water

MDPI

Article

Effects of Microalgae Grown in Membrane Treated Distillery Wastewater as Diet on Growth and Survival Rate of Juvenile Pearl Oyster (*Pinctada fucata martensii*)

David Kwame Amenorfenyo, Feng Li *, Yulei Zhang, Changling Li, Ning Zhang and Xianghu Huang *

College of Fisheries, Guangdong Ocean University, Zhanjiang 524088, China
* Correspondence: lifeng2318@gdou.edu.cn (F.L.); huangxh@gdou.edu.cn (X.H.)

Abstract: Microalgae serve as feedstock for bivalves and larvae in aquaculture. The production of microalgae in large quantities is, however, characterized by the high cost of major nutrients and vitamins and scarcity of freshwater. Wastewater is a cheap alternative medium for microalgae cultivation. The wastewater provides essential nutrients for microalgae growth and biomass production. This study examined the biomass productivity, nutrient removal, and biochemical content of *Chlorella vulgaris*, *Spiruna platensis*, and *Haematococcus pluvialis* biomass cultivated in membrane treated distillery wastewater (MTDW). The study further examined the use of cultivated biomass as a diet to analyze the growth and survival rate of *Pinctada fucata martensii*. The results showed 79.61% and 82.89%, and 41.73% of Total Nitrogen (TN) and 74.95%, 78.21%, and 29.05% of Total Phosphorus (TP) removal efficiency for *C. vulgaris*, *S. platensis*, and *H. pluvialis* respectively. Biomass productivity of 0.069 g L^{-1}, 0.086 g L^{-1}, and 0.057 g L^{-1}, 43.3%, 40.9%, and 34.9% (protein), 10.3%, 14.5%, and 13.8% (lipid), and 16.4%, 14.8%, and 20.8% (carbohydrate) for *C. vulgaris*, *S. platensis*, and *H. pluvialis* respectively. The specific growth rate and survival rate of pearl oysters were significantly ($p < 0.05$) higher (0.99 ± 0.12%, 87.3%) under *C. vulgaris* diet compared to *S. platensis* and *H. pluvialis* diets.

Keywords: wastewater treatment; microalgae; *Chlorella*; distiller wastewater; diet

Citation: Amenorfenyo, D.A.; Li, F.; Zhang, Y.; Li, C.; Zhang, N.; Huang, X. Effects of Microalgae Grown in Membrane Treated Distillery Wastewater as Diet on Growth and Survival Rate of Juvenile Pearl Oyster (*Pinctada fucata martensii*). *Water* **2022**, *14*, 2702. https://doi.org/10.3390/w14172702

Academic Editor: Antonio Zuorro

Received: 15 July 2022
Accepted: 28 August 2022
Published: 30 August 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

World aquaculture is expected to dramatically increase in the upcoming decade due to shortages in wild fish populations. As aquaculture becomes more popular, the need for feed will also, increase. Algae can serve as an optimal feed for various forms of aquaculture, including fish, crustaceans, and molluscs. Microalgae play a significant role in aquaculture. Algae are at the base of the aquatic food chain and food resources that fish eat are produced [1]. The use of microalgae feed in aquaculture provides essential benefits such as nutrients, color enhancement and biological activities, [2]. Due to their valuable contents, e.g., carotenoid and nutrients (protein, carbohydrate, vitamins, and fatty acid), microalgae are serving as a possible replacement for fishmeal in aquafeed. Microalgae also provide disease resistance and immunostimulant to aquatic animals. Microalgal species of the genera *Chlorella*, *Haematococcus*, *Schizochytrium*, *Isochrysis*, *Nannochloropsis*, *Arthrospira*, *Dunaliella*, and *Pavlova* are usually used as feedstock in aquaculture [3]. However, microalgae species for aqua feed must be non-toxic to both humans and fish, and more especially must have high nutritional values.

For microalgae to be used as a fish feed ingredient, it must be toxic free and should be easily cultured, and have high nutritional value with a digestible cell wall [4]. Cultivation of microalgae in large quantity may however be challenged by the high cost of

nutrients and as well as fresh water. Kadir et al. [5] suggested the cultivation of microalgae in wastewater as an alternative to overcoming the high cost of microalgae cultivation. According to Slade and Bauen [6], the cultivation of microalgae in wastewater could reduce production costs by more than 50%.

Cultivation of microalgae in wastewater provides not only valuable biomass but also served as wastewater treatment agent through absorption of nutrients [7]. Production of large-scale wastewater with its associated quantity of nutrients loads may lead to contamination of water bodies, and algal bloom. These affect human health and other recreational activities [8]. These have resulted to strict regulations to control the level of nitrogen, phosphorus and other organic loads in treated water [9,10].

The bioethanol industry generates large volume of wastewater that is made of high chemical (COD: 60–134 g L^{-1}) and biochemical (BOD: 16–96 g $L^{-1)}$, acidic (pH = 3–5) with average content potassium, nitrogen and phosphorus equal to 2–17.5 g L^{-1}, 0.55–4.2 g L^{-1} and 0.13–3.03 g L^{-1} respectively [11]. The utilization of microalgae as a pollutant removal agent as well as their convection into useful biomass is considered as one of the promising technologies for water remediation [12–14]. Liu et al. [15] examined the lipid content of *C. zofingiensis* for biofuel production by using sugarcane molasses as a carbon source. Furthermore, in 2019, Quintero-Dallos et al. [10] evaluated the growth of *C. vulgaris* UTEX 1803 by using vinasse as the growth medium.

Several works have been reported on microalgae cultivation in wastewater as a treatment agent and the application of its biomass, particularly in the area of biofuel production. However, to the best of our knowledge, no work has been done on its application in aquaculture as feedstock. Hence, this study aimed to investigate the biomass production, nutrient removal efficiency, and biochemical content of three microalgae cultivated on membrane-treated distillery wastewater and the influence of the biomass on the growth and survival of juvenile pearl oysters.

