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Effect of the Inclusion of Dried *Tetraselmis suecica* on Growth, Feed Utilization, and Fillet Composition of European Sea Bass Juveniles Fed Organic Diets

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Dried Tetraselmis suecica *was evaluated as a fish protein substitute when incorporated to replace (protein basis) 10% (TETRA10) and 20% (TETRA20) of the control diet. The diets were offered to nine groups of European sea bass (72 g) over 63 days. Test diets did not affect zootechnical performances nor carcass or fillet yields and proximate analysis of edible portion. Feeding TETRA20 resulted in lower apparent digestibility coefficients of protein, lipid, and organic matter, and hepatosomatic index compared to the control diet.* T. suecica *was able to replace up to 20% of fish protein without hampering growth performance and major quality traits of sea bass.*

Keywords organic aquaculture, *Tetraselmis*, European sea bass, fillet quality, nutrition

Introduction

Currently there is much interest in cultivating microalgae for the production of bulk products like lipids for bio-diesel or as feedstuff for industrial chemical processes. Several marine microalgal species offer a valuable source of long chain polyunsaturated fatty acids (PUFAs) that have the potential to become a sustainable alternative dietary source and therefore may help to meet the increasing demand for fish oil that is a critical limiting factor for the future expansion of aquaculture activities (Benemann, 1992; Hemaiswarya et al., 2011). Oils extracted from the algae have proven to successfully replace fish oil in aquafeed (Miller et al., 2007; Ganuza et al., 2008; Palmegiano et al., 2009). In addition, microalgae have a large potential as sources of protein, bio-active compounds, antioxidants, and vitamins to supplement the diet of high-value fish species or in organic-based

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aquaculture systems (Tredici et al., 2009). Microalgae as feed are currently used in relatively small amounts in aquaculture, mainly for the production of larvae and juvenile shellfish, as well as for raising the zooplankton required for feeding larval stages of finfish (Borowitzka, 1997).

In the last decade, research efforts have been directed toward the development of more efficient, high surface-to-volume ratio photobioreactors for microalgae cultivation (Tredici, 2004; Tredici and Rodolfi, 2004; Bosma et al., 2010). These recent technologies utilized in the massive production of microalgae significantly improved productivity and nutritional quality of the biomass in comparison to the traditional open culture systems. They also make microalgae production of interest for aquaculture beyond its consolidated role in hatcheries for most fish and shellfish and crustacean larval stages (Muller-Fuega et al., 2003). Some of these systems have been used to cultivate *Tetraselmis* spp. (Tredici et al., 1996; Borowitzka, 1997; Pedroni et al., 2004; Chini Zittelli et al., 2006), and several species also have been grown on an industrial scale under heterotrophic conditions with yields in excess of 100 g/L per day (Day et al., 1991). *Tetraselmis* spp. has been found to have a large spectrum of antimicrobial activity against important aquaculture pathogens (Austin and Day, 1990; Austin et al., 1992), and its members have shown high potential as probiotics (Irianto and Austin, 2002). Because of its high content of vitamin E, *Tetraselmis* has also been proposed as a source of this vitamin for human and animal consumption (Carballo-Càrdenas et al., 2003). Due to its high content of good quality protein (40–50% dry weight), *Tetraselmis* biomass represents a potential alternative ingredient to produce animal feed.

All these characteristics make *Tetraselmis* sp. a potential candidate to be used as an alternative ingredient in organic aquaculture in the attempt to mitigate the problems arising from a nonsustainable use of natural resources and to guarantee the consumer with a superior quality product (EU Commission Reg. 710/09).

In this context, the present study was carried out to evaluate growth response, feed utilization, and quality traits of the edible portion of sea bass (*Dicentrarchus labrax*) fed diets including substantial levels of dried *Tetraselmis suecica* as fish protein substitute.

