




# Exploring the potential of seaweed *Gracilaria gracilis* and microalga *Nannochloropsis oceanica*, single or blended, as natural dietary ingredients for European seabass *Dicentrarchus labrax*

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## Abstract

*Gracilaria gracilis* and *Nannochloropsis oceanica*, single or blended, were tested in European seabass (*Dicentrarchus labrax*) diets. A control (CTRL) diet was compared with experimental diets including either 8% *G. gracilis* (GRA8), 8% *N. oceanica* (NAN8), or a blend of 4% of each alga (NAN4GRA4). After 106 days of feeding, growth, nutrient utilization, antioxidant defense, immunological status, and end-product quality were evaluated. All fish exhibited similar feed intake (1.4–1.5%), body weight, growth, and feed conversion ratio (1.6). Dietary inclusion *N. oceanica* did not affect digestible N intake and gain. Fish fed GRA8 had the lowest digestible N and energy intake ( $P < 0.05$ ), and simultaneously the highest nitrogen retention efficiency and energy retention efficiency, resulting in a N and energy gain similar to all other treatments. All fish had well-preserved intestinal morphology; feeding NAN8 resulted in a significant increase in neutral goblet cells compared with GRA8. Fish fed the algal diets had significantly lower ( $P < 0.05$ ) hepatosomatic index (1.7–1.8 vs 2.1) and plasma triglyceride levels than CTRL, but whole body composition remained similar among treatments. The liver total antioxidant capacity of fish fed NAN8 was significantly higher than that of fish fed GRA8 but did not differ significantly from the CTRL group. NAN4GRA4 resulted in lower values of total glutathione, glutathione peroxidase, and alternative complement. *N. oceanica* decreased fillet springiness; however, with NAN4GRA4, the muscle fillet became less resilient. *G. gracilis* and *N. oceanica* biomass, either used single (8%) or blended (4% each), can be valuable natural ingredients for partial replacement of fish meal in European seabass diets.

**Keywords** Antioxidant capacity · *Gracilaria gracilis* · Gut integrity · Fish nutrition · *Nannochloropsis oceanica* · Novel ingredients

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## Introduction

In recent years, the use of both seaweeds and microalgae has attracted increasing interest from the feed, food, and pharmaceutical industries for the development of novel products, as well as sources of high-value-added compounds. Global trends point towards increasing demand for natural sustainable sources of nutrients and biologically active substances able to simultaneously promote fish health and function as a vehicle of valuable compounds for consumers.

Seaweeds can be natural sources not only of nutrients for aquafeeds, but also of bioactive compounds like pigments and water-soluble and liposoluble vitamins, as well as minerals (Valente et al. 2006; Holdt and Kraan 2011; Bellou et al. 2014; Silva et al. 2015; Sarker et al. 2016). Several studies have demonstrated the benefits of including seaweeds in fish diets in terms of growth, nutrient utilization, stress resistance, immune system, and fillet quality (Mohamed et al. 2012;

Araújo et al. 2016; Magnoni et al. 2017; Peixoto et al. 2019). Among seaweeds, red algae (Rhodophyta) such as *Gracilaria* are rich in polysaccharides being used for the production of valuable bioactive compounds (Torres et al. 2019). Besides, their protein content can increase up to 30–50%, DM basis, if cultivated under integrated multitrophic aquaculture systems (IMTA) (Abreu et al. 2011), making them more suitable ingredients for aquafeeds. According to Valente et al. (2006), *Gracilaria bursa-pastoris* can replace up to 10% fish-protein hydrolysate in seabass diets, without affecting growth, nutrient utilization or body composition, but the inclusion of *Gracilaria cornea* should be limited to 5% of the diet. In rainbow trout (*Oncorhynchus mykiss*), 10% *Gracilaria vermiculophylla* impaired growth and feed efficiency (Araújo et al. 2016), suggesting that responses are species dependent and also vary with dietary inclusion levels. *Gracilaria* spp. are rich in antioxidants such as carotenoids (Schubert et al. 2006) that may prevent flesh oxidation, increase shelf life, and affect flesh color (Husni et al. 2013; Araújo et al. 2016). In zebrafish (*Danio rerio*), up to 1% *Gracilaria gracilis* increased the expression of catalase intestinal mucosa and had a positive effect on fish immune system without compromising growth or feed efficiency (Hoseinifar et al. 2018). The inclusion of 5% IMTA-cultivated *Gracilaria* sp. also enhanced the innate immune response of rainbow trout (Araújo et al. 2016).

Microalgae have also attracted interest as important nutrient sources for aquafeeds (Kiron et al. 2012; Tulli et al. 2012; Qiao et al. 2014; Tibaldi et al. 2015; Valente et al. 2019). They have high protein content (up to 70%) and balanced amino acid profile and are also being rich lipid sources including long-chain n-3 PUFAS (Becker 2007; Hemaiswarya et al. 2011). Microalgae are also good sources of bioactive compounds, such pigments, vitamins, and minerals (Tibbetts 2018) with potential to improve stress response, immune status, antioxidant system, and nutritional value of fish species (Natrah et al. 2007; Sheikhzadeh et al. 2012; Vizcaino et al. 2018; Messina et al. 2019). *Nannochloropsis* spp. are among the most interesting marine microalgae due to their small size and high protein content, suitable amino acid profile, and particularly high lipid content (up to 40% of DM), normally rich in EPA and DHA (Becker 2007; Bellou et al. 2014; Molino et al. 2018; Ashour et al. 2019). Dietary supplementation with up to 10% *Nannochloropsis* sp. meal had no negative effects on fish growth and muscular amino acid profile in turbot (*Scophthalmus maximus*) (Qiao et al. 2019) or Atlantic salmon (*Salmo salar*) (Sørensen et al. 2017). Moreover, *Nannochloropsis* sp. defatted meal could replace up to 15% fish meal in European seabass (Valente et al. 2019) and up to 10% crude protein from fishmeal and soy concentrate in red drum (*Sciaenops ocellatus*) diets (Patterson and Gatlin 2013) without affecting growth, feed utilization, protein, and energy retention of the fish. The use of the eustigmatophyte

*Nannochloropsis* sp. has also been associated with diminishing lipid peroxidation and enhanced antioxidant capacity in turbot (Qiao et al. 2019) and Atlantic salmon (Sørensen et al. 2017).

Most studies in fish evaluated single algae species, but the supplementation of diets with a mix of seaweeds (*Gracilaria* sp., *Ulva* sp., and *Fucus* sp.) improved European seabass immune and antioxidant responses, without compromising growth (Peixoto et al. 2016a). Likewise, a substitution of 15% fishmeal by a mix of the microalgae *Tisochrysis lutea* and *Tetraselmis suecica* enhanced non-specific immune system response and induced greater villi height and upregulation of gene coding for certain brush border enzymes (Messina et al. 2019). The effects of concomitantly using microalgae and seaweed species in aquafeeds have never been explored before and may potentiate synergetic effects among distinct species, opening new opportunities for algae producers and the feed industry. Thus, the present work aimed to investigate for the first time the utilization of *G. gracilis* and *Nannochloropsis oceanica*, single or blended, in diets for European seabass. After 106 days of feeding, growth, nutrient utilization, antioxidant defense, immunological status, and end-product quality were evaluated.

## Materials and methods

The present study was performed by accredited scientists in compliance with the guidelines of the European Union (directive 2010/63/EU) and Portuguese law (Decreto-Lei no. 113/2013, de 7 de Agosto) on the protection of animals used for scientific purposes. All animal procedures were subject to an ethical review process carried out by the CIIMAR animal welfare body (ORBEA-CIIMAR) and further approved by our national competent authority.

## Algae and experimental diets

Commercial IMTA-cultivated *Gracilaria gracilis* (ALGApplus, Ílhavo, Portugal) and *Nannochloropsis oceanica* (Allmicroalgae, Pataias, Portugal) were dried by convection in a pilot-scale tray dryer (Armfield UOP8, England) with an air flow of  $0.6 \text{ m s}^{-1}$  maintained at  $50 \text{ }^\circ\text{C}$  until the constant weight of the sample, and by pilot-scale spray dryer (Niro Atomizer 2394, Denmark) with a vanned wheel rotating at high speed and a concurrent drying chamber (0.8 m in diameter and 0.6 m in height), respectively. The proximate composition, amino acid, and mineral content of each alga are presented in Table 1.

Four isonitrogenous (53% dry matter, DM) isolipidic (17% DM) diets were formulated and extruded by SPAROS Lda. (Olhão, Portugal), based on the nutritional requirements of European seabass (NRC 2011). A commercial-based diet was used as control (CTRL) and compared with three

**Table 1** Proximate composition, amino acid profile, mineral content, fatty acid, and energy content of algae used in test diets

	<i>Gracilaria</i>	<i>Nannochloropsis</i>
Dry matter (%)	91.0	95.6
Crude protein (% DM)	30.0	34.3
Crude fat (% DM)	0.9	9.8
Gross Energy (kJ g <sup>-1</sup> DM)	14.5	15.9
Carbohydrates <sup>1</sup>	31.8	16.5
Neutral detergent fiber (% DM)	29.7	28.8
Crude fiber (% DM)	5.2	1.1
Ash (% DM)	28.3	35.0
Amino acids (% DM)		
EAA	13.9	20.1
Arginine	1.3	2.0
Histidine	0.2	0.6
Lysine	1.6	3.2
Threonine	1.7	1.9
Isoleucine	2.3	2.4
Leucine	1.9	3.1
Valine	3.1	5.1
Methionine	0.2	vest
Phenylalanine	1.7	1.8
CEAA	1.4	1.7
Cystine	0.4	vest
Proline	1.0	1.7
NEAA	10.9	12.9
Alanine	1.9	2.1
Tyrosine	1.3	1.1
Aspartate	2.6	2.9
Glutamate	2.4	3.9
Glycine	1.1	1.7
Serine	1.6	1.3
Minerals (mg kg <sup>-1</sup> DM)		
Ca	1108.5	2272.8
Fe	574.1	388.2
K	53,293.9	21,200.0
Mg	1799.4	17,371.1
Mn	151.4	38.0
Na	8511.2	55,970.0
P	4642.3	7270.0
B	79.1	49.3
Zn	23.3	68.3
Cu	10.1	6.6
Se	0.6	<0.1
I	120.8	5.94

<sup>1</sup> Calculated by estimation: 100 – (ash + crude protein + crude fat + moisture). *EAA*, essential amino acids; *CEAA*, conditionally essential amino acids; *NEAA*, nonessential amino acids; *vest*, vestigial amount of amino acid (<0.1%)

experimental diets with the inclusion of either 8% *G. gracilis* (GRA8), 8% *N. oceanica* (NAN8), or a blend of 4% of each alga (NAN4GRA4). Algae were included in diets at the expense of fish meal and wheat meal. Diets were manufactured with a pilot-scale twin-screw extruder (CLEXTRAL BC45, France) to a pellet size of 2 mm, and oil was added after the extrusion process by means of vacuum coating (Dinnissen Pegasus vacuum mixer, PG-10VCLAB). All batches of extruded feeds were dried in a convection oven (OP 750-UF, LTE Scientifics, UK) and stored at 4 °C until use. Formulation and proximate composition of the experimental diets are presented in Table 2.