2. Materials and Methods

2.1. Microalgae Collection and Growth Conditions

The three freshwater algae *H. pluvialis S*, *platensis*, *and C. vulgaris* were acquired from Fisheries College, Guangdong Ocean University, Zhanjiang. The vegetative cells were pre-cultured photoautrophically under white light at 2000 lx for 7-days. The pre-cultured algal cells were then inoculated into 1 L BG11 medium in 3 L Erlenmeyer flasks for nine (9) days at 0.2 (OD_{680}) optical density in an illuminated incubator at 2500 lx and 25 °C under 12:12 h light/dark photoperiod. BG11 medium was purchased from Qingdao Hope Bio-Technology Co., Ltd., and its main components are as follows: $NaNO_3$ 1.5 g L^{-1}, $K_2HPO_4{\cdot}3H_2O$ 40 mg L^{-1}, $MgSO_4{\cdot}7H_2O$ 75 mg L^{-1}, $CaCl_2{\cdot}2H_2O$ 36 mg L^{-1}, $C_6H_8O{\cdot}H_2O$ 6 mg L^{-1}, Na_2EDTA, 1 mg L^{-1}, Na_2CO_3, 20 mg L^{-1}, H_3BO_3, 2.86 mg L^{-1}, $MnCl_2{\cdot}H_2O$ 1.81 mg L^{-1}, $ZnSO_4{\cdot}7H_2O$, 0.222 mg L^{-1}, $CuSO_4{\cdot}5H_2O$, 0.079 mg L^{-1}, $Na_2MoO_4{\cdot}2H_2O$, 0.39 mg L^{-1}, and $CoCl_2{\cdot}6H_2O$, 0.049 mg L^{-1}. For the preparation of BG11 working medium, weigh 1.70 g of the solid mixture, dissolve it in 1000 mL of distilled water by heating, and then sterilize it at 121 °C for 15 min.

2.1.1. Collection and Preparation of Wastewater

The membrane treated distillery wastewater (MTDw) contains Na^+ 269 mg L^{-1}, K^+ 1020 mg L^{-1}, Ca^{2+} 0.01 mg L^{-1}, Mg^{2+} 18.8 mg L^{-1}, Fe^{3+} 1.80 mg L^{-1}, As^{3+} 0.03 mg L^{-1}, B^- 0.3 mg L^{-1}, Mo^{2-} 0.07 mg L^{-1}, Pb^{2+} 0.03 mg L^{-1}, and Cu^{2+} 0.18 mg L^{-1}, which was collected from SDIC Guangdong Bio-Energy Company Limited, Zhanjiang, South China. The wastewater sample was collected about 7 am (Beijing time) in plastic container that was pre-washed with MTDw. The MTDw sample was stored at 4 °C to reduce the decomposition of a substrate before the wastewater characteristics analysis. The sample was then filtered by the aid of glass-microfiber Whatman filter (934-AH, 1.5 μm) to eliminate

turbidity and other particles and autoclaved for 30 min at 121 °C to remove algal growth inhibitors and bacteria.

2.1.2. Wastewater Nutrient Analysis and Removal Efficiency

Briefly, 10 mL of all samples were filtered using 0.22μm, Whatman filter paper and analyzed for total nitrogen (TN), and total phosphorus (TP). TN and TP were determined on the day of inoculation and every three days. The acid-persulfate digestion method and persulfate digestion method were used to analyze TP and TN respectively.

Nutrient removal efficiency (%) was calculated by the following Equation (1).

$$Nutrient\ removal\ efficicency = \frac{(C_0 - C_1)}{C_0} \times 100\% \quad (1)$$

where C_0 and C_1 = the nutrient concentrations of the influent and the effluent respectively.

2.1.3. Determination of Dry Weight

The Dry weight (DW) of algal biomass was measured every 3 days. The DW was determined by filtering 10 mL samples of the algal suspension through pre-weighed (m_1) filters (47 mm, 1.2 μm, Whatman). The filtered biomass was later dried overnight at 105 °C to a constant weight and weighed using microbalance (m_2). The DW (g L^{-1}) of the biomass was calculated as expressed in Equation (2).

$$DW = (m_2 - m_1) \times 10^3/10 \quad (2)$$

Biomass productivity (g L^{-1} day^{-1}) was calculated with Equation (3).

$$Biomass\ productivity = (DW_i - DW_0)/(t_i - t_0) \quad (3)$$

where DW_i and DW_0 represent the dry biomass (g L^{-1}) at time t_i and t_0 (day).

2.1.4. Carbohydrate, Protein and Lipid Quantification

According to Barbarino and Lourenço [16] and Ge et al. [17], a modified method was used to determine protein extracts of algal biomass. Thereby, 8 mL of distilled water was added to 30 mg powder of the three microalgae under study, soaked for 12 h, and centrifuged at 15,000 rpm (4 °C) for 20 min to collect the supernatant. The concentrated pellets were reextracted by adding 2.0 mL 0.1N NaOH and after centrifugation (15,000 rpm, 21 °C) for 20 min. The collected supernatant was mixed with the previous supernatant, and then extract (10 mL) was taken to measure protein concentration with Bio-Rad DC protein assay (Cat. 500–0111, Bio-Rad Laboratories, Hercules, CA, USA). The anthrone colourimetric method was employed to test the carbohydrate content and protein content of the supernatants with a Hach model DR 2800 spectrophotometer. Glucose (Stock—100mg of glucose was dissolved in 100mL distilled water and 10mL of the stock diluted to 100mL for working stock) and serum albumin [was formulated at 2 mg/mL in an ultrapure 0.9% sodium chloride (saline) solution] were utilized as the standard for testing carbohydrate and protein respectively.

The lipid content of algal biomass was determined gravimetrically. In brief, centrifugation was conducted to harvest algal suspension at 4 °C 5000 rpm, for 10 min then the harvested algal suspension was double-washed with distilled water, then oven-dried (60 °C) overnight. 0.1 g freeze-dried biomass was placed in 3 mL distilled water, vortex for 30 s at 3000 rpm, and immersed in a water bath (at 90 °C) for 20 min. Methanol/chloroform (1:2 *v*/*v*) was added at room temperature, the lipid content was extracted overnight then 1 mL of distilled water was added. After centrifugation (20 °C, 10 min), the organic phase was collected and transferred into a pre-weighed dish. The chloroform was evaporated at 50 °C, and extracted lipid was analyzed gravimetrically [18].

2.1.5. FAMEs Test and Amino Acids Determination

The sample was filtered and washed with 10 mL of methanol after 0.1 g of wet algal biomass was hydrolyzed and methylated with 2mL of 100% acetyl chloride in 20 mL of methanol solution at 90 °C. A rotary evaporator was used to evaporate the methanol after which 10 mL of hexane was added and vortex for 5 min. The hexane layer was pipetted and evaporated then the recovered FAME was analyzed using gas chromatography with flame-ionization detector (GC-2030, Shimadzu, Japan) by using an RT-2560 column (Shimadzu, Japan). Helium was used as a carrier gas at 260 °C for the injector and detector. The FAME peaks in the samples were identified by comparing their retention times with those of the standards (Supelco TM 37 component FAME mix, Sigma-Aldrich).

