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Microalgae biotechnology for simultaneous water treatment and feed ingredient production in aquaculture

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ABSTRACT

The use of *Tetraselmis chui* to produce sole feed ingredient and the recovery of nutrients from a recirculating aquaculture system (RAS) aquaculture facility was studied. The microalga was cultured in a pilot-scale (6 m^3) outdoor raceway reactor located in a sole production plant. First, the growth of the microalgae, the addition of phosphorus and the quality of the resulting water were studied. It was possible to cultivate in semi-continuous mode *T. chui* using the purge stream of a RAS, obtaining productivities of 15 mg L^{-1} d^{-1}, and reaching 36 mg L^{-1} d^{-1} when phosphorus was added. In terms of water quality of the effluent, the pollutants concentration were below discharge limits (suspended solids and total dissolved nitrogen, phosphorus and carbon) —directive 91/271/EEC. In the case of *Vibrio* sp., the concentration in the culture water at the end of the experiments remained below typical environmental concentrations (<100 CFU mL^{-1}). After harvesting, the biomass quality was analysed, being the microalgae produced in semi-continuous using phosphorus-enriched RAS remarkably similar to commercial *T. chui*. Biomass of *T. chui* was used in a sole feeding trial to supplement feeds (0, 10 and 20 % inclusion). Adding 10 and 20 % *T. chui* to the feed reduced the original raw material in fishmeal by 15 and 25 % without altering the gross composition. No significant differences were found in the pre-fattening test of *Solea senegalensis* in growth and fillet composition using the experimental feed with 20 % of *T. chui*. Finally, immune activity increased significantly in diets containing >10 % microalgae.

1. Introduction

While the contribution of fish catch has remained stable in the last 30 years, the contribution of aquaculture production is exponential, increasing annually by 3.1 % [1]. In the case of south Europe, the leading marine species farmed during the last years have been gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) [2]. Nevertheless, the farming interest of Senegalese sole (*Solea senegalensis*) is increasing since the number of catches has been reduced [3,4]. The significant problems in culturing *S. solea* were (1) the determination of the minimum duration of each life stage, (2) the search for a suitable natural diet, (3) the adaptation of aquaculture facilities to maintain optimal culture temperatures and photoperiods, and (4) problems related to the development of diseases during culture. The technological development of recirculating aquaculture systems (RAS) and the research into the ecology of this species enabled the commercial production on a large scale. However, although the production has improved, there are still many gaps that the market still faces [5].

One of the challenges currently being researched is the improvement of sole feed by increasing its quality and sustainability. The latest studies related to Senegalese sole feeding are focused on improving ration size [6], decreasing stress by addition of tryptophan [7] and evaluating alternative ingredients [8–11]. One of the trends in the world of fish feed is the inclusion of plant-based ingredients to reduce fishmeal (FM) or fish oil (FO) and, thus, reduce the fish in-fish out (FIFO) rate [12–14]. Soybean meal is a widely studied alternative, but it is beginning to be questioned due to possible adverse effects on the digestive tract and the sustainability of its production [15,16]. The use of microalgae biomass is an alternative that is increasingly being considered since the latest studies of the addition and substitution of FM and FO in fish feed showed no adverse effect on growth performance [17,18]. Although there are previous studies on the use of *Tetraselmis* sp. as an ingredient or feed additive for some fish [19–21], to the best of our knowledge, there is just one previous study for Senegalese sole, in which up to 15 % of the FM is replaced with biomass of *Tisochrysis lutea*, *Nannochloropsis gaditana* and *Scenedesmus almeriensis* [22]. Furthermore, previous studies proposed

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sustainable cultivation of microalgae so that fish farm streams were used to produce microalgae biomass that could be used as ingredients for fish feed and, simultaneously, treat the streams, removing nutrients such as nitrogen and phosphorus [23,24].

This work aims to study the use of microalgae biomass, more specifically *Tetraselmis chui*, cultured in a marine fish stream from a Senegalese sole recirculating aquaculture system (RAS) facility, to remove nutrients and partially replace FM of Senegalese sole diet, following the circular economy strategy. For this, the whole process was studied: the microalgae biomass growth kinetics were obtained on an outdoor pilot-scale 6 m^3 raceway reactor, the effluent quality after microalgae harvesting was analysed, the biomass quality was assessed, two experimental feeds were formulated by adding 10 and 20 % of microalgae biomass (15–25 % fishmeal replacement), and finally, feeding trials were carried out. The process proposed here is in line with the principle of resource hierarchy, as it is proposed to reuse the microalgae biomass produced in the facility as an ingredient in the feed. However, studies do not indicate whether its incorporation in the feed may have anti-nutritional effects, as observed with ingredients such as soybean meal.

To underline the novelty of this article, a bibliographic mapping of the subject matter was carried out ($n = 100$). Using the Elsevier search tool, it was found that 80 % of the results obtained related to "microalgae", "RAS" and "aquaculture" are studies in which microalgae biotechnology was applied to nutrient removal. Less than 5 % studied nutrient removal and biomass obtained simultaneously and, in all cases, these were laboratory-scale studies. However, the mapping did not find any studies that, besides combining the study of nutrient removal and biomass production on a pilot scale, also formulated and produced fish feed with microalgae as ingredient and included a fish feeding trial, which is the aim of the present study.

2. Material and methods

2.1. Experimental setup

2.1.1. Microalgae culture

2.1.1.1. Microalgae inoculum. The microalga species used was *Tetraselmis chui* (CCMM 03/0201), provided by the Marine Microalgal Culture Collection of the Institute of Marine Sciences of Andalusia (CSIC). The inoculum was maintained under sterile conditions in one-litre flasks in a culture room at 22 ± 1 °C and constant light (24 h) with a photosynthetic photon flux density (PPFD) of 100 µmol m^{-2} s^{-1}. The matrix used was UV-C sterilised seawater and f/2 culture medium. Under the same conditions, it was scaled up from 1 L to 4 × 2 L bottles and then to 5 × 8 L bottles. Then, microalgae were scaled up to five 18 L reactors at laboratory scale (airflow rate of 2 air volume per liquid volume per minute, 22 ± 1 °C and 24-h illumination, warm light 2700–3200K, LED panels 40 W, 131 µmol m^{-2} s^{-1}) and then to two outdoor 500 L raceway reactors to obtain the inoculum for the 6 m^3 pilot-scale reactor. Double nutrient (nitrogen and phosphorus) concentrated f/2 medium was used in the 500 L reactors.

2.1.1.2. Culture media. The microalgae culture was carried out using a stream from a RAS system of the fish farm Cultivos Piscícolas Marinos S. A. (CUPIMAR), located in the municipality of Puerto Real (Cádiz, southern Spain), with an annual production of approximately 450 t of Senegalese sole and a daily flow rate of 4704 m^3 day^{-1} (Fig. 1). The RAS system included a roto-sieve drum screen, biofiltration for aerobic nitrification, protein skimmer, and ozonation. Stream 1 (Fig. 1) was used as culture medium, with characteristics from Table 1.

