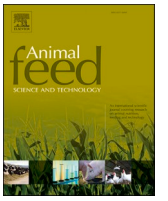
Animal Feed Science and Technology 320 (2025) 116199

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci



Evaluation of two blue mussel fractions for their ability to alleviate anaemia and improve the performance in common sole (*Solea solea* L.)

J. Kals [a,*], W. Muizelaar [a], A.M.J. Kootstra [b], E.W.J. Gerrits [c], C.W. Tao [c], H.M. de Vogel-van den Bosch [b], P. Geerdink [b], Y. Telleman [b]

[a] *Wageningen Livestock Research, Wageningen University & Research, PO Box 338, Wageningen 6700 AH, the Netherlands*
[b] *Wageningen Food & Biobased Research, Wageningen University & Research, PO Box 17, Wageningen 6700 AA, the Netherlands*
[c] *Wageningen Food Safety Research, Wageningen University & Research, PO Box 230, Wageningen 6700 AE, the Netherlands*

ARTICLE INFO

Keywords:
Hematology
Plasma biochemical parameters
Antioxidant activity
Vitamin B_{12}
Mytilus edulis

ABSTRACT

The main goal of this study was to test whether dietary supplementation of fractions from blue mussels shows a similar ability to alleviate anaemia and improve the performance of common sole like ragworm and blanched mussels. In addition, this study aimed to link the antioxidant activity of the fractions towards their ability to alleviate anaemia in common sole and evaluate the effect of processing on contaminants in the developed fractions. The experimental set-up contained six diets in triplicate: fresh ragworm (RW), the positive control, blanched mussel (BM) as the control for the fractions, a formulated diet with either 15 % of fraction A (DA), B (DB) or a combination of both (DAB) and a commercial pellet (CPL), the negative control. All diets, except BM, were well eaten, and fish growth exceeded expectations. Haemoglobin (Hb) levels rose with 24 %, 35 %, 44 %, 59 % and 156 % compared to levels at the start of the experiment for fish fed BM, DA, DAB, DB and RW respectively, while Hb for fish fed CPL remained similar. Growth was highest for fish fed RW (2.23 ± 0.08 % BW/d), lowest for fish fed BM (1.09 ± 0.14 % BW/d), and similar for all pelleted diets (1.75 ± 0.10–1.82 ± 0.04 % BW/d). Diets RW, BM and DB alleviated anaemia in common sole. From the formulated diets, DB showed the highest potential in increasing Hb levels compared to BM and the diets DAB or DA, suggesting the compound(s) responsible for alleviating anaemia are concentrated in fraction B. In addition, the production processes for making the fractions and pelleting did not noticeably affect their ability to alleviated anaemia, indicating that these diets are a reliable and effective of promoting Hb levels in common sole. The antioxidant activity of the diets was not related to their ability to alleviate anaemia in common sole. Except for total dioxins and dl-PCBs, all contaminants were not exceeding corresponding EU maximum levels for feed materials. The high plasma vitamin B_{12} levels found in common sole compared to other fish species suggest a specific metabolic or physiological role of vitamin B_{12} in common sole. These findings can have significant implications for the culture of common sole.

* Correspondence to: PO Box 338, Wageningen 6700 AH, the Netherlands.
E-mail addresses: Jeroen.Kals@wur.nl (J. Kals), Wouter.Muizelaar@wur.nl (W. Muizelaar), Maarten.Kootstra@wur.nl (A.M.J. Kootstra), gerrits@securefeed.eu (E.W.J. Gerrits), winnie.tao@wur.nl (C.W. Tao), heleen.vandenbosch@wur.nl (H.M.V.-v. den Bosch), peter.geerdink@wur.nl (P. Geerdink), yvette.telleman@wur.nl (Y. Telleman).

https://doi.org/10.1016/j.anifeedsci.2024.116199
Received 3 October 2024; Received in revised form 16 December 2024; Accepted 21 December 2024
Available online 24 December 2024

1. Introduction

Low trophic marine resources, like bivalves and worms, contain high levels of bioactive components (Macedo et al., 2021; Chakraborty and Joy, 2020; Stabili et al., 2019; Neves et al., 2016; Grienke et al., 2014). Bioactive components are compounds (e.g. peptides, alkaloids, or steroids) with a specific antibacterial, anti-inflammatory, antiviral, fungicidal or even anti-carcinogenic properties. When common sole is fed either blue mussel (*Mytilus edulis*) or ragworm (*Nereis virens*) the haematocrit (Hct) and haemoglobin (Hb) levels are increased, this reduces the anaemic conditions which are present when common sole is fed a commercial pellet (Kals et al., 2015). Anaemic conditions in common sole (*Solea solea*) causes reduced growth, increased sensitivity to diseases, low resilience and ultimately an economic loss for the farmer (Kals et al., 2015, 2016, 2017; Kals, 2017; Ende et al., 2014). The dietary levels of crude protein, crude fat, amino acids, calcium, phosphate, iron, or copper do not explain the differences in Hct between common sole fed ragworm or commercial pellets (Kals, 2017). The presence of (unidentified) bioactive components in these marine resources can explain the observed effects.

The development of dry feed alternatives containing the unidentified bioactive components present in fresh marine resources is a necessity, since the maturation of many marine fish and shrimp species are dependent on fresh marine resources (Slater et al., 2018; Balasubramanian et al., 2018; Ortiz, 2016). Fractionation of marine resources into a fraction that contains the (bioactive) components that, for example can alleviate anaemia in common sole, and one that mainly contains protein aimed at human consumption could have various benefits. For instance, providing the (bioactive) components for aquafeeds and at the same time contribute to the supply of human protein. This will reduce the food-feed competition when using low trophic marine resources for aquafeeds. Additionally, the use of fresh feed is not optimal in relation to the seasonal quality of the raw material, their effect on water quality, and the risk of introducing pathogens when feeding fresh feeds (Tacon, 2017; Ortiz, 2016). The heat sensitivity of bioactive components in general poses a challenge, since aquafeeds are often made through steam pelleting or cooking extrusion. Boiling had a negative effect on the ability of ragworm to increase Hct in common sole (Kals, 2017). Similar negative effects were also found for blue mussel (Baynes and Howell, 1993). A potential downside of processing is the concentration or dilution of respectively contaminants or bioactive components in the generated fractions. This should be carefully monitored to ensure maintaining bioactivity and to avoid food and feed safety hazards.

The main goal of this study was to test whether dietary supplementation of fractions from blue mussels shows a similar ability to alleviate anaemia and improve the performance of common sole like ragworm and blanched mussels. In addition, this study had two sub goals. The first subgoal aimed to link the antioxidant activity of the fractions with the ability to alleviate anaemia in common sole. The second subgoal evaluated the effect of processing on contaminants in the developed fractions. We hypothesize that fractionation of blue mussel concentrates the components responsible for reduced anemia in common sole in at least one of the fractions which yields similar results in haematology, plasma biochemical parameters and performance as ragworm and blanched mussel. Our second hypothesis is that a higher antioxidant activity of the fractions is related to an improved ability to reduce anemia in common sole. At last, this study evaluates the effect of processing blue mussel on the concentration of contaminants in the fractions.

2. Materials and methods

2.1. Experimental design, housing and husbandry

All animal procedures were in accordance with Dutch law and approved by the Central Committee for Animal Experiments (CCD), the Animal Experiments Committee (DEC) and the Authority for Animal Welfare (IvD) under project number AVD40100202114610 and experiment number 2020.D-0033.001, respectively. The experiment was conducted from October until December 2022 at the aquatic research facilities of Wageningen University & Research (Carus-ARF). The experiment lasted 65 days in total, with 7 days of adaptation to the system and 58 days of experimental treatments. During the adaptation period all fish received the same commercial pellet (1.5–1.9 mm, crude protein 56 %, crude fat 18 %, ash 8 %). The commercial pellet is based on fishmeal, wheat gluten, fish oil, maize gluten, and hydrolyzed fish proteins. The experiment followed a randomized complete block design with six dietary treatments in triplicate divided over three blocks, with tank as experimental unit. Common sole (18.5 ± 3.9 g), Stichting Zeeschelp, Kamperland, the Netherlands, reared on pellet for seven months and not previously fed with ragworm or mussel were randomly allocated in groups of 4 fish each to the 18 tanks (120 L; 0.90 m * 0.45 m * 0.45 m) to a total of 32 fish per tank integrated in a recirculation system at the start of the adaptation period. After weighing and sampling at the start of the experimental period, 126 fish were removed to allow for 25 fish per tank. Tanks were not provided with substrate to allow for monitoring of feed intake. Husbandry conditions were photoperiod 12L:12D, light intensity 11–15 lux, temperature 21.0 ± 0.4℃, oxygen 7.2 ± 0.2 mg/L, pH 7.8 ± 0.3, TAN 0.23 ± 0.23 mg/L, NO_2^- 0.3 ± 0.2 mg/L, NO_3^- 203 ± 105 mg/L, salinity 30.3 ± 3.1 g/L and flow 7 L/min. Temperature and oxygen were measured daily; flow, pH, TAN, NO_2^-, NO_3^- weekly.