Eighteen amino acids were quantified using HPLC System S433 (Sykam, Eresing, Germany). Briefly, 100 mg of freeze-dried biomass was re-suspended in 5 mL 1 N HCl with 1% (*w*/*v*) phenol then washed for 1 min with nitrogen. The probes were evaporated and dissolved in a sample solution buffer. Then injected on a cation separation column (4.6 × 150 mm, LCA K06/Na; Sykam, Eresing, Germany) and were detected at the wavelength of 440 nm and at 570 nm after the solutions were dried (110 °C for 24 h) and filtered. 60 min analysis cycle time at a flow rate of 0.45 mL min^{-1}, and 0.25 mL min^{-1} analyses conditions were run for buffer and ninhydrin respectively. The content of amino acids of algal biomass is stated as the summed content of proline (Pro), leucine (Leu), valine (Val), lysine (Lys), phenylalanine (Phe), tyrosine (Tyr), methionine (Met), threonine (Thr), and cysteine (Cys), aspartic acid (Asp), tryptophan (Try), alanine (Ala), arginine (Arg), glutamic acid (Glu), histidine (His), glycine (Gly), isoleucine (Ile), and serine (Ser). Due to acidic hydrolysis, the asparagine as well as aspartic acid content, and the glutamic acid content are provided as the summed content of both amino acids.

2.1.6. Experimental Diet and Feeding Procedures

Juvenile pearl oysters (*Pinctada fucata martensii*) were purchased from Breeding Base of Zhanjiang Fenglian Aquatic Products Co., Ltd., Houhong Village, Tandou Town, Leizhou City and were cultivated in natural seawater obtained from the South China Sea, Zhanjiang in tanks with erected mesh nets. The following water parameters were recorded: dissolved oxygen at 5.00 mg L^{-1}, the temperature at 27.5–29.5 °C, and salinity at 30‰ as described in [19]. Pearl oysters 29.39 ± 1.15 mm in mean shell Length (SL) were randomly sorted and assigned to three microalgae: *C. vulgaris*, *S. platensis*, and *H. pluvialis*. The pre-inoculated microalgae were grown in MTWD with 40% Vinoculation/Vmedia (OD_{680} = 0.6) under the same growth condition (25 °C, 2500 lx, 12:12 h light/dark photoperiod) for 9 days in batches to meet 60 days feeding-dose requirement. The experiment was conducted in three tanks for each group with each tank containing 100 pearl oysters with 1000 L water volume. The pearl oysters were fed every 4 h with 100 mL algal/media (3L) volume. Further, 500 L of water was replaced every morning for 60 days.

2.1.7. Survival Rate and Growth Rate

At the beginning and end of the experiment, the total number and growth performance of the pearl oysters in each replicate were determined. Shell length (SL) and shell width (SW) were measured with a digital caliper (0.01 mm accuracy). The total weight (TW) was obtained with an electronic balance (0.01 g accuracy), and survival rates were calculated according to [19]. The weight gain (WG, %), specific growth rate (SGR, %/days), and survival rate (SR, %) of pearl oysters were calculated at the end of the cultivation period using Equations (4)–(6), respectively.

$$WG = \left[100 \times \frac{(Final\ weight - Initial\ weight)}{Initial\ weight}\right] \quad (4)$$

$$SGR = \left[100 \times \frac{(Ln\ final\ weight - Ln\ initial\ weight)}{Test\ days}\right] \quad (5)$$

$$SR = \left[100 \times \frac{Final\ number\ of\ pearl\ oysters}{Initial\ number\ of\ pearl\ oysters}\right] \quad (6)$$

where, *Ln* = natural logarithm.

3. Results

3.1. Biomass Production and Nutrient Removal Efficiency

With initial biomass concentration (DW), coupled with growth conditions of this experiment, S. *platensis* showed higher daily biomass productivity of 0.086 ± 0.001 g L^{-1} day^{-1} than *C. vulgaris* and *H. pluvialis*, with 0.069 ± 0.012 g L^{-1} day^{-1} and 0.057 ± 0.006 g L^{-1} day^{-1}, respectively (Table 1). This demonstrates that the daily biomass productivity of *S. platensis* was significantly ($p > 0.05$) higher than *C. vulgaris* and *H. pluvialis*. However, *C. vulgaris* demonstrated higher biomass productivity than what was obtained in a study reported by Cho et al. [20] using autoclaved secondary treated wastewater.

The nutrient removal efficiency of TN and TP by *S. platensis* was significantly higher than *C. vulgaris* and *H. pluvialis*. *S. platensis* could remove as high as 82.89% of TN and up to 78.21% of TP, whiles *C. vulgaris* and *H. pluvialis* could remove up to 79.61% (TN) and 74.95% (TP), and 41.73% (TN) and 29.05% (TP) respectively. However, considering the initial biomass concentration of *S. platensis*, TP removal efficiency by *S. platensis* could no longer be significant compared to the removal efficiency by *C. vulgaris.* This indicates that nutrient removal rate and biomass productivity by *C. vulgaris* could perform better than *S. platensis* with the same biomass seeding density.

Table 1. Biomass production and TN & TP removal rates for *C. vulgaris* (CV), *S. platensis* (SP), and *H. pluvialis* (HP) during the cultivation period. Values within columns followed by different letters are significantly different ($p < 0.05$, Duncan's test). Standard deviation of the mean was calculated based on $n = 3$ (CV, SP & HP).

	Initial Density (g L^{-1})	Biomass Productivity (g L^{-1} day^{-1})	TN Removal Efficiency (%)	TP Removal Efficiency (%)
CV	0.031	0.069 ± 0.012^{b}	79.61 ± 2.61^{b}	74.95 ± 4.58^{b}
SP	0.046	0.086 ± 0.001^{c}	82.89 ± 0.35^{c}	78.21 ± 1.80^{c}
HP	0.038	0.057 ± 0.006^{a}	41.73 ± 2.09^{a}	29.05 ± 3.75^{a}

3.2. Biochemical Composition of the Microalgae

3.2.1. Protein, Carbohydrate and Lipid

At the end of 9-day culture, Figure 1 shows the biochemical composition of three microalgal biomass studied in this work. The study showed that protein content was the main composition of the three microalgal biomass.

