marine drugs

Article

Integrated multitrophic aquaculture by-products with high added value: the polychaete *Sabella spallanzanii* and the seaweed *Chaetomorpha linum* as sources of fatty acids

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Abstract: Aquaculture expansion is limited by the waste negative environmental impact and the need for alternative sources in the diet of reared fish. In this framework, for the first time, the polychaete *Sabella spallanzanii* and the macroalga *Chaetomorpha linum* were included with fish in an integrated multitrophic aquaculture system (IMTA) as bioremediators of aquaculture wastes. The survival rates and biomass gain of both species reared/cultivated in the IMTA were evaluated as well as their fatty acid profiles. Results showed that these organisms represent a natural source of ω3 with the ω6/ω3 ratio lower than 1. On account of these noteworthy results and the high biomass obtained as by-products, a preliminary study was performed employing both *S. spallanzanii* and *C. linum* as new dietary supplements to feed different sized *Dicentrarchus labrax*. Fish survival rate, biomass growth, and specific growth rate were determined resulting in no significant differences between control and treated fishes. Histological analyses showed no alterations of the stomach tunica mucosa and submucosa in treated fishes. The eco-friendly approaches applied in the here realized IMTA system could guarantee the proper waste management and the safety of rearing environment as well as the achievement of sustainable by-products represented by the bioremediators *S. spallanzanii* and *C. linum* as sources of high added value compounds beneficial to fish and human health.

Keywords: *Chaetomorpha linum*; *Dicentrarchus labrax*; fish growth; fish nutrition; innovative meal; *Sabella spallanzanii*

1. Introduction

Aquaculture currently provides almost 45% of the world's fisheries products and an increased production up to almost 62% is expected by 2030 [1]. However, its expansion is limited by several factors including the need to develop new alternative diets for reared fish and the reduction of the impact of this activity on the marine environment. In this context, in recent years, a substantial proportion of the research has been aimed at creating Integrated Multi-Trophic Aquaculture (IMTA) systems. Here, the simultaneous rearing of fed species with bioremediators, which can use for their growth the nutrient surplus, either inorganic (e.g., seaweeds or other aquatic vegetation) or organic (e.g. deposit- and suspension-feeders), can allow the attainment of a sustainable aquaculture [2]. Indeed, IMTA has the potential to produce economically exploitable biomasses and provide biomitigative services at the same time, which can be beneficial for both ecosystem and human health. In the light of a more sustainable aquaculture industry, the reduction of microbial pollution, within farms, by the use of co-cultured living organisms, represents a challenge. Several studies have shown that microbial contamination is reduced by some macroinvertebrates, in particular filter-feeders, able to process large volumes of waters for their food requirements efficiently retaining small particles including bacteria and thus acting as bioremediators [3–5]. Therefore, many of these macroinvertebrates, such as oysters, mussels, clams, polychaetes and sponges, are suitable to restore the environment [6,5,7,8,9]. Bioremediation is also accomplished by macroalgae used to reduce the nitrogen load, especially in ammoniacal form, produced by fish metabolism and by the processes of decomposition of uneaten feed. Algae are commonly used in co-colture with bivalves [10] although numerous variations to this basic scheme have been tested. The farming of fish with bioremediators allows the conversion of the uneaten feed, wastes and nutrients into biomass that can be removed and potentially managed as a valuable by-product. Indeed, marine biomass indeed has an enormous potential as a source for nutritional, therapeutic and functional ingredients, which may be used to make products for animal and human consumption [11]. At present, the interest of the food industry related to aquaculture activity is mainly focused on functional foods consisting of one or several functional ingredients of natural origin able to further supply fish and human health benefit compared to the common conventional food largely represented by fishmeal and fish oil [12,13]. The considerable content of high-quality proteins with all the essential amino acids makes fishmeal a very good ingredient in feeds and not substitutable with crop plants, in which proteins, conversely, lack most of these amino acids, such as lysine, methionine, threonine, and tryptophan [14]. Fishmeal is also rich in lipids with high-quality polyunsaturated ω-3 and ω-6 fatty acids (PUFAs), which are considered beneficial to human cardiovascular health. Although marine fish represents the main source of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the formulation of fish feeds, there is an urgent need for an alternative and sustainable source of ω-3 long chain PUFAs on account of the depletion of wild fish stocks and the pollution of the marine environment. Recently, Stabili et al. [15] suggested that the very common Mediterranean polychaete *Sabella spallanzanii,* obtained as by-product of bioremediation in aquaculture farms, could be employed as a dietary supplement for fish nourishment on account of the high protein content as well as the presence of certain amino acids that could improve palatability of the worms when included in fish feeds [15,14]. Indeed, *S. spallanzanii* shows an interesting amino acids profile including lysine, methionine and threonine in an amount comparable with fish meal other than some amino acids present in excess such as glycine, arginine, cysteine, histidine, and glutamic acid.