2.2. Diets, fractionation and feeding

The experimental setup consisted of six dietary treatments; fresh ragworm (*Nereis virens*, Sars; RW), blanched mussel meat (*Mytilus edulis*; BM), a formulated diet with fraction A (DA), a formulated diet with fraction B (DB), a formulated diet with the 1:1 combination of fractions A and B (on weight basis of freeze dried material; DAB) and a commercial pellet (1.5–1.9 mm, crude protein 56 %, crude fat 18 %, ash 8 %; CPL). On a weekly basis fresh ragworms were obtained from Topsy Baits (Wilhelminadorp, the Netherlands) and kept in a tank under the same conditions as the common sole until feeding. Fresh mussels for treatments BM, DA, DB and DAB were obtained in

one batch (batch L49624) from Driessen Food (Utrecht, the Netherlands). For treatment BM, whole mussels were blanched in boiling water for 30 s at 98 ºC (Type 1786S, nr 1946, Grosskuechen – Geraetebau Xanten/ANBO, Xanten, Germany) and transferred to an ice bath for 5 min, after which the meat was removed manually and stored in a − 20 ºC freezer until feeding. Unopened mussels and those with damaged shells were discarded. For treatments DA, DB and DAB blanched mussel meat was separated from the mussel shell by hand and fractions were produced according to proprietary protocols. The fractionation process was performed using techniques including, but not limited to homogenization, pH adjustment, and solid/liquid separation by centrifugation. The resulting pellet and supernatant fractions were freeze dried (EKS 30, model 4440, Zirbus, Tiel, the Netherlands), resulting in respectively fraction A and B. Based on the macro-nutritional and amino acid composition of fraction A and B (Table 1) isonitrogenous and isoenergetic diets were formulated for treatments DA, DB and DAB (Table 2). The diets were mixed, homogenized, extruded under low temperatures into a pellet and dried in corporation with Research Diet Services (Rijswijk, the Netherlands) and Tema & Partners (Wijchen, the Netherlands). The proximate composition of all treatments is presented in Table 3.

During the adaptation period all fish were fed CPL by hand to satiety (*ad libitum*) twice a day at 09.00 and 16.00. During the experimental period fish were fed the wet diets (RW and BM) twice a day at 09.00 and 16.00 in which the feed portion was added to the tank at once. The fish fed the formulated diets (DA, DB, DAB and CPL) were fed twice a day using belt feeders. The frozen BM was thawed in a refrigerator the day before feeding. Fresh RW and thawed BM were cut into equally sized pieces and sieved for one minute to drain excess fluids before weighing. Before the next feeding event, tanks were checked to see if all feed was eaten. If all feed was eaten, the amount of feed was raised by 5 % on dry matter basis. If feed was left, the amount of feed was reduced by 5 % on dry matter basis. Daily samples equal to the amount of feed given were taken and stored at − 20 °C for future analysis.

2.3. Sampling and growth rate calculation

Fish were weighed at the beginning, after 26 days, and at the end of the 56 day growth period. Before each weighing, fish were not fed for one day. From the individual weight data, average body weight at the start (BW_{t0}), after 26 days (BW_{t26}) and at the end (BW_{t56}) were calculated per tank. Growth rates are expressed in g/d, % BW/d and $g/kg^{0.8}$/d and were calculated using the formulas ($BW_{(t)}$-$BW_{(t0)}$))/t, (ln ($BW_{(t)}$)-ln ($BW_{(0)}$))/t * 100 and (($BW_{(t)}$-$BW_{(0)}$)/(($BW_{(t)}$ * $BW_{(0)}$)$^{0.5}$/1000$^{0.8}$)))/t, respectively. With t representing the duration of the growth period and ln the natural logarithm.

At the start of the experimental period, 20 fish were anesthetized with a low concentration of 2-phenoxy ethanol (1:3000) and euthanized using an overdose of phenoxy ethanol (1:1000) after being sampled for blood. Another 30 fish were euthanized immediately using an overdose of phenoxy ethanol and stored at − 20 ºC for proximate analysis. At the end of the trial after weighing, 15 fish per tank were sampled for blood using a similar procedure described for the fish at the start. The remaining fish per tank were

Table 1
The macro-nutritional and amino acid composition of fractions A and B in g/kg dry matter (DM), unless stated otherwise, produced from blue mussel (*Mytilus edulis*).

Item	Fraction A	Fraction B
Macro-nutritional		
Dry matter (g/kg product)	971	912
Ash	77	162
Crude protein	802	421
Crude fat	77	82
OC + CF[a]	16	206
Gross energy (MJ/kg DM)	22.4	18.8
Amino acid (g/kg product)		
Alanine	34.2	19.5
Arginine[b]	48.5	18.5
Aspartic acid	65.4	29.8
Cysteine	9.4	5.2
Glutamic acid	82.9	38.5
Glycine	45.5	36.5
Histidine[b]	13.9	6.0
Isoleucine[b]	28.1	9.5
Leucine[b]	45.3	13.8
Lysine[b]	53.9	18.2
Methionine[b]	14.4	4.6
Phenylalanine[b]	26.1	9
Proline	28.1	12.5
Serine	32	12.8
Threonine[b]	31.4	13.1
Tryptophan[b]	6.9	2.6
Tyrosine	25.7	8.9
Valine[b]	29.8	11.2
Total	621.5	270.2

[a] OC = other carbohydrates.
[b] Essential amino acid, CF = crude fiber.

Table 2
Ingredients of the formulated diets with fraction A (DA), a formulated diet with fraction B (DB) or a formulated diet with the 1:1 combination of fractions A and B (DAB) in % on weight basis and the calculated composition in g/kg dry matter (DM), unless stated otherwise.

	Diet		
Item	DA	DB	DAB
Total composition (%)			
Basal ingredients[a]	70	70	70
Test ingredients	30	30	30
Test ingredients (%)			
Fraction A	15.00		7.50
Fraction B		15.00	7.50
Casein	7.25	13.84	10.54
Soy oil	1.03	0.96	1.00
Diamol	2.76		1.38
Corn starch	3.96	0.2	2.08
Calculated composition			
Dry matter (g/kg product)	937	929	934
Crude protein	601.6	601.4	601.5
Crude fat	96.5	96.4	96.5
Gross energy (MJ/kg DM)	19.9	20.0	19.9

[a] Basal ingredient composition: Fish meal Danish LT 14.0 %, Soy protein concentrate 8.8 %, Maize gluten meal 8.1 %, Pea protein concentrate 7.7 %, Sonac 92P 5.1 %, Fish oil, 3.6 %, CPSP special G 3.5 %, Wheat gluten, 3.5 %, Squid meal 2.8 %, Casein 2 %, Soya lecithin 0.4 %, Cholesterol 0.2 %, Cyanocobalamin 0.3 %, MCP 1.1 %, Binder X^2 2.0 %, Binder Y^2 2.0 %, Binder Z^2 1.0 %, Salt 0.7 %, L-threonine 0.3 %, DL-methionine 0.2 %, L-iso leucin 0.2 %, Taurine 0.1 %, Lucantin Pink 0.4, $Premix^3$ 2.0 % (free from iron and vitamin B_{12}). [2]Pellet binders – in house composition 5 %; Salt 2.0 %, MCP 1.5 %; TMG 0.7 %, Yttrium oxide 0.02 %. [3]Vitamins (mg or IU per kg diet): vitamin A (retinyl acetate); 2.7 mg, 9000 IU; vitamin D3 (cholecalciferol), 0.04 mg 1700 IU; vitamin K3 (menadione sodium bisulphite), 10 mg; vitamin B1 (thiamine), 10 mg; vitamin B2 (riboflavin), 20 mg; vitamin B6 (pyridoxine hydrochloride), 12 mg; folic acid, 10 mg; biotin, 0.7 mg; inositol, 800 mg; niacin, 70 mg; pantothenic acid, 30 mg, choline chloride, 1500 mg; vitamin C, 500 mg; vitamin E, 300 mg. Minerals (g or mg per kg diet): Mn (manganese sulphate), 20 mg; I (potassium iodide), 1.5 mg; Cu (copper sulphate), 5 mg; Co (cobalt sulphate), 2 mg; Cr (chromium sulphate), 2.6 mg; Mg (magnesium sulphate), 500 mg; Zn (zinc sulphate) 60 mg; Se (sodium selenite) 0.3 mg; BHT (E300-321) 100 mg; Calcium propionate 1000 mg.