C vulgaris showed higher protein content (<45%) compared to *S. platensis* and *H. pluvialis*. *C. vulgaris* showed higher carbohydrate content compared to *S. platensis* and *H. pluvialis* with 20.8%,16.4%, and 18.4% of DW, respectively. The lipid content of *S. platensis* (14.5%) was significantly ($p > 0.05$) higher than that of *H. pluvialis* (13.9%) and *C. vulgaris* (10.3%) (see Figure 1).

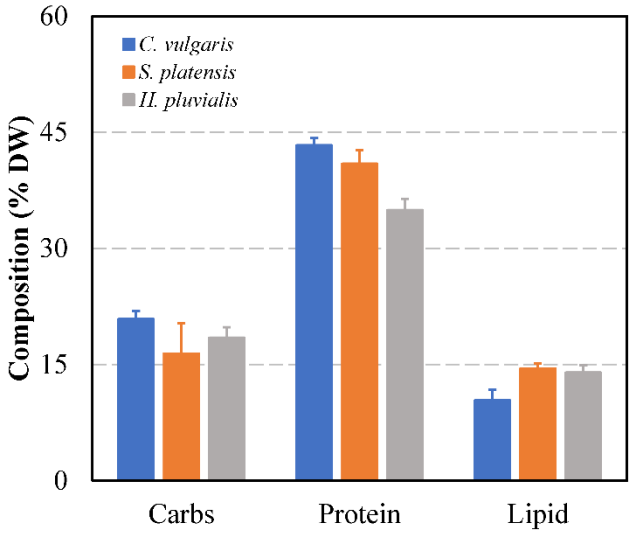

Figure 1. Biochemical compositions of cultivated microalgae.

3.2.2. Amino Acids and Fatty Acids

The amino acids content of algal biomass is shown in Table 2. Among the treatment, *S. platensis* contained highest total amino acids content with 47.9% compared to *C. vulgaris* and *H. pluvialis* with 47% and 15% respectively. Though *S. platensis* showed higher amino acids content compared to *C. vulgaris* and *H. pluvialis*, the essential amino acids (EAAs) content of *C. vulgaris* (18.5%) was higher compared to *S. platensis* (17.5%), with the lowest recorded by *H. pluvialis* (6.2%). *S. platensis* obtained 30.4% (highest) total non-essential amino acids (NAAs) content followed by *C. vulgaris* with 24%. *H. pluvialis* obtained 8.9% NEAAs, representing the lowest.

Table 2. Amino acids compositions and contents (g/100 g dry biomass) of cultivated microalgae.

	C. vulgaris	***S. platensis***	***H. pluvialis***
Pro	1.2 ± 0.46	3.6 ± 1.04	0.2 ± 0.63
Leucine *	1.4 ± 0.24	2.6 ± 0.59	1.9 ± 0.95
Valine *	2.3 ± 0.30	3.0 ± 0.23	0.9 ± 0.13
Lysine *	3.0 ± 0.72	1.4 ± 0.31	1.2 ± 0.40
Phenylalanine *	2.9 ± 0.92	3.4 ± 0.62	0.6 ± 0.45
Tyrosine	3.2 ± 0.81	3.3 ± 1.01	0.5 ± 0.04
Methionine *	2.1± 0.29	2.3 ± 0.56	0.3 ± 0.17
Threonine *	1.8 ± 0.05	2.1 ± 0.16	0.7 ± 0.33
Cysteine	0.6 ± 0.09	1.0 ± 0.04	ND
Aspartic acid	2.9 ± 0.03	0.8 ± 0.05	2.0 ± 0.04
Tryptophan	2.0 ± 0.63	0.4 ± 0.11	0.4 ± 0.15
Alanine	2.4 ± 0.40	3.1 ± 0.07	1.3 ± 0.09
Arginine	3.2 ± 0.69	4.3 ± 1.05	1.0 ± 0.03
Glutamic acid	3.8 ± 1.08	4.8 ± 0.54	2.2 ± 0.75
Histidine	2.0 ± 0.07	2.1 ± 0.09	ND
Glycine	3.9 ± 0.10	5.6 ± 1.03	1.1 ± 0.05
Isoleucine *	2.6 ± 0.98	1.0 ± 0.82	0.6 ± 0.01
Serine	1.2 ± 0.20	3.1 ± 1.08	0.2 ± 0.07

Notes: ND: not detected; *: Essential Amino Acids.

The percentage value of total unsaturated and saturated fatty acids of three microalgal biomass was displayed in Table 3. The percentage value of total saturated fatty acids of the three microalgae was less than 1%. However, *C. vulgaris* and *S. platensis* showed a little over 1% of total unsaturated fatty acids compared to *H. pluvialis* which was less than 1%.

Table 3. Fatty acids content (g/100 g dry biomass) of cultivated microalgae.

Fatty Acid	*C. vulgaris*	*S. platensis*	*H. pluvialis*
Myristic Acid (14:0)	0.122 ± 0.03	0.145 ± 0.06	0.093 ± 0.01
Palmitic Acid (16:0)	0.439 ± 0.52	0.521 ± 0.10	0.314 ± 0.93
Palmitoleic Acid (16:1)	0.015 ± 0.07	0.012 ± 0.09	0.003 ± 0.13
Oleic Acid (18:1)	0.237 ± 0.11	0.265 ± 0.23	0.157 ± 0.19
Stearic Acid (18:0)	0.024 ± 0.04	0.034 ± 0.08	0.031 ± 0.21
Linoleic Acid (18:2, ω -6)	0.481 ± 0.28	0.460 ± 0.33	0.527 ± 0.90
Arachidonic Acid (20:0)	0.033 ± 0.17	0.042 ± 0.54	0.045 ± 0.16
γ-Linolenic Acid (18:2)	0.440 ± 0.80	0.361 ± 0.39	0.198 ± 0.06
Linoelaidic acid	ND	0.001 ± 0.02	0.003 ± 0.13
Pentadecanoic acid	0.005 ± 0.12	0.003 ± 0.04	0.002 ± 0.01
Erucic Acid	ND	ND	ND
Docosadienoic Acid (22:6, ω -3)	0.020 ± 0.01	0.018 ± 0.11	0.025 ± 0.56
Eicosatrienoic Acid (20:5 ω -3)	ND	0.003 ± 0.05	0.001 ± 0.03
Total saturated	0.618	0.742	0.483
Total unsaturated	1.193	1.12	0.914

Note: ND: not detected.

