Algal Research 47 (2020) 101869

Contents lists available at ScienceDirect

Algal Research

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

Incorporation of defatted microalgal biomass (*Tetraselmis* sp. CTP4) at the expense of soybean meal as a feed ingredient for juvenile gilthead seabream (*Sparus aurata*)

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ARTICLE INFO

Keywords:
Biorefinery
Defatted microalgal biomass
Feed ingredient
Microalgae
Soybean
Sparus aurata

ABSTRACT

The forecasted growth of the aquaculture sector requires the use of novel and sustainable ingredients in aquaculture feeds. A study was undertaken to evaluate the effect of a 10% incorporation of defatted microalgal biomass (DMB) of *Tetraselmis* sp. CTP4, used at the expense of dehulled solvent-extracted soybean meal (SBM), on the growth performance, nutrient digestibility and physiological response to confinement stress in gilthead seabream juveniles. The trial comprised two dietary treatments: a control diet (CTRL) with relatively high levels of marine-derived proteins and 10% SBM; and a test diet (DMB10) with the incorporation of 10% DMB at the expense of SBM, while maintaining a fair constancy of all other ingredients. Triplicate groups of 30 fish, with a mean initial body weight of 6.0 ± 0.2 g were fed the experimental diets for 61 days. At the end of the trial, fish tripled their initial body weight, but the overall growth performance criteria (final body weight, daily growth index, feed conversion ratio and protein efficiency ratio), whole-body composition and nutrient retention were not significantly affected by the dietary treatments ($p > 0.05$). The DMB10 diet showed a significantly higher apparent digestibility coefficients (ADC) of dry matter, energy and phosphorus ($p < 0.05$). When measured as an isolated feed ingredient, the DMB had an ADC of protein, fat, energy and phosphorus of 87.9, 85.3, 75.5 and 41.4%, respectively. After an acute confinement stress test, fish fed with DMB10 diet displayed a significantly lower plasma cortisol response (120 ± 23 ng/mL) than those fed with the control diet (160 ± 33 ng/mL) ($p < 0.05$). Overall results showed that DMB, issued from biorefinery processes, could potentially spare the use of soybean meal in aquaculture feeds, contributing towards a reduction of the current protein deficit in the European market.

1. Introduction

There is increasing interest in large-scale production of microalgal biomass as a sustainable lipid feedstock for different biotechnological applications, which include human and animal nutrition as well as biodiesel production [1]. However, the downstream processing entailing the extraction of lipids from the biomass will generate massive amounts of defatted microalgal biomass (DMB) as a co-product. Several reports have investigated the suitability of upgrading these DMB into different biofuels to improve the net energy ratio of the whole production pipeline such as production of biogas, bioethanol and bio-oil (e.g., hydrothermal liquefaction or pyrolysis) in a biorefinery setting [2–5]. Although there is a high demand for renewable sources for global fuel supply from the market and policymakers, biofuels need to be relatively inexpensive in order to compete with fossil fuels. Therefore, to enable the commercial use of microalgae as feedstock for the generation of bioenergy, the production and processing costs have to be offset by higher-end commodities obtained from DMB and other residues.

Whole microalgal biomass (WMB) and DMB are feed ingredients not only as a solution to meet the high demand for feedstocks required by the feed industry, but also as a way to meet future demand caused by the expected growth of the human population in the forthcoming decades [6]. Thus far, most studies have focused on the incorporation of

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https://doi.org/10.1016/j.algal.2020.101869
Received 16 August 2019; Received in revised form 5 March 2020; Accepted 6 March 2020

WMB in feed without any processing, either as an additive or as a macro-ingredient [7–12]. Overall, most reports show that WMB is a promising feed ingredient with wide application in the farming of different livestock. Reports evaluating the applicability of DMB as a feed ingredient for land animals have been published [6,13–15], as well as in aquaculture species [16–22]. Indeed, over the last decades, alternative sources (e.g., vegetable protein sources, processed animal proteins, insect meals, krill meal) have been introduced in aquaculture feeds in order to reduce the dependency of aquafeeds on fishmeal. However, research is still needed for finding and fine-tuning innovative sources of feed ingredients for the aquaculture industry in order to decrease its dependence on non-sustainable feedstocks and thus ensure the future sustainability of commercial fish supply [23].

Among the alternatives proposed, soybean meal is one of the most used feedstocks for feed manufacturing. Dehulled solvent-extracted soybean meal (SBM) is a high-quality protein source with steady supply and competitive costs. This has triggered a significant rise in demand for soybean and derivatives (meal and oil) for livestock production and, more recently, for biodiesel production as well. However, sustainability concerns have been raised [24,25], as extensive and ever-increasing soybean farming areas have become a major driver for worldwide deforestation and loss of biodiversity in developing countries, along with other environmental and social concerns [26]. According to recent European Union (EU) reports [27,28], 60% of the world deforestation is related to the production of soybean and derivatives, which are mainly imported and consumed by EU countries. The EU animal feed market is highly dependent on protein feed imports and its self-sufficiency in soybean meal is extremely low (3%) [29]. This situation makes the animal feed sector highly vulnerable to trade distortions, availability and price volatility of soybeans [29,30]. A reduction of the EU protein deficit is a priority and requires the emergence of novel protein resources such as microalgae.

In this context, a nutritional study was undertaken to assess the effects of incorporating defatted microalgal biomass (from *Tetraselmis* sp. CTP4) at the expense of soybean meal, on the growth performance, digestibility and nutrient retention of gilthead seabream (*Sparus aurata*) juveniles. In addition, since microalgal biomass was previously suggested to be a promising feedstock with immunomodulatory and anti-stress effects in aquatic animals [16], an acute confinement test was performed to evaluate such effect.

2. Materials and methods

2.1. Defatted microalgal biomass

A defatted microalgal biomass of *Tetraselmis* sp. CTP4 was generated upon the extraction of lipids directly from wet microalgal paste using an ethanolic extraction. The detail procedure used for biomass growth has been previously described by Pereira et al. [31]. The methodology used for lipid extraction was based on the protocol of Yang et al. [32] with modifications. Briefly, wet microalgal paste was sequentially extracted (three times) with absolute ethanol at reflux temperature. After each extraction, the algae cake (DMB) was separated from the solvent by centrifugation (2000*g*, 10 min). Upon completion of the lipid extraction, DMB was air-dried at ambient temperature for 24 h. Further drying of the biomass was achieved at 40 °C using a forced air-circulating oven until constant weight. The cake was later milled to powder and stored under vacuum in a desiccator until the manufacture of the experimental diets. The composition of the experimental defatted microalgal biomass of *Tetraselmis* sp. CTP4 (DMB) and its comparison to soybean meal (SBM) and fishmeal (FM) is presented in Table 1.

Table 1
Composition of defatted microalgal biomass (DMB), dehulled solvent-extracted soybean meal (SBM) and fishmeal (FM) (values expressed on a fresh matter basis). n.a., not analysed.