The apparent digestibility coefficients (ADCs) of the experimental diets were determined by the indirect method, after the incorporation of 1% chromium oxide (Cr<sub>2</sub>O<sub>3</sub>, Merck KGaA, Germany), as an inert marker, to each experimental diet. Each extruded diet was ground and mixed with the marker before being dry pelleted through a 3.2-mm die at 50 °C using a laboratory pellet press (CPM, C-300 model, USA).

### Growth trial

European seabass from a commercial fish farm (SONRIONANSA S.L., Cantabria, Spain) were transported to CIIMAR (Matosinhos, Portugal). In order to adapt to the experimental conditions, fish were kept in quarantine for 2 weeks and fed with a commercial diet (AQUASOJA—49% crude protein, 20% crude fat). After acclimation, fish were fasted for 24 h, anesthetized with 75 mg L<sup>-1</sup> of MS222 (Sigma-Aldrich, USA) and individually weighed (g) and measured (total length, cm). Twelve homogeneous groups (average body weight of 29.7 ± 0.02 g, total length of 13.7 ± 0.08 cm, density of 11.3 kg m<sup>-3</sup>) of nineteen fish were distributed by 50-L fiberglass tanks within a saltwater recirculation system. Fish were adapted to the new conditions (water temperature of 21 °C, salinity of 35‰, flow rate at 4 L min<sup>-1</sup> and 12 h light/12 h dark photoperiod regime) for 2 days, and then each diet was randomly assigned to triplicate tanks and fed until apparent satiation, three times a day, by automatic feeders. The amount of feed supplied to each tank was daily adjusted based on the presence or absence of uneaten feed in the tank (Borges et al. 2009). Nitrogenous compounds and pH were monitored during the trial and kept at levels recommended for marine species. The trial lasted 106 days, and fish were bulk weighed monthly to monitor feed consumption and weight gain.

Before the growth trial began, ten fish from the initial fish stock were collected after a 24-h fasting period, sacrificed by anesthetic overdose (150 mg L<sup>-1</sup> of MS222), and kept at –20 °C, until initial whole body composition was analyzed. At the end of the growth trial, and after a 5-h fasting period, four fish per tank were anesthetized with 75 mg L<sup>-1</sup> of MS222, individually weighed (g) and measured (total length, cm), and

**Table 2** Formulation and proximate composition of the experimental diets

	CTRL	GRA8	NAN8	NAN4GRA4
Ingredients (%)				
Fishmeal 70 <sup>1</sup>	4.1	4.1	4.1	4.1
Fishmeal 60 <sup>2</sup>	16.5	13.4	13.1	13.2
Soy protein concentrate <sup>3</sup>	8.3	8.3	8.3	8.3
Pea protein concentrate <sup>4</sup>	1.9	1.9	1.9	1.9
Wheat gluten <sup>5</sup>	12.1	12.1	12.1	12.1
Corn gluten <sup>6</sup>	6.6	6.6	6.6	6.6
Soybean meal <sup>7</sup>	11.1	11.1	11.1	11.1
Rapeseed meal <sup>8</sup>	4.1	4.1	4.1	4.1
Wheat meal <sup>9</sup>	13.8	8.4	9.4	9.0
Sardine oil <sup>10</sup>	12.0	12.2	11.5	11.9
Vit and min premix <sup>11</sup>	0.8	0.8	0.8	0.8
Binder <sup>12</sup>	0.2	0.2	0.2	0.2
L-tryptophan <sup>13</sup>	0.1	0.1	0.1	0.1
DL-methionine <sup>14</sup>	0.4	0.4	0.4	0.4
Hemoglobin powder <sup>15</sup>	5.0	5.0	5.0	5.0
Porcine gelatin <sup>16</sup>	3.0	3.0	3.0	3.0
<i>Gracilaria</i> sp. <sup>17</sup>		8.3		4.1
<i>Nannochloropsis</i> sp. <sup>18</sup>			8.3	4.1
Chemical composition				
Dry matter (DM, %)	89.5	92.2	90.5	92.1
Crude protein	53.7	53.3	54.1	53.7
Crude fat (% DM)	16.9	17.4	16.6	16.6
Carbohydrates (% DM) <sup>19</sup>	11.6	12.6	10.5	12.6
Neutral detergent fiber (% DM)	25.3	12.9	13.1	13.1
Crude fiber (% DM)	1.7	1.2	1.3	1.3
Gross energy (kJ g <sup>-1</sup> DM)	22.7	22.3	22.5	22.3
Phosphorous (% DM)	0.9	0.9	0.9	0.9
Ash (% DM)	7.3	8.9	9.3	9.1

<sup>1</sup> Peruvian fishmeal LT: 71.0% crude protein (CP), 11.0% crude fat (CF), EXALMAR, Peru

<sup>2</sup> Fish meal 60: 60% CP, 12% CF, Savinor SA, Portugal

<sup>3</sup> Soy protein concentrate: 65% CP, 0.7% CF, ADM Animal Nutrition, The Netherlands

<sup>4</sup> Pea protein concentrate: Nutralys F85F, 78% CP, 1% CF, Roquette, France

<sup>5</sup> Wheat gluten: 84% CP, 1.3% CF, Roquette, France;

<sup>6</sup> Corn gluten meal: 61.0% CP, 6.0% CF, COPAM, Portugal

<sup>7</sup> Soybean meal 48: dehulled solvent-extracted soybean meal: 47.7% CP, 2.2% CF, Cargill, Spain

<sup>8</sup> Rapeseed meal: 36% CP, 2.7% CF, PREMIX Lda, Portugal

<sup>9</sup> Wheat meal: 10.2% CP, 1.2% CF, Casa Lanchinha, Portugal

<sup>10</sup> Sardine oil: Sopropêche, France

<sup>11</sup> Vitamin and mineral premix: INVIVO 1%, premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg kg<sup>-1</sup> diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulfite, 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 pantothenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg<sup>-1</sup> diet): cobalt carbonate, 0.65 mg; copper sulfate, 9 mg; ferric sulfate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulfate, 7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings

<sup>12</sup> Kielseguhr (natural zeolite): LIGRANA GmbH, Germany

<sup>13</sup> L-tryptophan: feed grade amino acids, SORGAL, SA. Ovar, Portugal

<sup>14</sup> DL-methionine: 99% Evonik, Degussa GmbH, Germany

<sup>15</sup> Porcine hemoglobin powder: 92% CP SONAC, The Netherlands

<sup>16</sup> Porcine gelatin: Lapi, Italy

<sup>17</sup> *Gracilaria* sp.: 30.01% CP, 0.45% CF, Algaplus Lda, Portugal

<sup>18</sup> *Nannochloropsis* sp.: 34.31% CP, 3.34% CF, Allmicroalgae, Portugal

<sup>19</sup> Calculated by estimation, 100 – (ash + crude protein + crude fat + moisture)



sacrificed with a sharp blow on the head. The intestinal tract of those fish was divided into pyloric caeca (PC), anterior intestine (AI; section below the tract with PC until the increase in diameter indicating the start of the posterior intestine), and posterior intestine (PI; the terminal part of the intestine with larger diameter until the anus), and samples were kept at  $-80\text{ }^{\circ}\text{C}$  until the determination of brush border membrane (BBM) enzyme activity. After a 24-h fasting period, all fish were anesthetized with  $75\text{ mg L}^{-1}$  of MS222, before individually weighed (g) and measured (total length, cm). Blood was collected from the caudal vein of four fish per tank with heparinized syringes, centrifuged ( $5000\times g$  for 10 min at  $4\text{ }^{\circ}\text{C}$ ), and the resulting plasma was stored at  $-80\text{ }^{\circ}\text{C}$  for the analysis of innate immune parameters and determination of postprandial metabolite levels. Fish were then sacrificed by a sharp blow on the head. Fish intestine and liver weights were registered to determine hepatosomatic and viscerosomatic indexes. The liver from each fish was immediately frozen in liquid nitrogen and then kept at  $-80\text{ }^{\circ}\text{C}$  until the determination of oxidative stress parameters. Left dorsal muscle was collected and immediately frozen in liquid nitrogen and then kept at  $-80\text{ }^{\circ}\text{C}$  until the determination of its nutritional value (DM; crude protein and crude fat). The right dorsal muscle was also collected for the instrumental texture determination. For intestinal histomorphological evaluation, approximately 0.5 cm of the anterior (after the pyloric caeca) and posterior (before the rectum sphincter) intestine was collected, washed, and fixed in 10% neutral-buffered formalin for 24 h. Five fish per tank, sacrificed by anesthetic overdose ( $150\text{ mg L}^{-1}$  of MS222), were collected for the whole body composition analysis and frozen at  $-20\text{ }^{\circ}\text{C}$  until analysis.

### Digestibility trial

After the growth trial, the remaining fish from each diet (12–18 fish) were individually distributed by tanks similar to those used for the growth trial, but equipped with individual feces sedimentation columns, in a system specially designed for *in vivo* digestibility studies (Guelph system) according to Cho and Slinger (1979). Fish were subjected to the same rearing conditions used during the growth trial. The experimental diets supplemented with 1%  $\text{Cr}_2\text{O}_3$  were fed to the fish until apparent satiation, twice a day (10:00 and 17:00) for 10 days to adapt to each new diet before the feces collection began. During the digestibility trial, about 30 min after feeding, each tank was carefully cleaned to assure that no uneaten feed remained in the tanks or in the sedimentation column. Daily collection of the feces was performed twice a day (9:00 and 16:00), before feeding, for 1 month. Feces were centrifuged (7200 rpm, 5 min,  $4\text{ }^{\circ}\text{C}$ ) to eliminate excess water, frozen at  $-20\text{ }^{\circ}\text{C}$  and freeze-dried prior to the analysis.