3.3. Growth Performance and Survival Rate of Juvenile Pearl Oyster (Pinctada Fucata Martensii)

After 60 days of feeding, *C. vulgaris*, showed significant ($p < 0.05$) growth in terms of weight gain and SL compared to *S. platensis*, and *H. pluvialis*. As shown in Table 4, *C. vulgaris* exhibited a significantly higher specific growth rate/day of 0.99%, whilst 0.87% and 0.46% were obtained by *S. platensis*, and *H. pluvialis* respectively. The FSL of pearl oysters exhibited under *C. vulgaris* diet was significantly ($p < 0.05$) higher compared to other diets (*S. platensis* and *H. pluviasis*). Although there was no significant difference between the *S. platensis* and *H. pluvialis* diet, the *H. pluvialis* diet showed a higher FSL of pearl oysters which could be as the result of its high carbohydrate content.

Table 4. Effects of microalgae biomass on growth performance of pearl oyster (*Pinctada fucata martensii*).

Index	*C. vulgaris*	*S. platensis*	*H. pluvialis*
IW (g)	2.135	2.135	2.135
FW (g)	2.87 ± 0.103 ^{c}	2.77 ± 0.19 ^{bc}	2.45 ± 0.10 ^{a}
WG (%)	34.58 ± 4.82 ^{c}	29.89 ± 8.90 ^{bc}	14.91 ± 3.94 ^{a}
SGR(%/day)	0.99 ± 0.12 ^{c}	0.87 ± 0.23 ^{bc}	0.46 ± 0.11 ^{a}
ISL (mm)	29.39	29.39	29.39
FSL (mm)	32.64 ± 1.42 ^{c}	30.91 ± 0.93 ^{abc}	31.65 ± 0.80 ^{abc}

Notes: Values are mean ± SD ($n = 3$). Values in the same row having a common superscript are not significantly different ($p > 0.05$). Where ISL = Initial Shell Length, FSL = Final Shell Length, IW = Initial weight, FW = Final weight, WG = Weight Gain, SGR = Specific Growth Rate.

At the end of the cultivation period, the survival rates of pearl oysters in the microalgae diet ranged from 77.7% to 87.3%. The pearl oysters under *C. vulgaris* diet exhibited significantly higher survival rates compared to *S. platensis* and *H. pluvialis* diets. ($p < 0.05$,

Figure 2). Although the study observed no significant difference between *S. platensis* and *H. pluvialis* diets, the highest survival was observed under *S. platensis.*

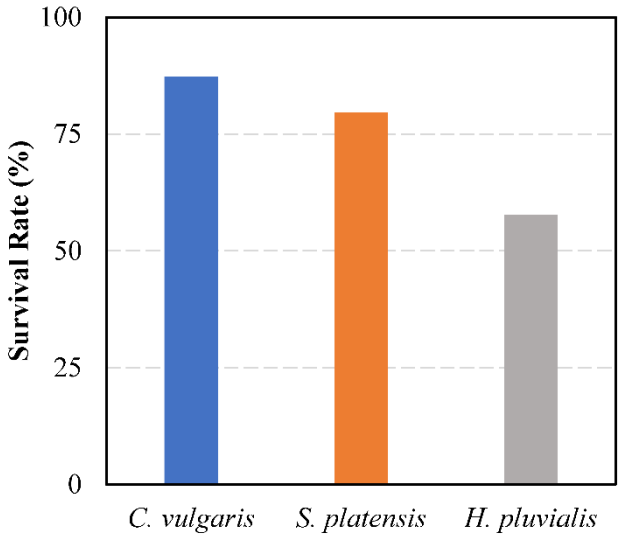

Figure 2. Effect of microalgal biomass (diet) on the survival rate of pearl oyster (*Pinctada fucata martensii*).

4. Discussion

In the last two or more decades, significant research has been conducted on the cultivation of microalgae in wastewater treatment and its remarkable effects on wastewater treatment and growth, as well as the utilization of its biomass. Wastewater, depending on the source, may contain the required amount of nutrients that support microalgae growth and biomass production. Nutrients, such as TN and TP concentration of wastewater, differ according to the wastewater type. Most microalgae absorb nitrogen in the form of ammonium [21] and phosphorus in the form of inorganic anions species such as $H_2PO_4^-$ and HPO_4^{2-} [22]. The result of the current studies showed that the two microalgae strains *C. vulgaris* and *S. plentesis*, absorb total nitrogen and total phosphorus efficiently, resulting in higher biomass productivity, which supports total nitrogen reduction of up to 1000 mg L^{-1} when the suitability of pretreated vinasse was assessed as a culture medium for *C. vulgaris* in a continuous photobioreactor [23]. In the case of *H. pluvialis* which showed relatively lower biomass productivity and TN and TP removal efficiency, carbon sources in the form of carbon dioxide (CO_2) and pH could be monitored during the growth phase to maximize biomass production [24,25]. Furthermore, the maximization of algal growth and biomass for wastewater-based algal cultivation could be largely influenced by C: N and C:P ratios [24].

Protein is considered a structural material found in plant cells that serves as the basis of an enzymatic reaction, cell growth and light-harvesting pigment [26]. The protein content of microalgae may depend on the availability of nitrogen in the culture medium and the ability of the microalgae strain to assimilate the nitrogen. The result of these studies was similar to those reported for *C. vulgaris*, *S. platensis*, and *H. pluvialis* [27]. However, Klin et al. [28] reported a decrease in protein content of green algae on the final day of culture due to depletion of nitrogen in the culture medium. It is shown from the result that the protein content of all microalgae tested in this study could be higher at the exponential growth phase than what was recorded since the samples were taken on the final day of cultivation. Nevertheless, it could not affect the trend significant among treatments. The microalgae examined in this study showed a higher protein content (26 to 35%) than known proteinaceous crops, e.g., soybean and pea (34% and 21%) [29,30]. However, the

protein content of *S. platensis* (32%) observed in this study was slightly lower than the results reported (45.31–55.15 of DW) by Lu et al. [31] when *S. platensis* was cultivated using raw piggery wastewater. Again, nitrogen content and the bioavailability of the culture medium could affect the biosynthesis of protein.