	DMB	SBM	FM
Proximate composition			
Moisture, %	0.90	9.20	6.60
Crude protein, %	40.63	42.04	71.85
Crude fat, %	1.29	2.00	6.90
Ash, %	14.57	5.54	13.07
Total phosphorus, %	0.93	0.60	1.92
Gross energy, kJ/g	17.10	17.45	19.78
Amino acids (%)			
Essential amino acids (EAA)			
Arginine	3.82	3.22	4.71
Histidine	0.86	1.14	1.75
Isoleucine	1.72	1.96	2.54
Leucine	3.16	3.32	5.10
Lysine	2.16	2.67	5.96
Methionine	1.10	0.58	2.61
Phenylalanine	2.79	2.18	3.38
Threonine	2.10	1.71	3.50
Tryptophan	n.a.	0.61	0.71
Valine	1.98	2.07	3.22
Subtotal	19.67	19.46	33.48
Nonessential amino acids (NEAA)			
Alanine	2.48	1.89	4.38
Aspartic acid	3.27	4.89	6.92
Cysteine	0.37	0.61	0.33
Glutamic acid	3.76	7.74	8.92
Glycine	2.28	1.82	5.20
Proline	1.85	2.21	2.93
Serine	2.18	2.18	3.15
Tyrosine	2.00	1.60	2.59
Subtotal	18.18	22.94	34.42
Total	37.85	42.40	67.90
Ratio (NEAA/EAA)	0.92	1.18	1.03

2.2. Formulation of experimental diets

The growth performance trial comprised two dietary treatments. A control diet (CTRL) containing relatively high levels of marine-derived proteins (fishmeal, fish hydrolysate and squid meal) and several plant proteins such as soy protein concentrate, wheat gluten, corn gluten meal and dehulled solvent-extracted soybean meal (SBM). Fish oil was used as the main lipid source. A second diet (DMB10) was formulated with the incorporation of 10% DMB at the expense of soybean meal, while maintaining a fair constancy of all other ingredients. Both diets were supplemented with monocalcium phosphate to avoid the risk of phosphorus imbalance: 0.5% and 0.3% for CTRL and DMB10 diets, respectively. Overall, these two diets were isonitrogenous (crude protein: 58% DM), isolipidic (crude lipids: 16.6% DM) and isoenergetic (gross energy: 18.7 kJ/g DM). A part of each experimental diet contained also 1% chromic oxide as an inert marker for digestibility measurements. One additional diet (DMB ADC) containing 70% of the same basal mixture of the control diet with 1% chromic oxide and 30% of the test ingredient (defatted microalgae biomass) was also manufactured to allow the measurement of the apparent digestibility of the individual test ingredient, according to the methodological approach recommended by NRC [33].

Experimental diets were manufactured by SPAROS, Lda. (Olhão, Portugal). Ingredients were mixed according to target formulation and ground (< 250 μm) in a micropulverizer hammer mill (Hosokawa-Alpine, 1SH, Germany). Powdered ingredients and fish oil were mixed in a paddle mixer (MAINCA, RM90, Spain) and the blend moisturized with 25% water. Diets were manufactured by low-shear and low temperature extrusion (Italplast P55, Italy) at a pellet size of 1.0 mm. Upon extrusion, pellets were dried in a vibrating fluid bed dryer (TGC Extrusion, DR100, France). Throughout the trial, experimental feeds were stored at room temperature, but in a cool and aerated emplacement. Samples of each diet were taken for proximate composition (Table 2).

Table 2
Formulation and composition of the three experimental diets (g/100 g): control diet (CTRL), a diet with 10% inclusion of defatted microalgal biomass (DMB10) and a diet for the ingredient apparent digestibility coefficient calculation (DMB ADC).

	CTRL	DMB10	DMB ADC
Fishmeal[a]	20.00	20.00	14.00
Fish hydrolysate[b]	5.00	5.00	3.50
Fish gelatin[c]	2.00	2.00	1.40
Squid meal[d]	12.50	12.50	8.75
Soy protein concentrate[e]	10.00	10.00	7.00
Soybean meal[f]	10.00	–	7.00
Defatted microalgal biomass[g]	–	10.00	30.00
Wheat gluten[h]	8.00	8.00	5.60
Corn gluten[i]	8.00	8.70	5.60
Wheat meal[j]	9.50	8.90	6.65
Fish oil[k]	12.00	12.10	8.40
Vitamin and mineral premix[l]	1.50	1.50	1.05
Soy lecithin[m]	0.50	0.50	0.35
Binder[n]	0.20	0.20	0.14
Antioxidant[o]	0.20	0.20	0.14
Sodium propionate[p]	0.10	0.10	0.07
Monocalcium phosphate[q]	0.50	0.30	0.35
Chromic oxide[r]	1.00	1.00	0.70
Dry matter (DM), %	96.5	97.4	97.2
Crude protein, % DM	58.3	57.8	54.1
Crude fat, % DM	16.5	16.7	16.0
Ash, % DM	6.07	6.41	7.89
Total phosphorus, % DM	1.20	1.20	1.29
Gross energy, kJ/g DM	18.7	18.8	19.2
Chromic oxide, % DM	1.28	1.18	1.22

[a] Fish meal NORVIK 70: 70.3% crude protein (CP) 5.8% crude fat (CF), Sopropêche, France.
[b] CPSP 90: 83% CP, 9% CF, Sopropêche, France.
[c] Fish gelatin: 96% CP, LAPI Gelatine SPA, Italy.
[d] Squid meal: 83% CP, Sopropêche, France.
[e] Soycomil P: 63% CP, 0.8% CF, ADM, The Netherlands.
[f] Dehulled solvent extracted soybean meal: 46% CP, 2.3% CF, CARGILL, Spain.
[g] defatted microalgal biomass from *Tetraselmis* sp. CTP4: 41% CP, 1.3 CF.
[h] VITAL: 80% CP, 1.7% CF, Roquette Frères, France.
[i] Corn gluten meal: 61% CP, 6% CF, COPAM, Portugal.
[j] Wheat meal: 10.2% CP; 1.2% CF, Casa Lanchinha, Portugal.
[k] Fish oil, Savinor UTS, Portugal.
[l] PREMIX Lda, Portugal (IU or mg/kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate, 7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings.
[m] Lecico P700IPM, LECICO GmbH, Germany.
[n] Guar gum: Seah International, France.
[o] Paramega PX, KEMIN EUROPE NV, Belgium.
[p] Sodium propionate: Disproquimica, Portugal.
[q] MCP: 22% P, 18% Ca, Fosfitalia, Italy.
[r] Chromic oxide: 98% Alfa Aeser, Germany.

2.3. Growth trial

All trials were performed at the experimental research facilities of SPAROS (Olhão, Portugal), and conducted by trained scientists (following category C FELASA recommendations) according to the European guidelines on protection of animals used for scientific purposes (Directive 2010/63/UE of European Parliament and of the European Union Council).