In this framework, in the present paper the rearing/cultivation of the two bioremediators *S. spallanzanii* and the macroalga *Chaetomorpha linum* in an integrated rearing fish system was realized, for the first time, in an aquaculture farm along a coastal site in the Mediterranean Sea. Both these bioremediation by-products were investigated as potential sources of lipids and essential fatty acids to be employed as dietary supplements in fish feed. Among the marine invertebrates, polychaete worms have been already used in aquaculture as feed [16–18]. In particular, nereids are commonly known as omegaworms due to a high omega-3 (ω-3) polyunsaturated fatty acid (PUFA) content [19]. Also macroalgae have been widely tested as dietary components in aquaculture plants [20–22]. Indeed, algae have been recognised as an obvious alternative source of these 'fish oil' fatty acids for use in fish feeds [23], especially EPA, and DHA, and arachidonic acid (ARA). In this perspective, in the present paper *S. spallanzanii* and *C. linum* biomass from the realized IMTA system were employed to prepare an experimental innovative fish feed utilized for preliminary feeding assays on the European sea bass *Dicentrarchus labrax* juveniles having market value especially in the Mediterranean countries. Moreover, in order to obtain a first insight on the potential stomach distress and damage of the epithelium due to the artificial feeding, histological analyses were performed on the sea bass fish juveniles.

2. Results

2.1. Rearing/Cultivation of Bioremediators in IMTA

The monthly monitoring of the well-being and growth of seaweeds placed in the realized IMTA system (Figure 1) showed, during the 6 months of *C. linum* cultivation, interesting cultivation performances.

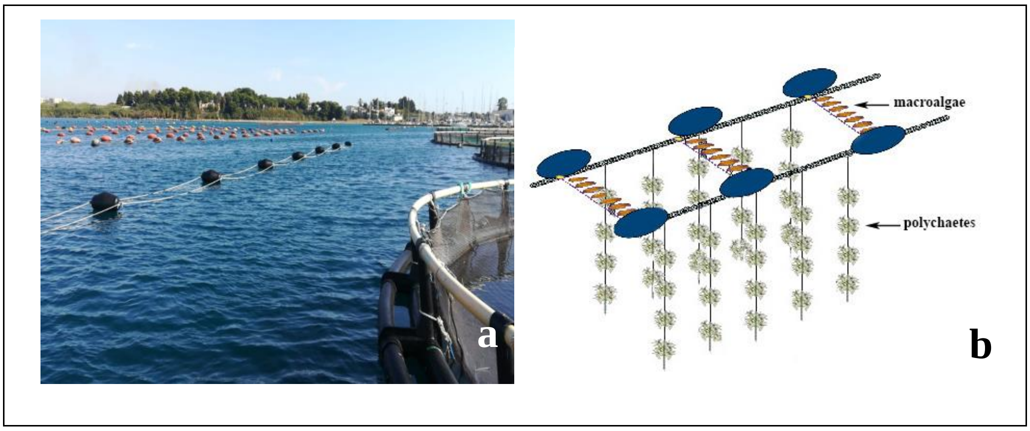

Figure 1. Rearing/cultivation of bioremediators s in the IMTA system: (**a**) Fish cages of the integrated multi-trophic aquaculture (IMTA) system in the Mar Grande of Taranto and long-line system; (**b**) Detail of the location of the bioremediators. As indicated by the arrows, the algae were horizontally arranged at 1 m of depth within a typical long-line system while polychaetes were placed vertically in polypropylene nets and placed around the fish cages.

High survival values and significant increases in biomass produced in short time intervals were indeed recorded. In particular, for *C. linum* a maximum SGR equal to 5% was calculated in a six month trial (Figure 2).

Figure 2. Cultivation trials of *Chaetomorpha linum* in the realized IMTA system: (**a**) and (**b**) Algae arranged in cultivation sockets; (**c**) *C. linum* located in the farm; (**d**) algae in net sacks hung at 1 m of depth within a typical long-line system.

In the IMTA system about 1428 specimens of *S. spallanzanii* were estimated in each collector for a total of 360,000 individuals in the whole system. After the 6 months of permanence the polychaete biomass was of 0.645 t (Figure 3).

