

RESEARCH PAPER

Microalgae Grown in Aquaculture Effluents and Their Effects on Mussels

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Abstract

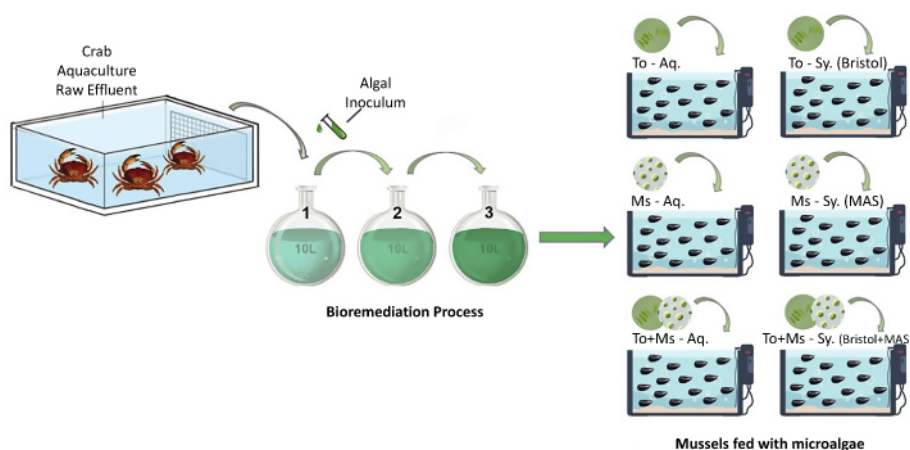
Global seafood consumption is rising, increasing the need for sustainable feed sources in aquaculture. Microalgae, such as *Tetrademus obliquus* (To) and *Microchloropsis salina* (Ms), offer a promising nutritional profile for animal feed, including mussels. This study evaluated the potential of these microalgae, cultivated in synthetic medium and aquaculture effluent, as feed for the saltwater mussel *Mytilus edulis*. The microalgae achieved productivity rates of 194.3 mg L⁻¹ day⁻¹ (To) and 156.4 mg L⁻¹ day⁻¹ (Ms), removing 100% of nitrogen and phosphorus and up to 91% of the chemical oxygen demand. Mussels were fed with microalgae grown in aquaculture effluent or in synthetic medium as their diet for 45 days. These mussels showed increases in protein (54.3–58.3%) and lipid (4.1–5.2%) content, with no significant differences between the microalgal diets. However, mussels fed microalgae



cultivated in aquaculture effluent exhibited significantly greater gains in weight and shell length than those fed with microalgae grown in synthetic medium. These findings highlight the potential of effluent-grown microalgae to enhance the growth and nutritional value of mussels while contributing to sustainable practices in aquaculture by using aquaculture effluent as a growth medium. This approach supports circular economy strategies, offering an innovative and environmentally friendly alternative for bivalve feed.

Keywords: aquaculture feed, bioremediation, *Microchloropsis salina*, nutrient recycling, *Tetrademus obliquus*

Graphical Abstract



1. Introduction

Global seafood consumption is rising, driving the need for sustainable feed sources in aquaculture, and microalgae, with their highly favourable nutritional profile, offer a viable solution for inclusion in both terrestrial and marine animal diets [1].

The expansion of aquaculture activities and the use of feed, including microalgae, further intensify pressure on water resources. This, coupled with the increasing waste from food production, highlights the critical need for innovative and eco-friendly water treatment methods to recycle nutrients and ensure environmental sustainability [2].

Biological treatments are considered very effective since they use microorganisms to remove organic matter and biodegradable compounds while producing biomass that can yield valuable chemicals and products for various applications. Among these approaches, microalgal bioremediation has been widely studied and is considered a sustainable technology due to its ability to capture carbon dioxide, recover nutrients such as nitrogen and

phosphorus, and maintain a low land-use footprint. Being naturally ubiquitous, microalgae can readily adapt to diverse environments, including aquaculture effluents [3, 4].

The aquaculture systems present some environmental issues as they tend to accumulate chemicals (e.g., from feed compounds and disinfectants used) normally administered in intensive aquaculture production. Whether intentionally administered or resulting from contamination during production, these substances can accumulate in the food chain when effluents are discharged, raising concerns about food safety and human health [5]. Dispersal of particulate and dissolved nutrients differs in scale and dynamics. While particles move a little more than 300 m away from the source before being deposited, dissolved nutrients can travel up to 100 km and are less prone to coagulation and deposition. Both are easily absorbed and metabolized by bacteria and phytoplankton [6]. For this reason, high risks of contamination arise when discharges from aquaculture production are made in natural aquatic environments.

Integrated multitrophic aquaculture systems combine the production of several species from different trophic levels, resulting in environmental benefits [5, 7]. These systems aim to recover nutrients derived from aquaculture inputs (e.g., feed losses and metabolic wastes from fish) and redirect them towards the production of extractive species such as microalgae, molluscs, and other filter feeders, thereby reducing the risk of eutrophication in surrounding ecosystems [6].

To reduce the risks to consumer health, several safety policies have been applied to achieve effective control of food production systems. These policies aim to implement international food quality and safety standards and to promote animal health, well-being, and nutrition, as well as plant health [3]. Over the years, the European Commission has established several restrictions on the use of harmful compounds in the production of food products [8] and maximum limits for dangerous chemicals in gaseous or liquid effluents that might contaminate the environment [9]. There is also legislation that controls the levels of minerals and organic load that can be released by discharged effluents. Each country in the European Union (EU) has incorporated this legislation into national laws that regulate maximum concentrations of nutrients and contaminants allowed for discharge in surface waters. In Portugal, the law that originated was Decree-Law No. 236/98 [10].

Microalgae have long been used in aquaculture as a primary feed source for larvae and filter-feeding organisms and have recently gained attention as ingredients in formulated feeds due to the rise in fish meal prices, the gradual decline of natural fishery resources, and their contamination with heavy metals, chemicals, and microplastics [11]. Microalgae have been demonstrated to be



viable substitutes for conventional feed sources due to their high content of proteins, polyunsaturated fatty acids (PUFAs), and pigments, among other bioactive compounds [12]. In addition, their production can be more sustainable than that of traditional feed sources such as terrestrial crops [13]. Recently, microalgae biomass has been grown in different wastewaters (e.g., food industry, municipal wastewater, agro-industrial wastewater, starch processing wastewater, and anaerobic digestion concentrate) and has been evaluated as a source of several bioproducts (e.g., biofuels, bioplastics, pigments, proteins, and amino acids for animal feed, antioxidants, and fertilizers) [14–19].