Gilthead seabream (*Sparus aurata*) juveniles, originated from a commercial hatchery (CUPIMAR, Cádiz, Spain), were adapted to the experimental conditions over a period of 15 days. Homogenous groups of 30 fish each, with a mean initial body weight of 6.0 ± 0.2 g, were stocked in 6 sub-square fiberglass tanks (volume: 60 L; water-flow rate: 3.5 L/min), supplied with thermo-regulated seawater, with 200% water renewal/h (temperature: 20.3 ± 1.1 °C; dissolved oxygen: 6.0 ± 0.5 mg/L; salinity: 35‰). A 12L:12D photoperiod was maintained with daybreak set at 7:00am. Each dietary treatment was tested in triplicate tanks over 61 :days. Fish were fed to apparent satiety, by hand, three times a day (930 am, 200 :pm and 500 pm) and utmost care was taken to avoid feed wastage and allow a precise quantification of feed intake. Light anesthetized fish (25 mg/L of MS-222, Germany) were group weighed at the start of the trial, at day 30 and day 61 for estimation of tank biomass. At the start of the trial, a pool of 15 whole fish from the initial stock and a pool of 5 whole fish per tank at the end of the trial were sampled and stored at −20 °C for subsequent analysis of whole-body proximate composition.

2.4. Acute confinement stress test

At the end of the growth trial and two days after all associated samplings, the remaining fish (average body weight: 20.8 ± 0.4 g) were subjected to an acute confinement stress test. Eight fish from each replicate tank were transferred from the 60 L tanks with a rearing density of 10 kg/m^3, to a plastic container at a density of 60 kg/m^3. The water level was maintained at a minimum, forcing dorsal fin exposure, and the test was carried out for 15 min. Afterwards, a sample of blood (1 mL) was collected from all fish by puncture of the caudal vein with a heparinized syringe. Blood was placed in cooled 1.5 mL plastic tubes and centrifuged at 6000*g* for 5 min at 4 °C. Upon centrifugation, supernatant plasma was transferred to Eppendorf tubes, snap-frozen in liquid nitrogen and stored at −80 °C until subsequent analysis of cortisol.

2.5. Digestibility measurements

Following the growth trial and the acute confinement stress test, the apparent digestibility of nutrients and energy of the test ingredient and of the experimental diets was measured by the indirect method. The remaining fish (n = 25 per tank) were maintained in the 60 L tanks equipped with a feces settling column. Each group of fish was fed the same diet and reared under identical water conditions as those described for the growth trial. Fish were fed once a day (9.00 am) by hand in slight excess. Upon a thorough cleaning of the rearing tanks from any feed residues, feces were collected daily for 10 consecutive days by means of a feces decantation column (Guelph system). Feces were collected approximately 18 h after the meal. After removal of excess water, daily feces were frozen at −20 °C. Pooled feces from each group of fish were freeze-dried prior to subsequent analysis.

Apparent digestibility coefficients (ADC) of dietary nutrients and energy in the experimental diets were calculated according to NRC [33]:

$$\mathrm{ADC}(\%) = 100 - \left[\frac{\%\text{marker diet}}{\%\text{marker faeces}} \times \frac{\%\text{nutrient faeces}}{\%\text{nutrient diet}}\right]$$

Subsequently, the apparent digestibility coefficients of the test ingredient were calculated according to NRC [33]:

$$\text{ADC Test Ingredient}(\%) = \mathrm{ADC_{TD}} + (\mathrm{ADC_{TD}} - \mathrm{ADC_{RD}}) \times (0.7 \times \mathrm{N_{RD}})/(0.3 \times \mathrm{N_{TI}})$$

$\mathrm{ADC_{TD}}$: ADC of test diet (%).
$\mathrm{ADC_{RD}}$: ADC of reference diet (%).
$\mathrm{N_{RD}}$: Nutrient content in the reference diet (% or kJ/g).
$\mathrm{N_{TI}}$: Nutrient content in the test ingredient (% or kJ/g).

2.6. Chemical analysis

The proximate composition analysis of the test ingredient, experimental diets, whole fish and feces was performed using the following analytical methods. Dry matter after drying at 105 °C for 24 h; total ash by combustion (550 °C during 6 h) in a muffle furnace (Nabertherm L9/11/B170, Germany); crude protein (N × 6.25) by a flash combustion technique followed by a gas chromatographic separation and thermal conductivity detection (LECO FP428); total lipids were quantified by a modified Bligh and Dyer [34] method, as described in Pereira et al. [35]; total phosphorus was determined according to the ISO/DIS 6491 method, using the vanado-molybdate reagent; gross energy in an adiabatic bomb calorimeter (Werke C2000, IKA, Germany); chromic oxide in feeds and feces was determined by spectrometry (SpectrAA 220 FS, Varian) according to Bolin et al. [36] after perchloric acid digestion. The amino acid profile was determined by ultra-performance liquid chromatography (UPLC) as reported by Aragão et al. [37]. The concentration of cortisol in the plasma was evaluated by a radioimmunoassay as described in Rotllant et al. [38].

2.7. Statistical analysis

Growth performance data and ADC were expressed as means ± standard deviation of three replicates. Statistical analyses were performed with R computing software [39]. Parameters expressed as percentage were subjected to arcsine square root transformation. Statistical significance was tested using a Student's *t*-test at a 0.05 probability level.

3. Results and discussion

3.1. Growth performance

The overall growth performance of fish fed with the experimental diets over a period of 61 days is presented in Table 3. At the end of the growth trial, fish showed a 3-fold increase in their initial body weight. No significant differences were found among the two dietary treatments in terms of final body weight (FBW), daily growth index (DGI), feed conversion ratio (FCR), protein efficiency ratio (PER) or survival ($p > 0.05$). However, fish fed the DMB10 diet showed a slight decrease in the daily feed intake (FI, $p = 0.018$).

Patterson and Gatlin [17] reported the inclusion of DMB from *Navicula* sp., *Chlorella* sp. and *Nannochloropsis salina* at the expense of fishmeal and soy protein concentrate in diets for juvenile red drum (*Sciaenops ocellatus*). The authors suggested a safe inclusion level of DMB up to 10% of dietary protein without affecting significantly the fish performance, since higher incorporations of DMB in the experimental diets led to decreased survival, weight gain and feed intake. Similarly, Ju et al. [16] tested the replacement of fishmeal by *Haematococcus pluvialis* DMB on the Pacific whiteleg shrimp *Litopenaeus vannamei*. In this study, it was shown that a dietary inclusion of DMB up to 12.5% did not affect the growth parameters as compared to those of the CTRL feed. More recently, it was reported that an inclusion level up to 20% of *Desmodesmus* sp. DMB and 10% *Nannochloropsis oceanica* DMB in the feeds of Atlantic salmon did not affect the growth performance and health parameters [19,21]. In addition, inclusion levels up to 15% of different whole microalgal biomasses in striped bass did not impact the growth performance [40].