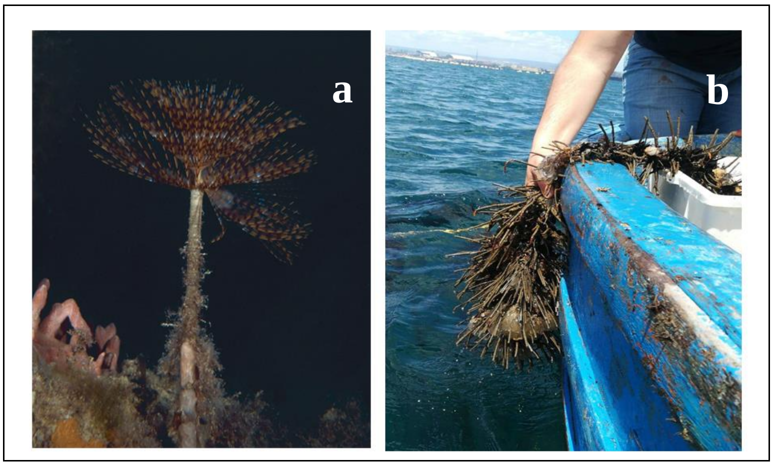

Figure 3. Rearing trials of the polychaete *Sabella spallanzanii* in the integrated multi-trophic aquaculture (IMTA) system: (**a**) Specimen of *S. spallanzanii;* (**b**) Polychaetes arranged in polypropylene nets, which were hung vertically within a typical long-line system.

Within the first three months of rearing a biomass increase of about 79.3% was recorded, while in the last three months the biomass mean value increased with a further gain of 20.7%. As far as the survivorship of *S. spallanzanii* in the farming plant, worms showed a very low mortality rate during all months of observations. In particular, after the six months of rearing a mortality rate of about 15% was recorded.

In addition to the high production of bioremediators biomass, thanks to the realized IMTA system a restoration of the aquaculture rearing environment was achieved in terms of microbial contamination (i.e. total coliforms and *Escherichia coli*) and nutrient concentrations (i.e. phosphorous and nitrogen salts) (L. Stabili unplished data).

2.2. Total Lipid and Fatty Acid Composition

The total mean lipid content of *C. linum* corresponded to 9.4 ±2.4 mg/g dry weight (DW). In the case of *S. spallanzanii,* the mean lipid content, expressed on a wet weight basis (WW), was 8 ± 0.42 g/100 g WW.

The fatty acid profile of total lipids extracted from *C. linum* is shown in Figure 4. Polyunsaturated fatty acids (PUFAs) were the most abundant accounting for 71.97% of total FAs, and the most abundant PUFAs were the linoleic acid (18:2 ω-6), linolenic acid (18:3 ω-6 γ), the ω-3 eicosapentaenoic acid (EPA, 20:5 ω3) and the ω-6 arachidonic acid (ARA, 20:4 n6) and accounting for 38.46%, 14%, 8.83%, and 8.14% of total FAs, respectively (Figure 4a). The ω-3 docosahexaenoic acid (DHA, 22:6 ω3) represented the 2.91%. Saturated fatty acids (SAFAs) represented 23.83% of total fatty acids (FAs). Palmitic acid methyl ester (16:0) was the prevalent SAFA (14.03% of total FAs), followed by myristic acid methyl ester (14:0; 9% of total FAs). Monounsaturated fatty acids (MUFAs) showed the lowest percentage (4.2% of total FAs) and among them oleic acid methyl ester (18:1 ω-9) prevailed (Figure 4b).