Material and Methods

Diets and Experimental Conditions

Three test isoproteic (50.3% DM) and isolipidic (17.9% DM) diets were compared. All preparations were formulated using "organic" ingredients. *T. suecica* was incorporated to replace 10% (TETRA10) and 20% (TETRA20) protein of dried fish trimmings (54.8%) which were the major protein source in the control diet (ORG) including wheat gluten (10%), mechanical extracted soybean meal (9%), wheat meal (12%), and fish oil (10.4%). The composition of the experimental diets is reported in Table 1. All diets were added with diatomaceous earth (Celite®, Sigma–Aldrich, Milano, Italy) as an indigestible marker for *in vivo* digestibility evaluation utilizing the settling column system as described in Cho et al. (1982). The chemical composition of the dried microalgae is reported in Table 2. The cold-pelleted experimental diets were offered to apparent satiety for 63 days to nine groups of 21 European sea bass juveniles (69.5 $\pm$ 0.2 g IBW) kept in a marine water tank (250 L) system under controlled environmental conditions ensuring nearly optimal parameters for the species (temperature 20.4°C; salinity 28 psu; dissolved oxygen 6.14 mg/L; N-NH_3

Table 1
Composition (g/kg) of the experimental diets

	ORG	TETRA10	TETRA20
Fish trimmings[1]	548	493	439
Wheat gluten[2]	100	100	100
Mech. extracted soybean meal[3]	90	90	90
Tetraselmis suecica dry powder		80	160
Wheat meal[4]	120	93	66
Fish oil[1]	104	106	107
Celite®	15	15	15
Mineral and vitamin mix	20	20	20
Carboxymethylcellulose	3	3	3

[1]Fish trimmings and fish oil from Vereinigte Fischmehlwerke Cuxhaven GmbH & Co. KG, Neufelder Straβe 44, 27472 Cuxhaven, Germany; [2]Wheat gluten from SACCHETTO S.p.A., Via Circonvallazione, 4 12030 Lagnasco (CN) Italy; [3]Mechanically extracted soybean meal from Strada Micaloro, 61020 Chiusa di Ginestreto, Pesaro (PU) Italy;[4]Wheat meal from Molino Di Giusto, Reana del Rojale (UD), Italy.

Composition of mineral mix (% mix): $CaHPO_4{}^*2H_2O$, 78.9; NaCl, 17.65; MgO, 2,725; FeCO3, 0.335; KI, 0.005; $ZnSO_4{}^*H_2O$, 0.197; $MnSO_4{}^*H_2O$, 0.094; $CuSO_4{}^*5H_2O$, 0.027; Na Selenite, 0.067.

Composition of vitamin mix (g/kg diet): Thiamine HCl Vit B1, 20; Riboflavin Vit B2, 26; Piridoxine HCl Vit B6, 20; Cyanocobalamine B12, 5.2; Niacin Vit PP, 156; Calcium pantotenate, 26; Folic Acid, 3; Biotin Vit H, 26; Myoinositol, 258; stay C Roche, 295; ?-tocoferol Vit E, 103; Menadione Vit K3, 58; Vit A (2500UI/kg diet) 1.3; Vit D3 (2400UI/kg diet) 2.5; choline clorure, 8.

0.11 mg/L; N-NO_2 0.09 mg/L; light-dark cycle 12L:12D). The three diets were tested in triplicate according to a completely random design. Feed intake as well as eventual mortality per tank/group of fish was registered on a daily basis. Water, ammonia, nitrogen, and nitrite were monitored weekly according to standard methods (American Public Health Association [APHA], 1980). Fish were tank/group weighed every 2 weeks under moderate sedation (ethanolic solution of clove oil 70% v/v) after an overnight fast.

At the end of the experiment, three fish per tank were sampled at random and sacrificed with an excess of anesthetic. They were subjected to individual biometry measurements and dissected in order to evaluate some major organ and tissue weights and slaughter yield. The edible portion was utilized for chemical characterization. The experimental diets were subjected to proximate analysis, lipid, and β-carotene content (Table 3), while the edible portion was analyzed for total lipid and quantitative fatty acid composition by gas chromatography.

Apparent Digestibility Coefficient

The apparent digestibility coefficients (ADCs) of the ORG and TETRA20 diets were measured by the indirect method using acid-insoluble ash (AIA; AOAC method 941.12) as external indigestible marker added (Celite®; 15 g/kg) to each diet before final mixing and dry pelletting. The digestibility measurements were carried out using the tank system and procedures outlined by Cho et al. (1982).

