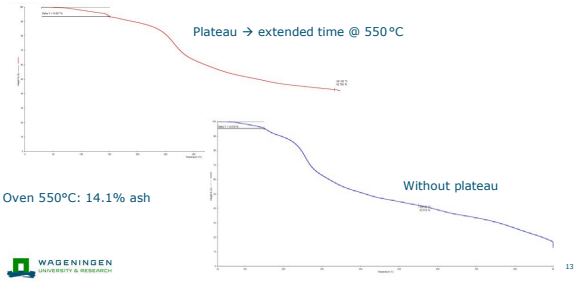
- TGA measurement on liquid samples leads to start weight <100%, due to initial evaporation of sample (open sample pans used)
- Accuracy of TGA for determination of dry matter content in model solutions is within 0.5%, with 0-30% deviation from true value
- TGA appears unsuitable for determining low dry matter and ash contents (<1% (w/w) in model solutions)
  - In theory, measuring DM or ash content of ≥0.1% (w/w) using TGA seems feasible (based on specs) → see next slide
  - Further optimization of TGA method (temperature-time profiles) to improve accuracy considered uncertain

Measurement limits derived from specs

- Based on specs of TGA equipment an estimate was derived for measurement limits in terms of minimum dry matter and ash content in samples
- Assumptions:
  - Specs TA Instruments TGA 55/550 and PerkinElmer STA 6000
  - Sample size: 50 µL (STA 6000)
  - Weight baseline drift: 8 µm (<25 µg over full temperature range)
- In theory, measuring DM or ash content of ≥0.1% (w/w) using TGA seems feasible (based on specs)

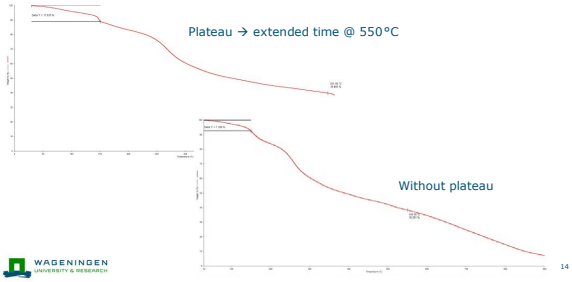
Min. DM or ash content	0.11	% DM or ash
Sample size	50	µL ~ mg
Min. sample weight	53	µg DM or ash
Inaccuracy (+/-)	15	%
Baseline drift (@ 0-600°C)	8	µg
Baseline drift (specs)	< 25	µg

NF feed (supernatant)

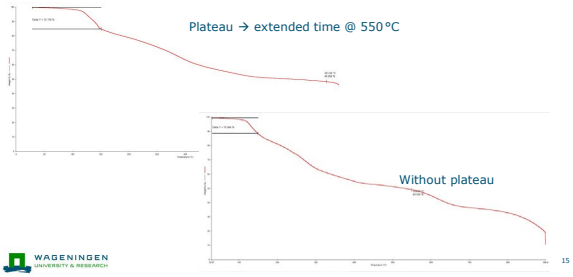


Oven 550°C: 14.1% ash

NF retentate



NF permeate



Conclusions NF samples (freeze-dried)

- Method adjustment ('standard' oven method translated into TGA temp-time profile) improves transitions
- However, with NF samples no constant weight is reached at 105 and 550°C
  → inaccurate determination of DM and ash content
- For freeze-dried NF samples (with complex composition), difference between standard oven method and TGA
  → no clear explanations found for these observed differences

Annex 2 Bioactive fraction

Sole, Liver and Spleen RNAseq analysis

Investigating the systemic effect of Mussel Extracts on Dover Sole via Liver and Spleen transcriptomic analysis part

Edoardo Zaccaria

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Disclaimers

The following results were obtained from the feed experiments carried-out in 2022

Both the feed experiment (2022) and the RNA-sequencing (2023-2024) are funded by the Dutch Ministry.

These activities are separate from the PPS Total Use Bivalves project.

This information constitutes WR background knowledge.