### Proximate analysis of algae, feeds, whole body, muscle, and feces

Fish collected from each tank for whole body composition were ground and pooled, and moisture was determined ( $105\text{ }^{\circ}\text{C}$  for 24 h). Afterwards, fish were freeze-dried, ground, and homogenized before being analyzed. Algae and experimental diets were also homogenized before analysis. All chemical analyses followed AOAC (2006) methods and were performed in duplicate. Samples were analyzed for DM ( $105\text{ }^{\circ}\text{C}$  for 24 h); ash, by combustion in a muffle furnace ( $500\text{ }^{\circ}\text{C}$  for 5 h); crude protein ( $\text{N} \times 6.25$ ), using a Leco nitrogen analyzer (Model FP-528, Leco Corporation, USA); lipid content, by petroleum ether extraction using a Soxtherm Multistat/SX PC (Germany) and after a pre-hydrolysis with hydrochloric acid for algae and diets; gross energy, by an adiabatic bomb calorimeter (Werke C2000, IKA, Germany); and phosphorous content by digestion at  $230\text{ }^{\circ}\text{C}$  in a Kjeldatherm block digestion unit followed by digestion at  $60\text{ }^{\circ}\text{C}$  in a water bath and absorbance determination at 820 nm (adapted from AFNOR 1992). Chromic oxide content in diets and feces was determined according to Bolin et al. (1952). Algae and feed crude fiber content was analyzed according to the intermediate filtration method (ISO 6865:2000) and neutral detergent fiber (NDF) according to ISO 16472:2006 (Robertson and Van Soest 1981; Van Soest and Robertson 1985); carbohydrates of the algae were calculated by deducting the sum of ash, CP, and total lipids from DM. The mineral content of the algae was determined according to USEPA (1995). Aliquots (0.3 g) of dry algae biomass were introduced in Teflon microwave vessels, and 9 mL of concentrated  $\text{HNO}_3$  and 1.0 mL aqua regia were added. Samples were processed in a microwave digester (CEM Mars Xpress Matthews, USA) at  $175\text{ }^{\circ}\text{C}$  and elevated frequency of 2450 MHz. The temperature was kept at  $170\text{--}180\text{ }^{\circ}\text{C}$  for 10 min. After cooling, digested solutions were filtered through a PTFE filter of  $0.2\text{-}\mu\text{m}$  size, transferred into 20-mL volumetric flasks, and stored at  $5\text{ }^{\circ}\text{C}$  for determination by inductively coupled plasma optical emission spectroscopy (ICP-OES). A Varian Vista Pro axial instrument (Varian Inc., USA) equipped with a cross-flow nebulizer and auto-sampler was used. The calibration was performed using an ICP-standard 23-element solution in 5%  $\text{HNO}_3$  (Merck solution IV) using yttrium (Y) as an internal standard. The calibration curve and two blanks were run during each set of analyses, to check the purity of the chemicals. The method detection limit (MDL) was calculated as  $3\text{SD}/S$  (where SD is the standard deviation of 10 replicate blanks and  $S$  is the slope of the calibration curve) for each element. For the amino acid profile, algae samples were subjected to acid hydrolysis (6 M HCl) in an oven for 18 h at  $110\text{ }^{\circ}\text{C}$ . The hydrolysis was performed using an amount of the samples corresponding to 5–10 mg protein per mL HCl. After hydrolysis, the samples were cooled to RT  $^{\circ}\text{C}$ , and  $100\text{ }\mu\text{L}$

was diluted with 1.5 mL 1 M NaCO<sub>3</sub> and filtered through a 0.2- $\mu$ m syringe filter (Q-max PTFE, Denmark) before derivatization using the EZ:Faast™ Amino Acid Analysis kit from Phenomenex (USA). The samples (50  $\mu$ L) were then analyzed by LC-(APCI)-MS (Agilent 1100, Agilent Technology) according to the procedure described by Sabeena Farvin et al. (2010).

### Intestine histomorphological evaluation

After fixation, intestine samples were preserved in ethanol 70%. Samples were further processed according to standard histological procedures and embedded in paraffin. Two fish per tank were then selected for histologic analyses (6 fish per diet). Cross intestinal sections (3  $\mu$ m) were obtained in a semi-automated rotary microtome (Leica RM 2245) and stained with specific Alcian blue/PAS (pH 2.5). Micrographs of each individual section were examined under a light microscope (Olympus BX51, Germany) with a camera (Olympus DP50). An imaging software (cell^B software, Olympus cellSens Dimension Desktop) was used to measure the following parameters in two sections of each sample, according to previous studies (Galeano et al. 2015; Batista et al. 2016; Ramos et al. 2017): cross-sectional area (mm<sup>2</sup>); villus length and width ( $\mu$ m); outer longitudinal and inner circular layers of muscularis externa ( $\mu$ m); and goblet cells (no. GC per fold divided into neutral and acid cells (magenta and blue cells, respectively). The muscularis externa thickness was measured in eight points of each transverse section analyzed, and the mean of the measurements was calculated. The fold's length and width were measured in the eight highest folds, with the width being measured at the base of the fold and length measured from the fold tip to the bottom, following the curves of the fold. Goblet cells (mucus-producing cells) were counted on the previous selected highest folds; the average number of total goblet cells per fold was determined as well as the number of each GC type.

### Brush Border Membrane (BBM) enzyme activities

The gut-stored sections (pyloric caeca, anterior and posterior intestine) were thawed, and whenever needed, the remaining content was gently squeezed out. Gut tissue was diluted 1:10 in iced saline buffer and crushed in a tissue-lyser disruption system (Tissue Lyser II, Qiagen, Germany) at 30 Hz for 1 min. Samples were centrifuged at 13,500 $\times$ g for 10 min at 4 °C, and the supernatant was used to measure the BBM enzyme activities (Messina et al. 2019). The hydrolysis of maltose and sucrose, by the BBM enzyme maltase and the complex sucrose-isomaltase (SI), was determined according to Harpaz and Uni (1999). Alkaline phosphatase (ALP) and  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GT) activity was determined using commercial kits (Paramedical, Pontecagnano Faiano, SA, Italy) as

indicated by the manufacturer. One unit (U) of enzyme activity corresponded to the amount of enzyme that transforms or hydrolyzes 1  $\mu$ mole of substrate mL<sup>-1</sup> min<sup>-1</sup>. The specific enzymatic activity was calculated as U of enzyme activity per g of tissue.

### Metabolic parameters

The glucose (Glu, mg dL<sup>-1</sup>), cholesterol (Chol, mg dL<sup>-1</sup>), triglycerides (Trig, mg dL<sup>-1</sup>), total proteins (TP, g dL<sup>-1</sup>), and albumin (Alb, g dL<sup>-1</sup>) were determined in plasma by an automated analyzer system for blood biochemistry (Roche Cobas Mira, Biosys, Milan, Italy) and commercially available kits (Biochemical Enterprise, Italy), following the manufacturer's protocols.

### Oxidative stress analysis

Liver samples were homogenized using phosphate buffer (0.1 M, pH 7.4) in a proportion of 1:10 (w:v). Protein content was determined according to Bradford (1976) and used to standardize antioxidant enzyme activities. The following methods were, without exception, measured in triplicate using a microplate reader. Concentration of total antioxidant was determined by using the total antioxidant capacity assay kit (Sigma MAK187) and measuring the formation of Trolox equivalents. Total glutathione (TG) was evaluated at 412 nm by the formation of 5-thio-2-nitrobenzoic acid (TNB), as detailed in Baker et al. (1990). Protein content in the post-mitochondrial supernatant (PMS) was precipitated with trichloroacetic acid (TCA; 12%) for 1 h and centrifuged at 12,000 $\times$ g for 5 min at 4 °C. TG was determined (in deproteinated PMS) adopting the enzymatic recycling method using GR excess, whereby the sulfhydryl group of GSH reacts with DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent) producing a yellow-colored TNB. Formation of TNB was monitored by spectrophotometry at 415 nm, for 7 min, and results were expressed as nmol TNB conjugated formed per min per mg of protein. Glutathione peroxidase (GPx) was evaluated based on NADPH oxidation at 340 nm (Mohandas et al. 1984) through an indirect method based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPx. The reaction was performed at 25 °C and pH 8.0, using H<sub>2</sub>O<sub>2</sub> and including sodium azide (NaN<sub>3</sub>) as a catalase inhibitor. The enzyme activity was calculated as nmol NADPH oxidized min<sup>-1</sup> mg<sup>-1</sup> protein. Glutathione s-transferase (GST) was determined as described by Habig et al. (1974). Total activity (cytosolic and microsomal) was determined by measuring the conjugation of CDNB with reduced glutathione (GSH). The change in absorbance was recorded at 340 nm and 25 °C for 5 min. Enzyme activity was calculated as mmol CDNB conjugate formed per min per mg of protein. Catalase (CAT) activity was measured according to

Claiborne (1985), but using hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) 30% as substrate. Changes in absorbance were recorded at 240 nm at 25 °C. CAT activity was calculated in terms of mmol  $\text{H}_2\text{O}_2$  consumed per min per mg of protein. Lipid peroxidation (LPO) was determined according to Bird and Draper (1984) by quantifying the presence of thiobarbituric acid reactive substances (TBARS) that induces the formation of malondialdehyde (MDA), quantified colorimetrically following its controlled reaction with TBA. The absorbance of each aliquot was measured at 535 nm and LPO was expressed as nmol of TBARS formed per gram of fresh tissue.

### Innate immune parameters analysis

Plasma lysozyme, peroxidase, and alternative complement (ACH50) pathway activities were measured in triplicate on a microplate spectrophotometer (BioTek Synergy HT, USA). Lysozyme activity ( $\text{EU min}^{-1} \text{mL}^{-1}$  plasma) was determined using a turbidimetric assay based on the method described by Ellis (1990) and adapted to microtiter as described by Hutchinson and Manning (1996). One lysozyme enzyme unit (EU) was defined as the amount of lysozyme that caused a decrease in 1 OD absorbance per minute. Total peroxidase activity ( $\text{EU mL}^{-1}$  plasma) was measured following the procedure described by Quade and Roth (1997) and Costas et al. (2011) and was determined by defining that one unit of peroxidase produces an absorbance change of 1 OD. Alternative complement pathway (ACH50) was based on the lysis of rabbit red blood cells ( $2.8 \times 10^8$  cells  $\text{mL}^{-1}$ ; Probiológica, Belas, Portugal) as target cells in the presence of ethylene glycol tetraacetic acid (EGTA; Sigma) and  $\text{Mg}^{2+}$  ( $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ; VWR) as described by Sunyer and Tort (1995). ACH50 units were defined as the concentration of plasma giving 50% lysis of cells.

### Instrumental texture and color analysis of fish skin and muscle

Skin and muscle color were measured using a CR-400 chromameter (Konica Minolta) with an aperture of 8 mm, at standard illuminant D65 using the CIE 1976 ( $L^*$ , brightness;  $a^*$ , redness;  $b^*$ , yellowness). Initially, the apparatus was calibrated with a white plate reference standard (Minolta Co, Ltd., Osaka, Japan). Color parameters were measured by applying the colorimeter onto the raw skin and flesh of 12 fish from each diet. Measurements were made above the lateral line and in three points of each fillet, and mean values were considered for each fish. After flashing,  $L^*$ ,  $a^*$ , and  $b^*$  reflected light values were recorded. From  $a^*$  and  $b^*$  values, the hue angle ( $h^* = \tan^{-1} b^*/a^*$ ) and the chroma ( $C^* = (a^{*2} + b^{*2})^{1/2}$ ) were calculated according to Choubert et al. (1997).

Fillet texture was analyzed immediately post-mortem using a TA.XT plus texture analyzer, fitted with a 5-kg load cell and

a 2.0-mm-diameter probe, and controlled by Exponent v6 software (Stable Micro Systems, UK). Texture profile analyses were obtained by double compression (constant speed and penetration depth of 1 mm  $\text{s}^{-1}$  and 4.0 mm, respectively) on the maximum thickness part of each raw fillet. Penetration depth was selected according to the maximum distance that did not induce fibers breaking, and the following measurements were determined: hardness (N), chewiness (J), adhesiveness (J), springiness, cohesiveness, and resilience.