Table 3
The proximate composition, including the iron content of the different diets on product basis and dry matter basis.

	Diet[a]					
Item	RW	BM	DA	DB	DAB	CPL
Product basis (%)						
Dry matter (DM)	19.8	22.2	90.4	89.3	89.2	92.6
Crude protein	14.8	16.8	59.3	59.1	58.4	57.6
Crude fat	3.2	2.3	9.0	8.7	8.8	18.3
Ash	2.0	2.0	11.0	9.6	10.2	9.0
Fibre	0.4	0.3	0.8	0.7	0.7	0.6
Gross energy (MJ/kg)	5.3	5.7	21.9	21.8	21.6	24.4
mg/kg						
Iron	75	77	1540	520	1000	192
µg/kg						
Vitamin B_{12}	306	244	1750	1770	1870	230
DM basis (%)						
Crude protein	74.9	75.6	65.6	66.2	65.5	62.2
Crude fat	16.2	10.3	10.0	9.7	9.9	19.8
Ash	10.1	9.0	12.2	10.7	11.4	9.7
Fibre	2.0	1.3	0.9	0.8	0.8	0.6
Other carbohydrates	− 3.2	3.8	11.3	12.6	12.4	7.7
Gross energy (MJ/kg)	27.0	25.8	24.2	24.4	24.3	26.4
mg/kg DM						
Iron	379	346	1704	582	1121	207
µg/kg DM						
Vitamin B_{12}	1584	1097	1936	1981	2096	248

[a] RW = ragworm, BM = blanched mussel meat, DA = diet with fraction A, DB = diet with fraction B, DAB = diet with 1:1 combination of fraction A and B, CPL = commercial pellet.

immediately euthanized using an overdose of phenoxy ethanol and stored at − 20 ºC for proximate analysis. All sampling was done in one day. Blood was sampled by caudal venous puncture using a heparinized low dead space syringe (ø 0.8 mm, length 30 mm needle). After collection, blood was transferred to tubes and stored on ice until further processing. The haematocrit (Hct) level was determined by using capillaries (ø 1.5 mm, length 75 mm) and a haematocrit centrifuge (Haematocrit 210, Hettich, Germany) to centrifuge for 5 min at 5000 × *g*. Haemoglobin (Hb) levels were determined using the method described by Kampen van and Zijlstra (1961). The remaining part of the blood sample was centrifuged (2050 × *g*, 4 ºC, 10 min) to collect plasma. The plasma samples were pooled per tank, using an equal amount of homogenized plasma per fish free of hemolysis, flash frozen in liquid nitrogen and stored at − 80 ºC until further analysis. Whole fish were directly stored at − 20 ºC until proximate analysis.

2.4. DPPH antioxidant assay

Antioxidant activity of the fractions was analyzed using an adapted DPPH assay (Sharma and Bhat, 2009; Shahidi and Zhong, 2015). MilliQ was added to freeze dried samples to a concentration of 5 mg/mL. Samples were gently shaken for 1–2 h at room temperature on an overhead shaker and centrifuged for 15 min at 18,000 RCF at room temperature. Afterwards 200 µL was pipetted in column 1 and 7 of row A – F of a 96-wells plate and diluted 1:1 in columns 2–6 and 8–12 using 50 % methanol. Row G was used for the control sample uric acid (5 mg/mL) and row H was used for 50 % methanol. In columns 7–12, 5 µL of 1800 mg/L vitamin C was added and 5 µL milliQ was added in columns 1–6. After that, 100 µL of a 200 µM DPPH solution was added to every well. The DPPH solution consisted of 8 mg per 100 mL buffered methanol (40 mL 0.1 M sodium acetate buffer with pH 5.5 plus 60 mL methanol). The plate was shaken in a plate shaker, covered with a sticker, and incubated for 30 min at 37 °C. The absorption was measured at 517 nm in a plate reader (Thermo Scientific Multiskan GO). The antioxidant activity of the fractions is reported as the EC50 which is defined as the effective concentration of the fraction that is necessary to halve the initial DPPH concentration.

2.5. Chemical analyses

Diets, fractions, and fish whole body composition were analyzed by Nutrilab B.V. (Giessen, The Netherlands), except for amino acids and energy content of the fractions. Amino acids were analyzed by the department of Animal Nutrition, (Wageningen, The Netherlands) and energy content was analyzed by the department of Aquaculture and Fisheries, (Wageningen, The Netherlands). Dry matter (DM) was determined by drying at 103 ºC (equivalent to EC-decree 152/2009), ash by combustion at 550 ºC (equivalent to EC-decree 152/2009), nitrogen by Dumas (NEN-EN-ISO 16634), crude fat after pre-extraction and hydrolysis (equivalent to EC-decree 152/2009), crude fibre which are insoluble in acid and alkaline media (equivalent to EC-decree 152/2009), iron by ICP-EOS (NEN-EN-15510) and vitamin B_{12} (cobalamin) by microbiological method (AOAC 952.20). Crude protein was calculated as N × 6.25 for all feedstuffs. Other carbohydrates are calculated by 100 minus percentages of crude protein, crude fat, ash and fiber on dry matter basis. Amino acids were determined after hydrolysis with 6 M HCl for 23 h. The hydrolysate was adjusted to pH 2.2 and amino acids were separated by ion exchange chromatography and determined by post column reaction with ninhydrin, using photometric detection at 570 nm and at 440 nm for proline (ISO 13903). Gross energy was determined by direct combustion using an adiabatic bomb calorimeter (IKA®, C7000; IKA Analyse Technik, Weikersheim, Germany; ISO 9831). In short, the sample was combusted with oxygen under standardized conditions. The measured temperature rise of the water in the calorimeter vessel and the effective heat capacity of the water was used to calculate the gross energy content after corrections for experimental conditions.

2.6. Plasma biochemical analyses

Plasma biochemical analyses were performed by University Veterinary Diagnostic Lab (UVDL, Utrecht, the Netherlands). Plasma was analyzed for osmolality by freezing point depression (OSMO STATION OM-6050, Gansuin-cho, Japan), iron content (End, AU680, Beckman Coulter, Brea, California, USA), total iron binding capacity (calculated), iron saturation (calculated), alanine aminotransferase (ALT, Rate, AU680, Beckman Coulter Brea, California, USA), aspartate aminotransferase (AST, Rate, AU680, Beckman Coulter, Brea, California, USA), cholesterol (End, AU680, Beckman Coulter, Brea, California, USA), triglycerides (End, AU680, Beckman Coulter, Brea, California, USA), folic acid (Solid-phase competitive enzyme immunoassay, (Immulite 2000 xpi, Siemens Healthineers, Germany) and vitamin B_{12} (Solid-phase competitive enzyme immunoassay, Immulite 2000 xpi, Siemens Healthineers, Germany).

2.7. Heavy metal, dioxin, PCB and PAH analyses

Starting material (raw and blanched mussel) and processed fractions (A, B and AB before freeze drying) were homogenized and analyzed with in-house validated methods at Wageningen Food Safety Research (Wageningen, the Netherlands). Moisture was analyzed conform NEN-ISO 1442:1997 based on a direct drying method by thorough mixing of the test portion with sand and drying to constant mass at 103 °C. Processed fractions were analyzed for heavy metals using ICP-MS after nitric acid and hydrogen peroxide addition and digestion in a microwave oven conform Commission Regulation (EC) No. 333/2007 of 28 March 2007. Processed fractions were analyzed for total dioxins, dioxin-like polychlorinated biphenyls (dl-PCBs) and non-dioxin-like polychlorinated biphenyls (ndl-PCBs) using GC-HRMS after homogenization. Extraction was performed using the Smedes method followed by purification and fractionation in an automated system of chromatographic columns. The method for total dioxins, dl-PCBs and ndl-PCBs was based on EPA method 1613 Revision B (1994) and work was performed according to Commission Regulation (EU) No. 2017/644. Processed fractions were analyzed for polycyclic aromatic hydrocarbons (PAHs) using GC-HRMS after homogenization, accelerated

solvent extraction (ASE) and gel permeation chromatography (GPC) to extract the PAHs from the fat, followed by clean-up with aluminum oxide SPE columns. Analyses on PAHs were performed with an in-house method conform Commission Regulation (EU) No 836/2011 of 19 August 2011.

2.8. Statistical analysis

All data related to haematology, plasma biochemical parameters and fish performance were averaged per tank. Data were subjected to a one-way analysis of variance (ANOVA) to test for diet effects with IBM SPSS statistics, version 25 (Armonk, NY, USA). Differences between treatment means were considered significant where $P < 0.05$, and trends were defined where $0.05 < P \leq 0.10$. When differences between treatment means were significant, means were compared using the Fishers LSD post-hoc test. Data are reported as means with the standard error of the means (SEM) and their effect size using η^2. An effect size ≥ 0.14 is considered large (Richardson, 2011).

