Article

Evaluation of *Aurantiochytrium mangrovei* Biomass Grown on Digestate as a Sustainable Feed Ingredient of Sea Bass, *Dicentrarchus labrax*, Juveniles and Larvae

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Abstract: The use of microalgae as a sustainable source of n-3 long-chain polyunsaturated fatty acids (LC-PUFA) as an alternative to fish oils from small pelagic fish (e.g., anchovy, sardine) has received growing interest in the past few years. The present study aimed to: (i) produce *Aurantiochytrium mangrovei* biomass by heterotrophic fermentation using a medium containing anaerobic digestion liquid effluent, and (ii) evaluate a biomass rich in n-3 LC-PUFA and good quality proteins as a feed ingredient for sea bass juveniles and larvae. Two 800 L bioreactors were used to produce *Aurantiochytrium* biomass in non-axenic conditions. Biomass was then filtered through a crossflow filtration system (300 Kda ceramic membrane) and freeze-dried. Sea bass juveniles (32.7 ± 4.2 g) were fed both a control diet and a diet containing 15% of freeze-dried *A. mangrovei* biomass for 38 days. Juvenile survival percentage was 90% on average in both dietary conditions. Similar growth was observed between fish fed with both diets, demonstrating the feasibility to replace 15% of a standard fish feed by *Aurantiochytrium* biomass. The liver of sea bass juveniles fed with the *A. mangrovei* diet contained significantly higher proportions of 22:6n-3, 22:5n-6, and 20:4n-6 than those fed with the control diet, while the proportions of 16:0, 16:1n-7, and 18:1n-9 were significantly lower. The secondary oxidation, as measured by malonylaldehyde (MDA) content, in the liver and muscle of juveniles fed with the microalgae diet tended to be higher than in fish fed the control diet, but the differences were not statistically significant. Although the larvae survival percentage was low for all the tanks after 41 days of rearing, the inclusion of 15% of hydrolyzed *A. mangrovei* biomass in the larvae micro-diet did not impair the development of sea bass larvae and only marginally affected their lipid composition. In the future, we have to further optimize a sustainable workflow between *Aurantiochytrium* cultivation and fish feed production and confirm the zootechnical and biochemical results.

Keywords: aquaculture; nutrition; sustainability; n-3 long-chain polyunsaturated fatty acids; Thraustochytrids; microalgae; fish oil

1. Introduction

Capture fisheries and aquaculture accounted for about 179 million tons in 2018, with aquaculture becoming dominant [1]. Farmed fish and shellfish production are major sources of seafood for human consumption, but this production partially depends on fishmeal and fish oil obtained from industrial fisheries of small pelagic fish. Fish meal and fish oil are key ingredients in fish feeds for farmed fish and shellfish species, as they are used as sources of

essential amino acids and n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) [2,3]. Most (74% in 2012) of the world's production of fish oils rich in n-3 LC-PUFA is used for aquaculture. The demand for fish oil is thus growing as the aquaculture sector grows [4] (Tacon and Metian, 2015).

Despite this, fishmeal and fish oil production are reaching their limits in terms of sustainability [5,6]. The use of these limited resources associated with the growing worldwide demand from the aquafeed industry is a threat to natural ecosystems and sustainable aquaculture. Thus, this results in a necessity to find alternative sources of these key ingredients to ensure more sustainable aquaculture production. Furthermore, as fishmeal and fish oil became more expensive and the awareness of aquaculture effect on small pelagic stocks rose, substitution of these ingredients by plant-based and animal-based ingredients increased during the last 30 years, potentially affecting the nutritional composition and value of the farmed fish [7]. Heterotrophic production of n-3 LC-PUFA by marine microorganisms as an alternative to fish oils from small pelagic fish (e.g., anchovy, sardine) has received growing interest in the past few years. The dinoflagellate *Crythecodinium cohnii* and Thraustochytrids are the most commonly grown marine protists for n-3 LC-PUFA production. Although the production cost under controlled heterotrophic fermentation of these microorganisms remains high, the resulting biomass is generally free of contaminants [8].

The Thraustochytrids, most notably the species of *Aurantiochytrium* and *Schizochytrium* genus, are particularly relevant for this type of application, mainly due to their high content in n-3 LC-PUFA [9,10]. Recently, oil and dry biomass from *Schizochytrium* sp. have successfully been incorporated into the diets of marine carnivore finfishes. It was found that substitution of marine fish oils by *Schizochytrium* sp. oil in the diet of Atlantic salmon parr did not impair fish growth [11]. Inclusion of whole *S. limacinum* cell biomass in Atlantic salmon diet was tested at various ratios, with 3–6% significantly improving growth and filet quality [12], 5% resulting in similar performance and a good availability of biomass n-3 LC PUFA [13], and 30% revealing a good digestibility [14]. Dried algae *Schizochytrium* sp. was also shown to be a good source of DHA for seabream *Sparus aurata* larvae [15]. The full replacement of fish oil by a high level of *Schizochytrium* sp. in the diet of Nile tilapia (*Oreochromis niloticus*) allowed for higher lipid digestibility, levels of DHA, and n-3 LC-PUFA as well as weight gain when compared to a control diet containing fish oil [16]. Including 2% of the dried algae *Schizochytrium* sp. in the diet of channel catfish led to a marked increase in n-3 LC-PUFA levels in the fish fillet [17]. A recent study demonstrated that a vegetal oil base supplemented with 5% and 10% of *Schizochytrium limacinum* biomass performed similarly to a fish oil-based diet in terms of growth and feeding efficiencies of *Dicentrachus labrax* juveniles [18].

Beyond providing high-quality n-3 LC-PUFA, *Schizochytrium* biomass could also contribute to the supply of good quality proteins and essential amino acids required in finfish aquaculture nutrition [19,20]. Furthermore, the inclusion of fish protein hydrolysates in marine fish feed improved fish growth, especially at their early stages of development [21]. For example, partial replacement of fishmeal by protein hydrolysates enhanced larval growth and/or survival performance in European sea bass *Dicentrarchus labrax* [22,23]. More recently, the total substitution of fish protein hydrolysate by yeast protein hydrolysate containing 44% of free amino acids (MW < 200 Da), 50% of di- and tripeptides (200 < MW < 500 Da) and 6% of larger polypeptides (500 < MW < 2500 Da) did not affect sea bream (*Sparus aurata*) larval performance [24]. However, the growth and survival of larvae fed with protein hydrolysates may vary according to the nature of the marine protein hydrolysate [25].

Oils and biomass from the industrial production of *Schizochytrium* are already commercialized; however, production costs remain high due to the need for expensive cultivation substrate. The use of industrial by-products and/or waste as carbon and nitrogen sources for microalgae cultivation can reduce production costs [18], but this novel source of nutrients has not been widely considered on an industrial scale [26].

The emergence of a circular economy allowing for the sustainable production of natural raw materials is now a preferred approach within the agricultural and food industries. In this context, the use of nutrient-rich effluents from anaerobic digestion aimed to reduce the cost associated with nutrient supplementation (notably nitrogen) for microalgal cultivation. The main challenge of such a circular economy approach is to identify the limiting nutrients of by-products and/or waste in order to maximize the growth rates of algal cultivated species. To date, most of the research on nitrogen and phosphorus recycling has been conducted at the laboratory scale. Cultivating *Aurantiochytrium* on a medium composed of digestate and a carbon source is possible and has been optimized in non-axenic cultivation conditions at a pilot scale (one m^3) [27,28].

The present study aims to: (i) apply a biomass production process for *Aurantiochytrium mangrovei* (formerly named *Schizochytrium*) by heterotrophic fermentation using a medium containing anaerobic digestion liquid effluent and (ii) evaluate a microalgal biomass rich in n-3 LC-PUFA and good quality protein in the form of feed ingredients for sea bass juveniles (whole biomass) and larvae (hydrolyzed biomass). The applicative objective is to partially replace fishmeal and fish oil ingredients generally obtained from the small pelagic fish industry in fish feed with 15% of microalgae biomass. It is meant to be a first step toward higher replacement percentage.

2. Materials and Methods

2.1. Pilot-Scale Cultivation and Harvesting of Aurantiochytrium Mangrovei on Liquid Effluent from Anaerobic Digestion

2.1.1. Preparation of *A. mangrovei* Inoculum

Initial inoculation was performed using 2 mL of a cryopreserved *A. mangrovei* (RCC893) master cultivation with a concentration of 1×10^7 cells/mL. First, 500 mL flasks were inoculated in 250 mL of Yeast Extract Peptone (YEP) medium containing 15 g/L sea salt (Sigma™, Saint Louis, MO, USA; Sigma S9883, 2 g/L peptone from casein (VWR™ Rosny-sous-bois, France, 84610.0500), 2 g/L yeast extract (VWR™ Rosny-sous-bois, France, 84601.5000) and 20 g/L glucose (TITOL chimica SpA™, Pontecchio Polesine, Italy, Glucose anhydrous pure CA:50-99-7). The culture was then maintained on a shaking platform (100 rpm) for 48 h at 23–25 °C. Then, 2 mL of the first culture was used to inoculate four new flasks in the same conditions for 64 h. The content of four cultures was transferred into two 20 L carboys to inoculate 8 L of YEP medium in each. The carboys were maintained for 24 h on the shaking platform (100 rpm), with air supply (4.8 L/min through a 4 mm diameter tubing) at the base of the carboy. After quality control, the two cultures of 8 L were finally transported from the laboratory (IUEM Plouzané) to the pilot site (Cooperl, Lamballe) for the inoculation of two 800 L poly(methylmethacrylate) (PMMA)-made cylinders for batch cultivation (500 L) at pilot scale.