The European Food Safety Authority recognizes some microalgae as a food ingredients that do not belong to the novel food category [20], as approvals date back to 2002 and 2014. The United States Food and Drug Administration has assigned the status of generally regarded as safe to various microalgae, namely *Chlorella vulgaris* and *Arthrospira platensis*, which means that these organisms are devoid of endogenous toxins and are safe for human and animal consumption. Several members of the *Chlorella* and *Haematococcus* genera are typically used as feed in aquaculture [21].

Mussels are bivalve molluscs that inhabit intertidal zones and feed on phytoplankton and detritus filtered from the surrounding seawater [22]. These animals represent over one-third of the EU's aquaculture production [23]. Mussels take between 12 and 24 months to reach their appropriate size (50–75 mm) to be harvested and traded [24]. There are some studies on the influence of mussels' diet on their composition and condition index (CI; percentage of meat in the shell), showing that physiological parameters tend to vary throughout the year depending on their reproductive status, food availability, salinity, temperature, oxygen concentration, and mobility level of these animals [25, 26]. Several studies have been carried out on the feeding of mussels with microalgae, and the results are generally positive, as is the case with feeding diatoms during the larval stage of mussels, which increases settlement success and reduces mortality [1, 27]. Additionally, Fernández-Reiriz et al. [28] investigated the use of three microalgal species in mussels feeding and found that *Rhodomonas lens* promoted the highest weight gain due to its better digestibility and a more appropriate size for filtration by the mussel gills. Previous studies recommend that the primary criterion for selecting microalgae as feed for mussels should be their suitability for ingestion and filtration by mussels at different developmental stages (larval or adult), considering key characteristics such as cell size, volume, and weight. The secondary criterion should focus on microalgae with high digestibility and a balanced biochemical composition to enhance nutrient absorption and metabolic conversion.

Another study evaluated four species of microalgae (*Cyclotella* sp., *Chlorella pyrenoidosa*, *Scenedesmus dimorphus*, and *Monoraphidium contortum*) as diet components for the mussel *Sinohyriopsis cumingii* and concluded that mussels



showed different growth, Ca content, and proximate and fatty acid compositions when fed with different microalgal diets [1]. Further research analysed the inclusion of several species of microalgae (*Tetraselmis chui*, *R. lens*, *Rhodomonas salina*, *Chlamydomonas* sp., *Chrysotila carterae*, *Prorocentrum cordatum*, *Dunaliella salina*, *Diacronema lutheri* (previously designated *Pavlova lutheri*), and *Tetraselmis marina*) in the diet of two species of molluscs (mussel and oyster) [29]. The authors concluded that wettability (which refers to the physico-chemical property of food particles – like algae – that dictates whether the mussel’s filtering organs – gills and labial palps – will easily capture, ingest, or reject them) is the most important factor in predicting the availability of the microalgae biomass to be filtered and absorbed by mussels [30]. Additionally, Pettersen et al. [28] studied the use of microalgae mixtures (composed of *Chaetoceros calcitrans*, *Isochrysis galbana*, *D. lutheri*, and *Chaetoceros muelleri*) in mussel feeding to understand their effect on larval growth, survival, and settlement success. All these studies were carried out with microalgae grown in synthetic culture media with UV-treated filtered seawater.

In a recent study, wastewater effluents were treated with the microalga *Tetrademus obliquus* (To) and then fed to *Dreissena bugensis* mussels as a binary bioremediation system. The goal was to evaluate the capacity of the mussels to consume the microalgae. This binary bioremediation system achieved high removal rates for nitrogen and phosphorus. Additionally, 94% of the microalgae could be harvested through settling and mussel filtration after only 24 h [29].

The present study evaluated the feasibility of coupling microalgae To and *Microchloropsis salina* (Ms) with *Mytilus edulis* mussels to recover nutrients from aquaculture effluents and to use the resulting algal biomass as a mussel diet. These two microalgal species were selected based on our previous work in which six microalgal species were tested in the same aquaculture effluent, with *T. obliquus* and *M. salina* showing the highest effluent remediation efficiency and biomass productivity [19]. Specifically, the study examined whether microalgae grown in aquaculture effluent, compared with a synthetic medium, differentially influenced mussel growth, weight gain, and biochemical composition. This study aimed to determine whether microalgae cultivated directly in aquaculture effluents can function as an effective circular-economy feed source for mussels while enhancing nutrient recovery within aquaculture systems.

2. Materials and Methods

2.1. Raw Materials

The aquaculture effluent was collected in November 2020 from Pesca Verde Lda., a seafood hatchery supplier located at Guincho (38°70' 98.6" N, 9°48' 45.3" W), Cascais, Portugal. The effluent consisted of seawater used for the transport of brown crabs (*Cancer pagurus*), which were shipped from France to Portugal in



refrigerated trucks during a 24-h journey. Upon arrival, the transport seawater was collected and used as crab aquaculture transport effluent in the present study. Annually, the company provides the effluent discharges around 100,000 L of this type of aquaculture effluent into the sea. The effluent was collected in 20 L plastic containers (three in total) and stored at 4°C to minimize chemical and biological changes. Physicochemical parameters of the effluent (as received) were analysed for total nitrogen, nitrate, nitrite, and total phosphorus using the following test kits: Hanna Instruments Kit tests, a Hanna Thermoreactor (HI8,39800-02, Romania), and a Hanna Photometer (HI8,3314-02, Romania). Chemical oxygen demand (COD) was determined through the high-range dichromate method using Hanna Instruments Kit tests (Romania). Biochemical oxygen demand (BOD₅) was determined using the WTW OxiTop IS 12 measurement system (Germany). Parameters, such as optical density (OD₅₄₀), pH, conductivity, and total and suspended solids, were analysed using the standard methodology [31]. All the analytical determinations and measured parameters were performed in triplicate.

The mussels (*M. edulis*), collected at an unimpacted site (Caparica coastal zone, Portugal), were brought to the laboratory, where they were acclimated for 7 days and fed daily with *To* grown in Bristol synthetic medium [32]. They were cleaned of the shell attachments, cultured in running seawater with aeration (temperature = 16 ± 1°C, pH = 8.1 ± 0.1, salinity = 37 ± 1, dissolved oxygen >7.0 mg/L, and photoperiod 12 h light/12 h darkness). On day 0 (*t*₀), a subsample of 10 mussels was used to determine their CI and biochemical composition (proteins, lipids, carbohydrates, ash, and moisture) at the beginning of the experiment (*n* = 3). Mussels were not fed 24 h before the beginning of the feeding experiments [33] and were randomly distributed across the aquariums.