The dietary inclusion of DMB at the expense of SBM had no significant effect on the whole-body composition of fish in terms of moisture, protein, fat, ash, phosphorus and energy ($p > 0.05$; Table 3). Values of whole-body composition are in accordance with those obtained in other experiments with seabream [41–44]. This absence of effects of dietary DMB inclusion on the whole-body composition of fish has been observed in several other studies [16,17,21,45]. Nonetheless, it is interesting to note that a reduction of whole-body fat associated with the dietary inclusion of microalgae, when used as whole biomasses, has been described in Japanese flounder [46], common carp [47,48], Atlantic salmon [48] and gilthead seabream [49]. The mechanisms underlying this lipid-lowering effect are not completely understood. Commonly, these algal biomasses contain liposoluble carotenoids (e.g., fucoxanthin in *Phaeodactylum tricornutum*) which have been associated with lower accumulation of abdominal white adipose tissue in rodents, due to a depression of lipogenic enzymes activities and an increase on fatty acid oxidation [50–52]. Conversely, lipid extraction process applied to the current microalgal biomass of *Tetraselmis* sp. CTP4 may probably result in the removal of these carotenoids and therefore eliminate such effect.

Based on data from feed intake and whole-body composition of fish, nutrient and energy retention (expressed as percentage of intake) were calculated (Fig. 1). Dietary treatments had no significant effect on the protein, fat, phosphorus and energy retention ($p > 0.05$). Similar findings have been reported by Valente et al. [45] in European seabass fed graded levels of a defatted *Nannochloropsis* sp. biomass. Conversely, it was previously reported that the retention of protein and energy was significantly reduced in experimental diets fed to *Sciaenops ocellatus*

Table 3
Growth performance and whole-body composition of fish (IBW[a] = 6.0 ± 0.2 g), fed both experimental diets: a control diet (CTRL) and a diet with 10% inclusion of defatted microalgal biomass (DMB10). Values are means ± standard deviation ($n = 3$). Values highlighted in bold present significant differences between dietary treatments.

	CTRL	DMB10	*p*-Value
FBW[b], g	20.9 ± 0.27	20.7 ± 0.54	0.60
FI[c], %ABW/d	2.59 ± 0.03	2.50 ± 0.02	**0.02**
DGI[d]	1.56 ± 0.02	1.55 ± 0.04	0.54
FCR[e]	1.42 ± 0.02	1.40 ± 0.06	0.67
PER[f]	1.26 ± 0.02	1.29 ± 0.06	0.45
Survival, %	97.8 ± 1.92	94.4 ± 6.94	0.64
Whole-body composition (%, wet weight)			
Moisture	69.4 ± 0.33	68.9 ± 0.34	0.18
Ash	3.01 ± 0.34	2.99 ± 0.43	0.83
Protein	15.3 ± 0.57	15.2 ± 0.30	0.38
Fat	8.09 ± 2.71	8.01 ± 2.67	0.77
Phosphorus	0.64 ± 0.03	0.61 ± 0.04	0.21

Body composition of initial fish (% wet weight): 70.9% Moisture; 3.30% Ash; 15.4% Protein; 8.46% Fat; 0.33% Phosphorus.

[a] Initial mean body weight.
[b] Final mean body weight.
[c] Feed intake per day: crude feed intake/average body weight/61 days.
[d] Daily growth index: ($FBW^{1/3} - IBW^{1/3}$) / 61 days) × 100.
[e] Feed conversion ratio: wet weight gain/dry feed intake.
[f] Protein efficiency ratio: wet weight gain/crude protein intake.

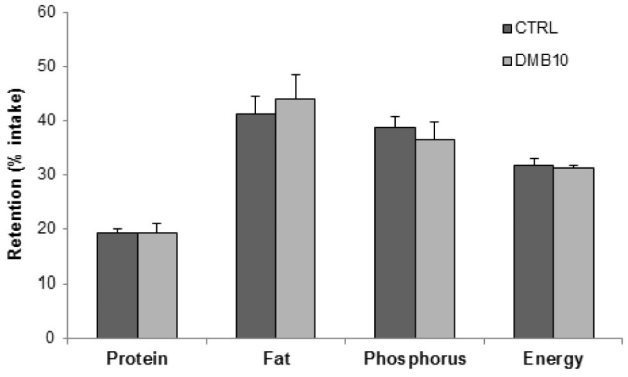

Fig. 1. Nutrient and energy retention in juvenile gilthead seabream, fed both experimental diets: a control diet (CTRL) and a 10% inclusion of defatted microalgal biomass (DMB10). Bars are means ± standard deviation ($n = 3$).

Table 4
Apparent digestibility coefficients (ADC) of control (CTRL) and 10% diet inclusion of defatted microalgal biomass (DMB10) as well as 30% diet inclusion of defatted microalgal biomass (DMB ADC). Values are means ± standard deviation (n = 3). Values highlighted in bold present significant differences between dietary treatments.

ADC (%)	CTRL	DMB10	p-Value	DMB ADC
Dry matter	69.4 ± 0.4	72.0 ± 0.3	**0.001**	25.3 ± 2.7
Protein	94.9 ± 0.2	95.3 ± 0.4	0.280	87.9 ± 0.4
Fat	87.9 ± 0.5	88.0 ± 0.1	0.860	85.3 ± 1.8
Energy	86.5 ± 0.8	88.0 ± 0.2	**0.036**	75.5 ± 5.4
Phosphorus	73.8 ± 0.2	75.9 ± 0.8	**0.017**	41.4 ± 2.9

containing 10% of DMB [17].

3.2. Digestibility of test ingredient (DMB) and experimental diets

Test ingredient, DMB from *Tetraselmis* sp. CTP4, showed apparent digestibility coefficients (ADC) of protein, fat and energy of 87.0, 85.3 and 75.5%, respectively (Table 4). A direct comparison with previously reported ADC values for SBM in *Sparus aurata* [53] shows that DMB had a similar protein digestibility and a slightly higher energy digestibility (72% in SBM). In gilthead seabream, the DMB from *Tetraselmis* sp. CTP4 showed a moderate phosphorus digestibility (41.4%). Data on the phosphorus digestibility of microalgae biomasses is extremely scarce, but this value of phosphorus ADC in *Tetraselmis* sp. CTP4 is higher than values previously reported for SBM in European seabass (36.1%), Senegalese sole (27.6%) and rainbow trout (22%) [54–56]. Approximately 70% of the total phosphorus in plant feedstuffs is present in the form of phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphosphate) and is largely indigestible by fish (NRC, 2011). Moreover, phytic acid has a strong binding affinity to other dietary minerals (e.g., calcium, iron, zinc) and proteins, inhibiting their absorption and therefore is generally considered as an antinutritional factor in fish [57]. Little information exists on the phosphorus forms present in microalgae [58,59]. Although requiring a thorough evaluation, there are indications that microalgae predominantly store inorganic phosphorus in vacuoles as polyphosphate granules, with variable positions of the phosphate groups on the inositol ring [59], and therefore may be more bioavailable for gastric liberation and intestinal absorption than phytic acid. Moreover, microalgae show the potential for tailoring their properties, and Erpel et al. [60] recently reported the development of a *Chlamydomonas reinhardtii* mutant that expressed phytase activity that could contribute to enhance phosphorus digestibility in monogastric animals.