2.1.2. Preparation of the Digestate for Integration in Culture Medium at Pilot Scale

The digestate used to grow *A. mangrovei* was the result of the anaerobic digestion of pig manure. Prior to microalgal cultivation, the digestate was first centrifuged to eliminate large particles and then using ultra-filtration at a pore size of 300 kDa to sanitize it and remove micro-particles. The composition of the raw digestate can be found in Table S1.

2.1.3. Cultivation of *A. mangrovei* at Pilot Scale

Two 800 L PMMA-made cylinders were used to produce about 10 kg of biomass in non-axenic conditions. Water (for process and cleaning) was supplied by a pump and delivered at the top of the cylinders through a rotating nozzle. Agitation and O_2 supply in each cylinder were provided by airflow bubbling from the bottom of the cylinder, at a rate of 0.4 volume of air per volume of culture per minute (air-lift system). The air was supplied through a ring of 16 mm diameter PVC tubing pierced with 500 1.5 mm diameter holes. All the other necessary inputs were introduced manually at the top of the two cylinders.

The pilot-scale medium for the batch cultivation was composed of industrial glucose syrup (final concentration: 24 g/L ISOSWEET 470 TEREOS), sterilized medium with tryptone and yeast extract at 2 g/L, digestate previously filtered as described above (final concentration 2.5% supplying 48.3 mg N/L, 2.6 mg P/L with N/P ratio of 18.7), and sea salt (final concentration 15 g/L, Le Saunier de Camargue, sel de mer non traité). Two 800 L cylinders were filled with 500 L of the pilot-scale medium and each inoculated with 8 L cultures as prepared above. Silicone-based anti-foam was added (final concentration: 1 mL/L, Antimousse 426R, LABOGROS) as soon as the yeast extract and peptone solution were poured into the cylinder. The microalgae *A. mangrovei* was then cultivated in non-axenic conditions for 62 h. The temperature of the culture was regulated between 28 and 30 °C by immersing a stainless steel electric heater (2 Kw for 500 L) while pH was maintained above 4.5 by regular addition of 10 N NaOH solution. After 62 h of cultivation, the biomass concentration reached about 10 g/L in both cylinders.

2.1.4. Harvesting of *A. mangrovei* Culture at Pilot Scale

Cultures from the two cylinders (1000 L) were pooled and then filtered through a crossflow filtration system. The filtration system from SIVA TM supports 3.5 m^2 of 300 Kda ceramic membrane, in two cartridges supporting 11 channels each (internal diameter 4.6 mm). Filtration conditions were as follows: 4 m/s for retentate velocity on membrane, 2500 L/h for retentate recirculation rate, 0.6 to 0.9 bars for transmembrane pressure, 300 to 180 L/h for permeate flow-rate. During crossflow filtration, samples were taken every hour in order to control the fatty acid composition, notably, their content in n-3 LC-PUFA. The final volume of retentate was approximatively 100 L at 90 g/L (equivalent DW) of microalgae biomass. The harvested biomass was frozen at −20 °C prior to further downstream processing (freeze-drying and enzymatic hydrolysis). The harvested biomass was freeze-dried externally by Eurolyo (https://www.eurolyo.fr/, (accessed on 27 September 2022)) (72 h at −40 °C and 250 µbar).

2.1.5. Monitoring of Culture Concentration and Cellular Parameters

Concentration and cellular parameters (size, complexity, lipid content) of *A. mangrovei* were measured using an Easy-Cyte Plus 6HT flow cytometer (Guava Merck Millipore®, Darmstadt, Germany) equipped with a 488 nm blue laser, detectors of forward (FSC) and side (SSC) light scatter, and three fluorescence detectors: green (525/30 nm), yellow (583/26 nm) and red (680/30 nm). Cell morphological variables, i.e., forward scatter (forward scatter, FSC), side scatter (side scatter, SSC), were used to identify and select the *A. mangrovei* cell population. FSC and SSC give, respectively, information on the relative size and complexity of cells [29]. The flow cytometry measurements were performed on fresh (living) samples. The BODIPY probe (BODIPY 505/515 FL; Molecular Probes, Invitrogen, Eugene OR, USA, final concentration of 10 mM), which stains lipid droplets/bodies within microalgae cells, was used as a proxy of lipid reserves [29]. The green fluorescence emitted is proportional to the quantity of lipid reserve present in the cells. Measurements were performed at a flow rate of 59 µL min^{-1}. The concentration of microalgae was given in cells per mL, and cellular parameters (FSC, SCC, and lipid reserve) were expressed in arbitrary units (A.U.).

2.2. Enzymatic Hydrolysis of A. mangrovei Biomass

The freeze-dried biomass was resuspended in distilled water at 10% (*w*/*v*), and it was then hydrolyzed with Alcalase 2.4 L (Sigma-Aldrich, a protease of *Bacillus licheniformis*) at an enzyme/substrate ratio of 1.8%. Hydrolysis was carried out in a 500 mL lab reactor heated with a water bath at 50 °C and stirred at 100 rpm with a 3-bladed propeller. After two hours of hydrolysis, when the degree of hydrolysis was around 14 ± 1%, the biomass was heated at 80 °C for 20 min to inactivate the enzyme.

To obtain enough hydrolyzed biomass (0.5 kg), hydrolysis was repeated ten times. The pooled hydrolysates were freeze-dried for 72 h using a freeze-dryer (Christ Alpha 1–4 LD plus).

2.3. Feed Formulation for Seabass Juveniles and Larvae

2.3.1. Seabass Juveniles Feed

Experimental juvenile diets, namely control and microalgae diets, were prepared in the INRAE facilities (Donzacq, Landes, France) and extruded as pellets of 3 mm. Gelatinized starch was included due to the extrusion process. The compositions of the control and microalgae diets are detailed in Table 1.

Table 1. Formulation and proximal composition of the experimental diets for sea bass juveniles.

	Experimental Juvenile Diets	
Ingredients (% of Dry Weight Diet)	**Control**	**Microalgae**
Fish meal	64	52
Microalgae biomass	0	15
CPSP 90 (pre-digest fishmeal)	10	10
fish oil	1	0
rapeseed oil	1	1
rapeseed lecithin	14	13
Starch	5	5
Vitamin mix [1]	3	3
Mineral mix [2]	1	1
Cellulose	1	0
Total	100	100
Calculated proximal composition (% dry weight)		
Proteins % DW	52.1	48.2
Lipids % DW	18.5	17.8
EPA+DHA % DW	1.1	1.1
EPA+DHA % lipids	6.1	6.0

Dietary ingredients, except microalgae biomass, were commercially obtained. Fishmeal, fish oil, and CPSP 90: Sopropêche 62,126 Wimille; Soya lecithin: Louis François 77,134 Marne La vallée; rapeseed oil: Oleandes, 40,250 Mugron; Starch: Roquette 62,136 Lestrem; Cellulose: Rettenmaier Saint Germain en Laye France 78,100; Vitamins and minerals: INRAE SAAJ 78,530 Jouy en Josas. [1] Per kg of vitamin mix: retinyl acetate, 340 mg; cholecalciferol, 2.5 mg; all-rac-α-tocopherol acetate, 4 g; menadione, 0.1 g; thiamin, 1 g; riboflavin, 2.5 g; D-calcium pantothenate, 5 g; pyridoxine HCl, 1 g; cyanocobalamin, 0.006 g; riacin, 10 mg; folic acid, 0.5 g; biotine, 0.1 g; meso-inositol, 100 g. [2] Per kg of mineral mix: KCl, 90 g; KI, 40 mg; $CaHP0_4$ $2H_2O$, 500 g; NaCl, 40 g; $CuSO_4$ $5H_2O$, 3 g; $ZnSO_4$ $7H_2O$, 4 g; $CoSO_4$ $7H_2O$, 20 mg; $FeSO_4$ $7H_2O$, 20 g; $MnSO_4$ H_2O, 3 g; $CaCO_3$, 215 g; Mg SO_4 $7H_2O$, 124 g; NaF, 1 g.

2.3.2. Seabass Larvae Feed

To produce larvae micro-diets, dietary ingredients (Table 2) were mechanically mixed with water, pelleted and dried at 50 °C for 20 min. The pellets were sieved to obtain two sizes of particles; 125–200 and 200–400 µm. The small micro-diet was used from 16 days after hatching as a co-feed of enriched *Artemia* nauplii at increasing ratios, from 20% to 100% using 20% increments for 5 days. Twenty days after hatching, the larger micro-diet was used until the end of the experiment (41 days post-hatching). Fish larvae were continuously fed in excess for 18 h/day using a belt feeder.

Table 2. Formulation and proximal composition of the experimental larvae micro-diet.

	Experimental Larvae Micro-Diets	
Ingredients (% of Dry Weight Diet)	**Control**	**Microalgae**
Fish meal	62	68
Hydrolyzed microalgae biomass	0	14
CPSP 90 (pre-digest fishmeal)	10	0
Tuna oil	1	0
Soya lecithin	13	13
Vitamin mix [1]	3	3
Mineral mix [2]	3	2
Cellulose	8	0
Total	100	100
Calculated proximal composition (% dry weight)		
Proteins % DW	50.7	50.1
Lipids % DW	16.5	17.6
EPA+DHA % DW	1.1	1.2

Dietary ingredients, except microalgae biomass, were commercially obtained. Fishmeal, fish oil, and CPSP 90: Sopropêche 62,126 Wimille; Soya lecithin: Louis François 77,134 Marne La vallée; rapeseed oil: Oleandes, 40,250 Mugron; Starch: Roquette 62,136 Lestrem; Cellulose: Rettenmaier Saint Germain en Laye France 78,100; Vitamins and minerals: INRAE SAAJ 78,530 Jouy en Josas. [1] Per kg of vitamin mix: retinyl acetate, 340 mg; cholecalciferol, 2.5 mg; all-rac-α-tocopherol acetate, 4 g; menadione, 0.1 g; thiamin, 1 g; riboflavin, 2.5 g; D-calcium pantothenate, 5 g; pyridoxine HCl, 1 g; cyanocobalamin, 0.006 g; riacin, 10 mg; folic acid, 0.5 g; biotine, 0.1 g; meso-inositol, 100 g. [2] Per kg of mineral mix: KCl, 90 g; KI, 40 mg; $CaHP0_4$ $2H_2O$, 500 g; NaCl, 40 g; $CuSO_4$ $5H_2O$, 3 g; $ZnSO_4$ $7H_2O$, 4 g; $CoSO_4$ $7H_2O$, 20 mg; $FeSO_4$ $7H_2O$, 20 g; $MnSO_4$ H_2O, 3 g; $CaCO_3$, 215 g; Mg SO_4 $7H_2O$, 124 g; NaF, 1 g.