### Calculations

ADCs of the experimental diets were calculated according to Maynard et al. (1979): DM ADC (%) =  $100 \times (1 - (\text{dietary Cr}_2\text{O}_3 \text{ level}/\text{feces Cr}_2\text{O}_3 \text{ level}))$  and nutrient ADC (%) =  $100 \times (1 - (\text{dietary Cr}_2\text{O}_3 \text{ level}/\text{feces Cr}_2\text{O}_3 \text{ level}) \times (\text{feces nutrient or energy level}/\text{dietary nutrient or energy}))$ ; condition factor =  $(\text{final body weight}/(\text{final body length})^3) \times 100$ ; daily growth index =  $100 \times ((\text{final body weight})^{1/3} - (\text{initial body weight})^{1/3})/\text{days}$ ; average body weight (ABW) =  $(\text{final body weight} + \text{initial body weight})/2$ ; voluntary feed intake (%) =  $100 \times \text{dry feed intake}/\text{ABW}/\text{day}$ ; feed conversion ratio =  $\text{dry feed intake}/\text{weight gain}$ ; hepatosomatic index =  $100 \times \text{liver weight}/\text{body weight}$ ; viscerosomatic index =  $100 \times \text{weight of viscera}/\text{body weight}$ ; digestible nitrogen (N) or energy (E) intake =  $(\text{dry feed consumption} \times \text{N} (\%) \text{ or } \text{E} (\text{kJ g}^{-1}) \text{ in the diet} \times \text{ADC N or E})/\text{ABW}/\text{days}$ ; N or E gain =  $(\text{final carcass N or E content} - \text{initial carcass N or E content})/\text{ABW}/\text{days}$ ; N or E retention efficiency (% digestible N or E) =  $(\text{N or E gain}/\text{digestible N or E intake}) \times 100$ .

### Statistical analysis

Data were tested for normality and homogeneity of variances by Shapiro-Wilk and Levene's tests, respectively, and transformed whenever required before being submitted to a one-way ANOVA with the statistical program IBM SPSS STATISTICS, 25.0 package (USA). When this test showed significance, individual means were compared using HSD Tukey's test. In all cases, the minimum level of significance was set at  $P < 0.05$ . The principal component analysis (PCA) was applied to assess the consensus among the variables that differed significantly among dietary treatments using XLSTAT 2019 system software (Addinsoft, USA).

### Results

The IMTA-produced *G. gracilis* and the *N. oceanica* biomass had high protein contents (> 30%) (Table 1), but the lipid content of *G. gracilis* was lower (< 1%) than that of *N. oceanica* (9.8% DM). Although *N. oceanica* seems to be a better source of essential amino acids (20%), *G. gracilis*

appears an excellent source of minerals, especially K ( $53.3 \text{ g kg}^{-1} \text{ DM}$ ). The neutral detergent fiber content was similar between the two algae, but *G. gracilis* had both higher crude fiber and carbohydrate contents.

The apparent digestibility coefficient (ADCs) of DM of fish fed NAN8 did not differ significantly either from the CTRL or from diet NAN4GRA4 (Table 3). Fish fed GRA8 diet displayed the lowest DM ADC value (16%) that differed significantly from all other treatments ( $P < 0.05$ ). Protein and energy ADC values of NAN8 did not differ from the CTRL and were significantly higher ( $P < 0.05$ ) than those observed in GRA8. The algae blend (NAN4GRA4) displayed a similar protein ADC value to the CTRL diet. Lipid and phosphorus digestibility was not affected by dietary algae inclusion.

All diets were well accepted by European seabass, resulting in similar voluntary feed intake (1.4–1.5%), final body weight, growth, and feed conversion ratio (1.6) among dietary treatments (Table 3). Moreover, survival rate was high (> 90%) and similar among groups. Fish fed GRA8 had a significantly higher condition factor (1.14) than fed CTRL diet (1.11;  $P < 0.05$ ). The dietary inclusion of algae resulted in a significant decrease (1.7–1.8;  $P < 0.05$ ) of the hepatosomatic index (HSI) compared with fish fed the control diet (2.1). Final whole body composition remained unaffected by the dietary treatments. Digestible N intake and N gain of fish fed *N. oceanica* diets did not differ from the CTRL group, resulting in similar nitrogen retention efficiency (NRE, %DN). Fish fed GRA8 had the lowest digestible N and digestible energy intake values ( $P < 0.05$ ), but highest NRE and ERE, resulting in a N and energy gain similar to all other treatments.

All fish had a very well-preserved intestinal morphology, without major differences among the experimental diets (Fig. 1 and Table 4). The only exception was the number of neutral GC per fold that was significantly higher in fish fed NAN8 than that in fed GRA8 ( $P < 0.05$ ), both in the anterior (17 vs 8 cells per fold) and posterior (22 vs 1 cell per fold) intestinal sections. In the anterior intestine, the dietary inclusion of the algae blend (NAN4GRA4) resulted in an increased number of neutral GC in relation to both the control and GRA8, but that could not be observed in the posterior intestine.

As shown in Table 5, the activities of the BBM enzymes maltase, SI, and  $\gamma$ -GT were affected by dietary treatments. Diet NAN8 resulted in the lowest activity of the disaccharases, maltase, and sucrose-isomaltase, compared with fish fed NAN8GRA8 diet ( $P < 0.05$ ) in the pyloric caeca. The highest activity of  $\gamma$ -GT was found in the posterior intestine, with the highest figures registered in fish fed NAN8 diet ( $12.99 \mu\text{mol min}^{-1} \text{ g}^{-1}$ ,  $P < 0.05$ ). The activity of the alkaline phosphatase was not affected by dietary treatments in the anterior and posterior intestinal tract ( $P > 0.05$ ) but significantly decreased in the pyloric caeca of fish fed NAN8 diet ( $P < 0.001$ ). The dietary inclusion of *Gracilaria* (diets

GRA8 and NAN4GRA4) resulted in enhanced IAP activity, with fish fed NAN4GRA4 displaying the highest and significant activity ( $88.0 \mu\text{mol min}^{-1} \text{ g}^{-1}$ ,  $P < 0.05$ ).

Feeding fish for 106 days with the algae-rich diets resulted in a general increase in circulating glucose level (Table 6), reaching statistical significance in fish fed diet GRA8 ( $149.6 \text{ mg dL}^{-1}$ ) relative to the control ( $120.9 \text{ mg dL}^{-1}$ ;  $P < 0.05$ ). Triglyceride levels decreased significantly in all fish fed the algae-based diets. Total cholesterol, protein, and albumin levels in plasma were not significantly affected by the experimental diets.

Considering the oxidative stress parameters (Table 7), all diets presented similar TAC content compared with the CTRL; however, fish from NAN8 groups showed the highest TAC content in the liver ( $19 \mu\text{L Trolox equivalents g}^{-1} \text{ w/w}$ ), being significantly different from GRA8 ( $16 \mu\text{L Trolox equivalents g}^{-1} \text{ w/w}$ ;  $P < 0.05$ ). The NAN4GRA4 diet leads to a significant decrease ( $P < 0.05$ ) of the TG ( $0.9 \text{ nanomol min}^{-1} \text{ mg}^{-1} \text{ protein}$ ) and GPx (1.6) activities in the liver, compared with the CTRL groups (2.2 and  $5.8 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$ , respectively). No differences were found for GST, GR, CAT, and LPO between dietary treatments.

The humoral non-specific immune parameters (Fig. 2) did not show any differences between treatments concerning the peroxidase and lysozyme activities. However, fish fed the blend NAN4GRA4 diet had lower alternative complement (ACH50) pathway activity ( $132 \text{ Units mL}^{-1}$ ) than fish fed CTRL diet ( $239 \text{ Units mL}^{-1}$ ).

No significant differences were observed in seabass skin color among dietary treatments by the end of the growth trial (Table 8). However, the fillet of fish fed the NAN8 diet had a significantly higher hue angle ( $h^*$ , 130) than those fed the GRA8 diet ( $h^*$ , 123;  $P < 0.05$ ). Fillets of fish fed the GRA8 diet had higher redness ( $a^*$ ,  $-2.6$ ) compared with fish from all other treatments ( $a^*$ ,  $-3.2$ ). Cohesiveness of the fillet was higher in fish fed NAN8 and GRA8 (0.3) than in those fed the CTRL diet (0.2). The dietary inclusion of *N. oceanica* (NAN8 and NAN4GRA4) significantly decreased the fillet springiness, while the inclusion of the algal blend (NAN4GRA4) decreased fillet resilience in relation to the CTRL. The fillet content of protein and lipids did not change among dietary treatments, but fish fed the algae-rich diets had significantly higher moisture content (> 76%) compared with the control (75%).

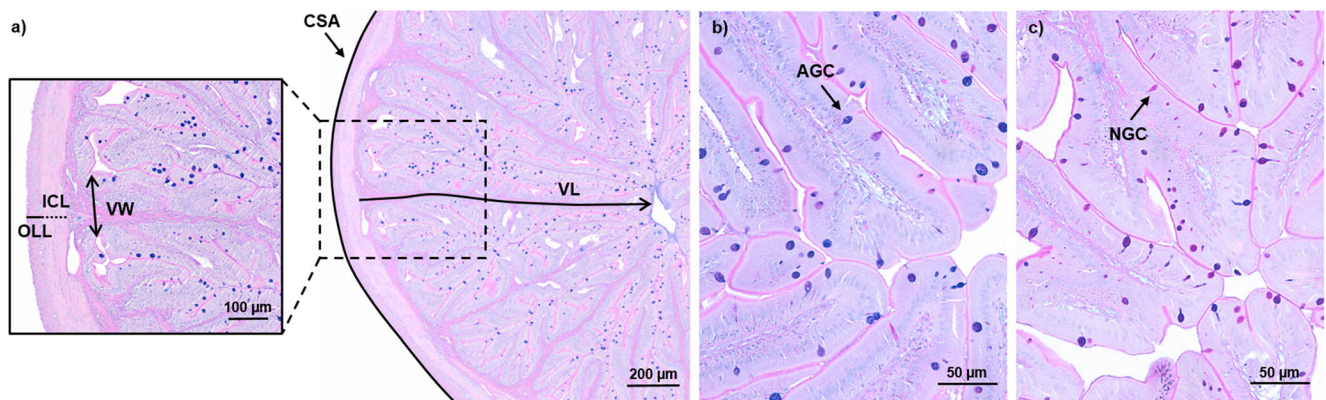
The PCA is often applied for data reduction to identify a small number of factors that explain most of the variance observed in a much larger number of manifest variables. This statistical methodology attempts to identify underlying variables or factors that explain the pattern of correlations within a set of observed variables. Figure 3 shows the two first dimensions of the PCA consensus, which explains 82.5% of the variability of the experimental data. The representation shows that although diets are projected into distinct



**Table 3** Apparent digestibility coefficients (ADCs) of the diets, growth performance, whole body composition, nitrogen, and energy balances of European seabass fed the experimental diets for 106 days