Table 2
Chemical composition of *Tetraselmis suecica*

N Kjeldhal * 6.25 (g/100 g dry weight)	48.7
Fiber (g/100 g dry weight)	3.4
Total lipid (g/100 g dry weight)	8.0
Ash (g/100 g dry weight)	17.5
Non-nitrogen extract (g/100 g dry weight)	22.4
Phosphorous (mg/g)	1.1
β-carotene (mg/kg)	267
AA composition (g/100 dry weight)	
Asp	5.3
Thr	2.5
Ser	2.4
Glu	7.1
Gly	2.8
Ala	4.4
Val	1.7
Ile	1.9
Leu	4.1
Tyr	0.7
Phe	3.3
Lys	3.0
His	1.4
Arg	3.5
Neutral lipid (% total lipid)	57.0
Polar lipid (% total lipid)	43.0
Saturated fatty acid (g/100 g dry weight)	1.02
Monounsaturated (g/100 g dry weight)	1.07
n-3 PUFA (g/100 g dry weight)	0.16
n-6 PUFA (g/100 g dry weight)	2.41

The apparent digestibility coefficient of dry matter, crude protein, crude lipid, and gross energy of the diets were computed as follows :

$$\begin{aligned}\text{ABCi}\,(\%) = \big[&(\%\ \text{nutrient in the diet}/\%\ \text{marker in the diet})\\ &-(\%\ \text{nutrient in the faeces}/\%\ \text{marker in the faeces})/\\ &(\%\ \text{nutrient in the diet}/\%\ \text{marker in the diet})\big]\ {}^{*}100.\end{aligned}$$

Proximate Analysis

Moisture analysis was carried out according to the Association of Official Analytical Chemists (AOAC, 1998) method 934.01. Total nitrogen content was determined by Kjeldahl method according to AOAC method 940.25 and multiplied by 6.25 to estimate the crude protein content. Ash content was gravimetrically determined using a muffle furnace by heating at 515°C to constant weight according to AOAC method 938.08. Fiber content was determined according to AOAC methods 985.29 and 991.43. Total lipid content

Table 3
Chemical analysis and fatty acid composition (% total fatty acids) of the experimental diets

	ORG	TETRA10	TETRA20
Dry matter (g/100 g)	93.5	93.9	94.5
N Kjeldhal * 6.25 (g/100 g DM)	51.3	49.9	49.9
Ether extract (g/100 g DM)	16.8	18.3	17.0
Crude fiber (g/100 g DM)	1.4	1.0	1.0
NDF (g/100 g DM)	3.6	4.8	3.5
ADF (g/100 g DM)	3.3	2.4	2.0
Ash (g/100 g DM)	15.9	16.2	16.4
Total phosphorus	1.92	1.79	1.94
β-carotene (ppm)	tr.	0.83	1.75
Neutral lipid (g/100 g DM)	14.73	15.55	15.60
Polar lipid (g/100 g DM)	2.14	2.73	3.24
Fatty acid composition[1]			
C14:0	5.4	5.5	5.3
C16:0	16.3	17.1	17.3
C16:1n-7	4.5	4.5	4.3
C18:0	2.7	2.3	2.3
C18:1n-9	16.0	16.1	16.6
C18:1n-7	2.4	2.2	2.2
C18:2n-6	11.9	10.6	10.9
C18:3n-3	2.4	4.0	4.5
C18:4n-3	2.2	2.5	2.6
C20:1n-9	6.7	6.6	6.4
C20:2n-6	0.2	0.3	0.3
C20:4n-6	0.5	0.5	0.5
C22:1n-11	8.3	8.5	8.2
C20:5n-3	5.8	5.8	5.8
C22:5n-3	1.2	1.0	1.0
C22:6n-3	9.9	9.2	8.6
Saturated	25.4	25.8	26.0
Monounsaturated	39.5	39.4	39.1
n-3 PUFA	21.5	22.5	22.6
n-6 PUFA	12.9	11.6	11.7
n-3/n-6 PUFA	1.73	1.95	1.93