In the experimental diets (Table 4), the ADC of dry matter varied between 69 and 72%, with fish fed diet DMB10 presenting a significantly higher digestibility, than those fed the CTRL diet ($p < 0.05$). Similarly, the ADC of energy for DMB10 (88%) was also significantly higher when compared to that of the CTRL diet (86.5%; $p < 0.05$). A similar result was previously reported for *Navicula* sp. DMB, where the ADC of energy of the diet containing 10% DMB was higher than the reference diet [17]. However, other studies have also shown that the inclusion of DMB or whole microalgal biomasses tend to reduce energy digestibility in fish [21,22,45,61,62]. This reduction of energy digestibility in microalgae-rich diets is often associated to an increase of dietary levels of complex carbohydrates, and particularly of non-starch polysaccharides [45,61,62]. Most of these studies targeted a scenario of replacing fishmeal by microalgal meals that consequently results in an increase of dietary non-starch polysaccharides levels. Although the starch content of DMB was not analysed in the present work, the whole microalgal biomass of *Tetraselmis* sp. CTP4 is known to be a source of starch-like polysaccharides [63], which can justify the slight increase in the ADC of energy in the DMB diet. Protein digestibility was similar in both CTRL and DMB10 diets, 94.9 and 95.3%, respectively ($p > 0.05$). The ADC of protein obtained in the present work was higher compared to previous reports (80–85%) using DMB from *Navicula* sp. in red drum [17], *Nannochloropsis* sp. and *Desmodesmus* sp. in salmon [22] and a blend of *Tisochrysis lutea* and *Tetraselmis suecica* in *Dicentrarchus labrax* [64]. Fat digestibility ranged 88.0% in both diets ($p > 0.05$). Phosphorus digestibility was significantly enhanced in fish fed the DMB10 diet ($p < 005$). The exact mechanisms underlying this effect are unknown, but as mentioned before there are indications that, even though microalgae are photosynthetic organisms, they might show an arrangement of phosphate groups around the inositol ring with a higher bioavailability than phytic acid [59].

The comparative carcass analysis combined with data on the ADC of both diets, allowed the calculation of the nitrogen and phosphorus mass balance (Fig. 2). Regarding the nitrogen mass balance, values for daily nitrogen gain (428 to 446 mg N/kg ABW/day) were not affected by dietary treatment. On the other hand, a 10% diet inclusion of DMB reduced fish total nitrogen losses (fecal and metabolic) when compared to fish fed with the CTRL diet, although only the fecal nitrogen losses were significantly lower ($p < 0.05$). These results are linked to a slightly lower nitrogen intake allied with a slightly higher nitrogen

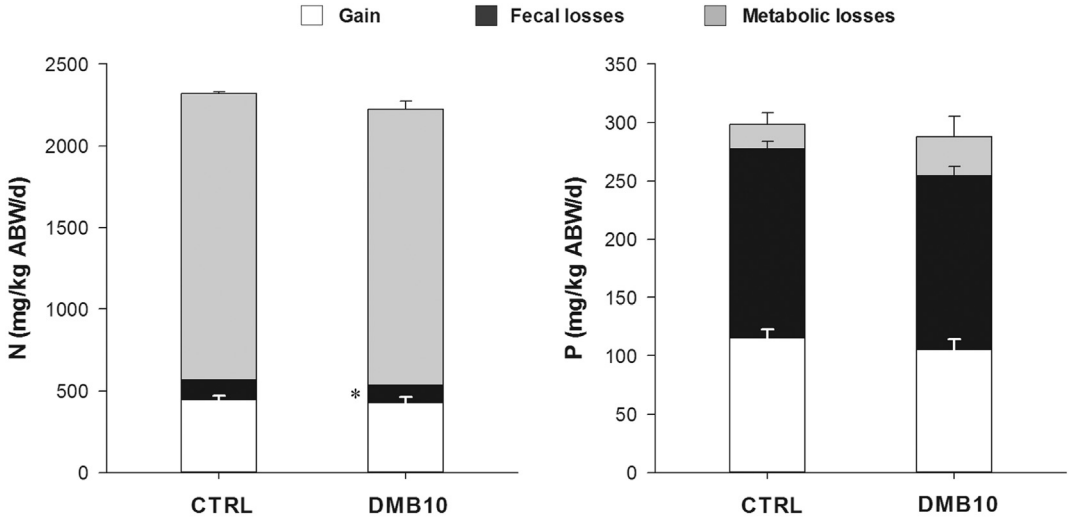

Fig. 2. Daily nitrogen and phosphorus balance in gilthead seabream fed experimental diets: control diet (CTRL) and a diet with 10% inclusion of defatted microalgal biomass (DMB10). Bars are means ± standard deviation (n = 3). N/P Gain: (final carcass N/P content − initial carcass N/P content)/ABW/Days. Fecal N/P loss: crude N/P intake (mg/kg ABW/day) × (100 − ADC Nitrogen/Phosphorus). Metabolic N/P losses: N/P gain – N/P fecal losses. * represent significant differences.

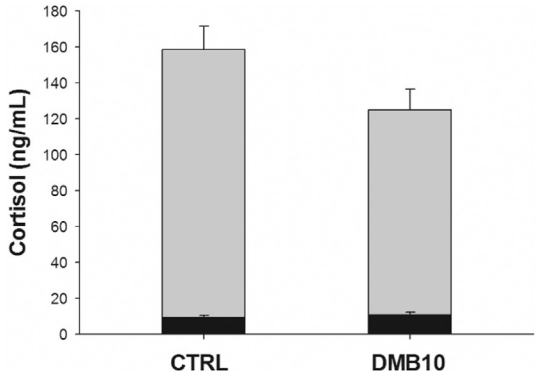

Fig. 3. Changes in plasma cortisol of seabream exposed to an acute confinement stress, fed with CTRL (control) and DMB10 (10% inclusion of defatted microalgal biomass) experimental diets. Black bars represent the basal values of cortisol in the plasma, while grey bars represent the cortisol response of stressed fishes. Asterisk denotes significant differences ($p < 0.05$). Bars are means ± standard deviation ($n = 15$).

digestibility, resulting in a lower fecal loss of fish fed DMB10 experimental diet. Regarding phosphorus mass balance, no significant differences were observed on phosphorus gain (115 and 105 mg P/kg ABW/Day, respectively), as well as the metabolic and fecal losses in fish fed with CTRL and DMB10 diets. The high fecal losses here obtained can be related with an excess of the total phosphorus supplied in both experimental diets [65]. Although this mineral is of the utmost importance for fish development (e.g., synthesis of phospholipids and nucleic acids), excess addition on the diets and consequent losses through feces can lead to negative impacts in the environment (e.g., eutrophication).