	CTRL	GRA8	NAN8	NAN4GRA4	P value
<b>ADCs (%)</b>					
Dry matter	50.1 ± 5.6 <sup>a</sup>	16.0 ± 2.1 <sup>c</sup>	47.4 ± 1.7 <sup>ab</sup>	33.2 ± 9.6 <sup>b</sup>	< 0.001
Protein	85.4 ± 0.4 <sup>a</sup>	67.7 ± 8.1 <sup>b</sup>	81.1 ± 1.2 <sup>a</sup>	78.1 ± 3.9 <sup>ab</sup>	0.01
Lipids	89.4 ± 1.0	86.3 ± 6.7	92.4 ± 2.3	92.1 ± 0.8	0.2
Energy	63.8 ± 2.0 <sup>a</sup>	37.6 ± 5.2 <sup>c</sup>	64.5 ± 1.7 <sup>a</sup>	52.9 ± 4.9 <sup>b</sup>	< 0.001
Phosphorus	58.5 ± 7.2	51.2 ± 2.9	46.9 ± 4.1	49.0 ± 1.2	0.1
<b>Growth and nutrient utilization</b>					
Initial body weight (g)	29.7 ± 0.02	29.7 ± 0.04	29.7 ± 0.03	29.7 ± 0.01	1.0
Final body weight (g)	80.9 ± 2.0	83.1 ± 3.8	81.7 ± 2.9	81.4 ± 4.3	0.9
Final condition factor	1.11 ± 0.1 <sup>b</sup>	1.14 ± 0.1 <sup>a</sup>	1.11 ± 0.04 <sup>ab</sup>	1.11 ± 0.1 <sup>ab</sup>	0.04
Daily growth index	1.2 ± 0.03	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	0.9
Voluntary feed intake (%)	1.4 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.04	0.4
Feed conversion ratio	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.03	1.6 ± 0.1	0.8
<b>Somatic indexes (%)</b>					
Hepatosomatic index	2.1 ± 0.3 <sup>a</sup>	1.7 ± 0.3 <sup>b</sup>	1.8 ± 0.3 <sup>b</sup>	1.8 ± 0.3 <sup>b</sup>	< 0.001
Viscerosomatic index	5.0 ± 1.1	4.8 ± 0.8	4.7 ± 0.7	4.6 ± 0.9	0.4
<b>Final whole body composition (% WW)<sup>1</sup></b>					
Dry matter	31.7 ± 0.7	32.0 ± 0.8	31.3 ± 0.3	30.9 ± 0.7	0.26
Protein	17.3 ± 0.3	17.7 ± 0.2	17.4 ± 0.3	17.5 ± 0.3	0.35
Fat	10.5 ± 1.0	11.0 ± 1.3	9.5 ± 0.6	9.5 ± 0.4	0.18
Energy (kJ g <sup>-1</sup> )	7.8 ± 0.3	7.9 ± 0.4	7.7 ± 0.1	7.6 ± 0.2	0.52
Ash	4.1 ± 0.4	3.9 ± 0.4	4.4 ± 0.04	4.2 ± 0.1	0.14
<b>Nitrogen (N) and energy (E) balance</b>					
Digestible N intake (mg (100 g) <sup>-1</sup> ABW d <sup>-1</sup> )	102.8 ± 2.1 <sup>a</sup>	85.3 ± 12.7 <sup>b</sup>	98.8 ± 2.6 <sup>ab</sup>	92.5 ± 2.6 <sup>ab</sup>	0.03
N gain (mg (100 g) <sup>-1</sup> ABW d <sup>-1</sup> )	22.9 ± 0.6	24.4 ± 0.7	23.4 ± 0.6	23.4 ± 0.6	0.1
NRE (% digestible N)	22.3 ± 0.7 <sup>b</sup>	29.0 ± 4.1 <sup>a</sup>	23.7 ± 0.5 <sup>b</sup>	25.4 ± 0.5 <sup>ab</sup>	0.01
Digestible E intake (kJ kg <sup>-1</sup> ABW d <sup>-1</sup> )	202.9 ± 7.3 <sup>a</sup>	123.9 ± 20.5 <sup>c</sup>	204.6 ± 6.7 <sup>a</sup>	162.1 ± 10.0 <sup>b</sup>	< 0.001
E gain (kJ kg <sup>-1</sup> ABW d <sup>-1</sup> )	66.1 ± 4.8	68.5 ± 7.6	64.5 ± 1.3	63.3 ± 6.0	0.7
ERE (% digestible E)	32.5 ± 1.2 <sup>b</sup>	56.4 ± 11.4 <sup>a</sup>	31.6 ± 1.0 <sup>b</sup>	39.1 ± 3.6 <sup>b</sup>	0.003

<sup>1</sup> Initial body composition (% ww): dry matter, 35.6%; protein, 18.8%; lipids, 12.0%; energy, 8.2 kJ g<sup>-1</sup>; ash, 4.5%. Values are means ± standard deviation. Means in rows with a different superscript letter differ significantly (*P* < 0.05). *RE*, retention efficiency



**Fig. 1** Histological sections (Alcian blue/PAS staining, pH = 2.5) of the intestine of European seabass showing. **a** Different parameters measured. CSA, Cross-section area; VL, villus length; muscularis externa (OLL and ICL, outer longitudinal and inner circular layer, respectively); VW, Villus

width; goblet cell (AGC and NGC, acid and neutral goblet cells respectively). **b** Fish fed GRA8 diet. **c** Fish fed NAN8 diet. Note the lower number of NGC in fish fed GRA8 diet compared with fish fed NAN8 diet. See Table 4 for more details

**Table 4** Intestinal morphology of European seabass after feeding the experimental diets for 106 days

	CTRL	GRA8	NAN8	NAN4GRA4	P value
<b>Anterior intestine</b>					
Cross-sectional area (mm <sup>2</sup> )	5.7 ± 1.0	5.6 ± 1.4	5.0 ± 1.1	5.5 ± 2.0	0.81
Villus length (mm)	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	0.29
Villus width (µm)	131.6 ± 16.5	113.2 ± 9.4	120.9 ± 24.1	116.2 ± 16.4	0.34
Muscularis externa (µm)	98.7 ± 18.5	107.8 ± 26.6	99.6 ± 21.9	91.1 ± 30.6	0.72
Outer longitudinal layer (µm)	32.8 ± 3.9	40.2 ± 11.0	36.7 ± 11.4	32.6 ± 13.9	0.61
Inner circular layer (µm)	62.5 ± 11.2	67.6 ± 18.0	62.9 ± 10.6	58.5 ± 16.8	0.76
Goblet cells (no. GC fold <sup>-1</sup> )	55.3 ± 15.8	65.6 ± 14.1	51.1 ± 16.5	79.2 ± 42.0	0.40
Neutral GC (no. GC fold <sup>-1</sup> )	8.9 ± 4.2 <sup>bc</sup>	7.5 ± 3.5 <sup>c</sup>	17.0 ± 6.6 <sup>ab</sup>	18.0 ± 7.0 <sup>a</sup>	0.01
Acid GC (no. GC fold <sup>-1</sup> )	46.4 ± 13.7	58.1 ± 16.1	34.1 ± 16.3	61.2 ± 40.3	0.23
<b>Posterior intestine</b>					
Cross-sectional area (mm <sup>2</sup> )	3.1 ± 0.7	4.2 ± 0.6	5.1 ± 1.4	4.7 ± 1.5	0.06
Villus length (mm)	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.71
Villus width (µm)	98.8 ± 9.5	97.2 ± 9.4	94.4 ± 1.3	97.4 ± 14.8	0.94
Muscularis externa (µm)	70.3 ± 15.4	75.3 ± 27.5	80.1 ± 35.6	88.2 ± 28.8	0.76
Outer longitudinal layer (µm)	33.5 ± 12.3	29.0 ± 10.5	25.7 ± 9.0	36.6 ± 12.5	0.42
Inner circular layer (µm)	41.1 ± 8.5	46.4 ± 17.1	47.9 ± 18.1	51.5 ± 17.6	0.76
Goblet cells (no. GC fold <sup>-1</sup> )	81.0 ± 24.6	100.7 ± 15.0	102.4 ± 17.3	96.9 ± 21.4	0.26
Neutral GC (no. GC fold <sup>-1</sup> )	5.6 ± 5.5 <sup>ab</sup>	0.9 ± 0.7 <sup>b</sup>	22.1 ± 20.4 <sup>a</sup>	8.4 ± 5.7 <sup>ab</sup>	0.02
Acid GC (no. GC fold <sup>-1</sup> )	75.5 ± 22.4	99.8 ± 15.1	80.4 ± 32.2	88.5 ± 22.9	0.34

Values are means ± standard deviation:  $n = 6$ . Means in rows with a different superscript letter differ significantly ( $P < 0.05$ )

quadrants, algae-rich diets tended to be separated from the CTRL. Fish fed the CTRL and NAN8 diets are separated from diets containing *G. gracilis* (GRA8 and NAN4GRA4) along

F1 with 50.1% of the variance. Fish fed diets with *N. oceanica* (NAN8 and NA4GRA4) are discerned in relation to the CTRL and GRA8 along F2 with 32.4% of the variance.

**Table 5** Specific activity (µm min<sup>-1</sup> g<sup>-1</sup> tissue) of maltase, sucrose-isomaltase (SI), intestinal alkaline phosphatase (IAP), and -glutamyltransferase (GT) in European sea bass intestinal tract

	CTRL	GRA8	NAN8	NAN4GRA4	P value
<b>Pyloric caeca</b>					
Maltase	2770.2 ± 251.8 <sup>ab</sup>	2441.4 ± 364.5 <sup>ab</sup>	2283.3 ± 503.7 <sup>b</sup>	2879.1 ± 856.0 <sup>a</sup>	0.04
SI	1023.3 ± 159.3 <sup>ab</sup>	822.2 ± 226.8 <sup>bc</sup>	729.3 ± 107.4 <sup>c</sup>	1080.1 ± 302.2 <sup>a</sup>	0.001
IAP	68.1 ± 10.1 <sup>b</sup>	82.3 ± 13.6 <sup>ab</sup>	47.6 ± 6.2 <sup>c</sup>	88.0 ± 26.2 <sup>a</sup>	<0.001
γGT	0.98 ± 0.23	1.17 ± 0.22	0.96 ± 0.32	1.33 ± 0.63	0.07
<b>Anterior intestine</b>					
Maltase	5793.5 ± 1444.6 <sup>a</sup>	4527.8 ± 1162.5 <sup>ab</sup>	4157.7 ± 958.7 <sup>b</sup>	5398.9 ± 1400.9 <sup>ab</sup>	0.01
SI	2238.5 ± 547.5 <sup>a</sup>	1499.0 ± 362.2 <sup>b</sup>	1476.3 ± 338.7 <sup>b</sup>	1636.5 ± 465.4 <sup>b</sup>	<0.001
IAP	123.6 ± 30.4	115.3 ± 39.9	98.4 ± 21.0	133.6 ± 44.0	0.10
γGT	1.54 ± 0.82	1.30 ± 0.48	0.98 ± 0.38	1.55 ± 0.45	0.05
<b>Posterior intestine</b>					
Maltase	4633.2 ± 944.1	4472.5 ± 958.0	5864.2 ± 1902.9	4520.4 ± 1463.2	0.05
SI	1821.7 ± 560.7 <sup>ab</sup>	1671.1 ± 462.9 <sup>b</sup>	2507.2 ± 982.3 <sup>a</sup>	1786.8 ± 591.1 <sup>ab</sup>	0.03
IAP	95.3 ± 21.1	94.6 ± 29.0	101.9 ± 34.4	81.5 ± 27.0	0.36
γGT	7.92 ± 3.72 <sup>ab</sup>	7.96 ± 4.64 <sup>ab</sup>	12.99 ± 6.32 <sup>a</sup>	5.63 ± 3.44 <sup>b</sup>	0.01