2.2. Microalgae and Culture Conditions

The freshwater microalga *To* (multicellular, Chlorophyta) and the marine microalga *Ms* (unicellular, Heterokontophyta) were selected for this study to test the aquaculture effluent remediation and mussel feeding. The microalga *T. obliquus* (ACOI 204/07 strain), formerly *Scenedesmus obliquus*, was obtained from the Algotec Culture Collection of the University of Coimbra, Portugal, while *M. salina* (NANNO-2 strain) was acquired from SERI Algotec. *M. salina* was cultivated in a 2 L Erlenmeyer flask of synthetic culture medium (Modified Artificial Seawater, MAS) appropriate for saltwater microalgae. The medium contained NaCl (35.08 g L⁻¹), MgSO₄·7H₂O (7.39 g L⁻¹), KNO₃ (1.0 g L⁻¹), K₂SO₄ (0.85 g L⁻¹), CaCl₂·2H₂O (0.8 g L⁻¹), and 1 mL L⁻¹ of ET-Chu trace element solution. The ET-Chu solution was prepared according to the Chu-10 trace metal stock formulation (g L⁻¹): H₃BO₃ (2.86), MnCl₂·4H₂O (1.81), ZnSO₄·7H₂O (0.222), Na₂MoO₄·2H₂O (0.390), CuSO₄·5H₂O (0.079), and Co (NO₃)₂·6H₂O (0.0494) [34]. *Tetrademus obliquus* was cultivated in Bristol



medium [32], a commonly used medium for freshwater microalgae. Both microalgal species were cultivated in aquaculture effluent using 10 L round-bottom flask reactors operated in semi-continuous mode, with three reactors per species ($n = 3$).

Although *T. obliquus* is a freshwater microalga, it was deliberately tested in a saltwater effluent given its resilience observed in previous work [19]. In the present study, *T. obliquus* was evaluated alongside *M. salina*, a marine species, to provide a comparative perspective. In fact, in an earlier study using the same seawater effluent, *T. obliquus* had already shown promising performance, which justified its inclusion here. The aim was therefore not only to test a marine species but also to assess the extent to which a versatile freshwater strain could provide efficient remediation in a saline effluent environment.

These inoculations were performed using 500 mL of stock solution (inoculum) with a concentration of 1.6 g L^{-1} . The reactors were aerated at a rate of 15.2 h^{-1} – air volume per culture volume per hour – (air pump Stellar 380 D, 5 W, China) that kept the culture stirred, preventing cell sedimentation, and sealed with hydrophobic cotton. The microalgae grew at room temperature ($20 \pm 2^\circ\text{C}$), under artificial lighting with LED tubular lamps at $\pm 200 \mu\text{E m}^{-2} \text{ s}^{-1}$ (digital luxmeter ROLINE, model RO 1,332A, China), 4,000 K, with an alternate 12 h light/dark cycle. In these trials, there was no experiment with a control in synthetic culture medium, as this comparison had been carried out in previous trials [19]. The experimental conditions (inoculum proportion, aeration, temperature, and illumination) were kept equivalent to those previously described in the study by Viegas et al. [19], with the only modification being the use of a 10 L round-bottom flask instead of a 2 L Erlenmeyer flask to accommodate a larger culture volume.

The experiment lasted 35 days and was conducted using a semi-continuous cascade system. Every 48 h, 1 L of raw effluent (10% of the reactor volume) was added to reactor 1, while an equivalent volume was simultaneously transferred from reactor 1 to reactor 2 and from reactor 2 to reactor 3, maintaining the same transfer frequency throughout the experiment (as in the study by Viegas et al. [19]) (Figure 1) (Figure S.M-1).

The hydraulic residence time was 60 days. Reactors 2 and 3 of both microalgae were supplemented with aqueous NaNO_3 to a final concentration of 10 mg N L^{-1} and with KH_2PO_4 to a final concentration of 5 mg P L^{-1} in the culture medium [2, 19].

2.3. Effluent Treatment Performance and Microalgae Growth

During the experiments, a sample of 10 mL of the effluent from the last reactor was collected weekly to analyse (in triplicate) total nitrogen, phosphorus, and COD in the medium according to the methods described in Section 2.1.





Figure 1. Schematic diagram of a semi-continuous cascade system, with three reactors of 10 L (addition and transfers of 1 L every 48 h).

Microalgae dry weight was determined by filtration of the samples through a Whatman GF/C 47 mm filter and drying for 24 h at 105°C, to evaluate algal growth. Microalgae productivity was calculated using Eq. 1.

$$Productivity (mg.L^{-1}.day^{-1}) = \frac{(Dw_f - Dw_i)}{V \times t} \quad (1)$$

Where Dw_f and Dw_i are the values of the final and initial biomass dry weight (mg), respectively, V is the reactor volume (L), and t is the duration time of the growth cycle (days).

Every other day, 1 L of treated effluent was collected from the last reactor for each microalga, which was placed to decant for 24 h. The supernatant was discarded, and the concentrated algal biomass was collected and frozen until its use in the mussel feeding bioassays. At the end of the experiment, all the cultures were harvested by decantation (allowing reactors 1 and 2 to complete the remediation treatment). All the analytical determinations (including biomass analysis: protein, sugar, and lipid content; composition of the lipidic fraction; moisture; and ash content) and measured parameters (total nitrogen, nitrate, nitrite, total phosphorus, COD, BOD₅, total solids, and suspended solids) were performed in triplicate.