2.1.1.3. Microalgae growth and nutrient removal. The microalgae culture experiment was carried out in a 6000 L raceway reactor. The reactor was located outdoor at the fish farm facilities. Evaporation in the reactor was compensated with fresh water. The paddlewheel rotational speed was adjusted to keep culture velocity at 0.3 m s^{-1} measured with a current meter (model 438110 hydro-bios). Daily radiation, water temperature, pH and turbidity were recorded in a data logger. Two microalgae growth experiments were conducted, the first experiment without phosphorus

Table 1
Characteristics of the RAS water from the CUPIMAR facility stream 1. Average and standard deviation ($n = 5$).

TDN (mg L^{-1})	N-NO_3^- (mg L^{-1})	N-NH_4^+ (mg L^{-1})	N-NO_2^- (mg L^{-1})	TDP (mg L^{-1})	TOC (mg L^{-1})	pH
19.3 ± 3.1	17.2 ± 0.7	0.7 ± 0.2	0.6 ± 0.6	0.26 ± 0.04	4.5 ± 2.0	7.5 ± 0.2

TDN, total dissolved nitrogen; TDP, total dissolved phosphorus; TOC, total organic carbon.

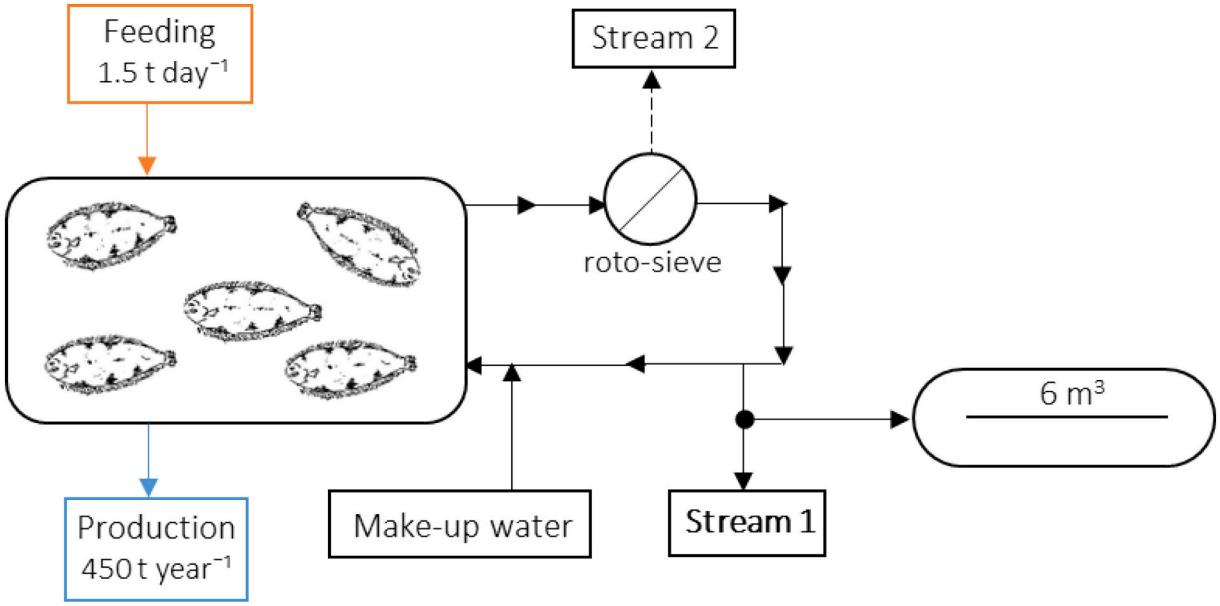

Fig. 1. Main flows of the RAS system of the fish farm CUPIMAR S.A.

enrichment in batch (RAS_B) and semi-continuous (RAS_SC). In the second experiment, the culturing media was phosphorus enriched (by adding 1 mg L^{-1} P-PO_4^{3-}) in batch (RAS + P_B) and semi-continuous (RAS + P_SC).

The commercial microalgae biomass used for the comparisons and the fish feed trials was purchased from Allmicroalgae - Natural Products, S.A. (Algafarm, Portugal). It was produced in horizontal tubular closed photobioreactors, pre-concentrated by filtration and spray dried (lot n° 201930183).

2.1.2. Fish feed test

2.1.2.1. Production system. The trial was carried out at CTAQUA's facilities in El Puerto de Santa María (Cádiz, southern Spain). The system in which the fish were placed during the entire test was a lab-scale RAS with the following characteristics: 9 tanks of 400 L each, 5–10 % daily water renewal, nitrification biofilter, UV-C lamps for water disinfection, roto-sieve and protein skimmer.

The roto-sieve was maintained and cleaned daily; oxygen, temperature and general health of the fish were monitored daily, and, finally, the salinity, ammonium, nitrite and pH of the culture water were monitored twice a week (Table 2).

2.1.2.2. Description of the test. A nutritional test was performed in which two experimental diets (TC_10%, TC_20%) were evaluated against a control diet for 12 weeks. All groups were evaluated in triplicate. The total number of individuals was 360, divided into nine tanks (three tanks per diet). The species tested was *Solea senegalensis*, a batch provided from Cultivos Piscícolas Marinos (CUPIMAR), with an average initial weight of 37.5 ± 1 g. The general status of the individuals was healthy, with normal behaviour. Before the initial stocking, all individuals that presented any deformity or injury were discarded. Fish were fed manually ad libitum four times per day for six days per week.

2.1.2.3. Stocking and sampling. Individuals were randomly stabled in the system to distribute fish in the tanks, obtaining similar initial fish biomass between tanks. For this purpose, a previous population survey was carried out to obtain mean weight and standard deviation data. A coefficient of variation below 15 % was obtained.

Once the individuals were stabled, regular weight sampling was carried out during the 12 weeks of the trial. All fishes were weighed individually using clove oil as an anaesthetic in the final sampling. In the intermediate samplings, the total biomass of each tank was estimated by sampling a representative sample from each tank (n = 20). Twenty-seven fishes (3 fishes per tank) were sacrificed during the final sampling to obtain samples for subsequent analysis.

2.2. Chemical analysis and methods

2.2.1. Microalgae growth

Microalgae biomass concentration was indirectly measured through turbidity. A calibration curve (n = 10) between suspended solids concentration and turbidity ($R^2 > 0.99$) was performed during the exponential growth phase of *T. chui*. A continuous record of photosynthetically active radiation (PAR), turbidity, pH and temperature was obtained. Radiation was measured using an Apogee SQ-420 PAR light sensor. Turbidity, pH and temperature were recorded by a controller (Hach SC200) using submersible sensors for pH and temperature (Hach pHD sc) and with a wiper for turbidity (Hach Solitax t-line sc). Quantum yield (QY) or light-processing efficiency (Fv/Fm) [25] analyses were performed five days a week using the FluorPen FP100 (Photon Systems Instruments).