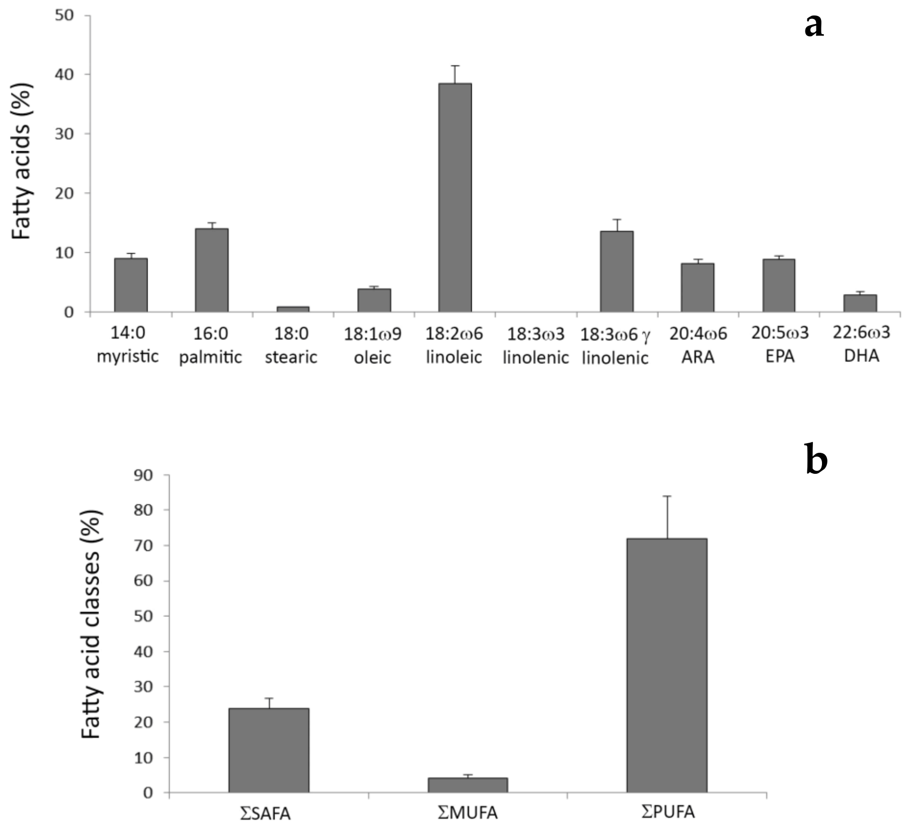

Figure 4. Fatty acid composition of *Chaetomorpha linum:* (**a**) Seaweed fatty acid profile as percentage of total fatty acids); (**b**) Percentage of saturated fatty acids (SAFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs).

The fatty acid profile of *S. spallanzanii* is shown in Table 1. Palmitic acid (16:0) was the predominant SAFA (accounting for 26.18% of total lipids) followed by myristic acid (14:0) and stearic acid (18:0). Palmitoleic acid (16:1) prevailed among MUFAs and 16-docosadienoic acid (22:2, n-6) was the most abundant PUFA.

In both the selected bioremediators the ratio of ω -3 to ω-6 fatty acids was <1.

2.3. Feed Formulation and Preliminary Fish Growth Trials

As shown in Table 2, both the prepared control (CTRL) and innovative (IM) feeds were isoenergetic, isoproteic and isolipidic. During both the preliminary fish growth trials (May-September), in which the experimental feeds were employed, the mean temperature ranged between 20.6 and 23 °C and dissolved oxygen between 6.2 and 7.4 mg/L. All the measured water parameters resulted in the physiological range for *D. labrax*. In both the experimental trials, differences on the biomass gain, specific growth rate, and survival rate between the control and the treatment were not statistically significant (Table 3).

Table 1. Fatty acid composition of *Sabella spallanzanii*.

Saturated fatty acid percentages (SAFA)	
14:00	9.64
15:00	0.25
16:00	26.18
17:00	0.20
18:00	8.95
20:00	1.66
22:00	7.62
23:00	0.34
24:00	2.43
∑	57.26
Monounsaturated fatty acid percentages (MUFAs)	
16:1 n-7	5.62
17:1 n-8	4.67
18:1 n-9	3.16
18:1 n-7	4.94
20:1 n-9	3.28
22:1 n-9	1.12
24:1 n-9	3.67
∑	26.46
Polyunsaturated fatty acid percentages (PUFAs)	
18:2 n-6	0.54
18:2 n-4	0.64
18:3 n-6	0.81
18:3 n-3	2.04
20:2 n-6	0.85
20:3 n-6	1.49
20:3 n-3	1.20
20:4 n-6	1.74
20:5 n-3	1.17
22:2 n-6	4.50
22:6 n-3	1.29
∑	16.28

Table 2. Proximate composition (% dry weight) of the experimental diets (n=3). Values are reported as mean ± S.E..

	[1] CTRL	[1] IM
Crude protein	46.0 ± 0.4	45.8 ± 0.3
Ether extract	15.5 ± 0.1	15.3 ± 0.1
Ash	11.5 ± 0.1	11.2 ± 0.2
Gross energy	20.79 ± 0.21	20.82 ± 1.01

[1] CTRL: Control feed; IM: Innovative feed.

Table 3. Survival rate (%), biomass growth, specific growth rate (n=3; mean ± S.D.) of *Dicentrarchus labrax* fed with the experimental feeds.

I Trial		
	[1] **CTRL**	[1] **IM**
Biomass gain (g)	0.62± 0.04	0.55 ± 0.01
Specific growth rate (%)	4.32 ± 0.21	4.14 ± 0.16
Survival rate (%)	87 ± 2	96 ± 2
II Trial		
	CTRL	**IM**
Biomass gain (g)	2.87 ± 0.15	2.1 ± 0.12
Specific growth rate (%)	2.96 ± 0.16	2.4 ± 0.11
Survival rate (%)	85 ± 3	94 ± 2

[1] CTRL: Control feed; IM: Innovative feed.

In particular, when the innovative meal was employed biomass gain reached a value of 0.55 ± 0.01 g in the first trial and 2.10 ± 0.12 g in the second trial. These values didn't differ statistically from those recorded for the control feed accounting 0.62 ± 0.04 g in the first trail and 2.87 ± 0.15 g in the second trial. As regards the specific growth rate, the value recorded for IM group in the first trial was 4.14 ± 0.16% and 2.4 ± 0.11% in the second trial.