Lipids, mostly triacylglycerols, serve as the major energy component for fatty acids in eukaryotic cells. Lipids have energy that is twice the density of carbohydrates and proteins. In fish meals, for example, lipids constitute about 17–15% and can be used as a substitute for protein [32]. However, the lipid content of algal biomass (in DW) largely depends on the strain's metabolic pathways, growth phase culture, and exposure to stress conditions, such as nitrogen depletion [28,33]. In our study, the lipid content of *S. platensis* (14.5%) was significantly ($p > 0.05$) higher than that of *H. pluvialis* (13.9%) and *C. vulgaris* (10.3%), which could be the result of the strain's metabolic pathways and culture growth phase (see Figure 1). This lipid content is also lower than what was obtained (35% lipid content at stationary phase dry biomass) by Schwenk et al. [34]. Microalgae are also known as a major source of polyunsaturated fatty acids (PUFAs). Polyunsaturated fatty acids, such as DHA and EPA, play a significant role in the food chain for aquatic ecosystems [35]. In the diet of both animals and humans, a concentration of fatty acids, mainly oleic acid, are synthesized for maintaining the fluidity and prevention of cell membrane [36]. Fatty acids are also known as one of the major sources of omega 6 which is used in the treatment of skin hyomegaperplasias [37]. These valuable components from microalgae are expected to be efficiently recovered in the future through innovative treatments, such as those based on the use of enzymes [38] and natural deep eutectic solvents (NaDEs) [39].

Carbohydrate (Carbs) performs many functions in a living organism in aquaculture, for example, carbohydrate constitutes an excellent source of energy in the formulation of feed. Carbohydrate content is one of the key pivotal indexes for the assessment of algal biomass energy potential [31]. *C. vulgaris* showed higher carbohydrate content compared to *S. platensis* and *H. pluvialis* with 20.8%, 16.4% and 18.4% of DW respectively. Although the carbohydrate content of *C. vulgaris* significantly higher, it is, however, lower than those reported in previous studies [40,41]. The low carbohydrate content recorded for all treatments demonstrates high nitrogen content in the culture medium. Several studies pointed to the fact that nitrogen starvation in a culture medium increases the lipid and carbohydrate content of microalgae [42–44]. For example, *H. pluvialis* during the red stage can accumulate higher carbohydrate content when exposed to stress conditions, such as nutrient deficiency, high acidity, and temperature variations [31,45]. Although in aquaculture, carbohydrates are not an essential nutrient component of aquafeed, about 10% of starch is required in high protein formula to achieve feed buoyancy. The carbohydrate content obtained in this study could be used as a carbohydrate source in the formulation of aquafeed.

In the pearl oyster industry**,** growth performance and survival rate the significant factors in measuring effect of diet [46]. Several studies showed the effective use of algal biomass as a fish meal substitute or feed additive on the growth rate of some aquatic species [47–49]. In this study, *C. vulgaris* exhibited a significantly higher specific growth rate/day of 0.99%, whilst 0.87% and 0.46% were obtained by *S. platensis,* and *H. pluvialis* respectively. This could be a reflection of the protein/carbohydrate balance in the diet [50]. Furthermore, studies have emphasized the importance of the high carbohydrate content of microalgae for the growth of *C. virginica* juveniles [51], *Crassostrea gigas* larvae [52], and scallop (*Patinopecten yessoensis*) larvae [53]. The FSL of pearl oysters exhibited under *C. vulgaris* diet was significantly ($p < 0.05$) higher compared to other diets (*S. platensis* and *H. pluviasis*). Although there was no significant difference between the *S. platensis* and *H. pluvialis* diet, the *H. pluvialis* diet showed a higher FSL of pearl oysters, which could be the result of its high carbohydrate content.

5. Conclusions

On the basis of growth and biomass production and nutrient removal efficiency, *C. vulgaris* and *S. platensis* showed better adaptability in the culture medium than *H. pluvialis*, thereby demonstrating higher biomass productivity and nutrient removal efficiency. Similarly, *C. vulgaris* and *S. platensis* showed an optimal protein/carbohydrate balance, suggested in the aquatic diet. In addition, *C. vulgaris* and *S. platensis* diets significantly influenced the weight gain, specific growth rate, and survival rate of *Pinctada fucata martensii*.

Based on the results obtained in this study, MTDW could serve as a cheap medium for cultivating *C. vulgaris* and *S. platensis* growth and biomass production as feedstock in aquaculture. However, feasibility in the case of large-scale application requires further research regarding culture techniques to maximize biomass production and improve the removal efficiency of nutrients in wastewater. Again, a feasibility study is required to ascertain economic viability of using un-autoclave (raw) wastewater on large scale production of algal biomass. Furthermore, research is needed to address the digestive enzyme activity, immunity, and antioxidant capacity of *Pinctada fucata martensii*.

Author Contributions: Data curation, D.K.A. and F.L.; methodology, F.L., Y.Z., and N.Z.; investigation, D.K.A.; project administration, F.L. and X.H.; resources, F.L., C.L., and X.H.; writing—original draft, D.K.A.; writing—review and editing, F.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was mainly supported by the National Key R & D Plan "Blue Granary Science and Technology Innovation" (grant number 2020YFD0900205) and partly funded by "the Program for Scientific Research Start-up Funds of Guangdong Ocean University" (grant number 060302022103 and 060302022102).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Mustafa, M.G.; Nakagawa, H. A Review—Dietary Benefits of Algae as an Additive in Fish Feed. *Isr. J. Aquac. Bamidgeh* **1995**, *47*, 155–162.
2. Dineshbabu, G.; Goswami, G.; Kumar, R.; Sinha, A.; Das, D. Microalgae–nutritious, sustainable aqua- and animal feed source. *J. Funct. Foods* **2019**, *62*, 103545.
3. Madeira, M.S.; Cardoso, C.; Lopes, P.A.; Coelho, D.; Afonso, C.; Bandarra, N.M.; Prates, J.A.M. Microalgae as feed ingredients for livestock production and meat quality: A review. *Livest. Sci.* **2017**, *205*, 111–121.
4. Patil, V.; Källqvist, T.; Olsen, E.; Vogt, G.; Gislerød, H.R. Fatty acid composition of 12 microalgae for possible use in aquaculture feed. *Aquac. Int.* **2007**, *15*, 1–9.
5. Kadir, W.N.A.; Lam, M.K.; Uemura, Y.; Lim, J.W.; Lee, K.T. Harvesting and pre-treatment of microalgae cultivated in wastewater for biodiesel production: A review. *Energy Convers. Manag.* **2018**, *171*, 1416–1429.
6. Slade, R.; Bauen, A. Micro-algae cultivation for biofuels: Cost, energy balance, environmental impacts and future prospects. *Biomass Bioenergy* **2013**, *53*, 29–38.
7. Leite, L.D.S.; Hoffmann, M.T.; Daniel, L.A. Microalgae cultivation for municipal and piggery wastewater treatment in Brazil. *J. Water Process Eng.* **2019**, *31*, 100821.
8. Stutter, M.I.; Graeber, D.; Evans, C.D.; Wade, A.J.; Withers, P.J.A. Balancing macronutrient stoichiometry to alleviate eutrophication. *Sci. Total Environ.* **2018**, *634*, 439–447.
9. Sheng, A.L.K.; Bilad, M.R.; Osman, N.B.; Arahman, N. Sequencing batch membrane photobioreactor for real secondary effluent polishing using native microalgae: Process performance and full-scale projection. *J. Clean. Prod.* **2017**, *168*, 708–715.
10. Quintero-Dallos, V.; García-Martínez, J.B.; Contreras-Ropero, J.E.; Barajas-Solano, A.F.; Barajas-Ferrerira, C.; Lavecchia, R.; Zuorro, A. Vinasse as a sustainable medium for the production of *Chlorella vulgaris* UTEX 1803. *Water* **2019**, *11*, 1526.
11. Olguín, E.J. Phycoremediation: Key issues for cost-effective nutrient removal processes. *Biotechnol. Adv.* **2003**, *22*, 81–91.
12. Ayala, F.J.; Bravo, R.B. Animal wastes media for *Spirulina* production. *Algological Studies/Archiv für Hydrobiologie.* **1984**, *67*, 349–355.
13. Costa, R.H.; Medri, W.; Perdomo, C.C. High-rate pond for treatment of piggery wastes. *Water Sci. Technol.* **2000**, *42*, 357–362.