2.4. Experimental Design

2.4.1. Sea Bass Juvenile Rearing

Sea bass juveniles, weighing on average 32.7 ± 4.2 g (approximatively 6 months old), were distributed into six 700 L tanks (three tanks per experimental condition) at a density of 100 juveniles per tank. Tanks were continuously filled at a flowrate of 500 L per hour with seawater at a salinity of 35.4 psu on average. Sea water prior to the experiment was passed through two sand filters (~500 µm), heated (tungsten, Plate Heat Exchanger), degassed using a column, filtered using a 2 µm membrane and finally UV sterilized assuring high water quality. Water temperature was increased progressively (17.5 °C to 20.5 °C) during the experiment due to the water network configuration. The fish were subjected to a photoperiod of 12 h:12 h light dark cycle by fluorescent ceiling lights (55–60 Lux). Temperature and water pH were measured weekly, and the tanks were cleaned regularly with a broom after a daily purge of 1/4 of the tank's volume (evacuation of fish feces).

Prior to the experimental feeding with the two diets described in Table 1, juveniles were acclimated for about a week with the fish feed "NEOSTART" 3 mm (Le Gouessant, Lamballe, France). After this acclimation, control and microalgae diets were distributed using belt feeders at 2% of the fish mass per day (ad libitum ratio at the applied rearing temperature [30]). The feeding quantities were adjusted with data obtained after weight measurements on days 14 and 29 of dietary conditioning.

2.4.2. Sea Bass Larvae Rearing

Two days post-hatching (PH) sea bass larvae were purchased from the marine hatchery of Gravelines Ichtus (France) and transferred to LEMAR rearing facility in Plouzané. Larvae were distributed into nine 35 L conical tanks (three tanks per experimental condition) at a density of 3000 larvae per tank. Conical tanks were continuously filled at a flowrate of 30 L per hour with seawater filtered at 10 µm at a salinity of 35.0 psu on average. Photoperiod was also adapted according to standard larval rearing (steady increase from 0.5 to 10 Lux between day 8 PH and day 31 PH, then a step increase to 31 Lux maintained until day 35 PH and a final increase to 59 Lux until day 41 PH). The temperature of each tank was controlled daily until the end of the experiment, showing a steady increase from 17 to 20 °C during the 41 days of the experiment. Tanks were siphoned once per week before micro-diet feeding and every two days after the trial began.

Larvae were fed *Artemia* nauplii (VNBS Brine Shrimp eggs *Artemia* cysts, Vietnam) from mouth opening (day 8 PH) until day 11 PH and then fed one day old *Artemia* nauplii (enriched with Larviva multigain from BioMar, Nersac, France) at day 12 until the co-feeding procedure. To condition larvae to more easily accept the experimental micro-diets, they were co-fed both enriched *Artemia* nauplii and micro-diets starting at day 13 PH. Starting at 16 PH during co-feeding, the micro-diet proportion was increased by a 20% increment each day for 5 days. From day 20 PH until the end of the experiment, the sea bass larvae were fed with the experimental micro-diets using a belt feeder. Feed quantities were calculated empirically: starting at 0.1–0.2 g micro-diet per day per 3000 larvae at day-15 PH and progressively increased up to 0.8–1 g at day 41 PH.

2.5. Sampling and Biometric Measurements

2.5.1. Juvenile Sampling

Prior to each sampling, and after a fast of 24 h, fish weights were recorded on day 0, 14, 29 and at the end of the experiment (day 38). On day 0, all individuals of each condition (100 juveniles) were gradually sedated through an intermediate tank (0.025 g/L of MS222, tricaine methanesulfonate), followed by deeper sedation (0.05 g/L MS222), fished and weighed in accordance with the regulations described in Directive 2010/63/UE. After sedation dissipation in an intermediate bucket, fish were re-introduced to their respective tanks. After 14 and 29 days of rearing, intermediate monitoring of fish weight was limited to a smaller sampling of 30 fish per condition. Mortality, malformations, or other peculiarities (macroscopically and visually assessed) were absent from the three tanks of both dietary conditions. At the end of the experiment, fish anesthesia was executed using euthanasian solution (0.125–0.250 g/L of MS222) according to Directive 2010/63/UE. Liver and muscle from 10 individuals per tank were sampled by dissection. Samples were then immediately submerged in liquid nitrogen and stored at −80 °C. Juvenile liver and muscle samples (5 individuals per tank × 3 tanks for each treatment) were freeze-dried for 72 h and stored at −20 °C until further analysis.

2.5.2. Larvae Sampling

Larvae sampling was performed to verify welfare and larvae development during the first two weeks prior to feeding the experimental micro-diets and two times during the experiment: on days 26 and 31. On day 0, larval density was estimated using alizarin-red staining. Larvae mouth opening and swim bladder formation were checked using a binocular microscope. During the experiment, sampling was processed as follows: water levels in the tanks were lowered, 10 to 15 larvae per condition were sampled using a sieve and sedated and then euthanized by submersion in euthanasian solution (MS222 tricaine methanesulfonate; 50 mg/L for sedation and 2 g/L for euthanization). Larvae were dried on an absorbent paper and then weighed using a precision scale (0.001 g precision). Mortality, malformations, food intake (thanks to larvae transparency) or other peculiarities were assessed under a binocular microscope. For the final sampling at 41 days PH, all remaining larvae of each tank were counted, euthanatized (as mentioned above), frozen in liquid nitrogen and stored at −80 °C. No weight measurement was performed on day 41 to limit air exposure and maximize the quantity and quality of biological material for biochemical analysis. Samples of 10–100 larva (n = 3 tank for those fed with the control diet and n = 3 tanks for those fed hydrolyzed microalgae diets) were freeze-dried for 72 h and stored at −20 °C for further analysis.

2.6. Biochemical Analysis

2.6.1. Lipid Extraction

The dried fish samples were manually grounded using a pestle prior to extraction. Then, 50 mg of powdered juvenile samples, and 10 mg of powdered larvae samples were approximately weighed and extracted in 6 mL of solvent mixture ($CHCl_3$:MeOH, 2:1, *v:v*) directly added into glass vials [31]. Extracts were flushed with nitrogen gas, vortexed, and

sonicated for 10 min to ensure complete lipid extraction and stored at −20 °C overnight for further analysis.

Freeze-dried hydrolyzed and non-hydrolyzed microalgae biomasses, as well as experimental diets and micro-diets, were similarly processed as juvenile samples using 50 mg of dried material for lipid extraction.

2.6.2. Lipid Class Analysis

Lipids classes of larvae samples were separated and quantified by high performance thin-layer chromatography (HPTLC). HPTLC glass plates coated with silica were cleaned and then activated by heating them at 120 °C for 30 min. Lipid extracts and a mixture of external standards of known concentrations were spotted on the plates with an automatic TLC sampler ATS4 (CAMAG, Switzerland). Lipid classes were separated by immersion of the plates in solvent mixtures of different polarities, which allow for lipid migration on the plates. First, the plate was immersed in a solution of *methyl acetate:isopropanol:chloroform:methanol:KCl* 0.25% (10:10:10:4:3.6; *v*/*v*), allowing for polar lipid (PL) separation. Subsequently, the plates were immersed first in a solution of *hexane:diethyl ether:acetic acid* (20:5:0.5; *v*/*v*) and then in a solution of *hexane:diethyl ether:* (97:3; *v*/*v*) for neutral lipid (NL) separation. Lastly, the plates were immersed in a solution of $CuSO_4$ 3% and H_3PO_4 (*w*/*v* in distilled water) and then heated at 180 °C for 30 min, to allow for final lipid class revelation. The plates were read using a scanner densitometer set at 370 nm (TLC Scanner 4, CAMAG). Lipid classes were identified and quantified by comparing the retention time and band intensity of each lipid class against those of known external standards using VisionCATS software (v2.4, CAMAG). The mean analytical variability for lipid class quantification was about 15%. Five classes of NL and six classes of PL were quantified: ALC—alcohols, FFA—free fatty acids, FS—free sterols, SE—sterol and wax esters, and TAG—triacylglycerides for NL; LPC—lyso-phosphatidyl choline, PC—phosphatidylcholine, SPG—sphingomyelin, PS—phosphatidylserine PI—phosphatidylinositol, and CL/PE—cardiolipin and phosphatidylethanolamine for PL. Total lipid concentration was computed as the sum of all lipid classes. Concentrations were expressed in $mg \cdot g^{-1}$ of dry weight.

2.6.3. Separation of Polar Lipids (PL) and Neutral Lipids (NL)

To fractionate NL and PL, 1 and 0.2 mL of total lipid (TL) extract (of larvae samples and microalgae biomass as well as the juvenile samples, respectively) were evaporated to dryness under nitrogen, recovered with 3 washes of 0.5 mL of $CHCl_3$:MeOH (98:2 *v*:*v*; final volume 1.5 mL) and spotted at the top of a silica gel column (40 × 4 mm, silica gel 60A 63–200 µm rehydrated with 6% H_2O, 70–230 mesh, Sigma-Aldrich, Darmstadt, Germany). The NL was then eluted using $CHCl_3$:MeOH (98:2 *v*:*v*; 10 mL) and PL fraction with methanol (20 mL). Both were collected in glass vials containing an internal standard (C23:0, 2.3 µg). Lipid fractions were then stored at −20 °C under a nitrogen atmosphere until further analysis.