These values were not statistically different from those recorded for the respective controls in both the trials. A negligible fish mortality was recorded for both the feeds at the end of each trial and in particular the survival rates were 87 ± 2% for CTRL and 96 ± 2% for IM with the lowest size of fish (first trial) and 85 ± 3% for CTRL and 94 ± 2 % for IM in the second trial.

2.4. Fish Histological Analyses

In the second trial the examined sections of fish stomach tunica mucosa and tunica submucosa showed normal histological pattern, without inflammations (lymphocyte infiltration) or degenerations in both CTRL and IM fish. Both the tunica mucosa and the tunica submucosa appeared regularly extended, without interruptions or alterations of the epithelium; and the cells were intact and didn't show any sign of suffering (Figure 5).

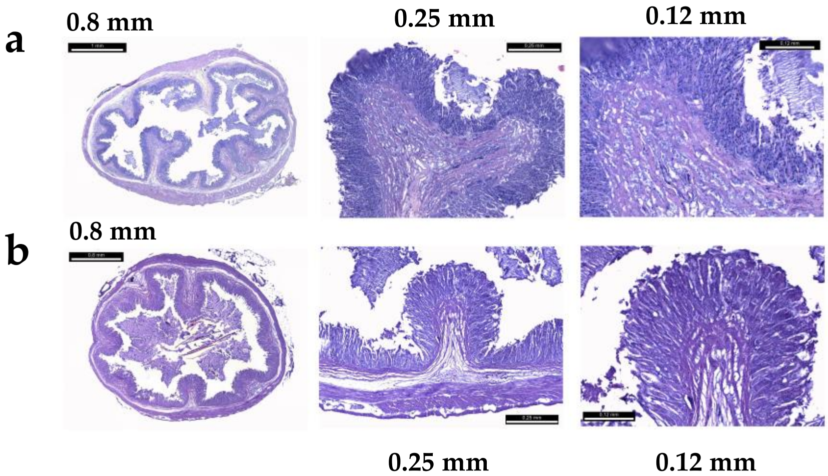

Figure 5. Stomach histological analyses of the fish feed with the experimental diets : (**a**) fish fed with the control meal; (**b**) fish fed with the innovative meal.

3. Discussion

The gradual increase in environmental eutrophication resulting from mariculture effluents represents a major issue imposing an urgent need to mitigate this negative impact to the marine ecosystem [24,25]. Bioremediation represents a valid solution, particularly in integrated mult-trophic aquaculture systems, where bioremediator organisms are employed providing the final self-purification [5], and transforming the wastes into useful biomass [2]. In the present study, we utilized an integrated bioremediation approach to realize an IMTA system involving polychaetes and macroalgae reared/cultivated in association with fish cages, achieving for both organisms consistent amounts of biomass, and opening several other interesting new horizons. From the obtained results several interesting issues arose:

-Both in the case of macroalgae and polychaetes consistent amount of biomass were achieved in the aquaculture plant. The algal species selected in the present study was cultivated in the multitrophic integrated aquaculture scenario to remove nitrogen and phosphorus surplus from waste and at the same time to become a valuable by-product of bioremediation. This species already showed to be an effective bioremediator in integrated cultivation systems with fish and crustacean, since the nutrient concentration in the surrounding environment resulted considerably reduced [26,27]. In the here realized IMTA system, the cultivation of *C. linum* resulted in a good biomass increase reaching about a 5% SGR. This result is noteworthy leading to suggest that this seaweed biomass could be employed as a potential source of nutritionally beneficial compounds for animal and human consumption as well as new dietary supplements. This is supported by the results obtained from the algal biochemical analyses on the total lipid and fatty acids content revealing a concentration of lipids corresponding to 9.4 ±2.4 mg/g DW with an interesting content of PUFAs. In particular, ω-3 and ω-6 fatty acids, which were mainly represented by oleic, linoleic, linolenic, docosahexaenoic (DHA), eicosapentaenoic acid (EPA), and arachidonic (ARA) acids. This issue is remarkable since fish oil, used for the formulation of fish feeds, is the most common and major source of the ω-3 fatty acids EPA and DHA making the fish more beneficial for consumers. However, since fish resource is decreasing whilst fish oil price is increasing as well as the commercial interest in these long chain fatty acids [28,29], there is an urgent need for an alternative source of essential PUFAs. In this regard, some farmers are opting to use cheaper alternatives with a high essential fatty acid content, for example vegetable oil, cotton seed oil and sun-flower oil. These substitutes, however, often result poor in terms of ω-3 EPA and DHA. By contrast, *C. linum* could be an optimal alternative as a source of these PUFAs. The commercial production of DHA and EPA from algae became a lively business in the last part of the 20th century due to the increase in awareness of their benefits for human health. In particular, DHA showed beneficial effects on preventing human cardiovascular diseases, cancer, schizophrenia, and Alzheimer's disease [30]. Moreover, this ω-3 fatty acids, as well as arachidonic acid, are necessary in the growth and functional development of the brain and the maintenance of the normal brain function in adults. Mammals are unable to produce them and consequently they must be supplied as food supplement, considering that an insufficient fatty acid consumption is the major cause of human chronic diseases.