3. Results

3.1. Body weight, feed intake and growth

Fish mortality was 0.7 % (n = 3 fish) and the water quality parameters remained within set limits. Body weight (g; BW) at the start was similar between treatments ($P = 0.12$; Table 4). After both 26 and 56 days, body weight of RW was higher and BM lower than all other treatments, with no difference between DA, DB, DAB and CPL ($P < 0.001$; Table 4). Apparent feed intake (g DM; aFI) was lower for fish fed BM compared to all other treatments, and was similar between RW, DA, DB, DAB and CPL ($P < 0.01$; Table 4). The apparent feed conversion ratio (aFCR) was similar between the treatments ($P = 0.11$). The protein efficiency ratio (PER) did not differ between treatments, but a trend was noticed with RW being the highest and BM the lowest ($P = 0.08$). After both 26 as well as 56 days growth (g/d), specific growth rate (% body weight/d; SGR) and growth on metabolic body weight ($g/kg^{0.8}$/d; GMBW) of RW was the highest and BM the lowest of all other treatments, with no difference between DA, DB, DAB and CPL ($P < 0.001$; Table 4).

3.2. Blood and plasma parameters

At the start of the experimental period, the average Hct and Hb levels were 9.7 ± 2.6 % and 19.2 ± 5.5 g/L, respectively. At day 56, both Hct and Hb were affected by diet ($P < 0.001$) with an effect size η^2 of 0.85 and 0.93, respectively. The Hct for RW (22.6 ± 2.2 %) was higher than for all other treatments, while CPL (12.6 ± 2.0 %) resulted in lower Hct levels than all other treatments except for DA (Fig. 1A). Treatments BM (15.5 ± 1.1 %), DA (15.2 ± 0.6 %), DB (17.4 ± 1.7 %) and DAB (16.4 ± 1.1 %) were all similar in their effect on Hct levels (Fig. 1A). The Hb level of RW (49.2 ± 2.4 g/L) was higher than all other treatments, and CPL (24.1 ± 2.7 g/L) resulted in lower Hb levels compared to all other treatments (Fig. 1B). The Hb levels of treatments BM (31.0 ± 4.0 g/L) and DA (30.4 ± 2.4 g/L) were comparable, DB (37.0 ± 2.3 g/L) and DAB (35.5 ± 1.3 g/L) were higher than DA, and DB was also higher than BM (Fig. 1B). The average Hct and Hb levels at the start of the experiment were comparable with the average Hct and Hb levels of CPL at the end ($P > 0.05$). Hemolysis during blood sampling occurred in 22, 42, 24, 22, 27 and 9 % of the samples of treatments RW, BM, DA, DB, DAB

Table 4
Average performance parameters and standard error of the means (SEM) of common sole fed the different diets at day 26 and at the end, day 56, with their effect size (η^2) and *P*-value.

		Diet*						SEM	η^2	*P-value*
Parameter	**Unit**	**RW**	**BM**	**DA**	**DB**	**DAB**	**CPL**			
Bodyweight										
Initial (BWt_0)	g	22.1	20.5	22.1	22.4	22.5	21.8	0.24	0.48	0.12
Day 26 (BWt_{26})	g	44.8^{a}	25.2^{c}	39.5^{b}	38.9^{b}	39.4^{b}	38.1^{b}	1.49	0.95	< 0.001
Final (BW_{t56})	g	77.2^{a}	37.7^{c}	61.3^{b}	61.6^{b}	61.7^{b}	58.3^{b}	2.89	0.94	< 0.001
At day 26										
Growth	g/d	0.87^{a}	0.18^{c}	0.67^{b}	0.64^{b}	0.65^{b}	0.63^{b}	0.05	0.96	< 0.001
SGR	% BW/d	2.71^{a}	0.79^{c}	2.24^{b}	2.12^{b}	2.15^{b}	2.15^{b}	0.14	0.97	< 0.001
GMBW	$g/kg^{0.8}$/d	31.2^{a}	9.0^{c}	25.3^{b}	24.5^{b}	25.0^{b}	23.8^{b}	1.69	0.95	< 0.001
At day 56										
Growth	g/d	0.98^{a}	0.31^{c}	0.70^{b}	0.70^{b}	0.70^{b}	0.65^{b}	2.73	0.95	< 0.001
SGR	%BW/d	2.23^{a}	1.09^{c}	1.82^{b}	1.80^{b}	1.80^{b}	1.75^{b}	0.08	0.96	< 0.001
GMBW	$g/kg^{0.8}$/d	12.6^{a}	5.4^{c}	9.8^{b}	9.7^{b}	9.7^{b}	9.4^{b}	0.52	0.96	< 0.001
aFI	g/DM	1100^{b}	481^{a}	1081^{b}	1178^{b}	1123^{b}	967^{b}	67.5	0.72	< 0.01
$aFCR_{DM}$	–	0.8	1.1	1.1	1.2	1.2	1.1	0.05	0.49	0.11
PER	–	1.7	1.2	1.4	1.3	1.4	1.5	0.06	0.52	0.08

* RW = ragworm, BM = blanched mussel meat, DA = diet with fraction A, DB = diet with fraction B, DAB = diet with 1:1 combination of fraction A and B, CPL = commercial pellet. With growth in g/d, SGR = specific growth rate, GMBW = growth on metabolic body weight, aFI = apparent feed intake, $aFCR_{DM}$ = apparent feed conversion rate of dry matter and PER = protein efficiency ratio. [a-c] Values with different superscripts indicate a significant difference between treatments using the Fishers LSD post hoc test ($P < 0.05$).

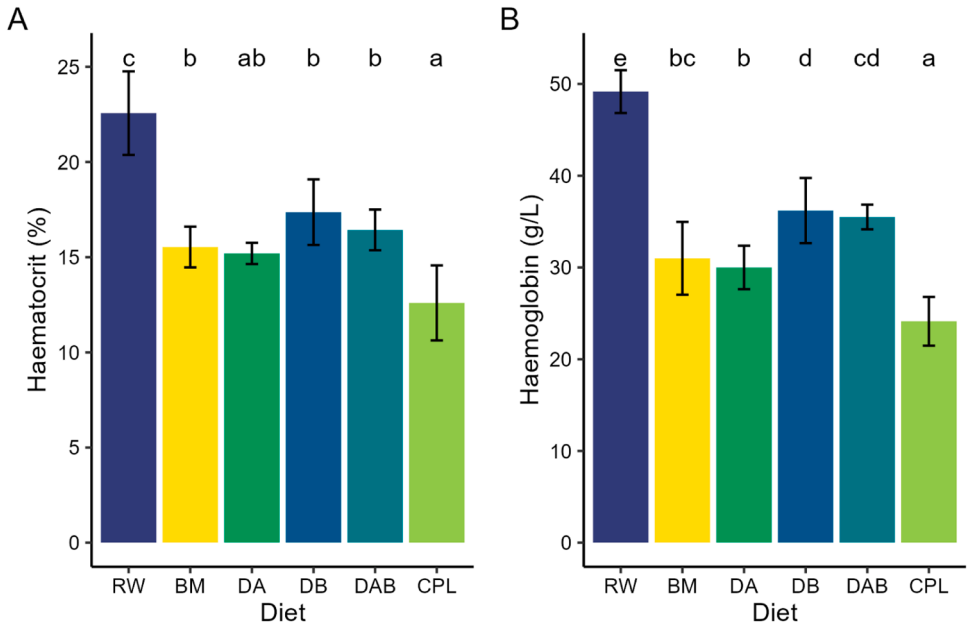


Fig. 1. Haematocrit (%; A) and haemoglobin (g/L; B) ± SD values at day 56 of the sole fed the different diets: RW = ragworm, BM = blanched mussel meat, DA = diet with fraction A, DB = diet with fraction B, DAB = diet with 1:1 combination of fraction A and B and CPL = commercial pellet. Bars with different superscripts indicate a significant difference between treatments using the Fishers LSD post hoc test ($P < 0.05$). The effect size (η^2) is 0.85 and 0.93 for Hct and Hb respectively.

and CPL respectively, and was not statistically different between treatments ($P > 0.05$).