2.6.4. Fatty Acid Analysis in Total Lipids (TL), Polar Lipids (PL) and Neutral Lipids (NL)

Polar and neutral fractions or total lipid extracts were dried under vacuum with an evaporator (Genevac). Dried lipid fractions were saponified with 1ml of KOH:MeOH (0.5 M) heated for 30 min at 80 °C; then, they were transesterified with 800 µL of MeOH: H_2SO_4 (3.4%; *v*/*v*) heated at 100 °C for 10 min. The fatty acid methyl ester (FAME) formed were recovered in hexane. FAME of the NL of all samples were separated from other unwanted compounds (e.g., sterols and alcohols, potentially present in the NL fraction) using high performance liquid chromatography (HPLC) equipped with two columns (LiChrospher Si 60 and LiChrospher 100 DIOL, both 5 µm). A Dionex HPLC system (P680 pump AS-100 auto sampler, UVD170U UV detector with deuterium lamp, Foxy fraction collector), was used. Details on the analytical methods (i.e., solvent proportions,

flow-rate) can be found in [32]. The purified FAME were recovered in new vials for gas chromatography analysis.

FAME composition was analyzed by gas chromatography coupled to a flame ionization detector (GC-FID; Varian CP8400 gas chromatograph, Agilent). Samples (2 µL) were injected at 250 °C in splitless mode at an oven temperature of 60 °C, with hydrogen as the carrier gas. The GC was equipped with a ZBWAX column (30 m in length, 0.25 mm internal diameter, 0.25 µm film thickness, Phenomenex). The oven temperature was raised to 150 °C at 50 $°C·min^{-1}$, to 170 °C at 3.5 $°C·min^{-1}$, to 185 °C at 1.5 $°C·min^{-1}$, to 225 °C at 2.4 $°C·min^{-1}$ and then to 250 °C at 5.5 $°C·min^{-1}$. FAME were identified by comparing their retention time to those of an external commercial standard mixture (S37 FAME Mix, PUFA No.1, and PUFA No.3, Supelco) using the software Galaxie 1.9.3.2 (Agilent). FAME peak area was converted into µg of FA based on the peak area of the internal standard C23:0. Concentrations were expressed in mg g^{-1} of wet weight, and FA compositions were expressed in percentage (%).

2.6.5. Analysis of Lipid Peroxidation

The level of lipid peroxidation was assessed by monitoring secondary oxidation using BIOXYTECH Malondialdehyde (MDA) 586TM kit (Tebu-bio, France). Free MDA or, after a hydrolysis step, total MDA (including protein-bound MDA and free MDA) concentration was determined in all samples through spectrophotometry, by measuring the absorbance of an MDA-chromogenic agent adduct at 586 nm. Sample weights were adapted to sample availability: 50 mg for all experimental diets and juvenile sea bass liver samples; 5–10 mg for larvae samples. Values (mean of three-repeated measurements) were expressed as µmol MDA/g DW tissue or diet.

2.6.6. Statistical Analysis

Data normality first was evaluated using the Shapiro–Wilk test, and then a t test for significant differences between dietary conditions in both juvenile and larvae feed trials was performed using Statgraphics Plus statistical software (Manugistics, Rockville, MD, USA). Homogeneity of variances was checked by means of the Barlett test. Differences were considered statistically significant if $p \leq 0.05$.

3. Results

3.1. Cellular Parameters of Microalgae Culture

The cell concentration increased rapidly from inoculation (7.5×10^5 cells mL^{-1}) to 38 h (3.0×10^8 cells mL^{-1}), reaching a plateau until the end of the cultivation at 62 h (3.3×10^8 cells mL^{-1}). Concomitantly, the dry matter of the culture increased from 1.4 g/L at 14 h up to 10.5 g/L after 62 h of cultivation (Table S2). Cell size decreased during cultivation, while cell complexity almost doubled between the beginning and the end of cultivation (Table S2). The proxy of cellular lipid content (green fluorescence of BODIPY staining) increased steadily from around 400 A.U. at 14 h up to 3790 A.U. after 46 h of cultivation and then decreased to 2600 A.U. after 62 h of cultivation (Table S2).

3.2. Biochemical Quality of Microalgae Culture and Concentrate

The total fatty acid content in A. mangrovei culture at the pilot scale increased with the duration of cultivation from 0.1 g/L after 14 h post-inoculation to > 2.3 g/L after 38 h post-inoculation and then slightly decreased to 1.6 g/L after 62 h of cultivation (Table S3). Total fatty acid content per DW increased from 8.6% at 14 h to 27.1% after 38 h and then decreased down to 15.3% at 62 h. Lipids were dominated by polar lipids (PL) during the first 23 h (73–74% of total lipids) and thereafter by neutral lipids (NL) until the end of the cultivation, accounting for more than 80% of total lipids (Table S3).

The percentage of DHA over total fatty acids, which is the targeted valuable compound, remained stable during the cultivation in both PL and NL (52–57% and 36–42%, respectively) (Figure 1A). When NL and PL were combined, the percentage of DHA over total fatty acids

ranged from 43% to 53% (Figure 1A). The second most dominant PUFA in A. mangrovei biomass was the n-6 DPA (22:5n-6), increasing from 12% to 17% in PL and from 7% to 11% in NL during the culture growth (Figure 1B, Table S3).

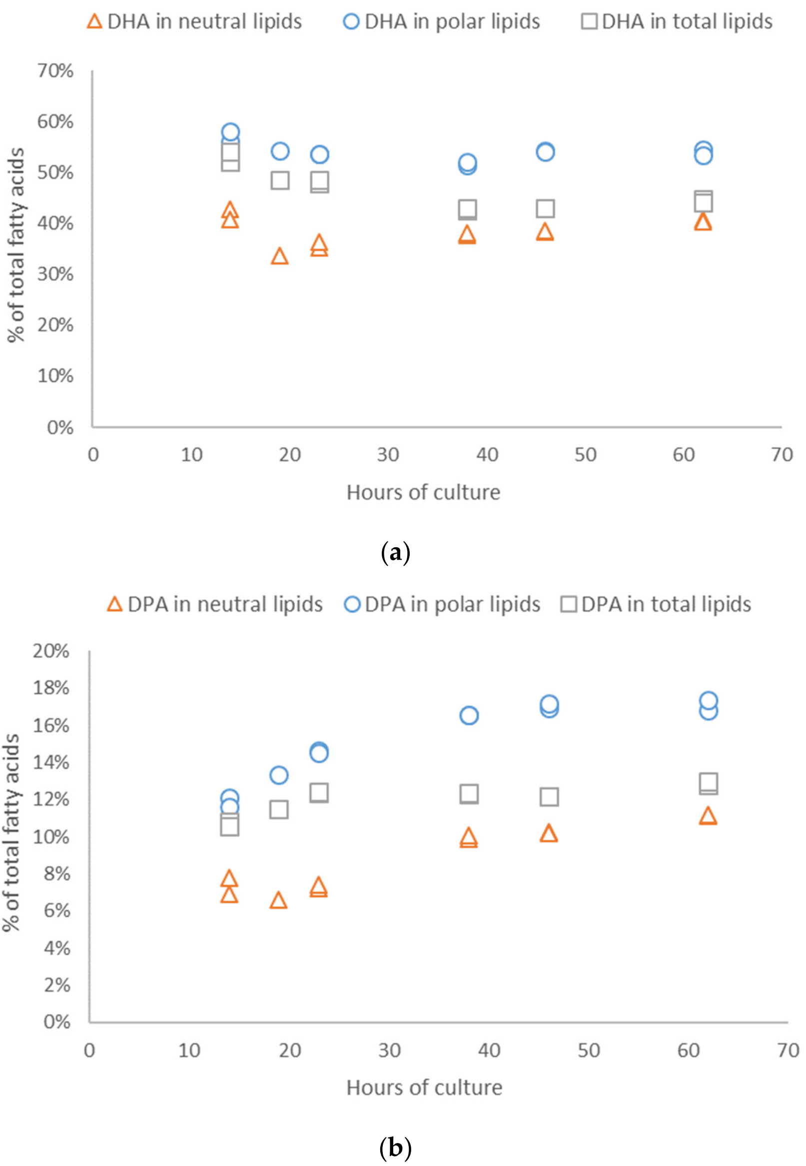


Figure 1. Percentages of the major PUFA, DHA (**a**) and DPA (**b**), expressed as percentage of total fatty acids in polar, neutral, and total lipids of Aurantiochytrium mangrovei biomass collected at 14, 19, 23, 38, 46, and 62 h of cultivation with yeast extract peptone medium supplemented with 2.5% digestate (Cooperl, France). At each sampling, the presented two values were obtained from the two 800 L cylinder cultures performed concomitantly.

At the end of cultivation (62 h), the biomass was concentrated to 90 g/L using crossflow filtration. The hourly control of fatty acid composition during filtration revealed that the PUFA content in the biomass was only slightly impacted by the harvesting process (Figure S1). The percentage of DHA in NL ranged between 39.4% and 42.8% independently of sampling time (every hour for five hours) and slightly decreased in PL from 60.0% to 53.1% after 5 h of cross filtration (Figure S1). When combining both NL and PL fractions, the percentage DHA in total lipids was 43.5% on average during the whole concentration process. The n-6 DPA was stable in both fractions at about 20% in PL and 11% in NL (Figure S1).