14. Jimenez-Perez, M.V.; Sánchez-Castillo, P.; Romera, O.; Fernandez-Moreno, D.; Pérez-Martınez, C. Growth and nutrient removal in free and immobilized planktonic green algae isolated from pig manure. *Enzym. Microb. Technol.* **2004**, *34*, 392–398.
15. Liu, J.; Hu, Q. *Chlorella*: Industrial production of cell mass and chemicals. In *Handbook of Microalgal Culture: Applied Phycology and Biotechnology*, 2nd ed.; Editor Richmond, A., Hu, Q., Eds.; John Wiley & Sons, Ltd.: Hoboken, NJ, USA, 2013; pp. 329-338.
16. Barbarino, E.; Lourenço, S.O. An evaluation of methods for extraction and quantification of protein from marine macro- and microalgae. *J. Appl. Phycol.* **2005**, *17*, 447–460.
17. Ge, S.; Qiu, S.; Tremblay, D.; Viner, K.; Champagne, P.; Jessop, P.G. Centrate wastewater treatment with *Chlorella vulgaris*: Simultaneous enhancement of nutrient removal, biomass and lipid production. *Chem. Eng. J.* **2018**, *342*, 310–320.
18. Ge, S.; Champagne, P. Nutrient removal, microalgal biomass growth, harvesting and lipid yield in response to centrate wastewater loadings. *Water Res.* **2016**, *88*, 604–612.
19. Yang, C.; Hao, R.; Deng, Y.; Liao, Y.; Wang, Q.; Sun, R.; Jiao, Y.; Du, X. Effects of protein sources on growth, immunity and antioxidant capacity of juvenile pearl oyster *Pinctada fucata martensii*. *Fish Shellfish Immunol.* **2017**, *67*, 411–418.
20. Cho, S.; Luong, T.T.; Lee, D.; Oh, Y.K.; Lee, T. Reuse of effluent water from a municipal wastewater treatment plant in microalgae cultivation for biofuel production. *Bioresour. Technol.* **2011**, *102*, 8639–8645.
21. Mularczyk, M.; Michalak, I.; Marycz, K. Astaxanthin and other nutrients from *Haematococcus pluvialis*—Multifunctional applications. *Mar. Drugs* **2020**, *18*, 459.
22. Razzak, S.A.; Hossain, M.M.; Lucky, R.A.; Bassi, A.S.; de Lasa, H. Integrated CO_2 capture, wastewater treatment and biofuel production by microalgae culturing—A review. *Renew. Sustain. Energy Rev.* **2013**, *27*, 622–653.
23. Travieso, L.; Benitez, F.; Dupeyrón, R. Algae growth potential measurement in distillery wastes. *Bull. Environ. Contam. Toxicol.* **1999**, *62*, 483–489.
24. Martínez, M.E.; Jiménez, J.M.; El Yousfi, F. Influence of phosphorus concentration and temperature on growth and phosphorus uptake by the microalga *Scenedesmus obliquus*. *Bioresour. Technol.* **1999**, *67*, 233–240.
25. Tran, H.D.; Do, T.T.; Le, T.L.; Tran Nguyen, M.L.; Pham, C.H.; Melkonian, M. Cultivation of *Haematococcus pluvialis* for astaxanthin production on angled bench-scale and large-scale biofilm-based photobioreactors. *Vietnam J. Sci. Technol. Eng.* **2017**, *61*, 61–70.
26. Lorenz, R.T.; Cysewski, G.R. Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. *Trends Biotechnol.* **2000**, *18*, 160–167.
27. Kim, G.; Mujtaba, G.; Lee, K. Effects of nitrogen sources on cell growth and biochemical composition of marine chlorophyte *Tetraselmis* sp. for lipid production. *Algae.* **2016**, *31*, 257–266.
28. Klin, M.; Pniewski, F.; Latała, A. Growth phase-dependent biochemical composition of green microalgae: Theoretical considerations for biogas production. *Bioresour. Technol.* **2020**, *303*, 122875.
29. Niccolai, A.; Chini Zittelli, G.; Rodolfi, L.; Biondi, N.; Tredici, M.R. Microalgae of interest as food source: Biochemical composition and digestibility. *Algal Res.* **2019**, *42*, 101617.
30. Pimentel, D. Energy inputs in food crop production in developing and developed nations. *Energies.* **2009**, *2*, 1–24.
31. Lu, W.D.; Lin, M.J.; Guo, X.L.; Lin, Z.Y. Cultivation of spirulina platensis using raw piggery wastewater for nutrients bioremediation and biomass production: Effect of ferrous sulfate supplementation. *Desalination Water Treat.* **2020**, *175*, 60–70.
32. Craig, S.; Kuhn, D.; Schwarz, M. *Understanding Fish Nutrition, Feeds, and Feeding Steven*; Virginia Cooperative Extention: Blacksburg, VA, USA, 2017; pp. 1–6.
33. Adams, C.; Godfrey, V.; Wahlen, B.; Seefeldt, L.; Bugbee, B. Understanding precision nitrogen stress to optimize the growth and lipid content tradeoff in oleaginous green microalgae. *Bioresour. Technol.* **2013**, *131*, 188–194.
34. Schwenk, D.; Seppälä, J.; Spilling, K.; Virkki, A.; Tamminen, T.; Oksman-Caldentey, K.M.; Rischer, H. Lipid content in 19 brackish and marine microalgae: Influence of growth phase, salinity and temperature. *Aquat. Ecol.* **2013**, *47*, 415–424.
35. Tang, B.; Liu, B.; Wang, G.; Zhang, T.; Xiang, J. Effects of various algal diets and starvation on larval growth and survival of *Meretrix meretrix*. *Aquaculture* **2006**, *254*, 526–533.
36. Mata, S.N.; de Souza Santos, T.; Cardoso, L.G.; Andrade, B.B.; Duarte, J.H.; Costa, J.A.V.; de Souza, C.O.; Druzian, J.I. *Spirulina* sp. LEB 18 cultivation in a raceway-type bioreactor using wastewater from desalination process: Production of carbohydrate-rich biomass. *Bioresour. Technol.* **2020**, *311*, 123495.
37. Kumar, R.; Ghosh, A.K.; Pal, P. Synergy of biofuel production with waste remediation along with value-added co-products recovery through microalgae cultivation: A review of membrane-integrated green approach. *Sci. Total Environ.* **2020**, *698*, 134169.
38. Zuorro, A.; Malavasi, V.; Cao, G.; Lavecchia, R. Use of cell wall degrading enzymes to improve the recovery of lipids from *Chlorella sorokiniana*. *Chem. Eng. J.* **2019**, *377*, 120325.
39. Mehariya, S.; Fratini, F.; Lavecchia, R.; Zuorro, A. Green extraction of value-added compounds from microalgae: A short review on natural deep eutectic solvents (NaDES) and related pre-treatments. *J. Environ. Chem. Eng.* **2021**, *9*, 105989.
40. Ferreira, G.F.; Ríos Pinto, L.F.; Maciel Filho, R.; Fregolente, L.V. Effects of cultivation conditions on Chlorella vulgaris and Desmodesmus sp. grown in sugarcane agro-industry residues. *Bioresour. Technol.* **2021**, *342*, 125949.
41. Wang, Y.; Guo, W.; Yen, H.W.; Ho, S.H.; Lo, Y.C.; Cheng, C.L.; Ren, N.; Chang, J.-S. Cultivation of *Chlorella vulgaris* JSC-6 with swine wastewater for simultaneous nutrient/COD removal and carbohydrate production. *Bioresour. Technol.* **2015**, *198*, 619–625.