[1]The fatty acids C12:0, C13:0, C14:1n-5, C15:0, C16:2n-4, C16:3n-4, C17:1, C16:4n-1, C18:2n-4, C18:3n-6, C18:3n-4, C18:4n-1, C20:1n-7, C20:3n-6, C20:3n-3, C20:4n-3, C21:5n-3, C22:4n-6, and C22:5n-6 were considered in the composite fraction but were not reported in the Table.

was determined by hydrolyzing the sample with hydrochloric acid in 100°C water bath and extracting the lipid fraction with diethyl ether and petroleum ether. The lipid fraction was obtained after heating on a 100°C hot plate and in an oven to remove the ether layers according to AOAC method 948.15. Total phosphorous content was determined spectrophotometrically according to AOAC method. The β-carotene content was measured according to AOAC method.

Determination of Amino Acid Composition

Amino acids analysis was performed using a LC 200 Perkin Elmer pump fitted with an ISS-100 auto sampler (20 μL loop) and a fluorimetric detector (Perkin Elmer, Norwalk, CT, USA), EX 250 nm and EM 395 nm. Separation was achieved by using one AccQ.Tag Amino Acid Analysis column (Waters Corporation, Milford, MA, USA) and one Waters pre-column filter. The column was thermostated at 31°C, and the flow rate was 0.8 mL/min (Liu et al., 1995). Mobile phase A consisted of acetate-phosphate aqueous buffer, and mobile phase B was acetonitrile 100%. Acid hydrolysis with HCl 6 M at 115–120°C for 22–24 h was used for all amino acids except cysteine (Cys) and methionine (Met) for which performic acid oxidation followed by acid hydrolysis was used. After borate buffer addition, filtered hydrolysated samples were derivatized at 55°C for 10 min with 20 μL of AccQ.Fluor reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) and injected in a high performance liquid chromatographer (HPLC; Bosch et al., 2006).

Determination of Fatty Acid Composition by Gas Chromatography

Fatty acids in the lipid fraction (Folch et al., 1957) of diet and edible portion were converted into fatty acid methyl esters (FAME) according to Morrison and Smith (1964), and their composition was determined by gas chromatography (GC). A Varian gas chromatograph 430-GC equipped with a flame-ionization detector and capillary column (Omegawax, 30 m × 0.32 mm i.d, 0.25 μm film; split ratio, 100:1; Supelco 24152) was used for analyzing FAME. Parameters of the GC system were set as follows: injector and detector temperatures, 220 and 300°C, respectively; the column temperature was set to 160°C for 1 min and gradually heated to 220°C at a rate of 2°C/min, then maintained isothermal for 9 min (40 min of total run); helium was used as a carrier and auxiliary gas. The fatty acid concentrations were calculated by comparison of their retention times with those of the reference standards (Supelco, Bellefonte, PA, USA). An internal standard (C23:0) was used to obtain absolute quantification.

Table 4
Growth performance, feed intake, feed efficiency, slaughter yield, and somatic indices of juvenile sea bass fed the experimental diets over 63 days

	ORG	TETRA10	TETRA20	MSE
Initial body weight (g)	69.4	69.5	69.5	0.049[1]
Final body weight (g)	117.7	118.3	116.1	7.518[1]
SGR (%)	0.84	0.84	0.81	0.002[1]
Feed intake (g/fish/day)	1.04	1.07	1.05	0.005[1]
Feed:gain ratio	1.35	1.41	1.43	0.002[1]
Carcass yield	88.96	88.27	88.04	1.760[2]
Fillets yield	44.68	44.68	44.35	25.988[2]
Viscerosomatic index	10.09	11.10	11.20	1.538[2]
Hepatosomatic index	1.84[a]	1.52[ab]	1.39[b]	0.132[2]

Mean values in the same row with different letter differ significantly ($p < 0.05$). [1]*MSE df* = 6; [2]*MSE df* = 24.

SGR (Specific Growth Rate) = {[Ln (final body weight) − Ln (initial body weight)]/days} * 100.

Carcass yield (%): (gutted body weight/body weight) * 100.

Fillet yield (%): (fillets with skin weight/body weight) * 100.

Viscerosomatic index (%): (viscera weight/body weight) * 100.

Hepatosomatic index (%): (liver weight/body weight) * 100.