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Experiment -> choosing the sites

- Analysis of the whole mRNA population
  - Two organs: Liver and Spleen
    - Liver Functions in Metabolism and Detoxification
    - Liver's Role in Erythropoiesis, particularly under stress or in pathological conditions
    - Spleen's Role in the Immune System and RBCs Lifecycle
- In general, looking for a:
  - Possible involvement in iron metabolism, erythropoiesis, and the inflammatory response.

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Experimental design

- 3 Dietary Treatments
  - Positive control -> Ragworm
  - Negative control -> Commercial PELlet
  - Experimental diet, Mussel extract B -> ME_B
- 15 Fishes / Treatment
- 3 Tanks / Treatment
- 2 Organs / Fish
- 90 samples in total

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Overview: Quality Control, pre-processing

- ~ 23 M reads / sample
- Mapped vs *solea solea* genome (fSolSol10.1, June 2023)
- OK, duplication rate, quality score, adapter content, etc
- S8 and S22 low GC* content (36% and 41% vs average 47%)
  - GC content is species-specific
- S8 very high number of over-represented sequences

*: Guanine and Cytosine

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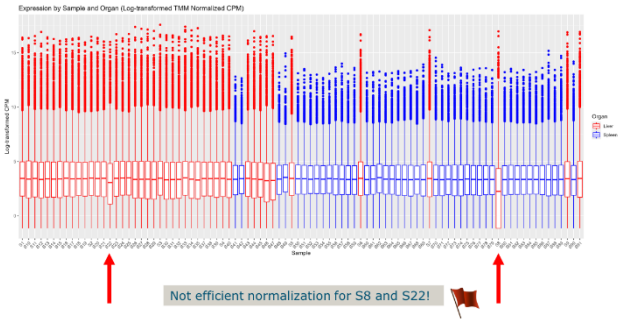
Overview: all samples



Not efficient normalization for S8 and S22!

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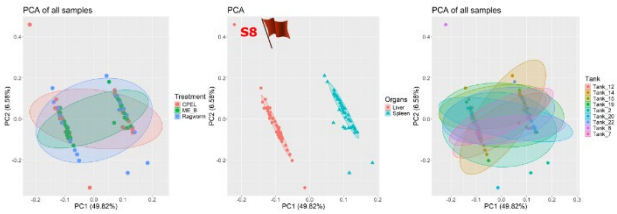
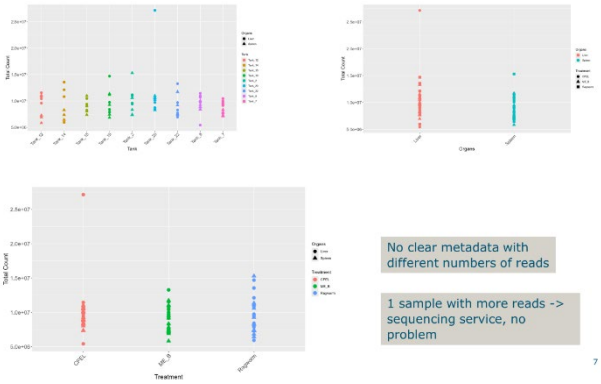
Overview: all samples



Clear clustering per organ

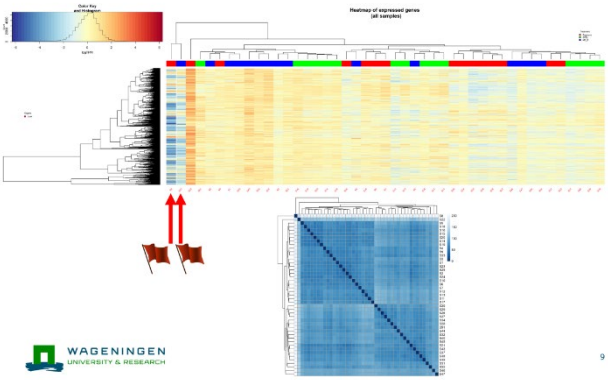
Overview: all samples



No clear metadata with different numbers of reads

1 sample with more reads -> sequencing service, no problem

Overview: Liver



Overview: Liver samples

- 36761 genes
- Filter the data by at least X samples with a count of 10 or more, where X is the sample size of the smallest group of samples after outlier removal (14 in this case)
- 14609 genes were kept
  - At least 10 counts in 14 samples