After cross-filtration, the biomass was freeze-dried. The final dried biomass contained 11% fats (8.3% total fatty acids), 6% carbohydrates, 33% proteins and 18% ash. Fatty acid and amino acid compositions are presented in Table S4. The fatty acid profile was dominated by the DHA, 16:0, and 22:5n-6 (40.5%, 34.3% and 12.3%, respectively) and remained very similar to the profile of the biomass right after the harvesting process (data not shown). The predominant amino acids were glutamic acid and aspartic acid above 10% followed by threonine, valine, leucine, lysine, arginine, serine, glycine, and alanine above 5%. Methionine, isoleucine, histidine, proline, and cysteine were below 5%.

3.3. Biochemical Quality of Experimental Juvenile Diets and Larvae Micro-Diets

As described in the material and methods, the fish feed formulation was complex and included several commercial ingredients. Including 15% of microalgae biomass in the fish feed allowed for replacement of the fish oil (usually supplied at 1% DW of fish feed), part of the fishmeal (reduced by 12%, from 64% to 52% DW) as well as part of the lecithin (reduced from 14% to 13% DW). Cellulose was not added into the experimental microalgae fish feed.

The fatty acid compositions of both fish feeds (control and microalgae) were compared (Table 3). The LC-PUFA DHA and 22:5n-6 contents were respectively two and four-fold higher in the microalgae diet than in the control diet. The saturated FA, 15:0 and 17:0, were also found in higher proportions in the microalgae diet, 3.4 and 1.8-fold, respectively. Furthermore, the LC-PUFA 20:5n-3 and 22:5n-3 were in lower proportions in the microalgae diet, 1.9 and 1.5-fold, respectively. The 16:1n-7, the C16 PUFA, and the 18:2n-4 were also found in lower proportions in the microalgae diet. The sum of the essential LC-PUFA EPA+DHA was 7.8% and 11.0% in the control and microalgae diets, respectively. Both were higher than expected according to the calculation in Table 3. The inclusion of the microalgae biomass in the fish diet could increase the DHA level and DHA/EPA ratio in the fish feed (from 4.4 to 9.0% and from 1.3 to 4.5%, respectively).

Total MDA (including MDA bound to proteins plus free MDA) and free MDA were slightly higher in the microalgae diet (2.0 and 1.0 µmol MDA/g DW, respectively) as compared to the control diet (1.5 and 0.75 µmol MDA/g DW) (Figure 2).

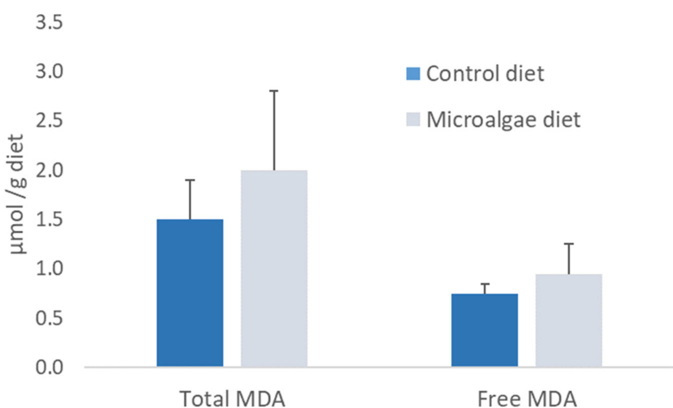


Figure 2. Total and free malondialdehyde (MDA) of control and microalgae diets for sea bass juveniles.

Fatty acid composition of the larvae control micro-diet was dominated by 16:0, 18:1n-9 and 18:2n-6 accounting for 20%, 17% and 25%, respectively (Table S5). The n-3 LC-PUFA, DHA and EPA were found at 44%, 8% and 4%, respectively. The n-3 LC-PUFA content of the hydrolyzed microalgae micro-diet was slightly lower than in the control micro-diets (41% vs. 44%) with DHA at 7%.

The larvae micro-diet containing 15% of hydrolyzed microalgae biomass had a higher level of total MDA (almost two-fold) than the control micro-diets (Figure 3). However, the content of free MDA was similar in the two tested micro-diets (Figure 3).

Table 3. Fatty acid composition of the control and microalgae diets for sea bass juveniles.

% of Total FA	Control Diet		Microalgae Diet	
	Mean	**S.D.**	**Mean**	**S.D.**
14:0	1.6	0.1	1.6	0.1
15:0	0.2	0.0	0.8	0.0
16:0	12.1	0.1	17.4	0.2
17:0	0.2	0.0	0.3	0.0
18:0	1.8	0.0	1.7	0.0
20:0	0.2	0.0	0.2	0.0
16:1n-7	2.2	0.1	1.4	0.0
18:1n-7	2.6	0.1	2.3	0.1
18:1n-9	39.2	0.2	35.5	0.2
20:1n-11	0.5	0.0	0.4	0.0
20:1n-7	0.1	0.0	0.1	0.0
20:1n-9	1.9	0.1	1.5	0.1
22:1n-11	1.8	0.1	1.6	0.1
22:1n-9	0.3	0.0	0.2	0.0
24:1n-9	0.7	0.0	0.6	0.0
16:2n-4	0.2	0.0	0.1	0.0
16:3n-4	0.1	0.0	0.0	0.0
16:4n-1	0.2	0.0	0.1	0.0
18:2n-4	0.1	0.0	0.0	0.0
18:2n-6	19.4	0.4	17.3	0.2
18:3n-3	3.2	0.0	2.8	0.0
18:4n-3	0.6	0.0	0.4	0.0
20:4n-3	0.2	0.0	0.2	0.0
20:4n-6	0.4	0.0	0.4	0.0
20:5n-3	3.3	0.0	1.7	0.1
22:5n-3	0.5	0.0	0.3	0.0
22:5n-6	0.4	0.1	1.8	0.1
22:6n-3	4.1	0.2	7.8	0.3
Total Branched	0.3	0.0	0.2	0.0
Total SFA	16.2	0.1	22.2	0.3
Total MUFA	49.9	0.4	44.0	0.3
Total PUFA	33.6	0.4	33.5	0.6
DHA/EPA	1.2	0.0	4.5	0.1

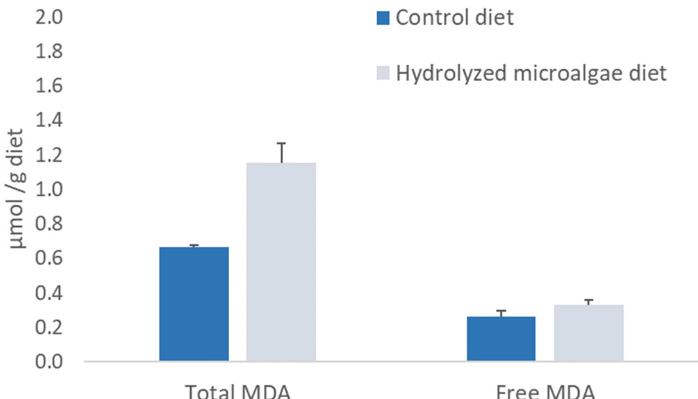


Figure 3. Total and free malondialdehyde (MDA) of sea bass larvae control and hydrolyzed microalgae micro-diets.

3.4. Sea Bass Juveniles Feeding Trial

After Sea bass juveniles were fed for 38 days, similar growth was observed in juveniles fed with both the control and microalgae diets (Figure 4), and survival percentage was 90% for both dietary conditions. This nutritional trial demonstrated the feasibility to replace 15% of a standard fish feed with microalgal biomass.

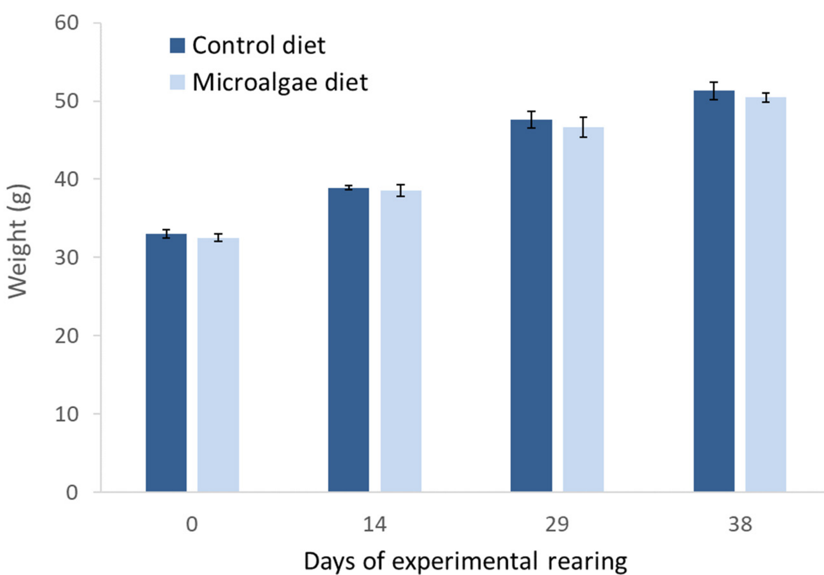


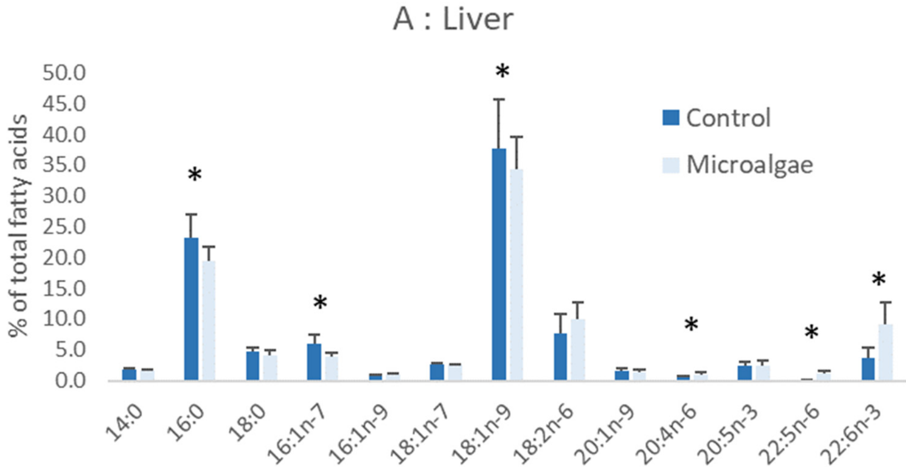
Figure 4. Growth of sea bass, Dicentrarchus labrax, juveniles fed with control and microalgae diets (described in Material and Methods) for 38 days.