2.4. Mussels Feeding Bioassays

The mussel feeding experiment was conducted in duplicate in 20 L parallelepiped aquariums containing 15 L of seawater with a salinity of 36 (filtered through 0.45 µm and treated with ultraviolet light) (Figure S.M-2). Fifteen mussels were placed per aquarium and six types of feeds were formulated – 6 aquariums × 2 replicas, making a total of 12 aquariums in the experiment (Figure 2): *T. obliquus* produced in aquaculture effluent (To – Aq.), *M. salina* produced in aquaculture effluent (Ms – Aq.), and a mixture in equal parts of the two previous microalgae (To + Ms – Aq.). As a control, the same microalgae were used but grown in a synthetic culture medium (To – Sy., Ms – Sy., and To + Ms – Sy.). The synthetic culture medium used for the growth



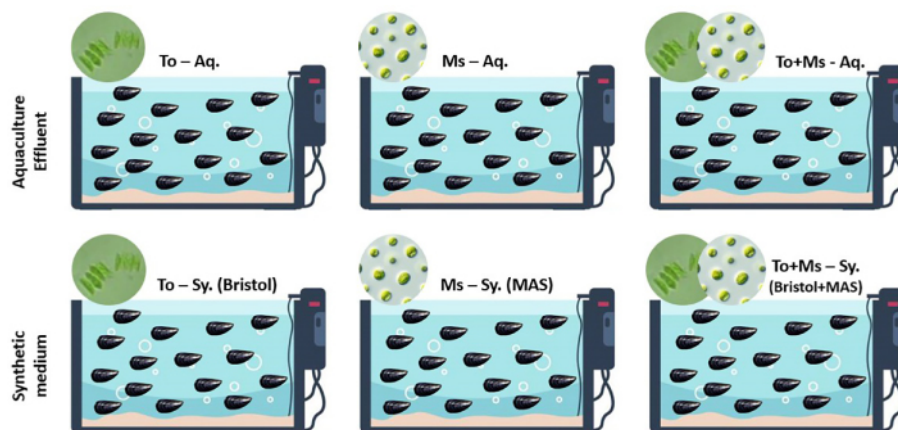


Figure 2. Mussel feeding bioassays experimental setup (6 aquariums $\times$ 2 replicas): To – Aq., *Tetrademus obliquus* grown in aquaculture effluent; Ms – Aq., *Microchloropsis salina* grown in aquaculture effluent; To + Ms – Aq., *T. obliquus* and *M. salina* (1:1) grown in aquaculture effluent; To – Sy., *T. obliquus* grown in Bristol synthetic medium; Ms – Sy., *M. salina* grown in modified artificial seawater synthetic medium; To + Ms – Sy., *T. obliquus* and *M. salina* (1:1) grown in synthetic medium, Bristol, and Modified Artificial Seawater.

of microalgae was Bristol medium for *T. obliquus* and MAS for *M. salina*. It should be noted that, unlike in the remediation assays, the synthetic medium-grown microalgae served only as a clean control feed for mussels, both to exclude potential effects of contaminants present in the effluent and to confirm that effluent-grown microalgae did not negatively affect mussel condition.

The concentrated biomass of each microalga, previously obtained through every other day collection and final decantation of the cultures, was diluted in distilled water to standardize all samples to 2.5 g L^{-1} of which 15 mL was added daily to each aquarium (corresponding to 2.5 mg L^{-1} of algal biomass per aquarium), according to the study by Galimany et al. [35]. Daily monitoring of temperature, pH, salinity, dissolved oxygen, and ammonia was carried out. For the maintenance of these parameters, $3/4$ of the water volume of each aquarium was replaced once a week.

The experiment was conducted for 45 days, with the mussels being weighed and measured at four different times: day 0 (t_0), day 15 (t_{15}), day 30 (t_{30}), and day 45 (t_{45}). At the two final sampling times (t_{30} and t_{45}), seven mussels were collected from each treatment, and the weight and length (total, shell, and edible tissue) were measured. Afterwards, the mussels were dissected, and the tissues were collected for further analyses of proteins, carbohydrates, lipids, and ash content (using the methods defined in Section 2.6). Analytical determinations and measured parameters were performed in triplicate.

2.5. Mussels' Condition Index and Growth Rate

The CI of mussels is defined as the ratio between the flesh (tissue) dry weight and the shell dry weight (Eq. 2). This index is commonly used to assess the health and the quality of the mussel [25, 26]:

$$CI(\%) = \frac{\text{Dry meat weight}}{\text{Shell dry weight}} \times 100 \quad (2)$$

The growth rate of mussels was determined in terms of length (GR_L) and weight (GR_W) using Eqs. 3 and 4, respectively:

$$GR_L(\%) = \frac{L_f - L_i}{L_i} \times 100 \quad (3)$$

$$GR_W(\%) = \frac{W_f - W_i}{W_i} \times 100 \quad (4)$$

Where L_f and W_f are the values of the final shell length and weight, and L_i and W_i are the values of the initial shell length and weight, respectively.

2.6. Microalgae and Mussel Biomass Characterization

The microalgae and mussel biomass were dried and ground in a coffee grinder (Bosch TSM6A011W, Germany) before analysis. Total nitrogen was quantified by an elemental analyser (Thermo Finnigan – CE Instruments Model Flash EA 112 CHNS series, USA). Total protein content was estimated by multiplying the measured total nitrogen by the conventional nitrogen-to-protein conversion factor of 6.25 [36]. Sugar content was determined by quantitative acid hydrolysis using the method of Miranda et al. [37], followed by the phenol-sulfuric spectrophotometric method [38]. The procedure was optimized for microalgae. Lipid content was determined gravimetrically after Soxhlet extraction with *n*-hexane for 6 h using 1 g of biomass. The fatty acid methyl esters were prepared by adding equal parts of the sample and methanolic KOH (2 N) [39]. The composition of the lipidic fraction in terms of fatty acids was determined. Analyses were performed with a GC-TOFMS analyser (gas chromatography coupled with a time-of-flight mass spectrometer; Agilent 7,890B, USA) equipped with a multi-mode inlet. An Agilent HP-5 MS UI fused silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) was used in all separations. An aliquot of the derivatized sample (1 μL) was injected via a LECO L-PAL3 autosampler in splitless mode at 250°C. The GC temperature program was set as follows. The initial temperature was 40°C and held for 1 min. It was then increased to 150°C at a rate of 10°C min⁻¹ and held for 15 min. Afterwards, the temperature was raised to 250°C at 5°C min⁻¹. Finally, the temperature was increased to 280°C at 10°C min⁻¹ and held for 10 min. The transfer line and ion source temperatures were 250 and 230°C, respectively. The



fatty acids' methyl esters present in the derivatized samples were identified by comparing their mass spectra with those in NIST and WILEY databases and with the retention time and mass spectra of corresponding standards.