For all blood plasma biochemical parameters there was a difference between treatments, except for plasma osmolality (Table 5). Plasma iron content was highest for RW and lowest for CPL, with BM higher than DA, DAB and CPL, and DB higher than CPL. ($P < 0.001$). The iron binding capacity was highest for CPL and lowest for BM, and no difference between the other treatments ($P = 0.012$). The difference in iron saturation between treatments was similar to iron content, with RW having the highest iron saturation and CPL the lowest ($P < 0.001$). The liver enzyme alanine aminotransferase (ALAT) level was highest for RW, then for CPL, and lowest for BM, with treatments DA, DB and DAB yielding similar results to CPL and BM ($P < 0.001$). The liver enzyme aspartate aminotransferase (ASAT) level was higher for RW and lower for BM compared to all other treatments, and no difference between treatments DA, DB, DAB and CPL ($P < 0.001$). The ratio ASAT/ALAT was lower for RW than all for other treatments, higher for DB than for both BM and CPL, and similar for BM, DA, DAB and CPL ($P = 0.002$). The plasma cholesterol level was highest for RW, then for CPL, lowest for BM and no difference between DA, DB and DAB ($P < 0.001$). The plasma triglyceride level was highest for RW, lowest for both BM and CPL, and no difference between DA, DB and DAB ($P < 0.001$). The folic acid level was highest for RW, lowest for BM, with all other treatments displaying intermediary levels ($P = 0.04$). Vitamin B_{12} levels for DA, DB and DAB were all higher than for RW and

Table 5
Average values of the plasma biochemical parameters and standard error of the means (SEM) analysed in the fish fed the different diets, with their effect size (η^2) and *P*-value.

		Diet*						SEM	η^2	*P-value*
Parameter	Unit	RW	BM	DA	DB	DAB	CPL			
Osmolality	mOsmol/kg	341	343	331	334	348	336	2.1	0.47	0.128
Iron	mol/L	28.5^{d}	20.5^{c}	16.0^{ab}	18.0^{bc}	16.1^{ab}	14.0^{a}	1.22	0.91	< 0.001
IBC†	µmol/L	85.9^{bc}	76.0^{a}	86.3^{bc}	80.0^{ab}	78.8^{ab}	91.8^{c}	1.59	0.73	0.012
Iron saturation	%	33.3^{d}	27.1^{c}	18.7^{ab}	22.4^{bc}	20.5^{ab}	15.3^{a}	1.54	0.86	< 0.001
ALAT	U/L	25.6^{c}	3.0^{a}	6.6^{ab}	5.6^{ab}	6.0^{ab}	8.7^{b}	1.89	0.93	< 0.001
ASAT	U/L	420^{c}	104^{a}	316^{b}	299^{b}	292^{b}	308^{b}	23.5	0.94	< 0.001
ASAT/ALAT	–	16.8^{a}	34.7^{b}	48.6^{bc}	53.5^{c}	50.3^{bc}	35.7^{b}	3.50	0.76	0.002
Cholesterol	mmol/L	10.9^{d}	4.8^{a}	6.8^{b}	6.6^{b}	6.9^{b}	8.8^{c}	0.48	0.97	< 0.001
Triglycerides	mmol/L	9.9^{c}	3.3^{a}	7.4^{b}	7.0^{b}	7.9^{b}	4.4^{a}	0.55	0.93	< 0.001
Folic Acid	mmol/L	160^{c}	39^{a}	96^{abc}	97^{abc}	81^{ab}	115^{bc}	11.5	0.58	0.04
Vitamin B_{12}	ng/mL	138^{b}	87^{a}	236^{e}	181^{cd}	213^{de}	169^{bc}	12.5	0.90	< 0.001

* RW = ragworm, BM = blanched mussel meat, DA = diet with fraction A, DB = diet with fraction B, DAB = diet with 1:1 combination of fraction A and B, CPL = commercial pellet.

† Iron binding capacity.

a-eValues with different superscripts indicate a significant difference between treatments using the Fishers LSD post hoc test ($P < 0.05$).

BM, with the value for DA being the highest, and the level for CPL was similar to that for both DB and DAB ($P < 0.001$).

3.3. Fish whole body composition

At the start of the experimental period the proximate composition of the whole fish was 622, 258, 124 g/kg DM for crude protein, crude fat, and ash respectively, and 12.4 mg/kg DM of iron. Whole body DM, crude protein, crude fat, gross energy and iron content were affected by diet ($P < 0.01$; Table 6). Whole body DM content was highest for RW and CPL and was lowest for BM. Whole body crude protein was highest for BM, lowest for CPL and similar for the other treatments. Treatments RW and CPL had the highest whole body crude fat content, crude fat content was lowest for BM, and similar for the other treatments. Gross energy was highest for RW and CPL, similar for DA, DB and DAB and lowest for BM. Whole body iron content was the highest for RW, followed by fish fed DB and DAB and the lowest for fish fed CPL. Whole body DM was positively correlated with whole body crude fat ($y = 2.64 x - 43.79$, $R2 = 0.69$, $P < 0.001$) and negatively correlated with whole body crude protein ($y = -2.06 x + 118.44$, $R2 = 0.59$, $P < 0.001$).

3.4. DPPH antioxidant assay

The antioxidant activity of the blue mussel fractions A and B were analyzed using the DPPH assay. As a control uric acid was measured. The EC50 of fraction A showed a higher numerical value than fraction B (2.2 mg/mL compared to 1.1 mg/mL), although the differences appeared not to be large.

3.5. Contaminants

Analyses of heavy metals, dioxins, PCBs and PAHs were performed on the starting material (raw and blanched mussel) and the processed material used in the feed formulation before freeze drying (fraction A, fraction B and fraction AB). Analyses results for these contaminants are indicative only (Table 7). The measured concentrations of lead, arsenic and cadmium did not exceed the maximum levels allowed in long-term supply formulations of feed for particular nutritional purposes. In addition, mercury concentrations did not exceed maximum levels allowed in feed materials. Concentrations of dl-PCBs and ndl-PCBs also did not exceed the maximum levels allowed in feed materials of animal (fish) origin. Although, not exceeding maximum levels for food fishery products and bivalve molluscs, concentrations of total dioxins and dl-PCBs found in the starting and processed material did exceed the maximum levels allowed in feed materials of animal (fish) origin. Measured concentrations of PAH4 and PAH16 were within similar range of PAH levels found in bivalves from European waters (Damir et al., 2022; Olenycz et al., 2015).

4. Discussion

4.1. Feed intake, growth and haematology

Body weight over treatments, on average, almost tripled during the experiment (Table 4). As far as we know, the growth rates achieved in our study by the formulated diets are one of the highest measured in experimental studies. Only the growth rate in this study with diet RW has been achieved before in studies using ragworm (Ende et al., 2014; Kals et al., 2015, 2017). Ende et al. (2014) concluded that the growth rate of common sole fed with mussel or ragworm is higher compared to the growth rate of sole fed an artificial diet because of a higher nutrient and energy intake combined with improved retention efficiencies. However, the growth rate of the fish fed diet BM was the lowest of all treatments and lower than the growth rate of 8.1 $g/kg^{0.8}/d$ observed by Ende et al. (2014) for sole fed fresh mussel meat. Similarly, the apparent feed intake (aFI) of BM was the lowest of all treatments. It is possible that the batch of mussels used in this experiment was low in attractivity and/or palatability and/or nutritional value, although the latter seems unlikely as the apparent feed conversion ratio (aFCRdm) was comparable to the formulated diets and in line with the ratio's reported by Ende et al. (2014). Baynes and Howell (1993) already reported that cooked mussel meat (3 min boiling) and deep-freeze storage

Table 6
Average whole body composition of the fish fed the different diets and standard error of the means (SEM), with their effect size (η^2) and *P*-value. Averages are expressed in g/kg DM, unless otherwise stated.

		Diet[†]						SEM	η^2	*P-value*
Parameter	Unit	RW	BM	DA	DB	DAB	CPL			
Dry matter	g/kg	281.4^{d}	249.4^{a}	265.3^{bc}	257.1^{ab}	266.7^{c}	278.3^{d}	0.29	0.89	< 0.001
Crude protein	g/kg DM	629.1^{b}	680.1^{c}	632.0^{b}	657.4^{bc}	632.7^{b}	590.5^{a}	0.77	0.76	0.002
Crude fat	g/kg DM	305.6^{c}	207.1^{a}	253.7^{b}	250.2^{b}	265.3^{b}	302.7^{c}	0.91	0.81	< 0.001
Ash	g/kg DM	105.4	117.6	109.1	108.9	107.4	107.7	0.23	0.17	0.78
Gross energy	MJ/g DM	26.3^{c}	24.2^{a}	25.1^{ab}	25.2^{b}	25.4^{bc}	26.0^{bc}	0.19	0.79	0.005
Iron	mg/kg DM	19.9^{d}	13.9^{ab}	13.1^{ab}	16.6^{c}	15.3^{abc}	11.9^{ab}	0.74	0.73	0.004

[†] RW = ragworm, BM = blanched mussel meat, DA = diet with fraction A, DB = diet with fraction B, DAB = diet with 1:1 combination of fraction A and B, CPL = commercial pellet.

a-dValues with different superscripts indicate a significant difference between treatments using the Fishers LSD post hoc test ($P < 0.05$).