3.3. Acute confinement stress

The basal cortisol values and response after an acute confinement stress of fish fed with both diets is presented in Fig. 3. The basal cortisol values (black bars) of both treatments in the tanks that were not subjected to the acute confinement stress were similar (~10 ng/mL), and the values obtained were within those normally observed for seabream [66,67]. However, after the acute confinement stress, an effective cortisol response with significant differences ($p < 0.05$) between both treatments was observed. Fish fed with DMB10 displayed a lower cortisol response (120 ± 23 ng/mL) compared to those fed with the control diet (160 ± 33 ng/mL). Nath et al. [68] also reported a slight decrease in cortisol values of guppy fry (*Poecilia reticulata*) fed with *Parietochloris incisa* compared to the CTRL diet after an acute confinement stress. Plasma cortisol values are normally used in fish physiology to study the effect of stress events, since cortisol is responsible for various physiological processes and is the main stress (corticosteroid) hormone in fish [69]; therefore, increased levels of plasma cortisol indicates higher physiological stress level. Even though significant differences were observed between both treatments, the analysis of a single hormone is not sufficient to claim a stress-protecting activity. However, the results here obtained are a preliminary indication that the DMB of *Tetraselmis* sp. CTP4 might reduce the stress in juvenile gilthead seabream. In fact, microalgae have previously been reported to contain anti-inflammatory and anti-stress bioactivities that seem to promote the health of aquatic animals [16].

4. Conclusions

In conclusion, the replacement of 10% SBM by DMB from *Tetraselmis* sp. CTP4 did not affect overall growth performance, whole-body composition and nutrient retention in gilthead seabream juveniles. In addition, the ADC of protein, fat and energy in DMB were also similar to those previously published for SBM. Therefore, if large-scale production of microalgae alongside with usage of edible oils or biofuels becomes a reality, DMB can be considered as a promising alternative to complement soybean usage. The use of DMB in the aquafeed sector appears to be a promising solution to decreasing the demand for soybean in the EU market, contributing to a higher sustainability of the aquaculture industry.

CRediT authorship contribution statement

Hugo Pereira: Conceptualization, Investigation, Formal analysis, Writing - original draft. **Manuel Sardinha:** Conceptualization, Investigation, Formal analysis, Writing - original draft. **Tamára Santos:** Investigation, Formal analysis, Writing - original draft. **Luísa Gouveia:** Formal analysis, Writing - review & editing, Funding acquisition. **Luísa Barreira:** Formal analysis, Writing - review & editing, Funding acquisition. **Jorge Dias:** Conceptualization, Formal analysis, Writing - original draft, Funding acquisition. **João Varela:** Conceptualization, Formal analysis, Writing - original draft, Funding acquisition.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors would like to thank Prof. Adelino Canário and Elsa Couto (Centre of Marine Sciences) for the kind determination of cortisol.

Funding

The present work was funded by Sparos, Lda., as well as by the Portuguese national budget, the UIDB/04326/2020 grant of the Foundation for Science and Technology (FCT) and project 24517_VALORMAR supported by Compete 2020, Lisboa 2020, CRESC Algarve 2020, Portugal 2020 and the European Union through FEDER/ERDF. H.P. (SFRH/BD/105541/2014) was funded by a PhD grant from FCT.

References

[1] R.H. Wijffels, M.J. Barbosa, An outlook on microalgal biofuels, Science 329 (2010) 796–799.
[2] D.R. Vardon, B.K. Sharma, G.V. Blazina, K. Rajagopalan, T.J. Strathmann, Thermochemical conversion of raw and defatted algal biomass via hydrothermal liquefaction and slow pyrolysis, Bioresour. Technol. 109 (2012) 178–187.
[3] N. Rashid, M.S.U. Rehman, J.I. Han, Recycling and reuse of spent microalgal biomass for sustainable biofuels, Biochem. Eng. J. 75 (2013) 101–107.
[4] T.S. Chandra, G. Suvidha, S. Mukherji, V.S. Chauhan, S. Vidyashankar, K. Krishnamurthi, R. Sarada, S.N. Mudliar, Statistical optimization of thermal pretreatment conditions for enhanced biomethane production from defatted algal biomass, Bioresour. Technol. 162 (2014) 157–165.
[5] L. Ou, R. Thilakaratne, R.C. Brown, M.M. Wright, Techno-economic analysis of transportation fuels from defatted microalgae via hydrothermal liquefaction and hydroprocessing, Biomass Bioenergy 72 (2015) 45–54.
[6] R.E. Austic, A. Mustafa, B. Jung, S. Gatrell, X.G. Lei, Potential and limitation of a new defatted diatom microalgal biomass in replacing soybean meal and corn in diets for broiler chickens, J. Agric. Food Chem. 61 (2013) 7341–7348.
[7] S. Fredriksson, K. Elwinger, J. Pickova, Fatty acid and carotenoid composition of egg yolk as an effect of microalgae addition to feed formula for laying hens, Food Chem. 99 (2006) 530–537.
[8] V. Dallaire, P. Lessard, G. Vandenberg, J. de la Noüe, Effect of algal incorporation on growth, survival and carcass composition of rainbow trout (*Oncorhynchus mykiss*) fry, Bioresour. Technol. 98 (2007) 1433–1439.
[9] A.B. Walker, D.L. Berlinsky, Effects of partial replacement of fish meal protein by microalgae on growth, feed intake, and body composition of Atlantic cod, N. Am. J. Aquac. 73 (2011) 76–83.
[10] R. Ekmay, S. Gatrell, K. Lum, J. Kim, X.G. Lei, Nutritional and metabolic impacts of a defatted green marine microalgal (*Desmodesmus* sp.) biomass in diets for weanling pigs and broiler chickens, J. Agric. Food Chem. 62 (2014) 9783–9791.
[11] P.K. Sarker, M.M. Gamble, S. Kelson, A.R. Kapuscinski, Nile tilapia (*Oreochromis niloticus*) show high digestibility of lipid and fatty acids from marine *Schizochytrium* sp. and of protein and essential amino acids from freshwater *Spirulina* sp. feed