The experience of rearing polychaetes in IMTA also gave encouraging results, which showed that a huge biomass can be achieved and thus suggesting the use of *S. spallanzanii* in fortified fish foods. The slight mortality recorded coupled with the high increase in volume and biomass of the reared polychaetes, demonstrated the feasibility of co-rearing *S. spallanzanii* with low costs of production and high profits. As a consequence, if the acquired technology is applied to a medium aquaculture farm, the produced polychaete biomass, coupled to algal and fish biomass, might reach values in the order of several tons/year in a completely non-fed culturing system. In addition, a reduction of the environmental impact due to aquaculture activity is accomplished on account of the well-known bioremediation capabilities of *S. spallanzanii* [7,31]. On the other hand, the capability of this polychaete to remove several bacterial groups from fish farm waste and the bioremediation service associated to the waste conversion into polychaete protein-rich biomass of potentially marketable value, had been already stated by Stabili et al. [32]. From the present study it can be inferred that the use of the here investigated invertebrate might ensure not only protein but also lipid and fatty acid supply for fish feeds. Obtained data indeed evidenced an interesting composition also in terms of fatty acids profile. In particular, long-chain polyunsaturated ω-3 fatty acids accounted for 5.6% of PUFAs and long-chain polyunsaturated ω-6 fatty acids for 9.9%. In addition, the presence of the essential α-linolenic acid (18:3 ω3) in the investigated worm is noteworthy not only for fish nutrition but also for humans eating fish potentially nourished with these polychaetes as supplements, since neither fish nor humans are able to synthesize this acid [33]. Moreover, *C. linum* if added to *S. spallanzanii* as a supplement, could represent a novel resource providing α-linolenic acid (18:3 ω6) but especially α-linoleic acid, since up to now the main known sources for these fatty acids are fish and vegetable oils. In addition, the profiles of both the here considered marine organisms regarding the ω-3 and ω-6 acids are interesting also in the light of the World Health Organization's recommendation indicating that ω-6:ω-3 ratio should not be higher than 10 in the human diet [34]. *Chaetomorpha linum* and *S. spallanzanii* showed a low ω-6/ω-3 ratio.

Even though the total lipid content is usually low in macroalgae, their employment as an ingredient in aquacultural feeds is well established on account of their high proportion of PUFAs, combined with other interesting secondary metabolites (e.g., polysaccharides, vitamins, proteins) [35–37]. In the present research, as new dietary supplements, *C. linum* was carefully mixed with *S. spallanzanii (*10% of polychaetes and 5% of algae) as new dietary supplements for the formulation of an innovative feed for farmed juveniles of seabass *D. labrax*. The analysis of the productive indexes obtained from the growth trials showed that:

-During both the trials, the physico-chemical parameters of water were in the normal range for optimal fish growth showing that the innovative feed didn't affect negatively the rearing conditions;

- Employing the innovative meal, the survival rate was 96 ± 2% with the lowest sized fish and 94 ± 2%. with the highest sized fish. Being these values higher than 80%, our results can be considered excellent in nursery operation [38]. Thus, our data indicate that the innovative meal did not affect the survival of treated fish and that the fish rearing was carefully managed. Moreover, high values of survival rate indicate that all fish had equal access to feed and uniform growth rates were achieved as demonstrated by the obtained indices of weight and length increase;

-In both the trials no statistical differences were evidenced in biomass gain and specific growth rate between fishes nourished with the control and the innovative meal. These results are of particular interest since demonstrated that a partial replacement of fishmeal with 10% of polychaete meal and 5% of algal meal does not produce negative effects on the fish growth. This result is in line with other studies showing positive effects on growth, feed utilization, lipid metabolism, liver function, body composition, stress responses and disease resistance determined by the addition of even small amounts of several algal-based meals to fish diets [39]. Moreover, the polychaete *S. spallanzanii*, due to its already investigated high protein content with noble amino acids [15] presumably contributed to improve palatability for the farmed fish species. This represents an added value in the hypothesis of fishmeal replacement since decrease of fish meal, and/or fish oil, can lead to a decrease in palatability in diets with, as an example, an increased vegetal content. The decrease of palatability can lead to a decrease of feed uptake, making these feeds less effective for fish growth and health;

- No histological alterations were observed in the stomach of fish fed with innovative meal and the conditions of stomach mucosa were comparable with those of fish fed with the control feed. These results indicated that the experimental feed containing polychaete and seaweed meal was comparable to a traditionally employed fishmeal with potential applicative relapses.