For the determination of total carotenoids in the algal biomass, about 15 mg of ground biomass, 10 mL of acetone, and 0.7 mg of glass microspheres were used. Vortexing was carried out, followed by an ice bath and centrifugation at 3,900 rpm (Hettich EBA 20), according to the study by Gouveia et al. [40]. The total pigments were calculated by scanning spectrometry (Analytik Jena AG – Spekol 1,500) between 380 and 700 nm. Quantification of total pigments was done with the Beer-Lambert equation (Eq. 5) with the maximum absorbance wavelength of the samples [40].

$$Total\ Pigments(\%) = \frac{A \times V \times f}{E_{1cm}^{1\%} \times m} \quad (5)$$

Where A is the absorbance (at the maximum absorption wavelength), V is the total volume of pigment extracted (mL), f is the dilution factor, $E_{1cm}^{1\%}$ is the extinction coefficient, and m is the sample mass (mg). A value of $E_{1cm}^{1\%} = 2,150$ was used based on the average value of the carotenoids mostly found in microalgae, according to Gouveia and Empis (2003) [41].

The moisture and ash content in the algal biomass and mussels were determined according to Standard Methods for the Examination of Water and Wastewater [31] by drying samples at 105°C to constant weight for moisture and subsequently incinerating the dried material at 550°C in a muffle furnace until constant weight, for ash content.

The microalgae biomass after treatment and the mussel biomass were calcined at 550°C for 2 h, and the ash was dissolved with concentrated HNO_3 (1:1). The resulting solutions were diluted with H_2O to an appropriate volume and filtered with a Whatman No. 42 filter paper. The mineral composition of the ash solutions was determined by ICP-AES – Horiba Jobin Yvon ULTIMA sequential ICP, using the Horiba Jobin Yvon ICP Analyst 5.4 software. A monochromator with a Czerny-Turner spectrometer was used, and the gas used was Argon.

2.7. Statistical Analysis

Experiments were performed in duplicate for microalgae effluent treatment and mussels feeding and in triplicate for all analytical determinations (including biomass analysis) and measured parameters. Data are reported as mean $\pm$ standard deviation (SD). The parameters, such as removal efficiencies, productivities, and biomass characterization, were compared using one-way analysis of variance by the software IBM SPSS Statistics version 23. The mean values were compared using the Tukey Honestly Significant Difference (HSD) test, and correlation was considered statistically significant when $p < 0.05$.



3. Results and Discussion

3.1. Production of Microalgae Biomass in the Aquaculture Effluent

The composition of the aquaculture effluent used for microalgae production and the algal biomass production rates are presented in Table 1. The pH of the effluent was 7.3, and the conductivity was 38 mS cm^{-1} , reflecting the high levels of salts present in this effluent, derived from seawater.

The effluent presented total solids and total ash contents higher than 30 g L^{-1} , indicating the presence of high levels of materials, mainly inorganic ones. Total nitrogen and total phosphorus levels are compatible with the growth of microalgae since these nutrients are present in non-vestigial concentrations and the ratio between nitrogen and phosphorus is 11. This value is within the range reported in previous works for *T. obliquus* [42, 43].

Table 1. Composition of raw aquaculture effluent (A), remediated aquaculture effluent (third reactor) by *T. Obliquus* (B1) and *M. Salina* (B2), and biomass production in aquaculture effluent – after remediation ended (C) (mean $\pm$ SD, $n = 3$).

	Total nitrogen (mg N L ⁻¹)	Total phosphorus (mg P L ⁻¹)	COD (mg O ₂ L ⁻¹)	BOD ₅ (mg O ₂ L ⁻¹)	Total solids content (g L ⁻¹)	Total ashes content (g L ⁻¹)	Optical density (540 nm)
(A) - Raw aquac. effluent	210.0 $\pm$ 5.3	18.4 $\pm$ 0.2	758.5 $\pm$ 6.3	620.0 $\pm$ 28.3	39.0 $\pm$ 1.3	33.0 $\pm$ 0.1	0.17 $\pm$ 0.01
(B1) - <i>T. obliquus</i> Remediated aquac. effluent	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	68.0 $\pm$ 1.2	10.0 $\pm$ 0.7	36.7 $\pm$ 0.9	35.2 $\pm$ 0.3	0.02 $\pm$ 0.00
(B2) - <i>M. salina</i> Remediated aquac. effluent	0.81 $\pm$ 0.1	0.0 $\pm$ 0.0	101.2 $\pm$ 1.9	0.0 $\pm$ 0.0	24.2 $\pm$ 9.3	36.1 $\pm$ 0.2	0.02 $\pm$ 0.00

(C) - Microalgae biomass	T. obliquus			M. salina		
	Average	Biomass	Total	Average	Biomass	Total
	productivity (Mg L ⁻¹ day ⁻¹)	concentration (g L ⁻¹)	mass produced (g)	productivity (Mg L ⁻¹ day ⁻¹)	concentration (g L ⁻¹)	mass produced (g)
	129.3 $\pm$ 55.3	4.5 $\pm$ 0.4	203.6 $\pm$ 3.2	107.8 $\pm$ 41.3	3.8 $\pm$ 0.3	169.8 $\pm$ 2.7



The microalgae *T. obliquus* and *M. salina* were chosen because of their ability to bioremediate effluents with respectively high loads of organic contaminants and high loads of inorganic salts [18, 44, 45]. The experimental results support that these microalgae could grow in this type of aquaculture effluent that combines both characteristics, presenting high concentrations of organic and inorganic contaminants. Both microalgae showed good development, reaching maximum productivities of 194.3 and 156.4 mg L⁻¹ day⁻¹ for *T. obliquus* and *M. salina*, respectively. Apandi et al. [46] reported productivities of 98.5 mg L⁻¹ day⁻¹ for *Scenedesmus* sp. grown in market wastewater from fish and seafood processing, with 1,754 mg O₂ L⁻¹ for COD, 480 mg L⁻¹ for TN, and 87 mg L⁻¹ for TP. Viegas et al. [19] used an effluent with the same origin as the one used in this work (aquaculture from crab transportation) to grow *T. obliquus* in semi-continuous mode, obtaining much higher productivity (811.7 mg L⁻¹ day⁻¹), probably due to its higher nutrient load (COD of 5.2 g O₂ L⁻¹ and TN of 737.8 mg L⁻¹).