After 38 days of dietary conditioning, the liver composition in FA of sea bass juveniles was significantly affected by the diets ($p < 0.05$; Figure 5). In the liver of fish fed with the microalgae diet, the content of DHA, 22:5n-6, 20:4n-6 over total fatty acids increased significantly, while the content of 16:0, 16:1n-7, and 18:1n-9 decreased significantly. Consequently, the PUFA proportion increased in juveniles fed with the microalgae diet as compared to the control diet (24% vs. 15.7%, respectively), and saturated and monounsaturated FA percentages decreased from 29.6% and 49% to 25.1% and 43.3%, respectively. A similar dietary imprint was observed in the juvenile muscle but to a lesser extent. Only the 18:1n-9, 20:1n-9 and the EPA proportions decreased significantly.

The total MDA and free MDA contents in the liver of juveniles fed with the microalgae diet tended to be higher than in the liver of fish fed with the control diet (Figure 6A), but the differences were not statistically significant. In muscle, only the free MDA tended to be higher in the muscle of juveniles fed with the microalgae diet than in those fed with the control diet (Figure 6B).

3.5. Sea Bass Larvae Feeding Trial

The larvae feed trial using control and hydrolyzed microalgae micro-diets started 20 days after hatching. Some mortalities, independent of dietary conditions, occurred when larvae were between 12 and 20 days post-hatching (during co-feeding with micro-diet from 20 to 100%) and between 31 and 37 days post-hatching (during feeding with 100% micro-diet). The measurement of larvae weight after 26 and 31 days of rearing revealed a large variability between the three tank replicates within one condition (Figure 7).



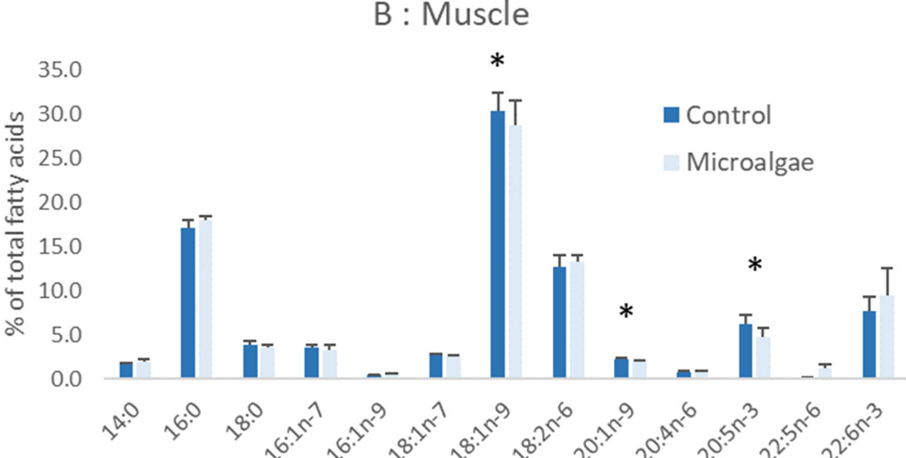


Figure 5. Fatty acid composition (expressed in percentage of total lipid fatty acids) of liver (**A**) and muscle (**B**) of sea bass juveniles fed control and microalgae diets for five weeks and a half. * indicates the significant differences between both treatments.

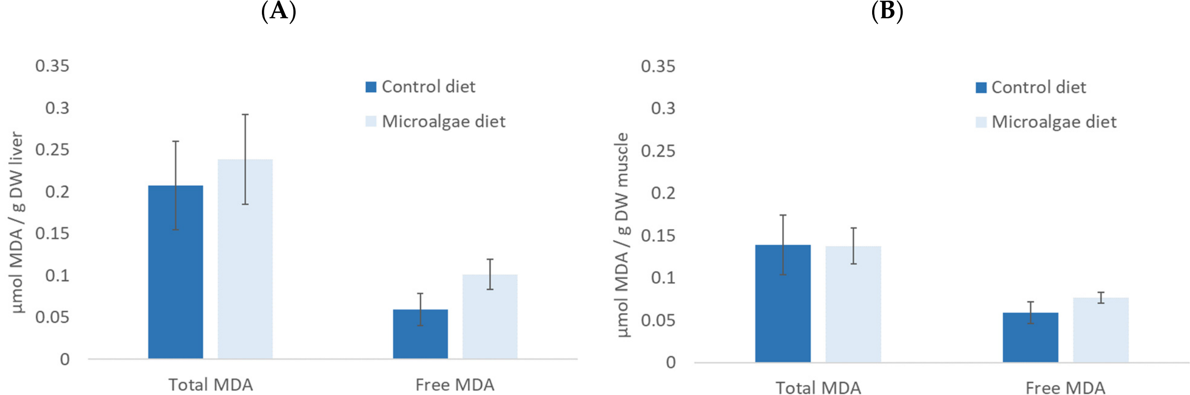


Figure 6. Total and free malondialdehyde (MDA) in the liver (**A**) and muscle (**B**) of sea bass juveniles fed control and microalgae diets for 5 weeks and a half.

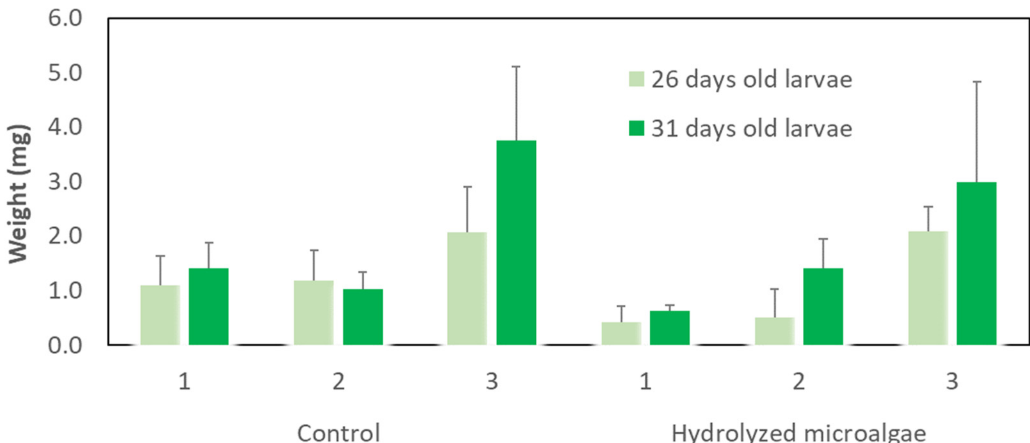


Figure 7. Weights of 26- and 31-day-old sea bass (Dicentrarchus labrax) larvae fed with the control, and hydrolyzed microalgae diets, in their respective 3 tank replicates.

After 41 days of rearing, the survival percentage was low for all the tanks (Table 4) and below 5% for most tanks. The highest number of live larvae was reported in one control tank having a survival percentage of 26%. Nevertheless, the chi-2 test comparing the six tanks did not reveal a statistically significant difference between dietary conditions.

Table 4. Survival of sea bass, Dicentrarchus labrax, larvae fed with control, and hydrolyzed microalgae diets from day 20 to 41 after hatching.

Micro-Diet Conditioning	Tank Number	Survival %
	1	1.4
Control	2	2.7
	3	26.4
	1	0.9
Hydrolyzed microalgae	2	1.7
	3	2.5

The statistical comparison of the fatty acid compositions in neutral and polar lipids of larvae according to the tested diets revealed only a few differences (Tables S6 and S7). The level of reserve lipids (47% of the FA present as neutral lipids) was higher in the larvae fed with the control micro-diet (Table S6). The percentage of saturated fatty acids in neutral lipids was higher in the larvae fed with the hydrolyzed microalgae micro-diet. The 18:2n-6, the most abundant FA in the neutral lipids, tended to be in higher proportion in larvae fed with the control diet while the 22:5n-6 tended to be higher in larvae fed with the hydrolyzed micro-diet (Table S6). The amount of DHA associated with neutral lipids expressed as mg/g DW was higher in the larvae fed with the control micro-diet (3.1 vs. 2.0 mg/DW, respectively), while the DHA percentage of total fatty acids was lower (9.2% vs. 9.9%, respectively) (Table S6).

In polar lipids, the proportions of 20:2n-6 and 22:5n-6 were significantly higher in larvae fed with the hydrolyzed micro-diet than in larvae fed with the control micro-diet (Table S7). The percentage of 15:0 in both neutral and polar lipids was higher in larvae fed with the hydrolyzed microalgae micro-diet than in larvae fed with the control micro-diet (Tables S6 and S7).

The level of secondary oxidation in the larvae partially reflects the level of secondary oxidation of their respective micro-diets, but differences were smaller and not statistically significant (Figure 8). The larvae fed with the control diet tended to have a lower level of total and free MDA than those fed with the hydrolyzed microalgae micro-diet (Figure 8).