Overview: L-samples, unsupervised analysis

- Per Treatment and per Tank clustering
  - High overlapping between ME_B and Ragworm

Differential expression analysis

- Performed via DESeq2 (FDR adjusted p-value < 0.05, |Log2FC| > 1)
- 3 contrast:
  - ME_B vs Ragworm
  - ME_B vs CPEL
  - Ragworm vs CPEL
- The Venn Diagram is available on request

DESeq2: 10.1186/s13059-014-0550-8

ME_B vs CPEL

Summary and Biological Implications

- **Iron Homeostasis/erythropoiesis:** Enhance iron homeostasis and absorption, boost erythropoiesis through direct and indirect mechanisms, protect cells from oxidative stress, and modulate metabolism.
- **Metabolic Shifts**: The upregulation of genes involved in lipid metabolism suggests an overall better metabolic status and energy management, crucial for growth and repair processes.
- **Inflammation and Stress Response**: The expression patterns indicate a potentially lower inflammatory state and better oxidative stress management, which might contribute to their improved health status compared to anemic animals.
- **Cellular Proliferation**: Enhanced expression of genes regulating cell proliferation points to active tissue regeneration and maintenance, essential for overall health and functionality.

ME_B vs Ragworm

Biological Implications

- **Lipid Metabolism:** Upregulation of ebpl and downregulation of *msmo1* in ME_B suggests significant differences in cholesterol metabolism and biosynthesis pathways compared to Ragworm.
- **Nitrogen Metabolism:** Upregulation of *arg2* in ME_B indicates alterations in nitrogen metabolism, specifically the urea cycle, which might be due to differences in dietary intake or metabolic needs between the two groups.
- **Muscle and Stress Response:** Upregulation of *fstl3* and downregulation of *bhlhe40* in ME_B suggest differences in muscle growth regulation and stress response mechanisms.

The comparison between ME_B and Ragworm reveals significant differences in gene expression related to lipid metabolism, nitrogen metabolism, muscle regulation, and stress response. These findings suggest that even in the absence of anaemia, ME_B and Ragworm have distinct metabolic and regulatory profiles.

Ragworm vs CPEL

Summary and Biological Implications

- **Iron Homeostasis:** The upregulation of *hepcidin* (*hamp*) and transferrin receptor (*tfr1a*) in Ragworm suggests better iron regulation, which is often impaired in anaemia. Anaemic animals might have disrupted iron homeostasis, leading to insufficient haemoglobin production.
- **Metabolic shift**: Genes involved in stress response and metabolism (like *klf9, lpin1a, foxo1a*) show differential expression, indicating that anaemia impacts metabolic pathways and cellular stress responses. Downregulation of genes like *fabp7a* in CPEL points to altered energy metabolism, which could be a response to the physiological stress of anaemia.

The differential expression analysis highlights significant gene changes related to iron metabolism, lipid metabolism, and cellular stress response. The upregulation of key iron homeostasis genes in Ragworm aligns with the need for efficient iron utilisation to prevent anaemia, while downregulation in CPEL suggests metabolic and stress-related adaptations to anaemic conditions.

Overview: spleen

- 36761 genes
- No outliers
- Filter the data by at least X samples with a count of 10 or more, where X is the sample size of the smallest group of samples after outlier removal (15 in this case)
- 16539 genes were kept
  - At least 10 counts in 15 samples

Overview: spleen, unsupervised analysis

- Per Treatment and per Tank clustering
  - Less overlapping than in the liver

Differential expression analysis

- Performed via DESeq2 (FDR adjusted p-value < 0.05, |Log2FC| > 1)
- 3 contrast:
  - ME_B vs Ragworm
  - ME_B vs CPEL
  - Ragworm vs CPEL

DESeq2: 10.1186/s13059-014-0550-8

Conclusion part 1

Overall Impact:

- There is a clear indication of the dietary treatment's effect on gene expression in both the liver and spleen.
- Liver transcriptomics shows that Iron Metabolism, Homeostasis, and other pathways related to the anaemic conditions are significantly and positively affected by both mussels extract and ragworm, suggesting a similar beneficial mechanism.
- The two dietary treatments show significant differences in gene expression related to lipid metabolism, nitrogen metabolism, muscle regulation, and stress response. These findings suggest that despite their common beneficial effect on the anaemic condition of the animals, ME_B and Ragworm have distinct metabolic and regulatory profiles.