Values are means ± standard deviation:  $n = 12$ . Means in rows with a different superscript letter differ significantly ( $P < 0.05$ )

**Table 6** Plasma 24 h postprandial metabolite levels in European seabass fed the experimental diets for 106 days

	CTRL	GRA8	NAN8	NAN4GRA4	P value
Glucose (mg dL <sup>-1</sup> )	120.9 ± 21.5 <sup>b</sup>	149.6 ± 26.3 <sup>a</sup>	142.5 ± 21.8 <sup>ab</sup>	127.1 ± 18.8 <sup>ab</sup>	0.01
Total cholesterol (mg dL <sup>-1</sup> )	169.4 ± 31.2	187.3 ± 22.0	204.8 ± 33.2	186.9 ± 40.6	0.08
Triglycerides (mg dL <sup>-1</sup> )	436.8 ± 146.6 <sup>a</sup>	316.2 ± 88.5 <sup>b</sup>	294.3 ± 83.5 <sup>b</sup>	320.9 ± 91.3 <sup>b</sup>	0.01
Total protein (mg dL <sup>-1</sup> )	3.90 ± 0.76	4.21 ± 0.93	3.79 ± 0.47	4.00 ± 0.55	0.55
Albumin (mg dL <sup>-1</sup> )	0.96 ± 0.17	1.07 ± 0.15	1.11 ± 0.22	0.96 ± 0.17	0.09

Values are means ± standard deviation; n = 12. Means in rows with a different superscript letter differ significantly (P < 0.05)

## Discussion

The sustainable growth of aquaculture largely depends on the use of new nutrient sources to partially replace fish meal, without compromising fish growth, health, and nutritional value of end products (Gatlin et al. 2007). The present study clearly shows that both *G. gracilis* and *N. oceanica* biomass, used either single or blended, can be valuable natural ingredients to partially replace FM in diets for European seabass without affecting fish growth. Both algae have high protein and mineral content, despite their low lipid level. Seaweeds such as *Gracilaria* concentrate minerals from seawater, reaching a mineral content 10–20 times higher than terrestrial plants (Moreda-Piñeiro et al. 2012). Among the macro minerals present in the *G. gracilis* biomass used in our study, K was the most abundant element, as corroborated by other studies (Reka et al. 2017; Radha 2018). The microalga *N. oceanica* also has been shown to be a good source of minerals, namely Na, K, Mg, Ca, and P; when compared with *G. gracilis*, this microalga also had a higher content of essential amino acids, confirming previous observations

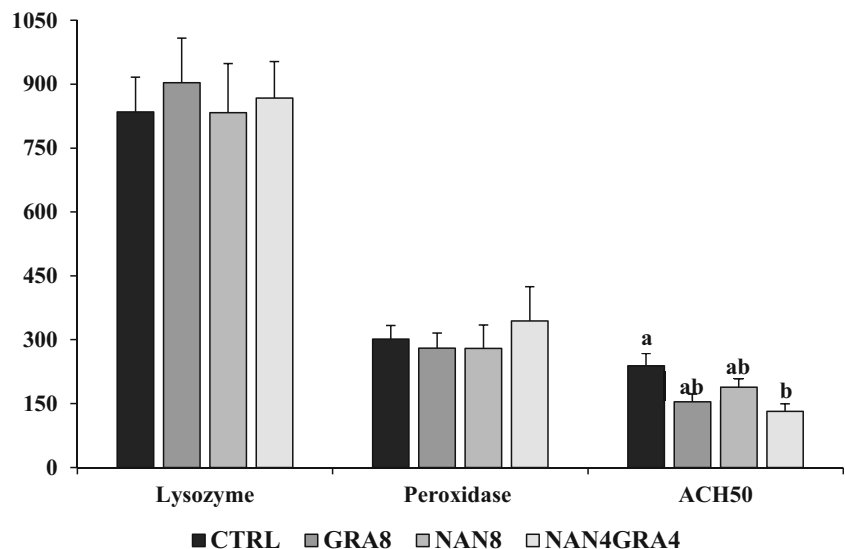
(Archibeque et al. 2009). Nevertheless, besides the nutritional value of each alga, their nutrient bioavailability is of major importance for nutritional studies. In the present study, the dietary inclusion of 8% *N. oceanica* did not affect nutrient digestibility, or nitrogen and energy retention efficiencies, but the diet with 8% *G. gracilis* had the lowest protein and energy ADC values. In European seabass, previous studies have reported that the inclusion of *G. bursa-pastoris* at 5–10% (Valente et al. 2006) resulted in protein ADC values similar to the control, but defatted *Nannochloropsis* sp. biomass up to 15% (Valente et al. 2019) impaired energy digestibility. The same defatted *Nannochloropsis* sp. included at 10% in diets for Atlantic salmon significantly reduced protein and energy ADCs (Sørensen et al. 2017). Moreover, a recent study in Atlantic salmon showed that the incorporation of 10% pre-extruded *N. oceanica* in plant-based commercial-like feeds did not affect protein digestibility but reduced the lipid digestibility (Gong et al. 2020). The significant reduction of energy ADC observed in diets containing *G. gracilis* (GRA8 and NAN4GRA4) might be explained by the presence of indigestible fibers in the seaweed cell wall, which may interfere with the digestion (Angell et al. 2016). Seaweeds are multicellular organisms with cell walls composed of complex polysaccharides. A recent study by Zheng et al. (2020) showed that red seaweeds are rich in sulfated polysaccharides of the carrageenan, agar, and agarose types which resist enzymatic degradation in the stomach and small intestine. The presence of such cell wall polysaccharides may limit the access of the digestive enzymes to algal proteins (e.g., phycobiliproteins) and can partially have contributed to the observed reduction of protein and energy ADC values in fish fed *Gracilaria*. Although seaweeds may have even higher levels of total dietary fiber than terrestrial plants, it is mostly soluble and, in the case of *Gracilaria*, comprises agar (Rajapakse and Kim 2011). This soluble fiber forms a viscous mass in the gut with binding properties that may trap digestive enzymes and some other nutrients, slowing down the digestibility and impairing nutrient absorption in the intestine. Although fish fed GRA8 had the lowest digestible N and digestible energy intake values, they also displayed the highest NRE and ERE, resulting in similar nutrient gain, final body size and whole

**Table 7** Oxidative stress parameters (TAC expressed in μL Trolox equivalents g<sup>-1</sup> ww; TG, GPx, GST, and GR expressed in nmol min<sup>-1</sup> mg<sup>-1</sup> protein; CAT expressed in μmol min<sup>-1</sup> mg<sup>-1</sup> protein; and LPO expressed in nmol TBARS g<sup>-1</sup> tissue) determined in the liver of European seabass after 106 days of feeding

	CTRL	GRA8	NAN8	NAN4GRA4	P value
TAC	16.2 ± 2.1 <sup>ab</sup>	15.5 ± 2.0 <sup>b</sup>	18.6 ± 4.1 <sup>a</sup>	17.7 ± 1.8 <sup>ab</sup>	0.04
TG	2.2 ± 1.3 <sup>a</sup>	2.0 ± 1.4 <sup>ab</sup>	1.1 ± 0.5 <sup>ab</sup>	0.9 ± 0.6 <sup>b</sup>	0.02
GPx	5.8 ± 4.4 <sup>a</sup>	5.6 ± 4.6 <sup>a</sup>	2.2 ± 1.1 <sup>ab</sup>	1.6 ± 0.6 <sup>b</sup>	0.001
GST	32.0 ± 8.9	28.5 ± 16.2	31.4 ± 7.7	27.1 ± 15.7	0.75
GR	0.6 ± 0.3	0.7 ± 0.2	0.6 ± 0.3	0.6 ± 0.3	0.86
CAT	66.0 ± 32.0	48.1 ± 24.1	54.5 ± 21.5	45.3 ± 17.0	0.18
LPO	48.4 ± 5.2	52.9 ± 5.4	51.8 ± 4.0	50.5 ± 4.6	0.14

Values are means ± standard deviation; n = 12. Means in rows with a different superscript letter differ significantly (P < 0.05). TAC, total antioxidant capacity; TG, total glutathione; GPx, glutathione peroxidase; GST, glutathione s-transferase; GR, glutathione reductase; CAT, catalase; LPO, lipid peroxidation

**Fig. 2** Effects of the experimental diets on humoral non-specific immune parameters of European seabass after 106 days of feeding. Lysozyme and peroxidase expressed in EU mL<sup>-1</sup> and alternative complement pathway hemolytic activity (ACH50) in Units mL<sup>-1</sup>. Values are means  $\pm$  standard error;  $n = 12$ . For each parameter, different superscript letters indicate significant differences between diets ( $P < 0.05$ )



body composition to all other treatments. According to Valente et al. (2006), macroalgae such as *Gracilaria* sp. have great potential as alternative ingredients in diets for European seabass juveniles at dietary inclusion levels up to 10%. In

rainbow trout, the dietary inclusion of 10–12% *G. vermiculophylla* meal or *G. pygmaea* impaired growth (Araújo et al. 2016; Sotoudeh and Mardani 2018), but in herbivorous fish such as Nile Tilapia (*Oreochromis niloticus*)

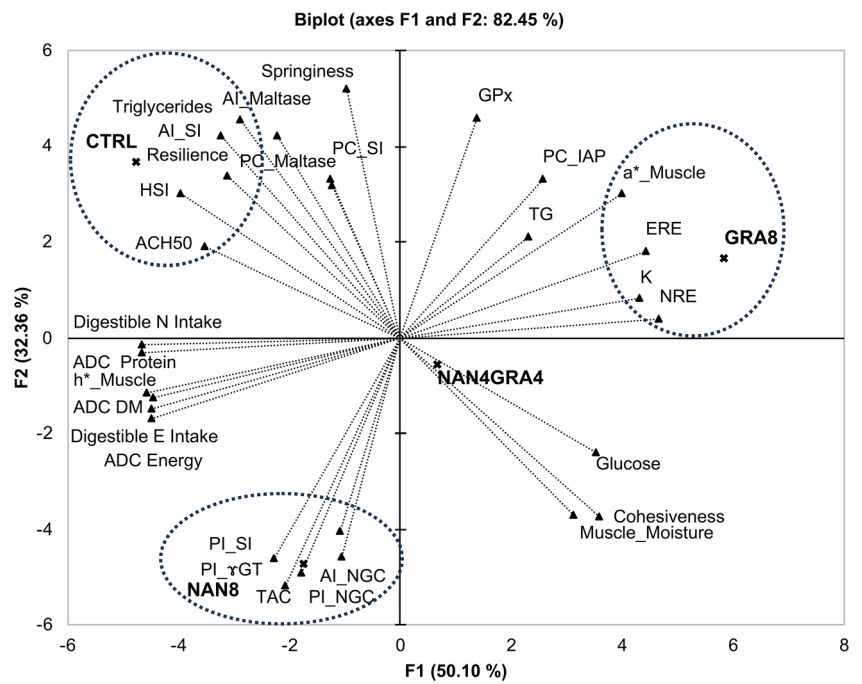
**Table 8** Color, texture, and composition (% wet weight, WW) of European seabass after 106 days of feeding the experimental diets