The remediation of the aquaculture effluent (third reactor) with both microalgae was remarkably high for all the parameters analysed (>86%). Both microalgae were able to completely remove the nitrogen and phosphorus present in the effluent (to levels below the limit of detection), with *T. obliquus* being in average values more efficient in COD removal (91.0% for To and 86.7% for Ms), but *M. salina* more efficient in average for BOD₅ (98.4% for To and 100% for Ms). Nevertheless, both microalgae reduced the parameters to levels that allow the effluent discharge (150 mg O₂ L⁻¹ for COD, 10 mg L⁻¹ for TP, and 15 mg L⁻¹ for TN), according to Portuguese legislation [10].

The supplementation of reduced amounts of nitrogen and phosphorus in the second and third reactors allowed the microalgae to reduce the COD parameter to levels below the discharge limits. Previous tests revealed that in situations with nitrogen and phosphorus below the detection limit, microalgae were unable to degrade other compounds in order to reduce COD to levels that would allow effluents to be discharged [14, 15, 47]. The treated effluent collected from the third reactor presented nutrient levels below the discharge limit and, therefore, could be considered treated and not susceptible to having negative impacts on surface waters [10].

3.2. Characterization of Microalgal Biomass

3.2.1. Microalgal Nutritional Composition

The nutritional composition of the algal biomass produced in synthetic medium versus aquaculture effluent was evaluated by quantifying its protein, carbohydrate, lipid, and ash contents (Figure 3).

There were significant differences ($p < 0.05$) between the composition of To biomass produced in synthetic culture medium and aquaculture effluent, in terms of carbohydrates (43.5% for To-Sy. to 30.4% in To-Aq.), lipids (16.6% for



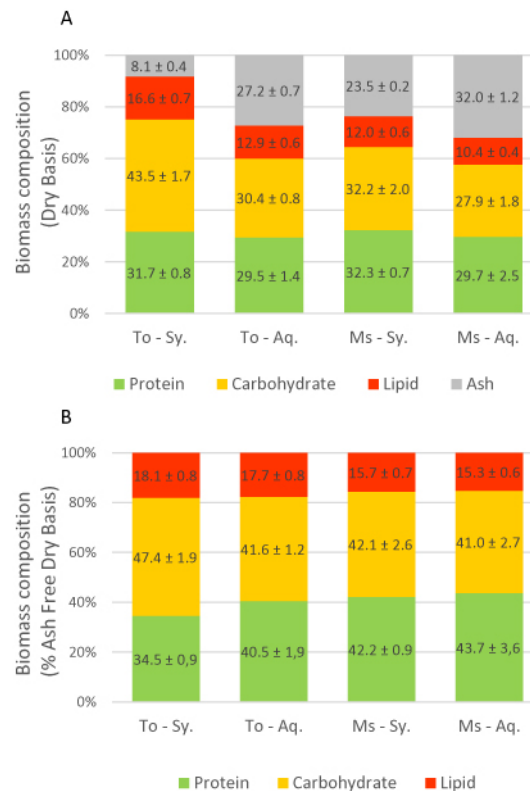


Figure 3. Biomass composition of the microalgae (mean and standard deviation, $n = 3$). (A) Percentage of dry weight. (B) Percentage of ash-free basis. To, *Tetrademus obliquus*; Ms, *Microchloropsis salina*; Aq., aquaculture effluent; sy., synthetic medium.

To-Sy. to 12.9% in To-Aq.), and ash contents (8.1% for To-Sy. to 27.2% in To-Aq.). Ms biomass also presented higher levels of proteins, carbohydrates, and lipids when produced in a synthetic medium because ash accumulation in the algal biomass was much higher when *M. salina* was grown in the aquaculture effluent (23.5% for Ms-Sy. and 32.0% for Ms-Aq.), owing to its high salinity. Regarding proteins, both microalgae present a higher amount in the synthetic medium compared to the aquaculture effluent, but the differences are not significant.

When comparing the same compositions on an ash-free basis, the microalgae produced in aquaculture effluent exhibited comparable concentrations of carbohydrates and lipids and slightly higher protein content than those produced in synthetic medium. These results indicate that this effluent created no nutrient limitations to the microalgae development but was a source of inorganic components that were further accumulated in the microalgae biomass (32.0 ± 0.5% for Ms-Aq, followed by To-Aq, with 27.2 ± 0.7%).

The increase in lipids and carbohydrates in the composition of microalgae was greatly influenced by the stress to which they were subjected (due to the lack of nitrogen and phosphorus in the medium in both microalgae, and additionally due to the salinity level in the microalgae *T. obliquus*, given its



freshwater origin). Several studies have been conducted to determine how the internal regulation of microalgae works when exposed to stress factors, namely a deprivation of nutrients such as nitrogen and phosphorus or an excess of salts in the environment, such as NaCl. Yang et al. (2024) stated that in the case of excess NaCl, reactive oxygen species have been widely assumed to promote lipid accumulation as an intermediary of oxidative stress. Furthermore, nitrogen feast–famine cycles were investigated and explored in order to increase the lipids and carbohydrates in microalgae with success [48–50].

3.2.2. Microalgal Fatty Acid Composition

In general, microalgae have a balanced fatty acid composition for animal feed, as they have a high content of monounsaturated and PUFAs [51].

Figure 4 presents the variations of fatty acids in the microalgae grown in aquaculture effluent and synthetic medium. Unsaturated fatty acids predominated in the fatty acid composition of the microalgae biomass. These included palmitoleic (C16:1), hexadecadienoic (C16:2), oleic (C18:1), linoleic (C18:2), linolenic (C18:3), and conjugated linoleic acid (CLA). Saturated fatty