ingredients, Aquac. Nutr. 22 (2016) 109–119.
[12] M. Sørensen, G.M. Berge, K.I. Reitan, B. Ruyter, Microalga *Phaeodactylum tricornutum* in feed for Atlantic salmon (*Salmo salar*) - effect on nutrient digestibility, growth and utilization of feed, Aquaculture 460 (2016) 116–123.
[13] S. Gatrell, K. Lum, J. Kim, X.G. Lei, Potential of defatted microalgae from the biofuel industry as an ingredient to replace corn and soybean meal in swine and poultry diets, J. Anim. Sci. 92 (2014) 1306–1314.
[14] X. Leng, K. Hsu, R. Austic, X.G. Lei, Effect of dietary defatted diatom biomass on egg production and quality of laying hens, J. Anim. Sci. Biotechnol. 5 (2014) 3.
[15] S. Vidyashankar, K.S. Venu Gopal, V.S. Chauhan, S.P. Muthukumar, R. Sarada, Characterisation of defatted *Scenedesmus dimorphus* algal biomass as animal feed, J. Appl. Phycol. 27 (2015) 1871–1879.
[16] Z.Y. Ju, D.F. Deng, W. Dominy, A defatted microalgae (*Haematococcus pluvialis*) meal as a protein ingredient to partially replace fishmeal in diets of Pacific white shrimp (*Litopenaeus vannamei*, Boone, 1931), Aquaculture 354–355 (2012) 50–55.
[17] D. Patterson, D.M. Gatlin, Evaluation of whole and lipid-extracted algae meals in the diets of juvenile red drum (*Sciaenops ocellatus*), Aquaculture 416–417 (2013) 92–98.
[18] N.A. Basri, S.R.M. Shaleh, P. Matanjun, N.M. Noor, R. Shapawi, The potential of microalgae meal as an ingredient in the diets of early juvenile Pacific white shrimp, *Litopenaeus vannamei*, J. Appl. Phycol. 27 (2015) 857–863.
[19] V. Kiron, M. Sørensen, M. Huntley, G.K. Vasanth, Y. Gong, D. Dahle, A.M. Palihawadana, Defatted biomass of the microalga, *Desmodesmus* sp., can replace fishmeal in the feeds for Atlantic salmon, Front. Mar. Sci. 3 (2016) 409.
[20] Z.Y. Ju, S. Davis, K. Ramm, M. Steck, F. Soller, B.K. Fox, Effects of microalgae-added diets on growth performance and meat composition of tilapia (*Oreochromis mossambicus*), Aquac. Res. 48 (2017) 5053–5061.
[21] M. Sørensen, Y. Gong, F. Bjarnason, G.K. Vasanth, D. Dahle, M. Huntley, V. Kiron, *Nannochloropsis oceania*-derived defatted meal as an alternative to fishmeal in Atlantic salmon feeds, PLoS One 12 (2017) e0179907.
[22] Y. Gong, H.A.D.S. Guterres, M. Huntley, M. Sørensen, V. Kiron, Digestibility of the defatted microalgae *Nannochloropsis* sp. and *Desmodesmus* sp. when fed to Atlantic salmon, *Salmo salar*, Aquac. Nutr. 24 (2018) 56–64.
[23] R.L. Naylor, R.J. Goldburg, J.H. Primavera, N. Kautsky, M.C.M. Beveridge, J. Clay, C. Folke, J. Lubchenco, H. Mooney, M. Troell, Effect of aquaculture on world fish supplies, Nature 405 (2000) 1017–1024.
[24] M.F. Milazzo, F.S. Spina, S. Cavallaro, J.C.J. Bart, Sustainable soy biodiesel, Renew. Sust. Energ. Rev. 27 (2013) 806–852.
[25] M.F. Milazzo, F.S. Spina, P. Primerano, J.C.J. Bart, Soy biodiesel pathways: global prospects, Renew. Sust. Energ. Rev. 26 (2013) 579–624.
[26] H. Steinfeld, P. Gerber, T. Wassenaar, V. Castel, M. Rosales, C. de Haan, Livestock's long shadow: environmental issues and options, food and agriculture Organization of the United Nations (FAO), Rome, (2006).
[27] Directive 2010/63/EU of The European Parliament and of the Council of 22 September 2010 on the Protection of Animals Used for Scientific Purposes, L276 Official Journal of European Union, 2010, pp. 33–79.
[28] European Commission Technical Report 2013-064, The Impact of EU Consumption on Deforestation: Identification of Critical Areas Where Community Policies and Legislation Could Be Reviewed, (2013).
[29] C. de Visser, R. Schreuder, F. Stoddard, The EU's dependence on soya bean import for the animal feed industry and potential for EU produced alternatives, O.C.L. 21 (2014) D407.
[30] M. Häusling, The EU Protein Deficit: What Solution for a Long-standing Problem, European Parliament, Strasbourg, European Parliament 2010/2111, (2011).
[31] H. Pereira, K.N. Gangadhar, P. Schulze, T. Santos, C. Bruno de Sousa, L. Schueler, L. Custódio, F.X. Malcata, L. Gouveia, J. Varela, L. Barreira, Isolation of a euryhaline microalgal strain, *Tetraselmis* sp. CTP4, as a robust feedstock for biodiesel production, Sci. Rep. 6 (2016) 35663.
[32] F. Yang, C. Cheng, L. Long, Q. Hu, Q. Jia, H. Wu, W. Xiang, Extracting lipids from several species of wet microalgae using ethanol at room temperature, Energy Fuel 29 (2015) 2380–2386.
[33] NRC, National Research Council, Nutrient Requirements of Fish and Shrimp, National Academy Press, Washington, USA, 2011, pp. 264–265.
[34] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[35] H. Pereira, L. Barreira, A. Mozes, C. Florindo, C. Polo, C.V. Duarte, L. Custódio, J. Varela, Microplate-based high throughput screening procedure for the isolation of lipid-rich marine microalgae, Biotechnol. Biofuels 4 (2011) 61.
[36] D.W. Bolin, R.P. King, E.W. Klosrerman, A simplified method for the determination of chromic oxide (Cr_2O_3) when used as an inert substance, Science 116 (1952) 634–635.
[37] C. Aragão, R. Colen, S. Ferreira, W. Pinto, L.E.C. Conceição, J. Dias, Microencapsulation of taurine in Senegalese sole diets improves its metabolic availability, Aquaculture 431 (2014) 53–58.
[38] J. Rotllant, P.M. Guerreiro, L. Anjos, B. Redruello, A.V. Canario, D.M. Power, Stimulation of cortisol release by the N-terminus of teleost parathyroid hormone-related protein in interrenal cells in vitro, Endocrinology 146 (2005) 71–76.
[39] R. Ihaka, R. Gentleman, R: a language for data analysis and graphics, J. Comput. Graph. Stat. 5 (1996) 299–314.
[40] C.R. Cruz, A. Lubrano, A.M. Gatlin, Evaluation of microalgae concentrates as partial fishmeal replacements for hybrid striped bass *Morone* sp, Aquaculture 493 (2018) 130–136.
[41] I. Lupatsch, G. Wm Kissil, D. Sklan, Comparison of energy and protein efficiency among three fish species: gilthead seabream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*) and white grouper (*Epinephelus aeneus*): energy expenditure for protein and lipid deposition, Aquaculture 225 (2003) 175–189.
[42] P. Gómez-Requeni, M. Mingarro, J.A. Calduch-Giner, F. Médale, S.A.M. Martin, D.F. Houlihan, S. Kaushik, J. Pérez-Sánchez, Protein growth performance, amino acid utilization and somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*), Aquaculture 232 (2004) 493–510.