Table 2
Water quality parameters during the growth test. Average and standard deviation, n = 24.

Oxygen (mg L^{-1})	Temperature (°C)	pH	Salinity (‰)	N-NH_4^+ (mg L^{-1})	N-NO_2^- (mg L^{-1})	photoperiod
6 ± 1	21 ± 1	7.5 ± 0.5	38 ± 1	< 1	< 3	12:12

2.2.2. Water analysis

During microalgae growth experiments, *Vibrio* sp. bacteria concentration was analysed in the effluent of the raceway reactor by duplicate immediately after sampling five days per week. For this purpose, TCBS (Thiosulfate Citrate Bile Salts Sucrose) agar and the membrane filtration method [26] were used to detect and enumerate bacteria. Serial dilutions were carried out to count high concentrations of bacteria (>150 CFU mL^{-1}). Total dissolved nitrogen (TDN), total dissolved phosphorus (TDP) and total organic carbon (TOC) were analysed in triplicate (n = 3) at the beginning and end of the batch and in the influent and effluents once the semi-continuous experiments reached steady-state. For this purpose, the samples were filtered (0.45 μm) and stored in plastic bottles at −20 °C. TDN and TDP were analysed by oxidation of all nitrogen and phosphorus to nitrate (NO_3^-) and phosphate (PO_4^{3-}) respectively and subsequently analysed [27,28]. A Shimadzu TOC-L analyser (non-purgeable organic mode) was used to analyse TOC. Five samples were taken, one per week, and analysed in triplicate to characterise the microalgae culture medium (RAS-stream 1; Fig. 1). For the water quality analysis, a sample was analysed at the end of the batch when the culture reached the stationary phase. Under semi-continuous operation, the sample was taken when the culture stabilised. The analysis was carried out in duplicate. N-NO_3^-, N-NH_4^+ and N-NO_2^- concentrations were measured using Spectroquant® colourimetric kit test (Code 1.14942.0001, Merck) for nitrates and the standard methods 4500-NO2- and 4500-NH3 for nitrite and ammonia, respectively [29,30]. Dissolved oxygen [31] and salinity [32] were measured according to standard methods.

2.2.3. Biomass and feed characterisation

Once the experiments were finished, the microalgae biomass was harvested. In the case of batch experiments, when the biomass concentration on three consecutive days differed by <10 %, and in semi-continuous experiments, when the steady-state was reached. In the latter case, the biomass samples from the last three days of operation were mixed.

The microalgae biomass was harvested using a GEA Westfalia centrifuge (model OTC 2-02-137). Microalgae biomass and the experimental fish feed were preserved at −20 °C and freeze-dried (Telstar Cryodos) before analysis. Moisture and ash analyses were performed gravimetrically by drying in an oven at 102 °C and subsequent incineration at 550 °C. Total protein analysis was performed by the Kjeldahl method after acid digestion [33]. The Soxhlet method was used for total lipid (fat) analysis after acid hydrolysis, which is the official Association of Analytical Chemists (AOAC)-recommended method [34]. The remaining fraction of the sum of moisture, ash, proteins, and lipids was considered as total carbohydrates, thus including fibre. The contained energy in the biomass (kcal g^{-1}) was calculated using the Atwater general factor system [35], with a conversion factor based on the combustion of protein (4 kcal g^{-1}), fat (9 kcal g^{-1}) and carbohydrate (4 kcal g^{-1}). Fatty acid (FA), amino acid and elemental analysis of CHNS were performed as previously described in [36]. In the case of the floatation test, ten pellets were used per, test, and the test was performed in triplicate for each diet. The pellets that floated after 10 s in the water were counted. The percentage of floatation was calculated as the ratio of (floating pellets/total pellets) × 100.

2.2.4. Fish characterisation

Proximal composition and fatty acid analyses were carried out in triplicate (technical replicates) using three samples of 150 g of whole individuals per tank, randomly chosen. Moisture, ash, total fat, and crude fibre analysis were determined using standard methods [34]. The crude protein was estimated through total Kjeldahl nitrogen concentration (conversion factor of 6.25) [33]. The fatty acid profile was analysed according to ISO 12966-2:2011 [37] and ISO 12966-4:2015 [38] by gas chromatography.

Blood samples of three fishes per tank were taken and subsequently analysed in triplicate to analyse plasma lysozyme activity. Analyses were performed according to the turbidimetric method using *Micrococcus lysodeikicus* lysis [39].

2.3. Data analysis

2.3.1. Microalgae parameters

2.3.1.1. Biomass production. To model the biomass performance during batch experiments, the Integrated Verhulst kinetic logistic equation [40] was used (Eq. (1)).

$$X = \frac{X_0 \cdot X_{max} \cdot e^{\mu \cdot t}}{X_{max} - X_0 + X_0 \cdot e^{\mu \cdot t}} \quad (1)$$

where X and X_0 are biomass concentration (mg L^{-1}) at instant t (d) and $t = 0$ respectively, X_{max} is the maximum biomass concentration reached (mg L^{-1}), and μ is the specific growth rate (d^{-1}).

For productivity (mg L^{-1} d^{-1}) under batch operation, Eq. (2) from Ruiz et al. [41] was used, where μ_{max} is the maximum specific growth rate (d^{-1}).

$$Productivity = \frac{\mu_{max} \cdot (0.9 \cdot X_{max} - 1.1 \cdot X_0)}{ln\left(\frac{9 \cdot (X_{max} - 1.1 \cdot X_0)}{1.1 \cdot X_0}\right)} \quad (2)$$

Productivity (mg L^{-1} d^{-1}) under semi-continuous operation was calculated as the difference between the biomass concentration in the effluent (X_e, mg L^{-1}) and the biomass concentration in the culture just after feeding the reactor (X_i, mg L^{-1}) divided by the hydraulic retention time (HRT).

HRT under semi-continuous operation was calculated as the reactor volume divided by the quotient of the volume withdrawn for harvesting and the time elapsed between feedings.

Photosynthetic conversion efficiency was calculated as the ratio between energy produced as microalgae biomass (kJ d^{-1}) and Solar PAR irradiation received in the reactor (kJ d^{-1}). To calculate the energy recovered as microalgae biomass, the analysed calorific value of the biomass (kJ g^{-1}) was used (see section "Biomass and feed characterisation"). The average PAR irradiation value registered in 24 h (μmol m^{-2} s^{-1}) and the reactor surface (20 m^2) were used to calculate the energy received.