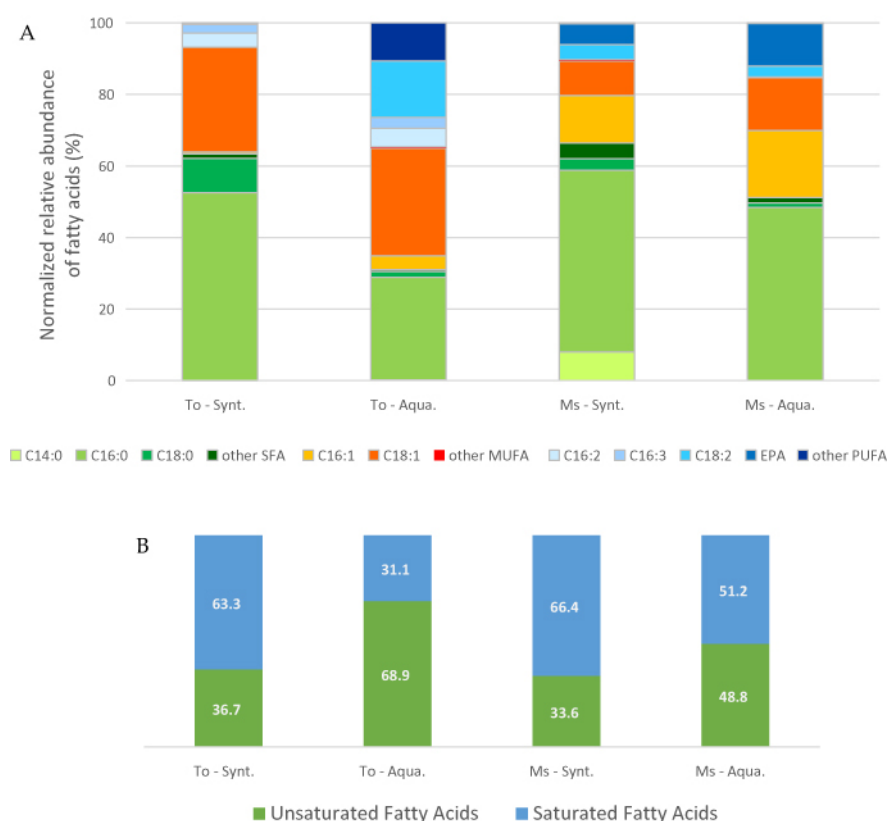


Figure 4. (A) Fatty acid profile in the microalgae grown in aquaculture effluent and synthetic medium. (B) Unsaturated fatty acids vs saturated fatty acids in the microalgae (To, *T. obliquus*; Ms, *M. salina*; Sy., synthetic medium; Aq., aquaculture effluent). SFA, saturated fatty acids; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.



acids were also present, including palmitic (C16:0), stearic (C18:0), behenic (C22:0), and lignoceric (C24:0). Higher levels of unsaturated and PUFAs were detected in Ms (both in synthetic and aquaculture media). A content of polyunsaturated and unsaturated fatty acids, namely linoleic acid (C18:2) and CLA, was observed in To produced in aquaculture compared to the synthetic medium. In the supplementary materials, Table S.M-1 compiles the information corresponding to Figure 4, with all the values of fatty acids. Daneshvar et al. [52] and Kuo et al. [53] reported higher lipid contents in microalgae cultivated in aquaculture effluents (8–23%) than those obtained in the present study, whether considering ash-free or ash-included values, while maintaining a similar proportion of saturated and unsaturated fatty acids.

For bivalves, including mussels, the essential fatty acids are mainly long-chain n-3 PUFAs, particularly eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), which cannot be synthesized again and must therefore be obtained from the diet. These fatty acids are known to support larval development, membrane fluidity, growth, and overall physiological performance [54]. Previous studies have also shown that diets rich in n-3 PUFAs, and with balanced ratios of saturated, monounsaturated, and PUFAs, enhance growth and feeding efficiency in mussels and other bivalves [54–56]. Accordingly, the high proportion of PUFAs in the biomass produced in this study is favourable for its use as feed.

3.2.3. Microalgal Pigments Analysis

The determination of total carotenoids was carried out for the two microalgae grown in the aquaculture effluent and the synthetic medium.

After extracting the pigments, the following results were obtained: 0.12, 0.20, 0.15, and 0.54 g_{carotenoids}/100 g_{microalgae} for To-Sy., To-Aq., Ms-Sy., and Ms-Aq., respectively. Analytically confirming what had been visually verified (Figure 5): microalgae grown in aquaculture effluent present a much darker green and significantly higher level of carotenoids. Although the determination of carotenoid content in mussels was not carried out in this study, literature indicates that a diet rich in carotenoids can improve animal performance through antioxidant effects and enhancement of colouration. Therefore,

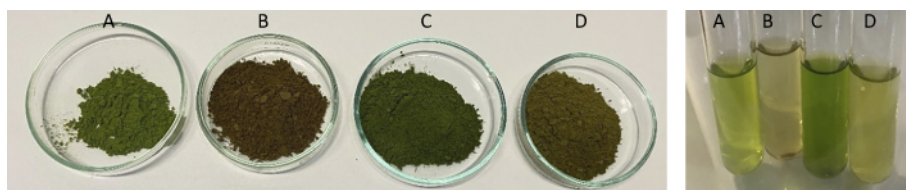


Figure 5. Colouration of the four dry biomass and the extracted pigments. (A) *T. obliquus* aquaculture effluent. (B) *T. obliquus* synthetic medium. (C) *M. salina* aquaculture effluent. (D) *M. salina* synthetic medium.

Table 2. Mineral composition of microalgae ash grown in aquaculture effluent (Aq.) and synthetic medium (Sy.) (concentration in microalgae mass in g kg⁻¹).

Sample	Al	Ba	Ca	Cu	Fe	K	Mg	Mn	Na	P	Si	Zn
To Aq.	0.382	0.135	17.067	0.053	0.630	4.138	4.799	0.080	6.741	9.715	1.131	0.174
To Sy.	1.272	0.074	7.433	0.030	4.675	2.475	6.281	0.161	4.087	19.396	1.476	0.294
Ms Aq.	0.286	0.016	22.377	0.046	0.223	7.646	16.912	0.017	122.537	27.294	0.488	0.122
Ms Sy.	0.055	0.011	11.150	0.024	0.500	3.209	14.542	0.088	23.310	15.029	3.311	0.125

Note: The presence of Ag, As, Bi, Cd, Co, Cr, Hg, Li, Mo, Ni, Pb, Sb, Sn, Tl, V, W, and Zr was not detected in the analysed biomass. B, Sr, and Ti were detected vestigially.

Each value corresponds to one analytical measurement per sample (no analytical replicates were performed); To, *T. obliquus*; Ms, *M. salina*.

feeding mussels with these microalgae may confer physiological benefits, although this remains to be directly assessed [57, 58].

3.2.4. Microalgal Mineral Composition

The mineral composition of microalgae shows some variation between the two microalgae species and the culture media in which they grew. Table 2 presents this information in detail, and the most abundant elements are calcium, sodium, phosphorus, magnesium, and potassium.