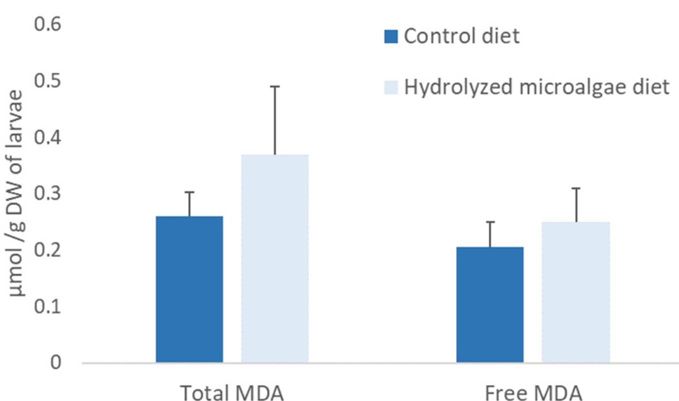


Figure 8. Total and free MDA of sea bass larvae fed control, and hydrolyzed microalgae diets from day 20 to 41 after hatching.

4. Discussion

The use of *Aurantiochytrium mangrovei* produced on a digestate-based medium was investigated as a microalgal ingredient in the nutrient formulation of sea bass (*Dicentrarchus labrax*) fish feed for juveniles and larvae. *Aurantiochytrium mangrovei* was selected in this study due to its richness in long-chain n-3 polyunsaturated fatty acids, especially DHA (or 22:6n-3). The sea bass juvenile experimental feed included 15% of non-hydrolyzed biomass. An inclusion level of 15% was targeted to have a significant impact on fishmeal and fish oil replacement in fish feed. The seabass larvae experimental feed included the same level (15%) of *A. mangrovei* biomass but in a hydrolyzed form, as protein hydrolysates were previously shown to enhance larval growth and/or survival performance of European sea bass *Dicentrarchus labrax* larvae.

4.1. From Microalgae Biomass Production to Fish Feed Formulation

4.1.1. Cellular and Biochemical Changes during Batch Cultivation

The Thraustochytrid *Aurantiochytrium mangrovei* was cultivated according to cultivation optimization performed by De La Broise et al. [28]. The culture grew exponentially for 38 h, resulting in up to 3.0×10^8 cell mL^{-1}, and reached a plateau until the end of the cultivation (62 h), obtaining 10.4 g L^{-1} of equivalent dry biomass.

Cell complexity and cellular neutral lipid content (as estimated by Bodipy staining) increased similarly to cell density, suggesting that increased complexity (or granularity) was likely related to lipid accumulation in the ageing culture. Bodipy fluorescence dye preferentially stained neutral (reserve) lipids [29]. Thus, it can be assumed that the increase in measured green fluorescence after Bodipy staining reflected the lipid body accumulation within the cells as it followed the increase in lipid content in the biomass (total fatty acid content per DW) (Tables S2 and S3). The cellular lipid content and lipid accumulation in biomass were particularly high after 38 h of cultivation, when vegetative division stopped or drastically slowed down. At this stage, most of the lipids were found in the form of neutral lipids (>80%). The predominant lipid class of neutral lipids is triacylglycerides in *Aurantiochytrium mangrovei* (data not shown).

An increase in lipid body was previously reported by Morita et al. [33] during synchronous growth of *Schizochytrium limacinum* SR21. Similarly, during the fed-batch fermentation of *Schizochytrium* sp., Zhao et al. [34] reported that lipid bodies were small during the lag phase, started to increase in number during the balanced growth phase and merged into larger lipid bodies during the lipid accumulation stage. They established a linear relationship ($R^2 > 0.98$) between the increase in cells full of lipid body and the lipid content of the biomass.

In our experiment, cellular lipid reserve (measured as green fluorescence after Bodipy staining) decreased between 46 and 62 h, suggesting that cells started to consume their energy reserve. It likely corresponded to the lipid turnover stage described by Zhao

et al. [34]. The authors observed a decrease in cells full of lipid body between 96 and 120 h of cultivation when glucose and nitrogen in the medium were almost exhausted.

The decrease in cell size reported in the present study when the culture is ageing may reflect the decrease in large zoosporangia and their replacement by cells full of lipid bodies as reported by Zhao et al. [34].

The fatty acid composition of our biomass was very similar to previous studies on Thraustochytrid [9,34–36], with 22:6n-3 (DHA) and 22:5n-6 (n-6 DPA) as major PUFA. In our study, 22:6n-3 (DHA) and 22:5n-6 (n-6 DPA) ranged from 43% to 53% and from 12% to 17% of total fatty acids, respectively. The DHA percentage in polar lipids (phospholipids) (52–57%) was consistently higher than in neutral lipids (36–42%). As DHA in the form of phospholipids is nutritionally preferred to DHA in the form of triacylglycerides, this could have some implication in future industrial cultivation strategies attempting to favor a biomass richer in phospholipids.

4.1.2. Biomass Downstream Processing

Following the cultivation of *A. mangrovei*, the influence of biomass concentration using cross-flow filtration (from 10 to 90 g L^{-1}) on the n-3 LC-PUFA was evaluated. Results showed that only the DHA in polar lipids slightly decreased from 60% to 53% while neutral lipids remained stable at 41%. This may reflect that DHA in the form of phospholipids are more sensitive to degradation and/or oxidation than in the form of triacylglycerides. The freeze-drying process allowed for the preservation of DHA content in the microalgal biomass to 40.5%, slightly lower than in the concentrated biomass. In addition to supplying high-quality n-3 LC-PUFA, the microalgae biomass contained all the essential amino acids required in finfish aquaculture in similar proportions to those found in fishmeal [37] (Table S8).

4.1.3. Fish Feed Formulation and Composition

The fish feed including the dried microalgal biomass was enriched with C22 PUFA, 22:6n-3 and 22:5n-6. It allowed the 22:6n-3/20:5n-3 to more than triple, reaching up to 4.5. It is widely recognized that this ratio has to be above 2 to ensure good survival and growth in farmed finfish. The fatty acids found in higher proportions in the control diet as compared to the microalgae reflect the natural origin of the fishmeal and oil. The C16 PUFA, the 16:1n-7 and 20:5n-3 are usually found in diatoms [38], which are at the base of the trophic chain supporting small pelagic fish generally harvested in upwelling zones [39].

Similar to other oleaginous microalgae species, the proportion of reserve lipids increased when the culture aged and reached the stationary phase. In the present study, the culture was stopped at the late exponential/early stationary phase in order to maintain a high level of n-3 LC-PUFA in the form of the phospholipids (29–36%). Kissinger et al. [19] highlighted that fishmeal is also important in supplying essential n-3 LC-PUFA for marine carnivores in the form of phospholipids, especially when the diets contain little or no fish oil. Commercially available *Schizochytrium* biomass is largely dominated by n-3 LC-PUFA in the form of triacylglycerides (data not shown). The biomass produced in the present study contained 20% of n-3 LC-PUFA in the form of phospholipids, which may represent a nutritional benefit, especially for fish in their early development stages. It could be interesting to shorten the cultivation duration in order to favor the amount of phospholipids in the biomass.

Although the inclusion of dried microalgae biomass at 15% in the fish diet resulted in an improvement of the essential n-3 LC-PUFA, 22:6n-3 + 20:5n-3, this may result in a higher susceptibility of this diet to oxidation [40]. Total and free MDA increased by 30% in the microalgae diet as compared to the control diet. Nevertheless, it did not appear to affect the zootechnical performance of sea bass juveniles.

The higher MDA content (secondary oxidation) in the microalgae diet (fed to Juveniles) may have non-exclusive origins. Firstly, the harvested biomass was not protected by the addition of any antioxidant, while this is generally the case for commercially available

ingredients containing a high proportion of PUFA. Secondly, after its manufacturing, the microalgae diet contained more long-chain PUFA (12.2%) than the control diet (8.9%). This may have increased the "targets" for oxidation during diet manufacturing. Although a high oxidation level of the microalgae diets did not impair the growth performance, the addition of commercial antioxidants could be considered to preserve the nutritional quality of the microalgal biomass.

The *Aurantiochytrium*-produced biomass was richer (in % DW) in proteins as compared to commercial Thraustochytrid biomass (30% vs. <10%, data not shown). This allows for a higher proportion of fishmeal to be replaced, potentially up to 30–40%, as it is well balanced in terms of essential amino acids in addition to its high DHA content. The amino acid profile of the dried microalgae biomass was very similar to the fishmeal amino acid profile (Table S4). Although the dried microalgae biomass contained fewer proteins (around 30%) than fishmeal (60–70%), it is a very good source of essential amino acids for the sea bass diet. To increase the inclusion percentage of dried microalgae biomass, an increase in the microalgal protein content would be necessary by optimizing cultivation conditions and maintaining the exponential phase. Additionally, the harvesting conditions could also be improved by coupling cross-flow filtration with centrifugation, improving solid matter percentage (up to 20%). This would reduce the ash content of the biomass prior to drying and allow for limiting the ash content in formulated diets.

4.2. Nutritional Value of Microalgae for Sea Bass Juveniles

Substitution of 15% of a standard juvenile sea bass feed resulted in similar growth performance after 38 days of experiment. The assimilation of the microalgal lipids was confirmed by monitoring specific FA. The C22 PUFA 22:6n-3 and 22:5n-6 increased 2.5- and 14.5-fold in the liver of fish fed with the microalgae diet. The FA supplied by the microalgae diet more intensively imprinted the liver than the muscle, probably linked to the liver being directly bound to the digestive system. It can also be expected that muscle, a bigger tissue, could take more time to reflect dietary changes. Overall, the results have demonstrated that the microalgal biomass was well incorporated/assimilated in juvenile tissues.