42. Belotti, G.; Bravi, M.; de Caprariis, B.; de Filippis, P.; Scarsella, M. Effect of nitrogen and phosphorus starvations on *Chlorella vulgaris* lipids productivity and quality under different trophic regimens for biodiesel production. *Am. J. Plant Sci.* **2013**, *04*, 44–51.
43. Recht, L.; Zarka, A.; Boussiba, S. Patterns of carbohydrate and fatty acid changes under nitrogen starvation in the microalgae *Haematococcus pluvialis* and *Nannochloropsis* sp. *Appl. Microbiol. Biotechnol.* **2012**, *94*, 1495–1503.
44. Uslu, L.; Işik, O.; Koç, K.; Göksan, T. The effects of nitrogen deficiencies on the lipid and protein contents of *Spirulina platensis*. *Afr. J. Biotechnol.* **2011**, *10*, 386–389.
45. Oslan, S.N.H.; Tan, J.S.; Oslan, S.N.; Matanjun, P.; Mokhtar, R.A.M.; Shapawi, R.; Huda, N. *Haematococcus pluvialis* as a potential source of astaxanthin with diverse applications in industrial sectors: Current research and future directions. *Molecules* **2021**, *26*, 6470.
46. Liao, Y.; Cai, C.; Yang, C.; Zheng, Z.; Wang, Q.; Du, X.; Deng, Y. Effect of protein sources in formulated diets on the growth, immune response, and intestinal microflora of pearl oyster *Pinctada fucata martensii*. *Aquac. Rep.* **2020**, *16*, 100253.
47. Hua, K.; Cobcroft, J.M.; Cole, A.; Condon, K.; Jerry, D.R.; Mangott, A.; Praeger, C.; Vucko, M.J.; Zeng, C.; Zenger, K.; et al. The future of aquatic protein: Implications for protein sources in aquaculture diets. *One Earth* **2019**, *1*, 316–329.
48. Kissinger, K.R.; García-Ortega, A.; Trushenski, J.T. Partial fish meal replacement by soy protein concentrate, squid and algal meals in low fish-oil diets containing *Schizochytrium limacinum* for longfin yellowtail *Seriola rivoliana*. *Aquaculture* **2016**, *452*, 37–44.
49. Vizcaíno, A.J.; López, G.; Sáez, M.I.; Jiménez, J.A.; Barros, A.; Hidalgo, L.; Camacho-Rodríguez, J.; Martínez, T.; Cerón-García, M.; Alarcón, F. Effects of the microalga *Scenedesmus almeriensis* as fishmeal alternative in diets for gilthead sea bream, Sparus aurata, juveniles. *Aquaculture* **2014**, *431*, 34–43.
50. Yang, C.; Hao, R.; Du, X.; Wang, Q.; Deng, Y.; Sun, R. Response to different dietary carbohydrate and protein levels of pearl oysters (*Pinctada fucata martensii*) as revealed by GC–TOF/MS-based metabolomics. *Sci. Total Environ.* **2019**, *650*, 2614–2623.
51. Flaak, A.R.; Epifanio, C.E. Dietary protein levels and growth of the oyster *Crassostrea virginica*. *Mar. Biol.* **1978**, *45*, 157–163.
52. Utting, S.D. A preliminary study on growth of *Crassostrea gigas* larvae and spat in relation to dietary protein. *Aquaculture* **1986**, *56*, 123–138.
53. Whyte, J.N.C.; Bourne, N.; Hodgson, C.A. Influence of algal diets on biochemical composition and energy reserves in *Patinopecten yessoensis* (Jay) larvae. *Aquaculture* **1989**, *78*, 333–347.