Table 7
The content of cadmium, lead, arsenic, mercury, dioxins, PCBs and polycyclic aromatic hydrocarbons in raw mussel, blanched mussel and mussel fractions A, B and AB.

	Substrate					
	Raw Mussel	Blanched	Fraction A	Fraction B	Fraction AB	EU-limits[a]
Moisture (%)	80.6	80.1	81.3	90.8	83.1	NA
Heavy metals (mg/kg, 12 % moisture)						
Cadmium	0.55	0.44	0.62	0.75	0.62	2
Lead	1.58	1.55	2.40	1.53	1.93	10
Arsenic	11.79	9.73	8.94	16.26	11.46	25
Mercury	0.11	0.12	0.18	< 0.007	0.15	0.5
Dioxins (12 % moisture)						
Total dioxins[b]	1.64	2.16	4.29	2.20	3.23	1.25
Sum of dioxins and dl-PCBs[c]	3.36	3.93	6.64	3.73	5.26	4.0
dl-PCB[d]	1.72	1.77	2.35	1.53	2.03	2.00
ndl-PCB[e]	16.65	16.81	20.66	15.11	18.54	30.00
PAHs (ng/g, 12 % moisture)						
PAH4	25.87	29.66	38.10	28.69	30.18	NA
PAH16	45.32	53.06	65.91	62.22	52.10	NA

DM = dry matter.
[a] Maximum content in mg/kg (ppm) relative to a feed with a moisture content of 12 %. Concentrations of dl-PCBs were assessed against its action threshold (Commission Directive (EC) No 2002/32).
[b] (ng WHO-PCDD/F-TEQ/kg DM (ppt)).
[c] (ng WHO-PCDD/F-PCB-TEQ/kg (ppt)).
[d] (ng WHO TEQ/kg DM (ppt)).
[e] (µg/kg DM (ppb)).

negatively affect growth and survival of sole. In the current study the mortality rate is not comparable to those of Baynes and Howell (1993), 0.7 % vs. 90 % respectively, and were not related to treatment. The difference with the findings of Baynes and Howell (1993) might be the duration of the boiling process (3 min vs. 30 s).

To the best of our knowledge, besides our group, little or no work related to the blood values of common sole has been performed, making comparison with blood data of common sole from other groups difficult. The fish at the start of the experiment are considered moderately anemic as discussed by Kals et al. (2015, 2016, 2019). In Kals et al. (2017) it is suggested that the slow growth of the sole fed commercial pellets might be due to the low Hct and Hb levels found in these fish. Low Hct and Hb levels limit maximum oxygen uptake and oxygen carrying capacity (Gallaugher and Farrell, 1998), preventing the sufficient uptake of oxygen and therewith lower the scope for growth. Even though hemolysis was observed, resulting in lower Hct values, and not different between treatments in this study, clear and similar patterns are observed for both Hct and Hb (Fig. 1). Both Hct and Hb are lowest for diet CPL and highest for RW, with approximately a 100 % difference, and with the values for CPL being not different from the start of the experiment, still pointing towards moderate anaemia. For the formulated diets with the mussel fraction (diet DA, DB and DAB) both Hct and Hb are higher than for CPL. These results support the hypothesis that at least one fraction of blue mussel yield similar or better results in haematology and performance than blanched mussel in this study. The ability of mussel to alleviate anaemia is not affected by the blanching and fractionation processes, or by incorporating the fractions in a formulated pellet. Adding 15 % mussel fraction resulted in similar or even higher Hct and Hb values than BM, indicating that the responsible compounds are concentrated due to the fractionation process. However, the diets containing fractions of blue mussel did not reach similar levels in haematology and performance as ragworm, and this part of the hypothesis can be rejected. The experimental setup considered the possibility of co-factors by evaluating diets DA, DB and DAB. The results showed either no co-factors were needed, or enough co-factor stayed in both fractions, otherwise larger differences on their effect on haematology would have been expected. The formulated diets with mussel fraction resulted in higher feed intake and growth rates than BM, but the feed intake and growth rates were similar to CPL, despite their higher Hct and Hb values. These results that the feed intake and growth are similar between DA, DB, DAB and CPL do not fit with the idea that the slow growth of sole fed with the commercial diet might be the consequence of low Hct and Hb levels, which limit the oxygen uptake and therewith lowers the scope for growth as described by Kals et al. (2017).

4.2. Plasma biochemical parameters

Anaemia in fish has not been well studied. In humans, multiple causes can underly anaemia, of which iron deficiency is a common condition linked to anaemia (Camaschella, 2015). The diagnosis of an iron deficiency is in most cases based on parameters like plasma iron, total transferrin saturation, plasma total iron binding capacity and plasma ferritin, but the clinical diagnosis can be difficult in the presence of inflammatory conditions (Camaschella, 2015, 2017). During an iron deficiency, both the plasma ferritin and plasma transferrin saturation are decreased and the total iron binding capacity increased. However, in animals ferritin is difficult to measure, species-specific, and tends to increase during inflammation (Thrall et al., 2012). Therefore, plasma transferrin saturation, which reflects the iron availability for erythropoiesis (Thomas et al., 2013), is often used as an alternative to diagnose iron deficiency. In addition, a decrease in plasma transferrin saturation is one of the earliest biomarkers of an iron deficiency, whether absolute or

functional, of which the latter is an iron deficiency due to inflammation (Peyrin-Biroulet et al., 2015). In the current experiment, the plasma iron content and saturation are the highest in sole fed with ragworm (Table 5). The iron plasma content and iron saturation show a similar pattern to Hb levels in sole fed the different diets e.g. rising Hb levels reflect the higher plasma iron contents and a higher iron saturation. The pattern of the total iron binding capacity is expected to show the opposite to the pattern of the plasma iron content and iron saturation, because the total iron binding capacity normally increases during an iron deficiency. However, this is not the case in this experiment. As expected, the total iron binding capacity is the highest in sole fed the commercial diet, but it is not different from sole fed with ragworm. We find it difficult to explain this contrast. When looking at the pattern of whole-body iron content (Table 3), using the low levels of the plasma iron content and transferrin saturation, the haematological parameters and the relation between whole body iron content and Hb, sole fed diet DA and CPL would be indicated as iron deficient and moderately anemic. This supports the finding of Kals et al. (2017) after evaluation of the expression of hepcidin, transferrin, ferritin, hsp70 and casp3 in the liver of common sole that the anaemia in common sole fed the commercial diet is a nutritional anaemia, and not a functional anaemia caused by inflammatory conditions. The hypothesis that at least one of the fractions yields similar results in plasma biochemical parameters related to anaemia as blanched mussel is confirmed for plasma iron content, iron binding capacity and iron saturation. Compared to ragworm the hypothesis is only confirmed for the iron binding capacity, but not for iron content and iron saturation. This suggests at least one of the fractions promotes the plasma biochemical parameters related to the alleviation of anaemia. The underlying mechanisms by which mussel, the produced mussel fractions and ragworm can alleviate this nutritional anaemia in common sole are not clear. Further research with the aim of gaining insight into the working principle of these phenotypic findings and which compounds are involved is expected to give more understanding.

Common sole kept under aquaculture conditions can experience nutritional anaemia, of which the levels of vitamin B_{12} in both the feed and plasma are important factors to consider. In general, fish obtain vitamin B_{12} from their diet and/or from vitamin B_{12} producing bacteria in the gut via absorption or diffusion (Pawlak et al., 2012; Nielsen et al., 2012). In the current study plasma vitamin B_{12} levels of the animals fed RW and BM diets are lower than when fed CPL, while the dietary vitamin B_{12} levels in RW, BM and CPL feed were 1548, 1097 and 248 μg/kg DM, respectively. This contrasts with Kals et al. (2019) which suggested that vitamin B_{12} absorption in sole is limited, and that sole may depend on high dietary levels of vitamin B_{12}. Maxwell (1951) suggested a specific metabolic role of vitamin B_{12} that aids in gas exchange or respiration by enhancing respiratory pigment e.g. Hb, especially in species that live in muddy environments often experiencing anoxic conditions. Common sole are well adapted to survive long times of hypoxia, or occasionally anoxia, and maintain relatively high ATP production (Dalla Via et al., 1994; Della Via et al., 1997). The plasma vitamin B_{12} levels (Table 5) of common sole in this experiment are on average about 16–78 times higher than plasma vitamin B_{12} levels reported for Channel catfish (*Ictalurus punctatus*), Nile tilapia (*Oreochromis niloticus*), grouper (*Epinephelus malabaricus*) and mullet which are between 2.2 and 10.7 ng/mL (Limsuwan and Lovell, 1981; Lovell and Limsuwan, 1982; Lin et al., 2010; Starr et al., 1957). This suggests a physiological difference between common sole and the other mentioned fish species. A possible direct role of vitamin B_{12} in the respiration of common sole is speculative, and as far as we know, no direct role is assigned to vitamin B_{12} in the binding or transport of oxygen. However, vitamin B_{12} does share structural similarities to porphyrins, such as the heme-group from haemoglobin (Valentin and Ruma, 2013). In addition, under physiological conditions, Co^{3+}, Co^{2+}, and Co^{+} states of cobalamin do occur and the conversion of aqua-Co^{3+}-corrinoids to Co^{2+}-corrinoids is possible (Derevenkov et al., 2016). Therefore, vitamin B_{12} might potentially function as an alternative oxygen source in common sole. This could partially explain that the growth on CPL and DB were not different, even though the Hb levels in the CPL feed were lower than in DB. The speculation that vitamin B_{12}, or other involved factors, can play a direct role in the respiration of sole needs further research.