Overview:

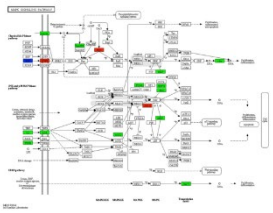
- Mapped vs *solea solea* genome (fSolSol10.1, June 2023)
  - Differential expression analysis <- Previous slides
  - Gene Ontology Enrichment analysis
- Mapped vs *solea senegalensis*
  - Differential expression analysis
  - Gene Ontology Enrichment analysis
  - KEGG Enrichment analysis

Overview:

- GO Terms:
  - Categorizes by function, location, and processes.
  - Hierarchical and structured vocabulary.
  - Used for gene product annotation and functional enrichment analysis.

Overview:

- KEGG:
  - Focuses on pathways and systems biology.
  - Includes pathway maps and modules.
  - Used for pathway mapping and understanding complex biological systems.



Overview: Liver samples

solea solea

- 36761 genes
- 14609 genes were kept
  - At least 10 counts in 14 samples

solea senegalensis

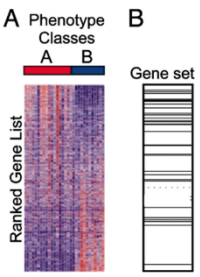
- 28924 genes
- 13539 genes were kept
  - At least 10 counts in 14 samples

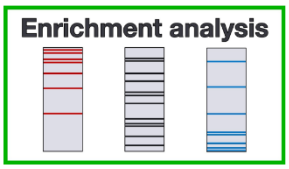
Fewer genes were mapped

Overview: L-samples, unsupervised analysis

- Per Treatment and per Tank clustering
  - High overlapping between ME_B and Ragworm

Gene Set Enrichment Analysis





GO terms
S. solea -> 19782 (53%) genes annotated
S. senegalensis -> 20697 (71%) genes annotated

KEGG
S. Senegalensis -> 7994 (27%)

KEGG, only *S. senegalensis*: Overall

The KEGG pathways enriched by both dietary interventions lead to

- Upregulation of pathways that enhance **protein turnover** (proteasome**), nucleotide availability** (nucleotide metabolism), and **cell membrane integrity** (fatty acid biosynthesis), all of which are **crucial for red blood cell health**.
- Downregulation of pathways associated with **stress and inflammation** (neuroactive ligand-receptor interaction and cytokine-cytokine receptor interaction) suggests a reduction in factors that could negatively impact red blood cell production and survival.

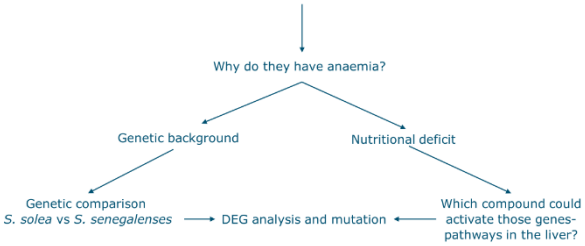
These combined effects create a favorable environment for red blood cell production and maintenance, helping to alleviate anemia through mechanisms that do not rely on iron supplementation.

KEGG, only *S. senegalensis*: Overall

- Fatty Acids: Given the upregulation of fatty acid biosynthesis and metabolism pathways, hypothesize that specific fatty acids or their derivatives in the diet are contributing to the improved red blood cell function and overall health.
- Antioxidants: The hydrogen peroxide catabolic process being upregulated suggests that antioxidants in the diet might be playing a role in reducing oxidative stress, protecting red blood cells.
- Amino Acids: Upregulation of nucleotide metabolism and proteasome pathways indicates that amino acids might be crucial for enhanced protein turnover and nucleotide synthesis, supporting cell proliferation and repair.

Final aim of this study?