4. Materials and Methods

4.1. Species Sampling

As regards macroalgae (Figure 6), the selected species was *Chaetomorpha linum* (Chlorophyta, Cladophorales) characterised by filamentous, uniseriate, unbranched thalli, of a pale bright green colour, 200-1000 μm wide and from 10 cm up to several metres long, commonly found both in the attached and in the unattached habitus (Figure 6a). Thalli of *C. linum* were hand-collected by means of a rake in the Mar Piccolo of Taranto (Mediterranean Sea, Ionian Sea, Italy) (Figure 6b) where it can make very thick drifting mattresses, in the period late autumn-late spring, with highly variable biomass values throughout the year.

Figure 6. *Chaetomorpha linum* from the Mar Piccolo of Taranto: (**a**) thalli; (**b**) hand-collection.

Specimens of *S. spallanzanii* were obtained from the natural recruitment on plastic nets used as collectors placed in the fish farm located in the Gulf of Taranto (Mediterranean Sea, Ionian Sea, Italy) (Figure 3a). Both macroalgae and worms were randomly divided in three sets The first set was placed around the fish cages in the realized IMTA system for bioremediation purposes, the second set was employed for the biochemical analyses and the third set was used for the preparation of the innovative fish feed.

4.2. Rearing/Cultivation of Bioremediators in IMTA System

In order to obtain high algal biomass, *C. linum* cultivation trials were realized with the first set of *C. linum*, in an IMTA system equipped with fish cages within the framework of the Remedia-Life Project (LIFE16 ENV/IT/000343). The collected seaweeds were transferred to the aquaculture farm to set up the cultivation sockets, each consisting in seaweeds enclosed into a net sack and hung with a festoon arrangement at 1 m of depth within a mussel long-line system located around 6 fish cages (Figures 1,2). A total of 252 cultivation sockets were allocated in the plant, hang for 6 months until seaweed reached the highest biomass, after that the surplus was collected. Later, since thalli fragmentation was observed, followed by a rapid decay, cultivation was stopped. Seaweed biomass growth was measured according to the standard formulation of Specific Growth Rate (SGR) for seaweeds as:

[(Wt/W0)1/t-1]x100% [40].

Polychaetes employed for the bioremediation purpose, as already specified, were obtained from the natural recruitment on plastic nets used as collectors immersed in the fish farm (Figure 3). A total of 252 collectors were placed around the fish cages suspended at about 12 m depth. The worm biomass was measured after 3 and 6 months of permanence in the realized IMTA system.

4.3. Total Lipid and Fatty Acid Analysis

The second set of macroalgae and polychaetes was transported to the laboratory under refrigeration. Here, macroalgal thalli were placed into suitable tanks, cleaned of possible epibionts and detritus, rinsed with seawater and then with a 8‰ sterile physiological solution. Macroalgae were then placed in a stove at a temperature of 50 °C for 24 h and subsequently were ground with the help of a mortar and a pestle. All the analyses were done with ground algal tissues. Afterwards, biochemical analyses including total lipids and fatty acids determination were carried out.

Animals were rinsed with seawater filtered with 0.45 μm Millipore filters to remove any possible epibionts and then extracted from their tubes, homogenized in a Polytron (Kinematica

Type PT/10/35), stored at −80°C until use and then employed for the biochemical analyses including total lipids and fatty acids evaluation.

All reagents and solvents (analytical grade) were acquired from Sigma (Sigma–Aldrich GmbH, Steinheim, Germany). Total lipids from macroalgae and polychaetes were extracted in accordance with the method described in Folch et al. [41]. All the samples (dry macroalgal tissues or polychaete homogenized tissues) were extracted with methanol/chloroform/water (1/2/1) in order to obtain a final volume 20 times the sample volume. Lipids were obtained after centrifugation and removal of the upper phase and collection of the lower chloroform phase. The evaluation of the total lipid content was obtained by a colorimetric enzymatic assay [42] employing a commercial kit (FAR, Verona, Italy).

The fatty acids composition of macroalgae and polychaetes was determined in accordance with the method described in Budge and Parrish [43]. Briefly, the fatty acids (FAs) of total lipids were transesterified to methyl esters as described by Stabili et al. [13,44]. The samples were cooled, and after the addition of 1 mL of distilled water, shaken vigorously. Fatty acid methyl esters (FAMEs) were collected in the upper benzene phase. Benzene phase was moved to a vial and subjected to dry by using a nitrogen stream at a very slow applied flow rate to avoid the loss of the sample. Gas chromatography using an HP 6890 series GC (Hewlett Packard, Wilmington, DE, USA) equipped with flame ionization detector was employed to perform the analyses of sample FAME extracts. In order to separate the FAMEs an Omegawax 250 capillary column (Supelco, Bellafonte, PA, USA) (30 mm long, 0.25-mm internal diameter, and 0.25-mm film thickness) was utilized. The column temperature program was the following: 150–250°C at 4°C/min. and then held at 250°C. The retention times of known standards (FAME mix, Supelco-USA) were used to attain the right FAMEs identification The results were indicated as percentages of total identified methyl ester fatty acids. The employed gas carrier was Helium at a flow of 1 mL/min. The injected volume was 1 mL.