4.3. DPPH antioxidant assay as an indicator to alleviate anaemia

The DPPH antioxidant assay showed a stronger antioxidant activity of fraction B, but the difference was not large between fraction A and fraction B. This is in line with animal performance, haematology, and biochemical parameters, where diets A and B often performed similarly as well, with the exception for Hb and Vitamin B_{12}. However, the antioxidant activity value of ragworm with an IC50 value $>$ 10 mg/mL measured in an earlier trial (unpublished data) was much lower than that of the mussel fractions. As both the Hct and Hb values, the plasma iron content and iron saturation are the highest in sole fed ragworm it seems that the antioxidant activity of the diets measured by the DPPH antioxidant assay is not directly related to the alleviation of anaemia in common sole. Therefore, the hypothesis that the antioxidant activity measured by the DPPH assay of the mussel fractions is an indicator for their ability to alleviate anaemia in common sole must be rejected.

4.4. The effect of processing on contaminants in mussel fractions

The processing of blue mussel changed the levels of heavy metals, dioxins, PCBs and PAHs in the extracts differently, depending on the product, processing technique and the contaminant. Relatively low levels of contaminants were found in the mussel fractions. Except for total dioxins and dl-PCBs, all measured contaminant levels did not exceed the maximum content allowed in complementary feed and feed materials in the EU (Commission Directive (EC) No 2002/32).

5. Conclusions

Dietary inclusion of 15 % mussel fraction B alleviates anaemia in common sole and increases haemoglobin levels more compared to blanched mussel, fraction A or AB, indicating the responsible compounds are more concentrated in fraction B. In addition, the

production processes for the fractions and pelleting did not noticeably affect their ability to alleviate anaemia, indicating that these diets are a reliable and effective means of promoting haemoglobin levels in common sole. The antioxidant activity of the fractions is not related to their ability to alleviate anaemia in common sole. Furthermore, relatively low levels of heavy metals and PAHs were found, which were not exceeding corresponding EU maximum levels for feed. The high plasma vitamin B_{12} levels found in common sole compared to other fish species suggest a specific metabolic or physiological role of vitamin B_{12} in common sole. These findings can have significant implications for the culture of common sole.

CRediT authorship contribution statement

Jeroen Kals: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **W. Muizelaar:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **A.M.J Kootstra:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **E.W.J. Gerrits:** Writing – review & editing, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **C.W. Tao:** Writing – review & editing, Formal analysis, Data curation. **H.M. de Vogel van den Bosch:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **P. Geerdink:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Y. Telleman:** Writing – review & editing, Methodology, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. This research was funded by the Wageningen University & Research Knowledge Base Programme KB34 “Circular & Climate Neutral Society” (KB Marine Resources (KB-34-2c-4), KB Biorefinery (KB34-3a-1) and High value ingredients from marine biomass for health (KB34-003-004)) that is supported by financing from the Dutch Ministry of Agriculture, Fisheries, Food security and Nature (LVVN). There are no additional activities to disclose.

Acknowledgements

The authors wish to thank the people from Carus-ARF in particular Menno ter Veld and Wian Nusselder for their help and caretaking of the experimental animals during the experiment, Ronald Booms, Piet van Wikselaar, Gisabeth Binnendijk, Edoardo Zaccaria, Elianne van der Valk, Annemarie Mens, Femke Bosman-van Schothorst and Eline Burgers for their support during sampling, Marjo Koets, Willem Drost, Nicole Engelen, Jacqueline Donkers, Ronald Vroon, Arnoud Togtema and Jan Jetten for their support with the processing of mussels and Elise Hoek for her support finalizing the contribution of WFSR. This research was funded by the Wageningen University & Research Knowledge Base Programme KB34 “Circular & Climate Neutral Society” (KB Marine Resources (KB-34-2c-4), KB Biorefinery (KB34-3a-1) and High value ingredients from marine biomass for health (KB34-003-004)) that is supported by financing from the Dutch Ministry of Agriculture, Fisheries, Food Security and Nature (LVVN).

Declarations

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

References

Balasubramanian, C.P., Shyne, A., Kannappan, S., Biju, I.F., 2018. Training manual on recent advances in farming of pacific white shrimp (*Penaeus vannamei*). Train. Man. Ser. 14, 126. ⟨https://krishi.icar.gov.in/jspui/bitstream/123456789/23385/1/NFDB%20training%20final%20manual.pdf⟩.

Baynes, S.M., Howell, B.R., 1993. Observations on the growth, survival and disease resistance of juvenile common sole, *Solea solea* (L.), fed *Mytilus edulis* L. Aquac. Fish. Manag. 24, 95–100. https://doi.org/10.1111/j.1365-2109.1993.tb00831.x.

Camaschella, C., 2015. Iron deficiency: new insights into diagnosis and treatment. Hematol. Am. Soc. Hematol. Educ. Program 1, 8–13. https://doi.org/10.1182/asheducation-2015.1.8.

Camaschella, C., 2017. New insights into iron deficiency and iron deficiency anemia. Blood Rev. 31 (4), 225–233. https://doi.org/10.1016/j.blre.2017.02.004.

Chakraborty, K., Joy, M., 2020. High-value compounds from the molluscs of marine and estuarine ecosystems as prospective functional food ingredients: an overview. Food Res. Int. 137, 109637. https://doi.org/10.1016/j.foodres.2020.109637.

Commission Directive (EC) No 2002/32. Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. ⟨https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02002L0032-20191128⟩.

Commission Regulation (EC) No. 333/2007 of 28 March 2007. Laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and polycyclic aromatic hydrocarbons in foodstuffs (Text with EEA relevance). ⟨http://data.europa.eu/eli/reg/2007/333/2012-09-01⟩.

Commission Regulation (EU) No 836/2011 of 19 August 2011. Amending Regulation (EC) No 333/2007 laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs Text with EEA relevance. ⟨https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=celex%3A32011R0836⟩.

Commission Regulation (EU) 2017/644 of 5 April 2017 laying down methods of sampling and analysis for the control of levels of dioxins, dioxin-like PCBs and non-dioxin-like PCBs in certain foodstuffs and repealing Regulation (EU) No 589/2014. ⟨https://eur-lex.europa.eu/eli/reg/2017/644/oj#:~:text=Commission%20Regulation%20(EU)%202017%2F,(Text%20with%20EEA%20relevance.%20)⟩.

Dalla Via, J., van den Thillart, G., Cattani, O., De Zwaan, A., 1994. Influence of long-term hypoxia exposure on the energy metabolisme of *Solea solea*. II. Intermediary metabolism in blood, liver and muscle. Mar. Ecol. Prog. Ser. 111 (1-2), 17–27. https://doi.org/10.3354/meps111017.