- Find the active compounds in Ragworm and mussels that may alleviate anaemia in Dover soles



Thank you

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Annex 3 Protein-rich fraction

Pellet towards food applications

Maaike Nieuwland, Jeroen Kals & Floor Boon

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Recap: Overall conclusions first trials

- Due to processing limited amount of soluble/native/functional protein (0.3-2.5%) is present (as expected)
- Some protein functionality observed & to be explored further
  - Emulsifying @pH 5: protein is dispersed in fat phase
  - Structuring by hand: "spaghetti" showed water stability for at least 5 hours
  - Structuring using a syringe: not conclusive, more tests needed

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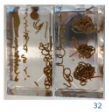
Next steps – Based on first trials

- Emulsifying at pH 5
- Applying shear as done by hand more automated
  - Dough kneading
  - Pasta machine
- Structuring using a syringe: extra tests with TG addition (concentration, reaction time, composition)
  - To be done



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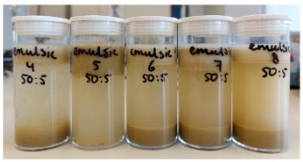
Recap Emulsifying

- 10% oil, 3% protein
- No emulsifying at pH 4-6-7-8
- pH 5: protein is dispersed in fat phase
- Next steps:
  - pH 5, 50% oil, 10% protein
  - Oil-in-water and water-in-oil emulsion



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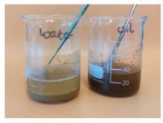
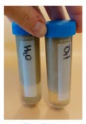
Emulsifying

- Two conditions
  1. 2.5 g mussel extract – 25 g water – set to pH 5 (HCl) – add oil – ultraturrax
  2. 2.5 g mussel extract – 25 g oil – add water and HCl (equal amount HCl to 1st condition) – ultraturrax



Before ultraturrax



Few hours

Over night

- Emulsion is unstable – do not pursue further for now, microscope pictures available

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Recap: effect of structuring by hand

- Using basic mix 25% pH as is (5.8)
- The "spaghetti" show water stability for at least 5 hours
- The pieces have been deliberately cut

5 min

5 hours

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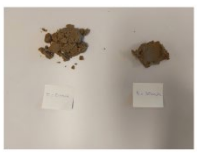
Kneading and sheating



- Farinograph

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Structure formation during kneading

- More structure formation (measured with farinograph) with less water
  - 67% water – 60% water – 55% water
- Preparing sheets using manual pasta machine
  - Thickness 3 for all three samples
  - Thickness 5 for 55% water sample
    - Thickness 5 did not work for the other crumbly

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Settings: 1 is thick, 7 is very thin

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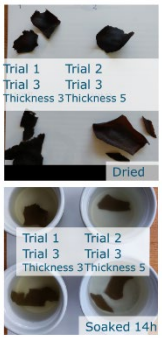
Experiments with mussel sheets

- Dry in the oven
  - Sheets curl and get dark
- Soaking dried sheets in water
  - After 19h:
    - Trial 1 breaks (strong, brittle
    - Trials 2&3 some elasticity
    - Thinner trial 3 is more clearly elastic



Experiments with mussel sheets

- Boil 5 min
- Sample is stable
- They all have some elasticity
- They are rather hard

Where do you find mussel extract?

- Use Innova database
- Search for
  - Fish protein
  - Mussel
  - Mussel extract
- Looked at groups like petfood, meat, fish and eggs, ready meals, snacks, spreads, sauces, supplements, ect.
- Next slides show only limited results

Meat, fish and eggs (89/628; 40 W-Europe)

South Africa

Spain, fish protein spaghetti
Fish protein 1st ingredient, 10.9% protein

UK, surimi

Mexico, shredded surimi

Spain,
fish protein 1st ingredient, 9.5% protein

Ready meals (59/628, 18 W-Europe)

Germany,
fish protein in surimi

Canada, hydrolysed fish protein, probably for flavour

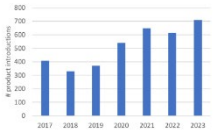
US, fish protein pasta
(fish protein, starch, egg white

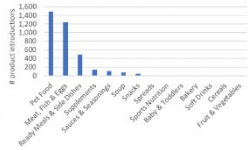
US, fish protein in fish cake extract

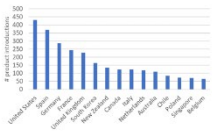
Spain, fish protein pasta
(fish protein, oil, wheat flour,...)