2.3.1.2. Nutrient removal. The percentage of nutrient removal (TDN, TDP, and TOC) was calculated using Eq. (3), being C_i (mg L^{-1}) the initial concentration in the culture and C_f (mg L^{-1}) the final concentration after the batch experiment or just before harvesting the biomass under the semi-continuous experiments.

$$Nutrient\ removal\ (\%) = \frac{C_i - C_f}{C_i} \cdot 100 \quad (3)$$

2.3.2. Fish growth parameters

The productive parameters of the fish culture were calculated as follows: Weight gain (g $fish^{-1}$) = $W_f - W_i$; being W_f, final weight (g $fish^{-1}$) and W_i, initial weight (g $fish^{-1}$). Specific growth rate (SGR) (% day^{-1}) = $((\ln W_f - \ln W_i)/t) \times 100$; being t, time (days). Feed conversion ratio (FCR) = total food supplied (g) / weight gain (g). Protein efficiency ratio (PER) = weight gain (g) / ingested proteins (g).

2.4. Statistical analysis

The confidence interval (CI) was calculated for mean samples with Student's *t*-test distribution at a significance level $\alpha = 0.05$. Shapiro-Wilk test ($p > 0.05$) was used to determine the normality of the data using R software.

The fish growth results were analysed with the IBM SPSS Statistics v.23 statistical software. The three experimental groups were compared by analysis of variance (one-way ANOVA) to determine if there were significant differences between the means of the parameters evaluated ($p < 0.05$). Using Tukey's test, significant differences between means were analysed two by two ($p < 0.05$). The Shapiro-Wilk and Levene tests were used to test normality and homogeneity of variances, respectively ($p < 0.01$).

3. Results

3.1. Microalgae culture

3.1.1. Biomass evolution

Since the N: P ratio of the RAS stream (Table 1) was high (74:1), a preliminary study using *Tetraselmis chui* and RAS stream was carried out in the laboratory (1 L photobioreactors, 1 volume air per liquid volume per minute and 0.45 μm filtered ambient air) under controlled conditions of radiation (100 μmol m^{-2} s^{-1}) and temperature (22 ± 1 °C) during ten days and testing six phosphorus concentrations. These conditions are in line with previous experiments performed by other authors [42–44].

The result (Fig. 2) showed that adding 1 mg L^{-1} of P-PO_4^{3-} to the RAS stream increased productivity by 69 %, while the addition of 1.8 mg L^{-1} (the same N:P ratio found in an f/2 medium) did not increase it substantially further (71 %). Therefore, it was decided to conduct the experiments in the pilot-scale raceway reactor under two conditions. The first experiment was performed under real environmental conditions without adding phosphorus—the unaltered RAS stream. While a second experiment involved the enrichment in phosphorus of this stream until a final P concentration of 1 mg L^{-1} (RAS + P).

On the one hand, the average daily irradiance (Fig. S1) during the RAS experiment (294 ± 82 μmol m^{-2} s^{-1}; CI $\alpha = 0.05$, $n = 13$) was lower than in the RAS + P experiment (440 ± 9 μmol m^{-2} s^{-1}; CI, $\alpha = 0.05$, $n = 8$) under batch operation. On the other hand, the temperature did not show significant differences (Fig. S2); 18.6 ± 3.0 °C for the RAS and 19.0 ± 3.8 °C for the RAS + P experiment. The pH in the culture (Fig. S3) ranged between 7.3–10.5 and 7.7–11.1 in the RAS and RAS + P experiments, respectively. Finally, photosynthetic activity measured as QY (Fig. S4) remained more stable throughout the batch operation when phosphorus was added (0.72 ± 0.02) than in the RAS experiment (0.67 ± 0.05).

Fig. 3 shows the evolution of *T. chui* during batch operation under both N:P ratios. As shown in Table 3, the specific growth rate (μ_{max}), maximum biomass concentration (X_{max}) and volumetric productivity increased by 58 %, 33 % and 72 %, respectively, when phosphorus was added. The optimal hydraulic retention time (HRT) for the batch operation was reduced by half. In the case of μ_{max}, these values are within the average described by other authors [45] operating *Tetraselmis* sp. in batch mode using raceway at pilot scale (0.027 ± 0.002 h^{-1}). Photosynthetic conversion efficiency PAR basis was calculated to avoid the difference in solar irradiance during both experiments. The results confirmed that the addition of P increases the raceway productivity as the efficiency rises from 1.5 to 2.8 %. These values are within the range obtained by Hase et al. [46] (2.2–8.1 % PAR basis) using raceways in a greenhouse for *Chlorophyta* sp. and *Chlorella* sp., and by Grobbelaar [47], who determined 8–9 % as the maximum value for optimal field conditions.

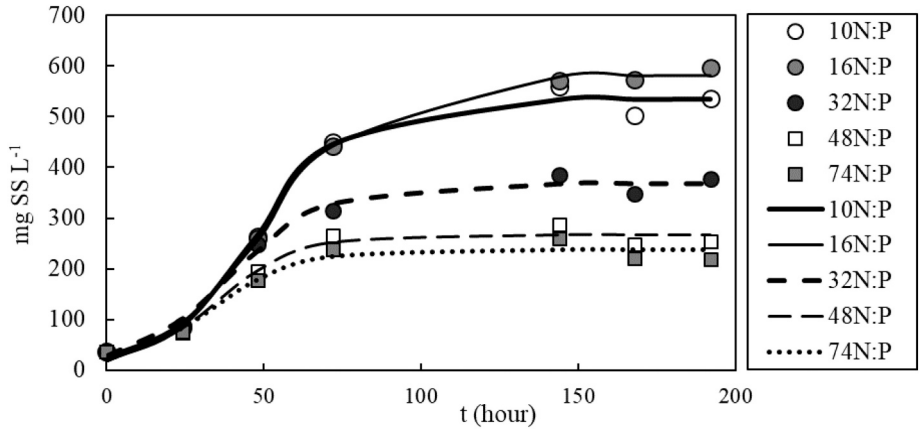

Fig. 2. Biomass evolution of *Tetraselmis chui* operated under batch operation and different phosphorus concentrations in the RAS stream. In the legend, N:P refers to mass ratios. Dots represent experimental data and lines predicted values.

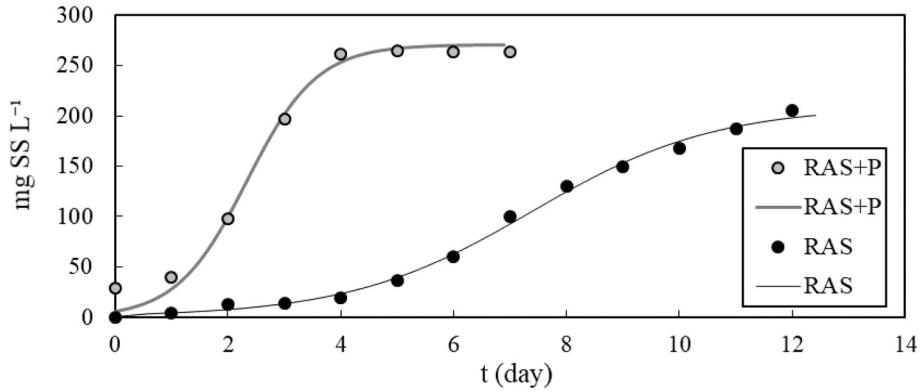

Fig. 3. Biomass evolution of *Tetraselmis chui* during batch operation using RAS stream (RAS) and RAS stream + 1 mg L^{-1} phosphorus (RAS + P). Dots are experimental data and line predicted values.