Damir, N.A., Coatu, V., Pantea, E.D., Galaţchi, M., Botez, E., Birghilă, S., 2022. Assessment of polycyclic aromatic hydrocarbons content in marine organisms of commercial interest from the Romanian black sea coast. Polycycl. Aromat. Compd. 42 (10), 7595–7606.
Della Via, J.D., van den Thillart, G., Cattani, O., Cortesi, P., 1997. Environmental versus functional hypoxia/anoxia in sole *Solea solea*: the lactate paradox revisited. Mar. Ecol. Prog. Ser. 154, 79–90. ⟨http://www.jstor.org/stable/24857845⟩.
Derevenkov, I.A., Salnikov, D.S., Silaghi-Dumitrescu, R., Makarov, S.V., Koifman, O.I., 2016. Redox chemistry of cobalamin and its derivatives. Coord. Chem. Rev. 309, 68–83. https://doi.org/10.1016/j.ccr.2015.11.001.
Ende, S.S.W., Kroeckel, S., Schrama, J.W., Schneider, O., Verreth, J.A.J., 2014. Feed intake, growth, and nutrient retention of common sole (*Solea, solea* L.) fed natural pray and an artificial feed. Aquac. Res. 1–8. https://doi.org/10.1111/are.12526.
EPA method 1613 Revision B, 1994. Tetra- through Octa-Chlorinated Dioxins and Furans by Isotope Dilution HRGC/HRMS ⟨https://well-labs.com/docs/epa_method_1613b_1994.pdf⟩.
Gallaugher, P., Farrell, A.P., 1998. Haematocrit and blood oxygen carrying capacity. In: Perry, S.F., Tufts, B. (Eds.), Fish Physiology, vol. 17. Fish respiration. Academic Press, New York, pp. 185–219. https://doi.org/10.1016/S1546-5098(08)60262-9.
Grienke, U., Silke, J., Tasdemir, D., 2014. Bioactive compounds from marine mussels and their effects on human health. Food Chem. 142, 48–60. https://doi.org/10.1016/j.foodchem.2013.07.027.
Kals, J., 2017. The Development of A Sole Diet Based on the Composition of Ragworm (PhD thesis). Wageningen University -ISBN 9789463431804 -136 p. ⟨https://doi.org/10.18174/412951⟩.
Kals, J., Blonk, R.J.W., Mheen, H.W. van der, Schrama, J.W., Verreth, J.A.J., 2015. Mussel (*Mytilus edulis* L.) and ragworm (*Nereis virens*, Sars) both alleviate anaemia in common sole (*Solea solea* L.). Aquac. Res. https://doi.org/10.1111/are.12871.
Kals, J., Blonk, R.J.W., Mheen, H.W. van der, Schrama, J.W., Verreth, J.A.J., 2016. Feeding ragworm (*Nereis virens* Sars) increases haematocrit and haemoglobin levels in common sole (*Solea solea* L.). Aquac. Res. 47 (10), 3346–3349. https://doi.org/10.1111/are.12767.
Kals, J., Blonk, R.J.W., Palstra, A.P., Sobotta, T.K., Mongile, F., Schneider, O., Planas, J.V., Schrama, J.W., Verreth, J.A.J., 2017. Feeding ragworm (*Nereis virens* Sars) to common sole (*Solea, solea* L.) alleviates nutritional anaemia and stimulates growth. Aquac. Res. 48 (3), 752–759. https://doi.org/10.1111/are.12919.
Kals, J., Blonk, R.J.W., Mheen, H.W. van der, Schrama, J.W., Verreth, J.A.J., 2019. Effect of vitamin B12 and taurine on the alleviation of nutritional anaemia in common sole (*Solea solea*). Aquac. Nutr. 25, 456–465. https://doi.org/10.1111/anu.12871.
Kampen van, E.J., Zijlstra, W.G., 1961. Standardization of hemoglobinometry II. The hemiglobincyanide method. Clin. Chim. Acta 6, 538–544. https://doi.org/10.1016/0009-8981(61)90145-0.
Limsuwan, T., Lovell, R.T., 1981. Intestinal synthesis and absorption of vitamin B-12 in channel catfish. J. Nutr. 1, 2125–2132. https://doi.org/10.1093/jn/111.12.2125.
Lin, Y., Wu, J., Shiau, S., 2010. Dietary cobalt can promote gastrointestinal bacterial production of vitamin B12 in sufficient amounts to supply growth requirements of grouper. Epinephelus malabaricus Aquac. 302 (1–2), 89–93. https://doi.org/10.1016/j.aquaculture.2010.02.008.
Lovell, R.T., Limsuwan, T., 1982. Intestinal synthesis and dietary non-essentiality of vitamin B12 for Tilapia nilotica. Trans. Am. Fish. Soc. 111 (4). https://doi.org/10.1577/1548-8659(1982)111%3C485:ISADNO%3E2.0.CO;2.
Macedo, M.W.F.S., Cunha, N.B., Carneiro, J.A., Costa, R.A., Alencar, S.A., Cardoso, M.H., ranco, O.L., Dias, S.C., 2021. Marine organisms as a rich source of biologically active peptides. Front. Mar. Sci. 8, 667764. https://doi.org/10.3389/fmars.2021.66776.
Maxwell, B.E., 1951. A Study of Vitamin B12 in Some Marine Invertebrates (Thesis). University of British Columbia, Canada. 71 p.
Neves, A.C., Harnedy, P.A., FitzGerald, R.J., 2016. Angiotensin converting enzyme and dipeptidyl peptidase-IV inhibitory, and antioxidant activities of a blue mussel (*Mytilus edulis*) meat protein extract and its hydrolysates. J. Aquat. Food Prod. Technol. 25 (8), 1221–1233. https://doi.org/10.1080/10498850.2015.1051259.
Nielsen, M.J., Rasmussen, M.R., Andersen, C.B.F., Nexø, E., Moestrup, S.K., 2012. Vitamin B12 transport from food to the body's cells—a sophisticated, multistep pathway. Nat. Rev. Gastroenterol. Hepatol. 9, 345–354. https://doi.org/10.1038/nrgastro.2012.76.
Olenycz, M., Sokołowski, A., Niewińska, A., Wołowicz, M., Namieśnik, J., Hummel, H., Jansen, J., 2015. Comparison of PCBs and PAHs levels in European coastal waters using mussels from the Mytilus edulis complex as biomonitors. Oceanologia 57 (2), 196–211.
Ortiz, J.A., 2016. Sustainable maturation protocol for shrimp using pellets as the main feeds. Aqua Cult. Asia Pac. 12 (4), 20–22. ⟨https://aquaasiapac.com/issue/july-aug-2016/⟩.
Pawlak, R., James, P.S., Raj, S., Cullum-Dugan, D., Lucus, D., 2012. Understanding vitamin B12. Am. J. Lifestyle Med. 7, 60–65. https://doi.org/10.1177/1559827612450688.
Peyrin-Biroulet, L., Williet, N., Cacoub, P., 2015. Guidelines on the diagnosis and treatment of iron deficiency across indications: a systematic review. Am. J. Clin. Nutr. 102 (6), 1585–1594. https://doi.org/10.3945/ajcn.114.103366.
Richardson, J.T.E., 2011. Eta squared and partial eta squared as measures of effect size in educational research. Educ. Res. Rev. 6 (2), 135–147. https://doi.org/10.1016/j.edurev.2010.12.001.
Shahidi, F., Zhong, Y., 2015. Measurement of antioxidant activity. J. Funct. Foods 18, 757–781. https://doi.org/10.1016/j.jff.2015.01.047.
Sharma, O.P., Bhat, T.K., 2009. DPPH antioxidant assay revisited. Food Chem. 113, 1202–1205. https://doi.org/10.1016/j.foodchem.2008.08.008.
Slater, M., D'Abramo, L., Engle, R.C., 2018. Editorial. Aquaculture research priorities for the next decade: a global perspective. J. World Aquac. Soc. 49 (1), 3–6. https://doi.org/10.1111/jwas.12503.
Stabili, L., Licciano, M., Giangrande, A., Gerardi, C., De Pascali, S.A., Fanizzi, F.P., 2019. First Insight on the mucus of the annelid *Myxicola infundibulum* (polychaeta, sabellidae) as a potential prospect for drug discovery. Mar. Drugs 17 (7), 396. https://doi.org/10.3390/md17070396.
Starr, T.J., Martinez, D., Fosberg, W., 1957. The vitamin-B12 activity of mullet and shark serum. Limnol. Oceanogr. https://doi.org/10.4319/lo.1957.2.2.0111.
Tacon, A.J., 2017. Biosecure shrimp feeds and feeding practices: guidelines for future development. J. World Aquac. Soc. 48 (3), 381–392. https://doi.org/10.1111/jwas.12406.
Thomas, D.W., Hinchliffe, R.F., Briggs, C., Macdougall, I.C., Littlewood, T., Cavill, I., 2013. British Committee for Standards in Haematology. Guideline for the laboratory diagnosis of functional iron deficiency. Br. J. Haematol. 161, 639–648. https://doi.org/10.1111/bjh.12311.
Thrall, M.A., Weiser, G., Allison, R.W., Campbell, T.W., 2012. Veterinary Hematology and Clinical Chemistry, 2nd ed. Wiley-Blackwell Oxford, UK, p. 792. https://doi.org/10.1177/1559827612450688.
Valentin, C., Ruma, B., 2013. Chapter 10 cobalt and corrinoid transport and biochemistry. In: Banci, Lucia (Ed.), Metallomics and the Cell. Metal Ions in Life Sciences. https://doi.org/10.1007/978-94-007-5561-10_9 (vol. 12).