	CTRL	GRA8	NAN8	NAN4GRA4	<i>P</i> value
<b>Skin color</b>					
L*	53.8 $\pm$ 4.6	56.2 $\pm$ 2.7	57.1 $\pm$ 6.7	56.6 $\pm$ 7.2	0.64
a*	-4.0 $\pm$ 0.7	-4.0 $\pm$ 0.8	-4.5 $\pm$ 1.0	-3.8 $\pm$ 0.9	0.32
b*	8.2 $\pm$ 2.1	8.7 $\pm$ 1.9	9.0 $\pm$ 1.1	9.1 $\pm$ 1.8	0.54
C*	9.6 $\pm$ 1.8	10.0 $\pm$ 1.6	9.9 $\pm$ 1.3	10.0 $\pm$ 1.8	0.89
h*	117.8 $\pm$ 5.2	115.1 $\pm$ 4.8	116.5 $\pm$ 4.5	114.2 $\pm$ 5.3	0.31
<b>Fillet color</b>					
L*	43.2 $\pm$ 0.7	42.3 $\pm$ 1.6	42.7 $\pm$ 1.4	42.9 $\pm$ 1.7	0.53
a*	-3.3 $\pm$ 0.3 <sup>b</sup>	-2.6 $\pm$ 0.4 <sup>a</sup>	-3.5 $\pm$ 0.3 <sup>b</sup>	-3.2 $\pm$ 0.4 <sup>b</sup>	<0.001
b*	3.9 $\pm$ 1.2	4.2 $\pm$ 0.9	4.3 $\pm$ 0.9	4.4 $\pm$ 0.5	0.60
C*	5.3 $\pm$ 0.6	5.0 $\pm$ 0.6	5.6 $\pm$ 0.8	5.5 $\pm$ 0.4	0.14
h*	129.7 $\pm$ 8.1 <sup>ab</sup>	122.8 $\pm$ 7.8 <sup>b</sup>	130.2 $\pm$ 4.5 <sup>a</sup>	125.5 $\pm$ 5.2 <sup>ab</sup>	0.02
<b>Fillet texture</b>					
Hardness (N)	1.1 $\pm$ 0.1	1.0 $\pm$ 0.2	1.1 $\pm$ 0.4	1.1 $\pm$ 0.3	0.86
Chewiness (J)	0.3 $\pm$ 0.04	0.3 $\pm$ 0.04	0.4 $\pm$ 0.1	0.3 $\pm$ 0.1	0.32
Adhesiveness (J)	-0.1 $\pm$ 0.03	-0.1 $\pm$ 0.02	-0.1 $\pm$ 0.04	-0.1 $\pm$ 0.02	0.09
Springiness	1.2 $\pm$ 0.2 <sup>a</sup>	1.1 $\pm$ 0.1 <sup>a</sup>	1.0 $\pm$ 0.04 <sup>b</sup>	1.0 $\pm$ 0.04 <sup>b</sup>	0.001
Cohesiveness	0.2 $\pm$ 0.1 <sup>b</sup>	0.3 $\pm$ 0.1 <sup>a</sup>	0.3 $\pm$ 0.1 <sup>a</sup>	0.3 $\pm$ 0.04 <sup>ab</sup>	0.02
Resilience	0.7 $\pm$ 0.4 <sup>a</sup>	0.3 $\pm$ 0.1 <sup>ab</sup>	0.4 $\pm$ 0.2 <sup>ab</sup>	0.2 $\pm$ 0.1 <sup>b</sup>	0.03
<b>Fillet composition (%WW)</b>					
Moisture	74.6 $\pm$ 1.2 <sup>b</sup>	75.7 $\pm$ 0.7 <sup>a</sup>	75.7 $\pm$ 1.1 <sup>a</sup>	75.9 $\pm$ 0.7 <sup>a</sup>	0.01
Protein	21.5 $\pm$ 0.3	20.9 $\pm$ 1.7	21.2 $\pm$ 0.3	21.5 $\pm$ 0.3	0.76
Fat	3.5 $\pm$ 0.1	2.9 $\pm$ 0.5	2.6 $\pm$ 0.5	2.5 $\pm$ 0.7	0.13

Values are means  $\pm$  standard deviation;  $n = 12$ . Means in rows with a different superscript letter differ significantly ( $P < 0.05$ ). L\*, lightness; a\*, redness; b\*, yellowness; C\*, chromaticity; h\*, hue angle



**Fig. 3** Principal component analysis biplot of the mean scores and loadings for all the variables with significant difference ( $P < 0.05$ ) between European seabass fed the experimental diets (CTRL, GRA8, NAN8, and NA4GRA4). ADC, apparent digestibility coefficient; AI, anterior intestine; ERE, energy retention efficiency; GPx, glutathione peroxidase; GT, -glutamyltransferase; HSI, hepatosomatic index; IAP, intestinal alkaline phosphatase; IAP, intestinal alkaline phosphatase; K, final condition factor; NGC, neutral goblet cells; NRE, nitrogen retention efficiency; PC, pyloric caeca; PI, posterior intestine; SI, sucrose-isomaltase; TAC, total antioxidant capacity; TG, total glutathione



inclusion levels could go up to 20% (Stadtlander et al. 2013; Younis et al. 2018). Few studies have evaluated the inclusion of whole *Nannochloropsis* sp. in diets for European seabass, but defatted *Nannochloropsis* sp. biomass was successfully used up to 15% without affecting feed intake, fish growth, or whole body composition (Valente et al. 2019). Likewise, a study with turbot (*S. maximus*) carried out by Qiao et al. (2019) showed that *Nannochloropsis* sp. meal could be successfully used as a feed ingredient, contributing up to 10% of the diet without negative effects on fish growth performance. In Atlantic salmon, incorporation of 10% pre-extruded *N. oceanica* in plant-based commercial-like feeds did not affect the growth, feed utilization, or body proximate composition (Gong et al. 2020). However, reduced feed intake was observed in Atlantic cod *Gadus morhua* fed with 14 and 28% of a *Nannochloropsis* sp. and *Isochrysis* sp. blend (Walker and Berlinsky 2011).

Reduced growth reported in many fish species fed algae-rich diets was often associated with decreased intestinal absorption area due to a reduction in villi length or width (Silva et al. 2015; Araújo et al. 2016; Moutinho et al. 2018). Although intestinal morphology was not affected by the experimental diets used in the present study, the inclusion of *N. oceanica* (NAN8 and NAN4GRA4) resulted in a significant increase of neutral GC in the anterior intestine compared with fish fed GRA8. Goblet cells reside throughout the length of the small and large intestine and are responsible for the production and maintenance of the protective mucus blanket by synthesizing and secreting high-molecular-weight glycoproteins known as mucins (Specian and Oliver 1991). The increased number of total GC observed from the anterior to

posterior intestine also indicates increased mucus production and lubrication for nutrient passway. Moreover, the mucin-containing mucus layer coating the gastrointestinal epithelium is the front line of innate host defense (Kim and Khan 2013). Mucins are likely to be the first molecules that invading pathogens interact with at the cell surface and thus can limit binding to other glycoproteins and neutralize the pathogen (Kim and Khan 2013). Changes in goblet cell functions and in the chemical composition of intestinal mucus are detected in response to a broad range of luminal insults, including alterations of microbiota (Deplancke and Gaskins 2001). It has been suggested that acidic mucins protect against bacterial translocation (Deplancke and Gaskins 2001), whereas neutral mucins have been related to digestion and absorption processes (Grau et al. 1992). The higher number of neutral GC associated with a higher protein and energy ADC values in fish fed *N. oceanica* (NAN8 and NAN4GRA4) compared with fish fed GRA8 suggests that *N. oceanica* inclusion is positively influencing the digestion and absorption processes in the anterior intestine, mitigating the negative effect of *G. gracilis* in the blended diet (NAN4GRA4). However, this warrants further studies for clarification. In Atlantic salmon, increased cell proliferation (PCNA) was observed in the distal intestine of fish fed 10% *N. oceanica* but without causing any histomorphological changes (Gong et al. 2020).

The present study has investigated the effect of dietary algae inclusion on the activity of a panel of BBM enzymes 5 h after the last meal, that is, during the digestive process, when the presence of the substrates could trigger their activity. The presence of *N. oceanica* in the diet has significantly affected the activity of both the disaccharidases and the

intestinal alkaline phosphatase. In particular, diet NAN8 decreased the activity of maltase and sucrase-isomaltase in the pyloric caeca and anterior intestine and such effect could be due to the lower carbohydrate content of *N. oceanica* and consequently of diet NAN8, compared with other diets. The alkaline phosphatase (IAP) is expressed in mature enterocytes and is generally considered a marker of intestinal cell differentiation (Hinnebusch et al. 2004). The activity of IAP in the pyloric caeca was enhanced in fish fed seaweed-containing diets while the diet including *N. oceanica* resulted in a significant decrease, indicating that the dietary substitution of fishmeal with algae can modulate IAP gut activity. The IAP decrease observed in the anterior intestine of fish fed NAN8, although not statistically significant, is consistent with the increase of the neutral mucins in the same tract of the gut, thus describing a likely positive effect of *N. oceanica* on gut physiology. A different role of IAP has been proposed in zebrafish by Bates et al. (2007) where the alkaline phosphatase of the intestinal brush border has been shown to act as a detoxifier agent when dephosphorylates the lipopolysaccharides produced by the luminal microflora. Anyway, the interaction between algae and microflora is poorly investigated and was not considered in the present study.

The  $\gamma$ -GT enzyme participates in the final steps of the dietary protein digestion in the microvilli surface. Its activity was not significantly affected by dietary treatments, and the highest level of activity was registered in the posterior intestine as already reported by Tibaldi et al. (2006) and Messina et al. (2019). These results are consistent with total protein concentration in plasma, observed 24 h postprandial, suggesting that dietary algae inclusion may have limited effects on protein metabolism of European seabass.