Table 3
Batch model and semi-continuous kinetics parameters under RAS stream (RAS) and P-enriched RAS stream (RAS + P) conditions. For measurements (semi-continuous), average and standard deviation ($n = 3$ for RAS and $n = 5$ for RAS + P).

	RAS	RAS + P
Batch kinetics parameters		
μ_{max} (h^{-1})	0.022	0.053
X_{max} (mg L^{-1})	165	246
Productivity (mg L^{-1} d^{-1})	21.6	77.8
R^2	0.992	0.991
Semi-continuous kinetics parameters		
X_i (mg L^{-1})	114 ± 17	112 ± 4
X_e (mg L^{-1})	133 ± 8	165 ± 22
Productivity (mg L^{-1} d^{-1})	15 ± 11	36 ± 8
HRT (d)	6	3

μ_{max}, specific growth rate; X_{max}, maximum biomass concentration; X_i, concentration after feeding the reactor; X_e, concentration before feeding the reactor; HRT, hydraulic retention time.

After the batch, both experiments were conducted under semi-continuous operation for six days (four feeding cycles) in RAS and eight days (six feeding cycles) in RAS + P. The solar irradiances were similar to those under batch conditions. 248 ± 51 μmol m^{-2} s^{-1} (CI, α = 0.05, $n = 7$) and 445 ± 24 μmol m^{-2} s^{-1} (CI, α = 0.05, $n = 8$) for RAS and RAS + P experiments. There was no major difference in temperature between both experiments, ranging between 12.7–25.0 °C and 12.2–25.5 °C in RAS and RAS + P experiments, respectively. Similarly to the batch experiment, the photosynthetic activity was more variable (0.58 ± 0.04) in RAS than in the phosphorus-added experiment (0.73 ± 0.01).

The results (Table 3) for biomass concentration (X_e) and volumetric productivity are comparable with those observed by other authors [45] using *Tetraselmis* and raceways under semi-continuous operation, 160–200 mg L^{-1} and 36–39 mg L^{-1} d^{-1}.

The operating strategy in both semi-continuous experiments consisted of maintaining the same biomass concentration after being fed (X_i), being the objective X_i of 80 ± 10 mg TSS L^{-1}. For this, the feeding volume was different; the HRT in the case of the RAS + P experiment was half that of the RAS experiment (Table 3). In combination with the higher average maximum biomass concentration (X_e), the lower HRT resulted in the RAS + P experiment achieving semi-continuous productivity 2.4 times higher. When the photosynthetic efficiencies were compared, the differences between both experiments were lower, reaching 1.8 % during RAS and 2.4 % in RAS + P. Therefore, the observed increase in biomass volumetric productivity in the semi-continuous RAS + P experiment is due to a combination of phosphorus enrichment and solar irradiance.

Finally, during the end phase of the RAS and RAS + P experiments, zooplankton contamination appeared, resulting in a significant decrease in productivity. Other authors have already described this drawback using long-term open cultivation of *Tetraselmis* sp. [48]. Nevertheless, it has not compromised the results nor the relevance of the experiments, as the tests ended shortly after the appearance.

3.1.2. Water quality

In Europe, the Water Framework Directive (MFD) and the EU Marine Strategy Framework Directive (MSFD) do not contain explicit obligations for aquaculture, the resulting discharges can be regarded as point-source inputs. Thus, monitoring information is likely to be required. Therefore Directives 91/271/EEC and 98/15/EEC concerning the treatment and discharge of urban wastewater and wastewater from specific agro-food industrial sectors have been used to analyse the results of this section. Total suspended solids (TSS), biological oxygen demand (BOD_5)—calculated considering a BOD_5: TOC ratio of 2 [49]—, total dissolved phosphorus (TDP) and total dissolved nitrogen (TDN) concentrations in the microalgae treatment effluents were compared with the discharge limit concentrations. Table 4 shows the concentration and percentage removal of TOC, TDN and TDP at the end of the treatments under batch and semi-continuous operation.

In the case of TOC, the feeding stream initially doesn't exceed the limit of 25 mg BOD L^{-1} (Directive 91/271/EEC). Also, the lowest concentration (1.7 mg TOC L^{-1}) was obtained in the RAS + P experiment under semi-continuous operation and the highest (9.5 mg TOC L^{-1}) in the RAS experiment in the same operation regime.

Depletion of TDN was not complete, with removal efficiencies ranging from 41 to 56 %. When compared with the regulation (Directives 91/271/EEC), the nitrogen concentration in all cases (Table 4) was lower than the limit (<15 mg N L^{-1}). The best result was obtained in the RAS experiment under semi-continuous operation, where the nitrogen concentration decreased to 7.6 mg L^{-1}. In other studies, using the same microalgae, >90 % removal rates were obtained using bubble column reactors and operating in batch and semi-continuous operation [24]. However, in other long-term semi-continuous pilot-scale raceway studies using *Tetraselmis* sp. [48], a remaining nitrogen concentration between 6.3 and 12.5 mg L^{-1} was also observed. Therefore, the effect of the operation time and type of reactor influences the nitrogen removal. Future studies should consider whether a more extended reactor operation (>2 months) could positively affect the adaptation of the microalgae to the environment and, therefore, increase the nitrogen uptake capacity.

In the case of phosphorus, almost all the TDP was removed in each experiment (Table 4). It complied with the current urban wastewater discharge standard that requires >80 % removal or a concentration in the effluent below 2 mg P L^{-1}.

Since the centrifuge capture efficiency was at 95 %, the TSS concentration in the effluent was between 7 and 12 mg L^{-1}, values which were below the limit set by the directive (35 mg L^{-1}).

Finally, the evolution of *Vibrio* sp., a bacteria pathogenic to certain marine animal species [50], was analysed in the effluent. The concentrations of these bacteria found in a RAS system can reach 10^6 CFU mL^{-1} [51]. During the batch operation, the evolution of *Vibrio* sp., concentration in both experiments took the form of a normal distribution (Fig. 4). Initially, the concentrations were <100 CFU mL^{-1} in both experiments, typical concentrations in coastal waters [52]. After the first few days of experimentation, the concentration of bacteria in both experiments increased during the exponential growth phase of microalgae until it reached a maximum. When the microalgae biomass reached the maximum concentration (stationary phase), the bacteria concentration dropped below 30 CFU mL^{-1} in the RAS experiment and 1 CFU mL^{-1} in the RAS + P. The major difference between both experiments was that the maximum bacterial concentration reached a value of 800 CFU mL^{-1} in the RAS, while the bacterial population in RAS + P was below 300 CFU mL^{-1}. This may be because the rapid growth of *T. chui* in the RAS + P experiment could inhibit bacterial growth until it finally reached a low concentration.