Statistical Analysis

Experimental data were subjected to statistical analysis (analysis of variance, ANOVA) according to a completely random experimental design, and average values were compared using the Duncan's multiple range test for $p < 0.05$ using the SPSS-PC Release 17.0 statistical software (SPSS Inc., Chicago, IL, USA).

Results and Discussion

No mortality was observed during the experimental trial. Feeding the test diets over 63 days resulted in similar feed intake (1.04 g/day fish, $p > 0.05$), fish growth rate (SGR 0.82%; weight gain 68.8% initial body weight; $p > 0.05$), and feed utilization (feed:gain ratio 1.39; $p > 0.05$) (Table 4). Dietary algae inclusion significantly affected the apparent digestibility

Table 5
Water, nitrogen, lipid, and fatty acids composition[1] (% total fatty acids) of the edible portion of juvenile sea bass as affected by feeding the experimental diets for 63 days

	ORG	TETRA10	TETRA20
Water (g/100 g w.w.)	64.6	64.6	64.8
N Kjeldhal * 6.25 (g/100 g w.w.)	24.1	24.1	24.4
Total lipids (g/100 g w.w.)	7.26	7.16	7.26
Fatty acids (% total fatty acids)			
C14:0	4.3	4.3	4.2
C16:0	18.3	18.3	18.2
C16:1n-7	5.0	5.0	4.9
C18:0	3.6	3.6	3.6
C18:1n-9	19.8	20.1	19.9
C18:1n-7	2.5	2.5	2.5
C18:2n-6	12.0	12.5	12.4
18:3n-3	1.9[b]	2.2[b]	2.5[a]
18:4n-3	1.2[b]	1.4[a]	1.4[a]
C20:1n-9	3.8	3.8	3.6
C20:2n-6	0.5	0.6	0.5
C20:4n-6	0.5	0.5	0.5
C22:1n-11	4.2	4.0	3.8
C20:5n-3	7.7	7.8	7.7
C22:5n-3	1.4	1.3	1.3
C22:6n-3	10.8	9.7	10.1
Groups			
Saturated	27.01	27.01	26.92
Mono	36.06	36.08	35.58
n-3 PUFA	22.95	22.39	23.09
n-6 PUFA	13.16	13.68	13.59
n3/n6	1.75[a]	1.64[b]	1.70[ab]

Different letters on the same row indicate significant differences (*MSE df* = 24, $p < 0.05$).
[1]The fatty acids C12:0, C13:0, C14:1n-5, C15:0, C16:2n-4, C16:3n-4, C17:1, C16:4n-1, C18:2n-4, C18:3n-6, C18:3n-4, C18:4n-1, C20:1n-7, C20:3n-6, C20:3n-3, C20:4n-3, C21:5n-3, C22:4n-6, and C22:5n-6 were considered in the composite fraction but were not reported in the Table.

coefficients as measured *in vivo* for diets ORG and TETRA20 (data not shown). Compared to the control diet, fish fed diet TETRA20 resulted in significantly lower ADCs of protein (93.3 vs. 95.3%; $p < 0.05$), lipid (79.7 vs. 99.3%; $p < 0.05$), and organic matter (87.4 vs. 89.1%; $p < 0.05$). So, techniques of cell disruption should be tested to increase the microalgae digestibility, thus reducing fecal outputs and making its use in aquafeeds more environmentally sustainable. Dietary algae inclusion resulted in a significant decrease of the hepatosomatic index compared to the control diet related to the decrease of lipid/energy uptake due to the graded inclusion of *Tetraselmis* associated with the decrease of the diet ADC of lipid but also to possible modulating effects of microalgae compounds on lipid metabolism. No differences were observed in viscerosomatic index or in carcass and fillet yields. As shown in Table 5, feeding the test diets for 63 days to the sea bass juveniles did not affect the major flesh components as fish edible portion exhibited similar water (on average 64.4%) and lipid content (on average 7.2%). Also, the fatty acids composition of the edible portion resulted in similar findings among groups—27.1% saturated,