Mussel as an ingredient

- 3618 introductions from Jan 2017 to 19 Sept 2023
- Increasing introductions in the past years
- Most introductions in US
- Most introductions on PetFood







Mussels: meat, fish, eggs (1239/3618)

UK, mussels

Argentina, mussels

UK, mussels

UK, mussel extract

Spain, mussels

UK, mussel meat

UK, mussel powder

Ready meals and side dishes (492/3618)

Indonesia,
mussel, mussel extract

US, mussel

France,
mussel cooking juice

UK, mussels

UK, mussel extract

Spain, fish stock (mussels)

India, seasoning (ao mussel)

South Korea,
mussel concentrate powder

Supplements (143/3618; 42 in NL; 6 in UK)

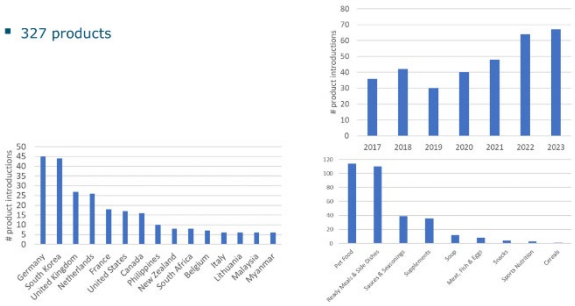
Ingredient: mussel – snacks (42 products)

Mussels - conclusion

- Products with whole mussels
- Ready made products with pieces of mussels (size is less important)
- Pet food is really big – nutritional value is of importance
- Mussel extract flavouring
- Health remarks (green-lipped mussel, sometime in combination other ingredients)
  - Joint health
  - Anti-inflammatory
  - Gastrointestinal health

Mussel extract (free text search)

- 327 products



Mussel extract - summary

- Petfood – small quantities – for health effects?
- Ready meals and side dishes: as flavouring
- Sauces, Seasonings, Soups: as flavouring
- Supplements
  - Cartilage/joints
  - Healthy immune system
  - Support for the airways
  - Source of … (*The green-lipped mussel belongs to the best natural sources of omega-3 fatty acids, glycosaminoglycans, glucosamine, chondroitin and amino acids and also contains minerals and vitamins; post exercise recovery, joint health and mobility*)

Mussel extracts – what do we know?

- Low functional
  - Can serve as filler, not as gelling agent
  - Can increase nutritional value
  - Colour and flavour are strong
- Fish protein used as surimi (but need gelling properties)
- Fish protein used as flavour
- Mussel used in pasta, as flavour

Mussel extracts – possible application routes

- As a Filler
  - Pasta
  - Cookies
  - Kroepoek
  - Crackers
  - Bread
  - Meat replacer
  - Coating
    - Breadcrumbs
    - Batter
  - Extruded snacks
- As a Flavour
  - Flavouring in
    - Sauce
    - Soup
    - Crisps
  - Savoury drink
  - Spread
- Other
  - Pet food
  - Jerky-type products/biltong (ziltong?)
  - Supplements

Annex 4 Chemical safety

Microplastics analyses in mussel fractions

Anna Undas, Eelco Sijtsma & Winnie Tao

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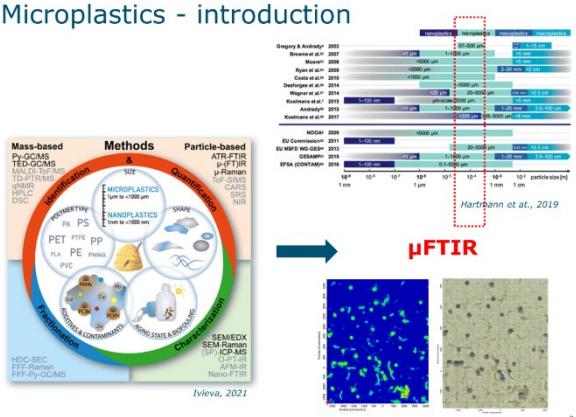
Microplastics - introduction