Similarly, in the literature, the inclusion of *Schizochytrium* sp. in the fish diet up to 16% of DW (fully replacing fish oil) resulted in higher weight gain and DHA deposition, as well as higher DHA/EPA in Nile Tilapia fillets [16]. Additionally, *Schizochytrium limacinum* has been proven effective as the main lipid source in diets for giant grouper without significantly affecting fish performance or condition [20].

Channel catfish (*Ictalurus punctatus*) fed with diets containing 1.0% and 1.5% dried algae *Schizochytrium* gained significantly more weight than fish fed with diets containing 0% and 0.5% dried algae [17]. Concomitantly, the content of 22:6n-3 and 22:5n-6 of channel catfish fillet increased as dietary levels of dried algae increased. Change in PUFA composition was also observed in salmon fed a diet with Thraustochytrid oil, revealing higher percentages of 22:6n-3 and 22:5n-6 (33% and 7% in white muscle, respectively) as compared to salmon fed a control diet with fish oil (20.4% and 0.4%, respectively).

Although arachidonic acid (20:4n-6) was absent from the microalgal biomass, its percentage increased in the liver of fish under the microalgae diet. This likely reflects the retro-conversion (or beta oxidation) of the 22:5n-6 into 20:4n-6. Similar observations were reported by Miller et al. [11], in which a higher concentration of arachidonic acid found in red and white muscles of salmon fed a diet containing Thraustochytrid oil originated from the retro-conversion of DPA-6 (22:5n-6) present in high proportions in such oil. Similarly, both ARA (20:4n-6) and DPA (22:5n-6) contents in seabream larvae fed *Schizochytrium*-containing diets were higher than in larvae fed with the standard diet with fish oil [15]. The authors also suggested that DPA was retro-converted to ARA by oxidation. *Schizochytrium* oil and biomass may have an additional benefit to DHA supply by contributing to ARA supply.

Hart et al. [14] reported a very good digestibility of *Schizochytrium* sp. biomass by Atlantic salmon (*Salmo salar*) juveniles. Apparent digestibility coefficients of protein, lipid, and gross energy of *Schizochytrium* biomass were 93.9%, 67.1% and 70%, respectively. PUFA

and DHA were more digestible in the diet containing *Schizochytrium* biomass (96.7% and 96.3%) than in the reference diet (96% and 93.1%, respectively). A blend of soybean meal, soy protein concentrate and algal meal from *Schizochytrium limacinum* could replace 40% of marine proteins from fishmeal and squid meal [20].

As fatty acids with a high degree of saturation are more prone to lipid peroxidation, MDA was measured in the juveniles' liver and muscle at the end of the dietary experiment. Feeding the sea bass juveniles with the microalgae diet tended to increase the amount of total and free MDA, showing that the higher oxidation level of the microalgae biomass diet was translated into in the fish tissues fed this diet.

Although tested in shrimp diets, the inclusion of commercial Thraustochytrid biomass from 2.5% to 7.5% did not result in an increase in MDA content in the tail muscle of shrimp [41]. However, commercial Thraustochytrid biomasses are generally protected from oxidation by the addition of antioxidants.

Some studies explored the change of microbial composition upon feeding with a diet containing Thraustochytrid biomass. The partial substitution of fish oil component by the microalgae *Schizochytrium limacinum* (5% inclusion) in the diet of the farmed rainbow trout (*Oncorhynchus mykiss*) resulted in a greater level of microbial diversity in the distal intestinal microbiota than in the trout fed with a control diet without microalgae [42]. Inclusion of Thraustochytrid biomass in the Tilapia fish diet modulated its microbiome and increased the red blood cell and lymphocyte concentrations [43]. It would be interesting to confirm similar effects in sea bass fed with a diet enriched with Thraustochytrid biomass.

4.3. Nutritional Value of Microalgae for Sea Bass Larvae

To maintain fast growth and optimal visual and neural development during the early life stages, fish larvae require a high level of n-3 LC-PUFA and especially DHA [44,45]. Thus, we tested the influence of substituting fish oil in sea bass larvae with DHA-containing Thraustochytrid biomass. As the inclusion of fish protein hydrolysates in marine fish feed improves larvae fish growth and survival [21], we included hydrolyzed microalgae biomass in the micro-diet. Tanks receiving the micro-diet containing 15% of hydrolyzed microalgae biomass performed similarly to the tanks receiving the control micro-diets. The fact that the 18:2n-6 in neutral and polar lipids (Tables S5 and S6), as well as triacylglycerides in neutral lipids (Table S9), were found in higher proportions in larvae fed with control micro-diets as compared to larvae fed with hydrolyzed microalgae diet may reflect slightly better assimilation of the artificial micro-diet.

As the inclusion of 15% of hydrolyzed microalgae biomass in the micro-diet did not impair the development of sea bass larvae, it is tempting to consider that the hydrolyzed microalgae biomass has the potential to substitute both fish oil and pre-digest fishmeal (CPSP 90) in larvae artificial micro-diets. Nevertheless, the low general survival percentages of larvae in this dietary conditioning experiment limit further exploitation of the obtained biochemical results. In the present study, high mortalities occurred when an artificial micro-diet was introduced in their regime. Feeding with artificial micro-diets, particularly before the complete maturation of the digestive functions, led to poor larval performance compared to live prey [21,23]. Survival percentages between 15–35% were usually reported in the present experimental rearing facility with sea bass larvae fed exclusively with an artificial micro-diet as compared to >50% for larvae fed with live prey [21,46]. The commercial hatcheries introduce artificial food generally around the 30th day of development of sea bass larvae [47] in order to ensure good survival, which may suggest that we should not have tested our experimental feed in only 13-day-old larvae. Nevertheless, this could only partially explain the low survival percentages reported in the present study. Such low survival may also reflect some anterior weakness or weakening due to their transportation from the marine hatchery of Gravelines Ichtus (France). Although the experimental system used allows for good larval growth and survival, the rearing conditions for this batch of larvae may have been sub-optimal.

5. Conclusions

The feed trials on sea bass juveniles and larva indicated that microalgal biomass can partially replace fishmeal (pre-digest fishmeal) and fish oil in sea bass feed. This allowed for an equivalent growth rate and improved DHA delivered to sea bass juveniles. However, the high performance variability between larvae rearing tanks did not allow for a clear and definitive conclusion and would require further testing, introducing the experimental feed later in the sea bass feeding sequence, around day 20 of development. Overall, feeding carnivore fish species with diets containing Thraustochytrid oil or biomass increased the amount of DHA and n-3 LC-PUFA in fish fillets, which would make them nutritionally beneficial to the human consumer.

Considering that fish oil has higher levels of EPA than DHA, the inclusion of Thraustochytrid oil or biomass may improve the DHA/EPA ratio as required in certain aquaculture applications such as in marine fish larvae rearing in husbandry. Beyond supplying a high level of n-3 LC-PUFA, Thraustochytrid may also bring other nutritional compounds such as essential amino acids, vitamins, and pigments. Thus, it cannot be excluded that part of the ability of Thraustochytrid biomass to replace fish meal and oil may also be due to other nutrients than n-3 LC-PUFA.

Finally, the introduction of digestate as a source of nitrogen in the culture medium resulted in good quality microalgae biomass, with the needed biochemical composition, and including it in fish feed to replace fish meal and oils in fish feed did not cause any detrimental effect on the animals. However, European regulation states that animal-based digestates cannot be used for feed production, including aquafeed (de la Broise et al., 2022). Today, only crop-based digestates can be used in the developed process. Alternatively, other by-products from the food industry could be investigated as potential sources of carbon and/or nitrogen to produce Thraustochytrid biomass.

In the future, further optimization of the workflow between digestate or by-product processing, microalgae cultivation and fish feed production needs to be performed. Additionally, we will need to confirm the zootechnical and biochemical results.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su142114573/s1. Table S1: Chemical characterization of digestate from COOPERL Organic biological waste (pig manure), Brittany (FR) https://www.nweurope.eu/projects/project-search/alg-ad-creating-value-from-waste-nutrients-by-integrating-algal-and-anaerobic-digestion-technology/publications/the-alg-ad-project-reports-and-deliverables/ (accessed on 27 September 2022). Table S2. Cellular parameters (concentration, size, complexity, lipid content) as measured by flow cytometry on two 500 L culture tanks. Table S3. Fatty acid composition (expressed as % of total fatty acids in the fraction; n = 2 batches) in neutral lipids (NL) and polar lipids (PL) and percentage of total lipids per dry weight of *Aurantiochytrium mangrovei* cultivated on yeast extract peptone medium enriched with liquid effluent from anaerobic digestion. Figure S1. Percentages of the major PUFA, DHA (A) and DPA (B), expressed as percentage of total fatty acids in polar, neutral, and total lipids of *Aurantiochytrium mangrovei* biomass collected every hour during 5 h of cross filtration. Table S4. Amino acid and fatty acid profiles of the dried microalgae biomass included in the experimental microalgae diet for juveniles and micro-diet for larvae. Table S5. Fatty acid composition expressed in percentage of the control, and hydrolyzed micro-diets for sea bass larvae. Table S6. Fatty acid composition of neutral lipids (expressed as % of total fatty acids of the fraction) of sea bass (*Dicentrarchus labrax*) larvae fed control, and hydrolyzed microalgae diets 41 days after hatching. Table S7. Fatty acid composition of polar lipids (expressed as % of total fatty acids of the fraction) of sea bass (*Dicentrarchus labrax*) larvae fed control, and hydrolyzed microalgae diets 41 days after hatching. Table S8. Comparison of the amino acid profile (expressed in % of total amino acids) of *Aurantiochytrium mangrovei* with this of fish meal. Table S9. Lipid class composition of sea bass (*Dicentrarchus labrax*) larvae fed control and hydrolyzed microalgae diets 41 days after hatching.

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