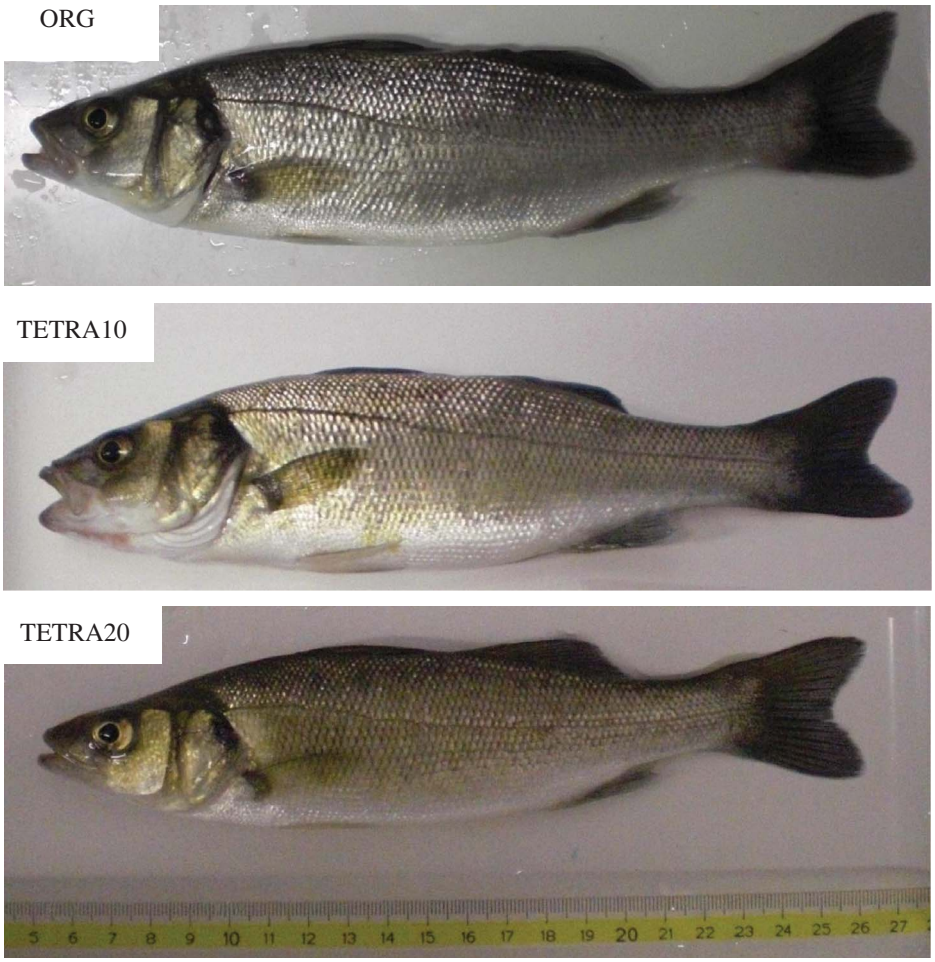

Figure 1. Effect of feeding the experimental diets for 63 days on juvenile sea bass skin pigmentation (color figure available online).

35.9% monounsaturated fatty acids, 22.8% n-3 PUFA, and 13.5% n-6 PUFA—values consistent with recent data reported for cultured sea bass flesh (Grigorakis, 2007). On the contrary, some differences were observed in n-3 PUFA/n-6 PUFA ratio that resulted in higher values in the ORG group than in the TETRA10 group (1.75 vs. 1.64; $p < 0.05$), while the TETRA20 group showed an intermediate value (Table 5).

An interesting side effect observed after feeding diets including *Tetraselmis suecica* to sea bass juveniles was a change in the skin pigmentation. An enhanced greenish pigmentation was observed in fish fed diets including microalgae, similarly to what was observed in *Carassius carassius* (Gouveia and Rema, 2005; Figure 1). This side effect can be a valuable characteristic of the algal biomass to be considered if wild-like fish are the expected objective of the aquaculture practice.

As a concluding remark, the results of this trial show that dried *Tetraselmis suecica* can replace up to 20% of fish protein without hampering growth performance and major quality traits of sea bass when diets are offered near to satiety. The microalga has the potential to become an alternative dietary ingredient to be used in organic feed production.

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