PE standard

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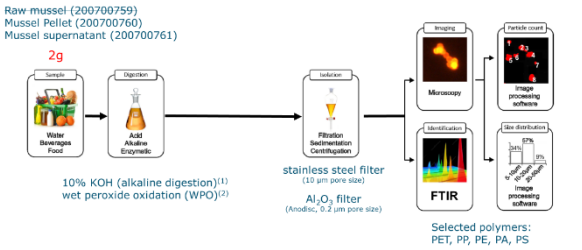
Microplastics - methods



(1) @ 50 °C overnight with mild shaking
(2) 30% H_2O_2 method with Fenton's reagent used as a catalyst, at pH=2.5

modified Vitali et al. 2022

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Microplastics – methods (1/2)

- Sample amount: 2 grams
- Digestion method:
  1) Potassium Hydroxide (10%) alkaline digestion
     - At 50 degrees overnight with mild shaking
  2) Wet peroxide oxidation (WPO) method with Fenton's reagent
     - Iron(II)sulphate is used as a catalyst (at pH=2.5)
     - Applied in combination with 30% H_2O_2
- Vacuum filtration
  - Stainless steel filters (mesh, 10 μm pore diam.)
  - Aluminum Oxide (Anodisc, 0.2 μm pore diam.)
  - Final filtration step. Filter used for acquisition on FTIR

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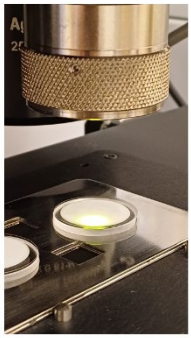
Microplastics – methods (2/2)

- Analytical method
  - μFTIR
  - Agilent Carry 620 with FPA detector
    - Nitrogen cooled
- Parameters:
  - Mode: Transmission
  - CaF_2 crystal
  - IR range: 3400 – 1250 cm^{-1}
    (self absorption of AlO_3 <1250 cm^{-1})
  - #scans: 8
  - Resolution: 8 cm^{-1}



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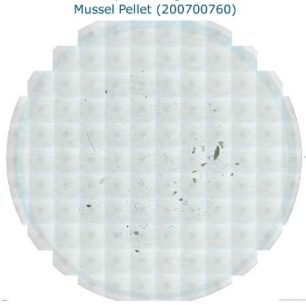
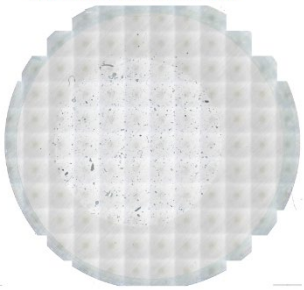
Microplastics – main results

Optical image of
Mussel Pellet (200700760)



Optical image of
Mussel supernatant (200700761)



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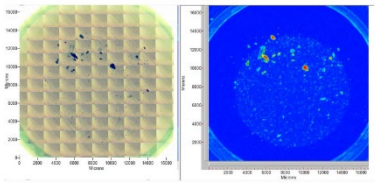

(1) Mussel Pellet (200700760)

Class Name	Area [µm²]	Length [µm]	Width [µm]
PP	50625	312.05	241.70
PP	11250	150.00	75.00
PP	16875	225.00	75.00
PE	5625	75.00	75.00
PS	28125	241.26	179.15
PS	95625	449.65	333.65
PA	5625	75.00	75.00

Class ID	No. of Particles
PP	3
PE	1
PS	2
PA	1

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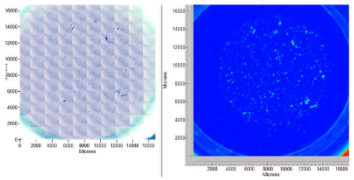

(2) Mussel supernatant (200700761)

Class Name	Area [µm²]	Length [µm]	Width [µm]
PS	95625	813.0	212.4
PS	84375	569.6	450.0
PP	11250	150	75
PP	5625	75	75
PP	5625	75	75
PP	5625	75	75
PP	22500	150	150
PP	5625	75	75
PP	11250	150	75
PP	5625	75	75
PP	11250	150	75
PP	28125	281.5	153.9
PA	5625	75	75