[43] L. Benedito-Palos, A. Saera-Vila, J.A. Calduch-Giner, S. Kaushik, J. Pérez-Sánchez, Combined replacement of fish meal and oil in practical diets for fast growing juveniles of gilthead sea bream (*Sparus aurata* L.): networking of systemic and local components of GH/IGF axis, Aquaculture 267 (2007) 199–212.
[44] F. Kokou, G. Rigos, M. Henry, M. Kentouri, M. Alexis, Growth performance, feed utilization and non-specific immune response of gilthead sea bream (*Sparus aurata* L.) fed graded levels of a bioprocessed soybean meal, Aquaculture 364–365 (2012) 74–81.
[45] L.M.P. Valente, M. Custódio, S. Batista, H. Fernandes, V. Kiron, Defatted microalgae (*Nannochloropsis* sp.) from biorefinery as a potential feed protein source to replace fishmeal in European sea bass diets, Fish Physiol. Biochem. 45 (2019) 1067–1081.
[46] K.W. Kim, S.C. Bai, J.W. Koo, X. Wang, S.K. Kim, Effect of dietary *Chlorella ellipsoidea* supplementation on growth, blood characteristics, and whole-body composition in juvenile Japanese flounder *Paralichthys olivaceus*, J. World Aquacult. Soc. 33 (2002) 425–431.
[47] M.C. Nandeesha, B. Gangadhar, T.J. Varghese, P. Keshavanath, Effect of feeding *Spirulina platensis* on the growth, proximate composition and organoleptic quality of common carp, *Cyprinus carpio* L, Aquac. Res. 29 (1998) 305–312.
[48] V. Kiron, W. Phromkunthong, M. Huntley, I. Archibald, G. De Scheemaker, Marine microalgae from biorefinery as a potential feed protein source for Atlantic salmon, common carp and whiteleg shrimp, Aquac. Nutr. 18 (2012) 521–531.
[49] A.R. Ribeiro, A. Gonçalves, M. Barbeiro, N. Bandarra, M.L. Nunes, M.L. Carvalho, J. Silva, J. Navalho, M.T. Dinis, T. Silva, J. Dias, *Phaeodactylum tricornutum* in finishing diets for gilthead seabream: effects on skin pigmentation, sensory properties and nutritional value, J. Appl. Phycol. 29 (2017) 1945–1956.
[50] A.W. Ha, W.K. Kim, The effect of fucoxanthin rich power on the lipid metabolism in rats with a high fat diet, Nutr. Res. Pract. 7 (2013) 287–293.
[51] H. Maeda, Nutraceutical effects of fucoxanthin for obesity and diabetes therapy: a review, J. Oleo Sci. 64 (2015) 125–132.
[52] J. Peng, J.P. Yuan, C.F. Wu, J.H. Wang, Fucoxanthin, a marine carotenoid present in brown seaweeds and diatoms: metabolism and bioactivities relevant to human health, Mar. Drugs 9 (2011) 1806–1828.
[53] I. Lupatsch, G. Wm Kissil, D. Sklan, E. Pfeffer, Apparent digestibility coefficients of feed ingredients and their predictability in compound diets for gilthead seabream (*Sparus aurata*), Aquac. Nutr. 3 (1997) 81–90.
[54] J. Gomes da Silva, A. Oliva-Teles, Apparent digestibility coefficients of feedstuffs in seabass (*Dicentrarchus labrax*) juveniles, Aquat. Living Resour. 11 (1998) 187–191.
[55] S.J. Kaushik, Besoins et apport en phosphore chez les poisons, INRA Prod. Anim. 18 (2005) 203–208.
[56] J. Dias, M. Yúfera, L.M.P. Valente, P. Rema, Feed transit and apparent protein, phosphorus and energy digestibility of practical feed ingredients by Senegalese sole (*Solea senegalensis*), Aquaculture 302 (2010) 94–99.
[57] F. Kokou, E. Fountoulaki, Aquaculture waste production associated with antinutrient presence in common fish feed plant ingredients, Aquaculture 495 (2018) 295–310.
[58] C. Mukherjee, R. Chowdhury, K. Ray, Phosphorus recycling from an unexplored source by polyphosphate accumulating microalgae and cyanobacteria—a step to phosphorus security in agriculture, Front. Microbiol. 6 (2015) 1421.
[59] W. Feng, Y. Zhu, F. Wu, Z. He, C. Zhang, J.P. Giesy, Forms and lability of phosphorus in algae and aquatic macrophytes characterized by solution 31P NMR coupled with enzymatic hydrolysis, Sci. Rep. 6 (2016) 37164.
[60] F. Erpel, F. Restovic, P. Arce-Johnson, Development of phytase-expressing *Chlamydomonas reinhardtii* for monogastric animal nutrition, BMC Biotechnol. 12 (2016) 16–29.
[61] E. Teuling, J.W. Schrama, H. Gruppen, P.A. Wierenga, Effect of cell wall characteristics on algae nutrient digestibility in Nile tilapia (*Oreochromis niloticus*) and African catfish (*Clarus gariepinus*), Aquaculture 479 (2017) 490–500.
[62] P.K. Sarker, A.R. Kapuscinski, A.Y. Bae, E. Donaldson, A.J. Sitek, D.S. Fitzgerald, O.F. Edelson, Towards sustainable aquafeeds: evaluating substitution of fishmeal with lipid-extracted microalgal co-product (*Nannochloropsis oculata*) in diets of juvenile Nile tilapia (*Oreochromis niloticus*), PLoS One 13 (2018) e0201315.
[63] H. Pereira, J. Silva, T. Santos, K.N. Gangadhar, A. Raposo, C. Nunes, M.A. Coimbra, L. Gouveia, L. Barreira, J. Varela, Nutritional potential and toxicological evaluation of *Tetraselmis* sp. CTP4 microalgal biomass produced in industrial photobioreactors, Molecules 24 (2019) 3192.
[64] G. Cardinaletti, M. Messina, M. Bruno, F. Tulli, B.M. Poli, G. Giorgi, G. Chini-Zitttelli, M. Tredici, E. Tibaldi, Effects of graded levels of a blend of *Tisochrysis lutea* and *Tetraselmis suecica* dried biomass on growth and muscle tissue composition of European sea bass (*Dicentrarchus labrax*) fed diets low in fish meal and oil, Aquaculture 485 (2018) 173–182.
[65] J.D. Kim, S.J. Kausshik, J. Breque, Nitrogen and phosphorus utilization in rainbow trout (*Oncorhynchus mykiss*) fed diets with or without fish meal, Aquat. Living Resour. 11 (1998) 261–264.
[66] R.J. Arends, J.M. Mancera, J.L. Munoz, S.E. Wendelaar Bonga, G. Flik, The stress response of the gilthead sea bream (*Sparus aurata* L.) to air exposure and confinement, J. Endocrinol. 163 (1999) 149–157.
[67] P.M. Guerreiro, J. Rotllant, J. Fuentes, D.M. Power, A.V.M. Canário, Cortisol and parathyroid hormone-related peptide are reciprocally modulated by negative feedback, Gen. Comp. Endocrinol. 148 (2006) 227–235.
[68] P. Nath, I. Khozin-Goldberg, Z. Cohen, S. Boussiba, D. Zilberg, Dietary supplementation with the microalgae *Parietochloris incisa* increases survival and stress resistance in guppy (*Poecilia reticulata*) fry, Aquac. Nutr. 18 (2012) 167–180.
[69] S.E. Wendelaar Bonga, P.K.T. Pang, Control of calcium regulation hormones in the vertebrates: parathyroid hormone, calcitonin, prolactin and stanniocalcin, Int. Rev. Cytol. 128 (1991) 139–213.