At the end of the feeding trial, the physiological status of fish was assessed through the analysis of certain blood biochemical parameters which influence energy metabolism and are considered important diagnostic parameters (Coz-Rakovac et al. 2005). The algae-rich diets resulted in a general increase in circulating glucose level, reaching statistical significance in fish fed diet GRA8 relative to the control diet. Also, Belal et al. (2012) observed that Nile Tilapia fingerlings fed with diets containing 1% of *Spirulina* exhibited higher glucose values in fish serum. However, Vizcaino et al. (2016) found an inverse relationship of glucose concentration when increasing dietary macroalgae (*G. cornea* and *Ulva rigida*) levels in *S. aurata* diets. Algae inclusion had no effect on blood cholesterol and protein levels which is in accordance with a previous study in *S. aurata* fed 5% of seaweed biomass (Guerreiro et al. 2019). However, triglyceride levels were significantly decreased in fish fed the algae-based diets and could probably account for the reduced HSI observed in those fish. In red sea bream (*Pagrus major*) fed *Spirulina* sp., reduced total lipids both in serum and liver were associated with elevated activity of carnitine palmitoyltransferase, which is

related to fatty acid  $\beta$ -oxidation, activating lipid mobilization (Nakagawa et al. 2000). In addition, 1% *Chlorella* extract could improve lipolysis in *Plecoglossus altivelis* by a synergistic effect with vitamin C (Mustafa et al. 1997). In humans, DHA supplementation from algal oil reduced serum triglycerides and increased HDL and LDL cholesterol (Bernstein et al. 2012). It seems that algae may modulate lipid metabolism although in the present study fish whole body composition and muscle fat remained unchanged. Moreover, lipid peroxidation (LPO) also remained unaffected by the dietary inclusion of algae. Further studies are warranted to better clarify the effect of algae biomass on fish intermediary lipid metabolism.

Marine algae are rich sources of bioactive compounds able to modulate oxidative stress (Lee et al. 2013) which occurs when the production of excessive reactive oxygen species (ROS) is not balanced by an endogenous antioxidant defense system or exogenous dietary antioxidants that maintain these oxidizing compounds at acceptable levels (Biller and Takahashi 2018). Exogenous antioxidants provided by microalgae uptake can prevent oxidative stress by activating a wide range of enzymes, as previously observed in fish fed *Chlorella vulgaris* (Rahimnejad et al. 2017), *Spirulina platensis* (Teimouri et al. 2019), and *N. oceanica* (Sørensen et al. 2017). In the present study, the inclusion of algal biomass in fish diets had limited effects on oxidative stress, confirming previous studies using seaweeds (Guerreiro et al. 2019). All diets induced similar total antioxidant capacity (TAC) in the seabass liver, when compared with the CTRL. However, fish fed NAN8 had a higher TAC than fish fed GRA8, suggesting that *N. oceanica* might have higher levels of exogenous compounds with antioxidant capacity than *G. gracilis*. In Atlantic salmon, the dietary inclusion of defatted *Nannochloropsis* sp. had no effect on TAC (Kiron et al. 2016), but in rainbow trout, the TAC increased concomitantly with increasing levels of *S. platensis* (Teimouri et al. 2019). This effect was attributed to a possible role of carotenoids with recognized antioxidant potential in *S. platensis*. In fact, both the chemical composition, mainly the carotenoid type and content, and the inclusion level of each alga will certainly determine the effect on each fish species. Antioxidant enzymes such as GPx indicate the scavenging capacity for ROS and the ability to protect cells from oxidative damage, thus preventing lipid peroxidation. The antioxidant function of GPx is largely dependent on its interaction with TG (Lu 2009), reducing H<sub>2</sub>O<sub>2</sub> and lipid peroxide as GSH is oxidized to GSSG. Contrarily to what was observed by Peixoto et al. (2016a), who reported increased GPx activity associated with the inclusion of *Gracilaria* sp. extracts, seabass fed NAN4GRA4 had a lower activity of GPx and lower bioavailability of TG in the liver. This implies that these fish may be less prepared to cope with eventual biotic stressors. Data from humoral non-specific immune parameters also reinforces that hypothesis, as fish fed NAN4GRA4 diet had a lower ACH50 activity, which further points to an

increased vulnerability to biotic stressors. Moreover, our study showed that lipid peroxidation (LPO) did not vary among dietary treatments, indicating that algae inclusion did not have any major effect in terms of the antioxidant status of European seabass. Likewise, Peixoto et al. (2016a) did not find any influence on LPO in the liver of seabass fed diets supplemented with two different *Gracilaria* sp. extracts, as well as on the activity levels of the antioxidant bioindicators CAT and GR. Different results were observed in turbot where increasing inclusion levels (2.5–10%) of *N. oceanica* were associated with diminished rates of LPO (Qiao et al. 2019). Moreover, Magnoni et al. (2017) reported a downregulation of genes of antioxidant enzymes in gilthead sea bream (*S. aurata*) fed heat-treated *Gracilaria* sp. and subjected to hypoxia challenge, suggesting a protective role against oxidative stress. Further studies, perhaps involving a pro-oxidant challenge, are necessary in order to fully assess the potential of algae in fish oxidative stress.

The ability of fish to cope with stress events will definitely be a priority for aquaculture in the coming years, and the dietary inclusion of dietary sources rich in biologically active substances seems a promising strategy to improve fish robustness. In this study, some humoral innate immune parameters were evaluated in seabass plasma after being fed the experimental diets. Despite the fact that humoral peroxidase and lysozyme activities were not affected by dietary treatments, fish fed NAN4GRA4 had a lower alternative complement pathway (ACH50) than fish fed the CTRL diet. The complement system, made up of a large number of distinct plasma proteins that react with one another, plays an essential role in alerting the host of the presence of potential pathogens, as well as in their clearing (Boshra et al. 2006). It seems that the algae blend may be contributing towards the depletion of plasma serum hemolytic activity, perhaps due to the inhibition of the protein cascade that composes the alternative pathway of the complement system. This lack of certain complement proteins may lead to an increased susceptibility to infections, as well as the development of autoimmune diseases in fish. A similar trend was observed in seabass fed with 7.5% *Gracilaria* sp. that resulted in a significant reduction of ACH50 (Peixoto et al. 2016b). Opposite results were reported in rainbow trout, where the dietary inclusion of 5% *Gracilaria* sp. increased both ACH50 and peroxidase activity (Araújo et al. 2016). A dose-dependent stimulation of ACH50 was also observed with the inclusion of defatted *Nannochloropsis* sp. up to 10%, in spite of results not differing significantly from the control (Valente et al. 2019).

Besides their nutritional properties, algae are also good sources of pigments that may affect the appearance of fish, namely in terms of skin and muscle color. Such attributes are known to influence market value, flavor perception, and acceptability of fish food products (Spence et al. 2010). Although fish used in the present study were not commercial

sized, the instrumental color of skin and muscle textural properties were evaluated to predict any possible effect of the algal biomass that could compromise these parameters at later stages. The dietary inclusion of *N. oceanica* or *G. vermiculophylla* did not influence the skin color of seabass. However, Peixoto et al. (2018) reported a significantly lighter (L\*) skin in fish fed diets with *Gracilaria* sp. extracts compared with control groups. This same author did not find any effect of the *Gracilaria* sp. on the fillet color, but in our study fish fed GRA8 had higher redness (a\*) compared with fish from all other treatments and lower hue angle (h\*) than fish fed NAN8. Increased a\* was also previously reported in raw and cooked fillets of rainbow trout fed *G. vermiculophylla*, which were perceived by a sensory panel as having increased color intensity (Valente et al. 2015; Araújo et al. 2016). Moreover, in large-sized seabass, increased skin greenish and fillet redness were observed in fish fed *Isochrysis* sp., but the sensory panel was not able to discriminate among samples (Tibaldi et al. 2015). The extent of such pigmentation patterns on the impact on consumer preferences remains to be clarified.

The texture and structure of fish muscle are important freshness quality attributes that depend on several parameters such as hardness, cohesiveness, springiness, chewiness, resilience, and adhesiveness, as well as the internal cross-linking of connective tissue and the detachment of fibers (Cheng et al. 2014). In our study, muscle cohesiveness and moisture content were higher in fish fed NAN8 and GRA8, compared with CTRL. According to Jonsson et al. (2001) expressible moisture of salmon fillet was positively correlated with textural properties being of major importance for commercial value and consumer acceptance. Water holding capacity and expressible water content significantly affect the tenderness of the fish muscle (Varghese and Mathew 2017). Furthermore, the dietary inclusion of *N. oceanica* somehow decreased the fillet springiness while the blend of both algae (NAN4GRA4) decreased fillet resilience. This suggests soft flesh traits in the NAN4GRA4 group and a lower elasticity in the NAN8 fillets. Water holding capacity of muscle is greatly influenced by changes in protein structure, and it was demonstrated that the water binding capacity of the proteins shows a noticeable decrease due to the increase in the moisture content of meat (Aaslyng et al. 2003). Collagen is the most significant constituent of the connective tissues, playing a vital role in maintaining fillet integrity and muscle cohesiveness (Aussanasuwannakul et al. 2012). In yellowtail and red sea bream fed with algae (such as *Spirulina*, brown algae, and *Chlorella*), high muscle total collagen was associated with a firm raw meat texture (Nakagawa and Montgomery 2007). In the present study, fillet hardness was not affected by the dietary treatments, but a longer trial until fish reach commercial size is required to fully understand the impact of algae on fish textural properties.



According to the PCA plot, all diets are projected into distinct quadrants, but algae-rich diets tended to be separated from the CTRL. Diets containing *N. oceanica* (NAN8 and NAN4GRA4) were negatively loaded along F2 and projected in opposite quadrants in relation to CTRL and GRA8. NAN8 diet demonstrated a strong correlation with the number of posterior and anterior intestinal neutral GC, and also with the total antioxidant capacity and activity of both  $\gamma$ -GT and SI in the posterior intestine. GRA8 dietary group was positively loaded along F1 and F2 and associated with the condition factor, N and energy retention efficiency, and muscle redness. CTRL dietary group positively loaded along F1 and was associated with the HSI, plasma triglycerides, and activity of both SI and maltase in the anterior intestine. Moreover, PCA analysis also unveiled the effects of the dietary treatments on the nutrient and energy ADC where the NAN8 and CTRL diets were correlated with better digestibility.

In conclusion, the present study clearly shows that both *G. gracilis* and *N. oceanica* biomass, used single or blended, can be valuable natural ingredients for partial replacement of FM in diets for European seabass, as they do not affect growth performance, feed efficiency, N gain, or whole body composition. However, triglyceride levels significantly decreased in fish fed the algae-based diets and could probably account for the reduced HSI observed in those fish. The dietary inclusion of *N. oceanica* did not affect nutrient digestibility, but *G. gracilis* led to the lowest protein and energy ADC values enlightening the need for some processing prior use. *N. oceanica* had higher total antioxidant capacity than *G. gracilis*, despite not differing from the control diet. Moreover, the algae blend (NAN4GRA4 diet) resulted in lower values of TG, GPx, and ACH50, suggesting that these fish may be less prepared to respond in case of possible infection or stress. However, an infection and/or pro-oxidant challenge would be necessary to further assess the implications of these results. Finally, an impact of algal biomass of muscle textural properties was demonstrated: the inclusion of *N. oceanica* decreased fillet springiness and elasticity, but when *G. gracilis* was added (NAN4GRA4), the muscle fillet became softer. A longer feeding trial is recommended to ascertain these effects on commercial-sized fish.

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### Compliance with ethical standards

The present study was performed by accredited scientists, in compliance with the guidelines of the European Union (directive 2010/63/EU) and Portuguese law (Decreto-Lei no. 113/2013, de 7 de Agosto) on the

protection of animals used for scientific purposes. All animal procedures were subject to an ethical review process carried out by CIIMAR animal welfare body (ORBEA-CIIMAR) and further approved by national competent authorities.

**Conflict of interest** The authors declare that they have no conflict of interest.

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