Other studies [53] showed that the marine diatom *Phaeodactylum tricornutum* could inhibit bacterial growth in reactors, more concretely pathogenic *Vibrio* sp. The genus *Tetraselmis* may also achieve this, although it has not yet been reported to the best of our knowledge. This is interesting because the biomass harvested afterwards will also have a low bacterial load. Therefore, the direct use of the fresh biomass as fish larval feed could be considered. During semi-continuous production, the control of *Vibrio* sp. in both experiments was very similar: between 11 and 56 CFU mL^{-1} in the RAS experiment and between 13 and 62 CFU mL^{-1} in RAS + P. Consequently, microalgae treatment used can be considered adequate to maintain the concentration of these pathogenic bacteria below those found in the environment.

Table 4
Water quality (mg L^{-1}) of *T. chui* experiments in batch and semi-continuous operation under RAS stream (RAS) and RAS stream + 1 mg L^{-1} phosphorus (RAS + P) conditions. In brackets is the percentage of nutrient removal (%). Average and standard deviation ($n = 2$).

	RAS_B	RAS + P_B	RAS_SC	RAS + P_SC
mg TDN L^{-1}	10.1 ± 0.3	10.4 ± 0.5	7.6 ± 0.2 (56)	10.6 ± 0.3
(%)	(41)	(52)		(51)
mg TDP L^{-1}	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01
(%)	(99)	(99)	(99)	(99)

B, batch; TDN, total dissolved nitrogen; TDP, total dissolved phosphorus; SC, semi-continuous.

3.2. Biomass quality and fish feed formulation

3.2.1. Biomass composition and quality

Table 5 shows the biomass composition (gross composition, essential amino acids and primary fatty acids) obtained during batch and semi-continuous operation. The proteins and lipids concentrations increased when the process was optimised by adding 1 mg L^{-1} of phosphorus both in batch and semi-continuous. However, the concentration of total carbohydrates increased in P-limited conditions. Other authors [54] have also observed that a phosphorus deficiency in the culture medium promotes carbohydrate accumulation. No differences were observed in the case of essential amino acids originating from the phosphorus addition. The total sum of amino acids was slightly higher under semi-continuous (50 ± 1 g 100 g^{-1} proteins) than in batch (47 ± 1 g 100 g^{-1} proteins) experiments. In general, similar results were observed between operational modes, except for arginine (Arg), histidine (His) and lysine (Lys). During batch operation, a lower proportion of arginine (−38 % for RAS and −16 % for RAS + P) and histidine (−39 % for RAS and −10 % for RAS + P) and a higher proportion of lysine (+91 % for RAS and +34 % for RAS + P) were observed. Regarding the fatty acid (FA) profile, the main differences were observed in the oleic (OLA) and docosahexaenoic (DHA) acids content, which increased under phosphorus deficiency, as well as the polyunsaturated fatty acid (PUFA) content, which was higher in the P enriched experiment. The whole amino-acid and FA profiles, as well as the elementary compositions (C, H, N, S and P), can be found in the supplementary material (Tables S1, S2 and S3).

Since insufficient biomass was obtained during the experimentation to produce sole feed, commercial biomass of *T. chui* was used. When this biomass is compared with that got in the RAS + P experiment under semi-continuous operation (Table 5), it can be seen that the gross composition was similar in terms of the organic fraction. Nevertheless, the highest difference was in the ash content, 34 % in the commercial biomass and 43 % in RAS + P_SC.

This is because the production of experimental *T. chui* was carried out in an open raceway so that deposits of inorganic matter such as dust and sand happen, as other authors described using long-term open cultivation of *Tetraselmis* sp. [48].

3.2.2. Fish feed formulation

The feed formulation, ingredients and gross composition of the control diet and the two experimental diets are shown in Table 6. In addition, the amino acid and FA profiles of the three diets were included in Tables S1 and S2. The experimental diets consisted of 10 (TC10) and 20 % (TC20) addition of commercial freeze-dried *T. chui* biomass, which

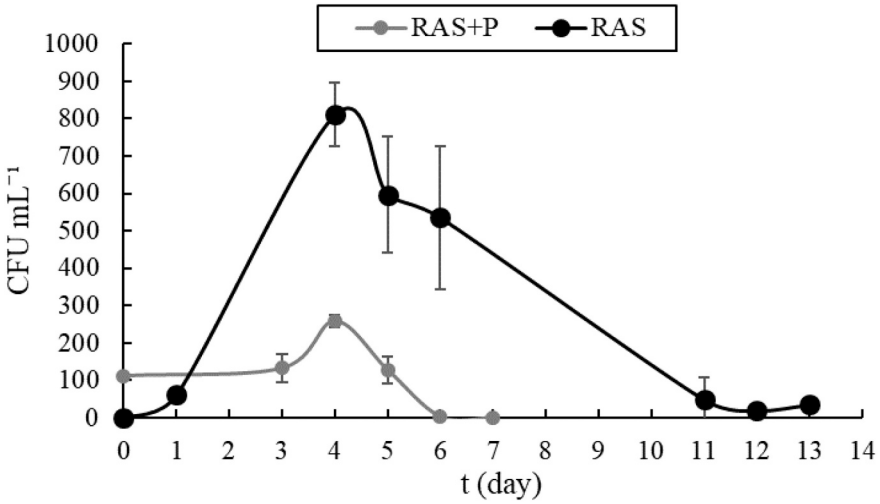

Fig. 4. Daily *Vibrio* sp. concentration (CFU mL^{-1}) in batch operation under RAS stream (RAS) and RAS stream + 1 mg L^{-1} phosphorus (RAS + P) conditions. Average and standard deviation ($n = 2$).

Table 5
Biomass composition (g 100 g^{-1} volatile matter), essential amino acid (g 100 g^{-1} protein) and fatty acids (FA) profile analysed in *T. chui* biomass produced under different experimental conditions tested and commercial *T. chui* biomass.