All assays were conducted in replicate samples of the macroalgae and worms.

4.4. Experimental Feed Fish Formulation and Proximate Composition

The third set of macroalgae and polychaetes was placed on aluminium foils and dried in oven at t 60 °C for 48 h. Dried material was then ground to obtain algal (AS) and polychaete (PS) powder to be used as supplement to prepare the experimental fish feed. For this purpose, oil and dry ingredients were thoroughly mixed whereby water was then blended into the mixture to attain an appropriate consistency for pelleting using a meat grinder. Pellets were dried overnight at 50 °C and refrigerated at 6 °C until utilization. Two feeds were prepared: a control fishmeal based feed (CTRL) and an innovative feed (innovative meal = IM) containing 10% of polychaete supplement (PS) and 5% of algae supplement (AS).

The prepared feeds (CTRL and IM) were also analysed (n= 3) to determine their proximate composition in accordance with standard methods [45].

The gross energy (GE) content was determined by an adiabatic calorimetric bomb (IKA C7000, Staufen, Germany). Total nitrogen content was determined in accordance with the Dumas method, using a nitrogen analyzer (Rapid N III, Elementar Analysensysteme GmbH, Hanau, Germany). The crude protein was calculated as total N × 6.25.

4.5. Fish Growth Trials

The innovative feed as well as the control feed were utilized for feeding juveniles of European sea bass *Dicentrarchus labrax*. The study was carried out between May 2019 and September 2019 for 4 months in experimental tanks at the fish farm. In a first trial, juveniles of *D. labrax* at 36 days of age with an initial mean body weight of 0.05 ± 0.001 g were randomly stocked (200 fish for tank) in 6 fibreglass tanks (80 L) supplied by an open water circuit: three tanks for the control feed (CTRL) and three tanks for the innovative feed (IM). The first trial lasted 60 days from May 2019 to July 2019. Fish were singly weighted after 30, 45 and 60 days, in order to check the fish biomass gain. The fish were fed to satiation by hand twice a day 7 days per week. Temperature and dissolved oxygen were determined daily in the morning and in the afternoon with a digital oximeter (YSI 55 Hexis).

The same procedures described above were also utilized in a second trial, which lasted 30 days from August 2019 to September 2019. In this case, *D. labrax* juveniles at 153 days of age with an initial mean body fish weight of 2.00 ± 0.001 g were employed.

At the end of both experimentation trials, survival rate (%), biomass growth, specific growth rate, and coefficient of variation for length were evaluated with the following formulas [46]:

Survival rate (%) = (number of fish at the end/number of fish at the beginning) x 100;

Biomass gain (g) = final individual weight – initial individual weight;

Specific Growth Rate (%) = (ln final weight – ln initial weight) x 100/feeding days.

4.6. Fish Histological Analyses

In order to control the histology of the stomach tunica mucosa and tunica submucosa, six fish for each experimental tank (CTRL and IM) were sampled at the beginning and at the end of the second trial. The stomachs were excised from fish and samples were fixed in buffered formalin in order to assess eventual alterations induced by the experimental diet. After dehydration and embedding in paraffin wax following standard histological techniques, the histological samples were cut (5 μm thickness) by a microtome and stained with Haematoxylin/Eosin after 24 h in a laboratory thermostat.

4.7. Statistical Analysis

Analysis of variance ANOVA-1-way was used to test for differences in the weight, length, survival rate, biomass gain and specific growth rate between fish fed with the innovative feed and fish fed with the control feed. All the analyses were performed by using GMAV 5 computer programme (University of Sidney, Australia).

5. Conclusions

The rearing/cultivation of *Sabella spallanzanii* and *Chaetomorpha linum* in the realized IMTA system allowed to restore the farming environmental quality and, at the same time, to obtain as a by-product, high biomass of both macroalgae and polychaetes intended to be utilized as sources of functional ingredients such as fatty acids beneficial to fish and human health. On the basis of our results from the experimental feeding trials on *Dicentrarchus labrax* juveniles, we can conclude that the prepared innovative feed containing polychaetes and algae as supplements represents a potential meal for the European seabass practical diets. The employment of the examined species as dietary supplement in fish feed can contribute to overcome the major concerns over fish as a declining resource and the consequent rising cost of fish feeds worldwide. Work is ongoing to explore some of these issues in detail.

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