Class Name	Class ID	No. of Particles
PP	PP	11
PS	PS	2
PA	PA	1

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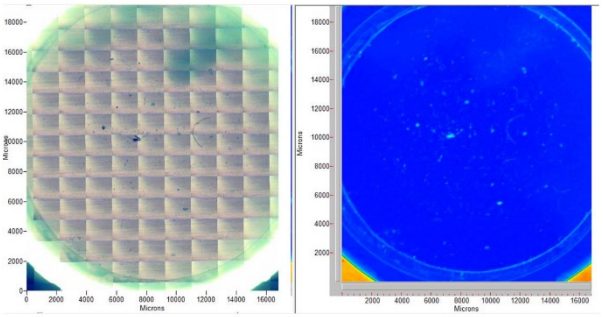
Microplastics – main results Bl Chem



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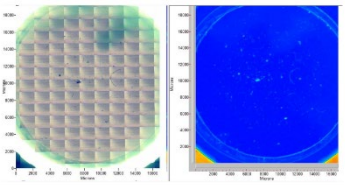
QA/QC
Blank sample (NEG control)



Class Name	Area [µm²]	Length [µm]	Width [µm]
PP	28125	281.46	153.86
PP	11250	150.00	75.00

Class ID	No. of Particles
PP	2

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Microplastics – discussion and conclusions

- Measured in 4x => spatial resolution limited to 75 cm^{-1}
  - Caused by software limitations
- LOD_{size} ~ 50 µm
- KOH succesfully digested most of sample material
- Consecutive oxidation step was necessary to prevent clogging of the Anodisc filter => peroxide combined with Fentons reagent
- No spike was included => no information on degradation
- PP the most prominent in the analysed samples

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PFAS analyses in mussel fractions

Ruben Kause & Winnie Tao

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PFAS

- Focus on 'EFSA 4' legacy PFAS
  - perfluorooctanoic acid (PFOA), perfluorooctane sulfonate (PFOS), perfluorononanoic acid (PFNA), perfluorohexane sulfonic acid (PFHxS)
- Raw mussel, pellet and supernatant: analysed using a method applied for fish products and animal feed

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Results PFAS (wet weight)

- PFOSA not commonly measured

	PFOSA	PFOS	PFOA	PFNA	PFHxS	Sum of PFOS, PFOA, PFNA and PFHxS (EFSA 4)
Measured in fresh mussel (µg/kg WW) (200700759)	-	0.22	<0.1	<0.1	<0.025	0.22
Measured in pellet (µg/kg WW) (200700760)	1.87	0.93	0.06	0.08	0.03	1.10
Measured in supernatant (µg/kg WW) (200700761)	1.44	0.38	0.05	<0.08	<0.03	0.43
Maximum limit (µg/kg WW) in Crustaceans and bivalve molluscs*	**-**	**3.0**	**0.70**	**1.0**	**1.5**	**5.0**

*Commission Regulation (EU) 2023/915

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Results PFAS mass balance (dry weight)

- Results below LOQ → deemed as zero

Proximate composition	g/kg
	Mussel (raw)
Dry matter	161.0
Crude protein	102.0
Crude fat	14.0
Ash	26.0
Carbohydrates	19.0
	Pellet
Dry matter	981.0
Water	19.0
	Supernatant
Dry matter	959.7
Water	40.3

	Raw mussel (ug/kg DW)	Pellet (ug/kg DW)	%	Supernatant (ug/kg DW)	%	SUM%
PFOA	0.00	0.06	-	0.05	-	-
PFNA	0.00	0.08	-	0.00	-	-
PFHxS	0.00	0.03	-	0.00	-	-
PFOS 99	1.37	0.95	69.20	0.40	29.06	98.26

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Wageningen Food & Biobased Research
Bornse Weilanden 9
6708 WG Wageningen
The Netherlands
E info.wfbr@wur.nl
wur.eu/wfbr

Report 2655

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