	RAS_B	RAS + P_B	RAS_SC	RAS + P_SC	Commercial
g 100 g^{-1} volatile matter					
Energy value (kcal)	431.5	450.4	452.0	460.2	450.6
Total protein	35.6	41.6	38.3	50.4	55.2
Total lipids	6.3	10.1	10.4	12.0	10.1
Total carbohydrates	58.1	48.3	51.3	37.6	34.7
Essential amino acids (g 100 g^{-1} proteins)					
Arg	6.17	7.75	9.99	9.27	14.96
His	2.48	3.31	4.05	3.67	2.82
Ile	4.65	4.20	4.45	4.14	4.13
Leu	9.15	8.87	8.55	8.79	8.57
Lys	4.35	2.60	0.38	1.73	3.01
Met	1.54	1.86	1.95	2.35	1.44
Phe	6.23	7.64	9.05	7.81	7.00
Thr	5.17	5.30	5.59	5.16	4.61
Val	6.56	6.25	6.11	6.23	5.95
FA (% of total)					
18:1 n-9 OLA	20.42	15.37	19.85	13.00	7.20
20:4 n-6 ARA	0.46	0.61	0.59	0.58	0.83
20:5 n-3 EPA	4.96	5.57	6.27	5.87	4.93
22:6 n-3 DHA	0.24	–	0.71	–	–
Σ SFA	41.30	40.19	40.53	43.65	49.23
Σ MUFA	30.57	23.21	31.14	22.59	16.15
Σ PUFA	28.13	36.60	28.33	33.76	34.62

Arg, arginine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Thr, threonine; Val, valine; OLA, oleic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

resulted in 15 and 25 % FM reduction compared with the control diet, respectively. To formulate the diets, it was not necessary to increase the fish oil (FO) content (Table 6). On the other hand, to achieve an equivalent gross composition, it was required to modify the content of other ingredients due to the addition of microalgae, such as an increase of monocalcium phosphate (MCP) or the reduction of wheat meal and rapeseed oil. The addition of MCP has negative aspects related to the loss

Table 6
Ingredients (%) and gross composition (% of dry weight, DW) of the control and experimental diets, with an addition of 10 % (TC10) and 20 % (TC20) of commercial *T. chui* biomass. The highlighted values correspond to ingredients that vary between diets. Pellet size of 3 mm.

	Control	TC10	TC20
Ingredient composition (%)			
Fishmeal Super Prime	**30.00**	**25.50**	**22.50**
Squid meal	3.00	3.00	3.00
Tetraselmis chui	**0.00**	**11.50**	**19.20**
Soy protein concentrate	15.00	15.00	15.00
Pea protein concentrate	3.50	3.50	3.50
Wheat gluten	10.00	10.00	10.00
Corn gluten meal	9.50	9.50	9.50
Wheat meal	**15.05**	**7.90**	**3.10**
Vitamin and mineral premix	1.00	1.00	1.00
Vitamin C35	0.05	0.05	0.05
Vitamin E50	0.10	0.10	0.10
Betaine HCl	0.20	0.20	0.20
Antioxidant	0.20	0.20	0.20
Monocalcium phosphate (MCP)	**0.45**	**0.90**	**1.20**
L-Tryptophan	0.10	0.10	0.10
L-Taurine	0.20	0.20	0.20
Soy lecithin	0.25	0.25	0.25
Binder (carboxymethylcellulose)	0.50	0.50	0.50
Fish oil	7.00	7.00	7.00
Rapeseed oil	**3.90**	**3.60**	**3.40**
g 100 g^{-1} DW			
Energy value (kcal)	452	439	424
Ash	8.0	11.3	13.7
Total proteins	54.5	54.3	55.6
Total lipids	16.8	16.9	15.7
Total carbohydrates	20.7	17.5	15.1

of water quality due to the input of phosphorus from the unconsumed MCP [55]. However, as discussed in Section 3.1.1, the addition of phosphorus in the fish farming streams improves microalgae productivity. Therefore, the increase of this ingredient could be positive, avoiding adverse environmental effects since, as mentioned in Section 3.1.2, 99 % of phosphorus is assimilated by microalgae. In addition, the reduction of ingredients of terrestrial plant origin reduces risks related to their content of anti-nutritional substances [56]. Concerning the microalgae addition, previous studies have included 8–16 % *Tetraselmis suecica* to replace 10.0–19.9 % FM; nevertheless this strategy implies an increased FO content (1.9–2.8 %) in European seabass trials [21]. 5–10 % of *T. suecica* was used to reduce 5.2 and 11.9 % FM in gilthead

seabream trials [57]. For Senegalese sole trials [22], the inclusion of *I. galbana* (15 %), *Nannochloropsis* sp. (15 %) and *Scenedesmus* sp. (15 %) has been tested to replace 11.5–14.5 % FM and 3.3–50 % FO. However, in that study, the protein and lipid content in the diets were lower, more specifically between 46.6 and 48.1 % of fresh weight (FW) crude protein and 8.9–9.5 % of FW crude lipids. In these studies, the microalgae were added so that the gross composition of the feed was the same as the control. This addition had similar results in fish growth, feed conversion and fatty acid composition compared with the base feed. To the best of our knowledge, there are no previous studies on *T. chui* and Senegalese sole diets.

Finally, a floatation test of the three diets was carried out. Since soles are benthic organisms, the pellets must reach the bottom of the tank and are accessible to the individuals. In this way, food wastage will be avoided, the feed intake rate will increase, and loss of water quality will be prevented. During the first 10 s, the control diet had the higher percentage (10 %) of afloat pellets followed by TC20 (3 %). All TC10 pellets sank in less than 10 s. After 10 s, all pellets of all diets sank, being available to individuals. Although the differences were minimal in the tests, the wheat meal content, MCP or ash content could slightly affect the different buoyancy of the pellets [58].

Table 7
Production parameters, proximal composition (% of fresh weight) and fatty acid (FA) profile (% of total) of Senegalese sole fillet at the end of the trials using one control diet and two experimental diets with an addition of 10 % (TC10) and 20 % (TC20) of commercial *T. chui* biomass. Average values and standard deviation ($n = 120$ for growth performance and $n = 9$ for fillet composition).

	Control	TC10	TC20	*p*-Value
Growth performance				
Average initial weight (g fish^{-1})	37.59 ± 0.07	37.39 ± 0.19	37.37 ± 0.20	0.277
Average final weight (g fish^{-1})	58.36 ± 2.87	59.79 ± 1.17	60.50 ± 2.75	0.569
Weight gain (g fish^{-1})	20.77 ± 2.81	22.40 ± 1.36	23.13 ± 2.93	0.525
SGR (% day^{-1})	0.52 ± 0.06	0.56 ± 0.03	0.57 ± 0.06	0.444
FCR	1.15 ± 0.08	1.03 ± 0.02	1.11 ± 0.08	0.179
PER	1.68 ± 0.12	1.86 ± 0.04	1.74 ± 0.12	0.171
Fillet proximal composition				
Moisture (%)	75.83 ± 0.46	76.02 ± 0.57	76.12 ± 0.43	
Ash (%)	0.90 ± 0.13	1.10 ± 0.05	1.22 ± 0.15	
Total proteins (%)	19.00 ± 0.30	20.20 ± 0.52	22.05 ± 0.33	
Total lipids (%)	8.05 ± 0.44	7.32 ± 0.78	7.10 ± 0.50	
FA (% of total)				
18:1 n-9 OLA	15.88 ± 0.32	14.60 ± 0.33	14.22 ± 0.34	
20:4 n-6 ARA	1.44 ± 0.12	2.10 ± 0.16	2.05 ± 0.07	
20:5 n-3 EPA	5.80 ± 0.19	6.33 ± 0.11	6.40 ± 0.25	
22:6 n-3 DHA	18.20 ± 0.51	21.40 ± 0.70	21.54 ± 0.20	

SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio; FA, fatty acids; OLA, oleic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

3.3. Senegalese sole trials

3.3.1. Growth performance and fillet composition

The production parameters of the tests carried out with Senegalese sole and three types of diets throughout the 84-day trial are shown in Table 7. The fish accepted the feed and showed no rejection of the experimental diet samples. In general, no significant differences were observed between experimental groups for any of the production parameters evaluated at a confidence level of 95 %. The final body weight of the fish was not different in the diets with the addition of microalgae ($p = 0.569$). This corresponds to a daily weight gain of 0.27, 0.28 and 0.25 g day^{-1} for TC10, TC20 and control, respectively. These values are within the range (0.22–0.43 g day^{-1}) found by other authors [22] for Senegalese sole using control diets and the addition of 15 % microalgae with FM and FO substitution between 11.5–14.5 % and 3.3–50 %, respectively (*Nannochloropsis* sp., *Tisochrysis* sp. and *Scenedesmus* sp.). Specific growth rate (SGR), and feed utilisation parameters, feed conversion ratio (FCR) and protein efficiency ratio (PER), did not differ significantly between the experimental groups either. A comparison of the values with other studies carried out with Senegalese sole juveniles [8,22] showed that the SGR (1.12–1.69 %) was lower in our experiments, but FCR (1.01–1.92) and PER (0.95–1.77) were on average. Therefore, it was observed that the addition of up to 20 % microalgae biomass in diets to replace fishmeal was successfully carried out with no negative effects on sole growth performance, as previously described by other authors for species such as European seabass (16.7–33.3 % FM and 30–60 % FO substitution) [18], rainbow trout (*Oncorhynchus mykiss*) (2.5–10 % FM substitution) [59] or gilthead seabream (15.7–46.9 % FM and 6.3–25 % FO substitution) [60].

According to the proximal analyses of the fish fillets, all groups show a very similar nutritional profile to that obtained in a recent study [10] involving an experimental diet adding 5 % of the macroalga *Ulva ohnoi*. The main difference observed was in the protein content. For the same species with a similar weight (50.1–55.5 g), the protein concentration in the muscle was between 19.95 and 20.16 % of fresh weight. In this case, the control diet and TC10 were on average, while in those fishes fed with the diet TC20, the protein concentration in the fillet increased by 2 %. Saez et al. [10] founded a similar proximal composition to this study (55.1 % crude protein, 12.2 % crude lipids) although using twice as much FM and half as much FO.

Focusing on the primary fatty acids, the proportion of oleic acid (OLA) decreased in the experimental diets. In contrast, the other three fatty acids, arachidonic (ARA), eicosapentaenoic (EPA) and docosahexaenoic (DHA), increased significantly in the diets with the inclusion of microalgae biomass. All values were similar to those obtained by Sáez et al. [10]. Other trials also increased these fatty acids [22] with Senegalese sole juveniles fed diets that included microalgae (15 % inclusion). In that case, differences were not significant, most probably because the inclusion of microalgae did not significatively increase these fatty acids in the diets. The complete fatty acid profile can be found in Table S4.

This study shows that the addition of microalgae increases, to a certain degree, the retention of certain PUFAs in the tissues of Senegalese sole. This also occurs in diets that include other algae [10]. Specifically, this selective fixation of fatty acids like ARA, EPA or DHA was also observed in previous studies in the same fish species [61,62]. Furthermore, some authors [61,63] show that diets low in DHA increase the fatty acid content in the muscle of Senegalese sole, suggesting the existence of specific mechanisms of fatty acid storage.

3.3.2. Immunological activity

Plasma lysozyme activity is an indicator of antimicrobial activity and therefore, the immune status of the fish. As shown in Fig. 5, in the control diet the lysozyme activity was lower (4.02 ± 0.10 μg mL^{-1}) than in the TC10 (5.15 ± 0.12 μg mL^{-1}) and TC20 (5.33 ± 0.20 μg mL^{-1}) diets. The increase in this activity in both experimental diets and compared to the control diet, indicates that the inclusion of microalgae

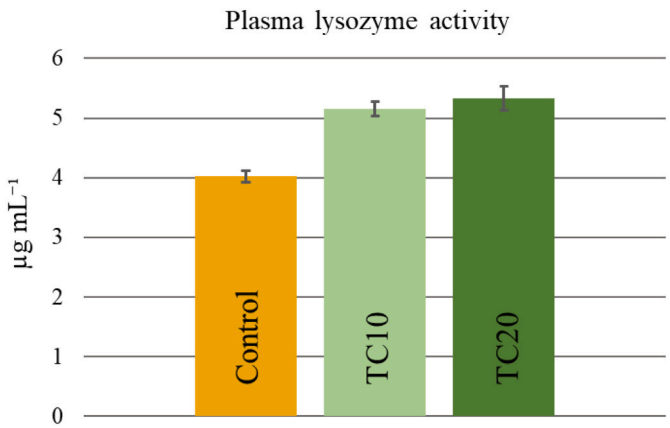

Fig. 5. Plasma lysozyme activity analysed in individuals fed the control diet, TC10 and TC20. Mean and standard deviation ($n = 9$).

enhances disease resistance and, therefore, could reduce bacterial disease loss rates in aquaculture productions. These results are in line with other studies indicating that the addition of herbal compounds [64] or other microalgae such as *Dunaliella salina* [65], *Tisochrysis lutea* and *Tetraselmis suecica* [66] increase plasma lysozyme activity in rainbow trout or European seabass.

4. Conclusions

The culture of the microalgae *T. chui* has been effectively achieved outdoors in a 6 m^3 raceway in semi-continuous operation using the effluent from a RAS of a Senegalese sole farm. The addition of phosphorus resulted in a productivity of 36 mg L^{-1} d^{-1}, comparable to commercial microalgae production systems with raceway reactors. The process resulted in an effluent with a concentration of dissolved phosphorus, nitrogen and organic carbon, and *Vibrio* sp., which could be safely discharged into the environment.

The quality of the harvested biomass is comparable to commercial *T. chui* in terms of proximate analysis and lipid and amino acid profiles, provided that the ash content of the microalgae biomass obtained experimentally is reduced. The use of greenhouses can overcome this aspect. Such biomass allowed to formulate sole fattening feeds with a composition similar to that of a commercial feed but potentially more sustainable by reducing the fishmeal content by 25 %.

The sole fattening trials demonstrate that the use of this formulation does not present significant differences in terms of growth or product quality, but it also improves the fish's immune system by increasing plasma lysozyme activity.

Declaration of competing interest

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

Data availability

No data was used for the research described in the article.

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Statement of informed consent, human/animal rights

The animal test was carried out according *Real Decreto* 53/2013 of 1 February, establishing the basic rules applicable to the protection of animals used for experimental and other scientific purposes.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jwpe.2022.103115.

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