Figure 6 expresses the ratio between the minerals present in the biomass of algae grown in the different media. This figure illustrates that using aquaculture effluent can lead to higher accumulation of certain minerals in the microalgae biomass. *M. salina* grown in aquaculture effluent tends to accumulate higher levels of minerals (i.e., sodium, calcium, and phosphorus) than when grown in synthetic medium. Similarly, *T. obliquus* exhibits higher accumulation of

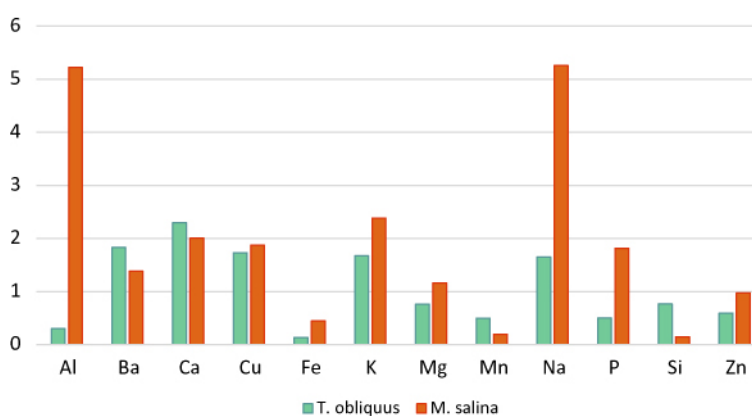


Figure 6. Ratio between the minerals present in the two microalgae grown in aquaculture effluent in relation to growth in synthetic medium.



calcium, potassium, sodium, and barium when cultivated in aquaculture effluent. These differences reflect the influence of the nutrient composition of the aquaculture effluent on mineral accumulation patterns in both microalgae species.

3.3. Mussel Feeding Assay

3.3.1. Weight and Length Analysis

The 190 mussels collected from their natural and uncontaminated environment had an average total weight of 5.91 ± 0.28 g and an average shell length of 37.8 ± 1.1 mm at the beginning of the experiment (t_0).

Figure 7 shows the mussels being fed in their aquariums and then during characterization.

The average weight and length gain of mussels subjected to different diets over time (t_{15} , t_{30} , and t_{45}) are shown in Figure 8 (relative to measurements on t_0). Weight and length gains were measured at 15-day intervals (T_{15} , T_{30} , and T_{45}) and are reported for each period independently, rather than as cumulative totals.

After 15 days, the average weight of mussels increased similarly in all treatments, varying between 0.51% ($T_0 + M_s - Sy.$) and 0.98% ($T_0 + M_s - Aq.$) relative to mussels at t_0 . There were no significant differences between them except for $T_0 + M_s - Sy.$, which was the lowest. The increase in shell length (GR_L) was lower in mussels subjected to the mixture of both microalgae ($T_0 + M_s$) in synthetic and aquaculture media. After 30 days, the effect of the $T_0 - Aq.$ on weight gain was higher (0.91%); however, no significant differences were observed between diets, except for $T_0 + M_s - Sy.$ (0.27%). For the length, the growth was similar between the several diets; however, the mixed diets showed lower growth.

In the final evaluation, t_{45} , the difference in weight gains (GR_W) between aquaculture and synthetic medium diets was more evident, with significant differences between them. Regarding shell length, the greatest GR_L was found

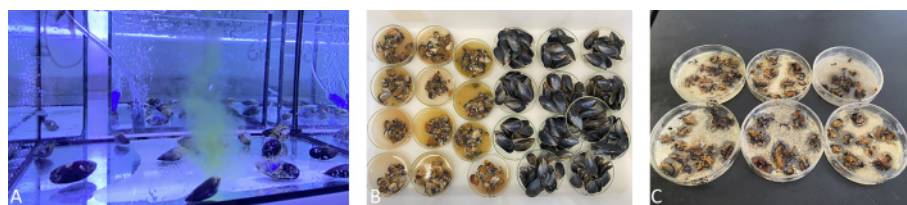


Figure 7. (A) Feeding time for mussels in one of the aquariums, with the diet of *Tetrademus obliquus* produced in aquaculture effluent ($T_0 - Aq.$). (B) Separation of mussel meat from its shells to determine the condition index (CI). (C) Mussels after drying to be further characterized.

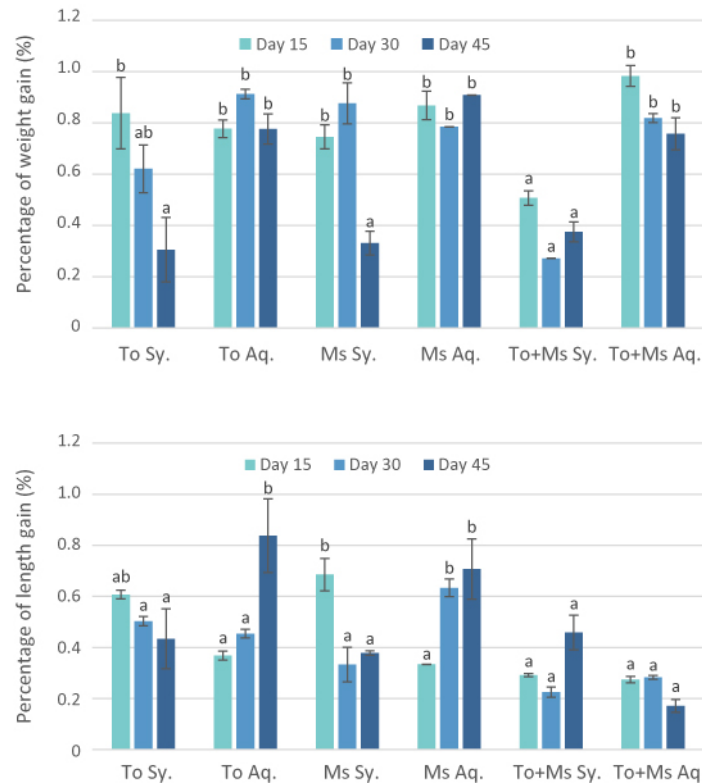


Figure 8. Average percentage of weight and shell length gain for mussels, by type of diet at days 15, 30, and 45 (mean $\pm$ SD; $n = 2$; To, *Tetrademus obliquus*; Ms, *Microchloropsis salina*; Aq., aquaculture effluent; Sy., synthetic medium). Values with different index letters show significant differences with $p < 0.05$.

for the two pure culture diets grown in aquaculture, with significant differences from all others. The mussels exhibited the poorest growth and performance when cultivated in the To + Ms-Aq mixture.

After 45 days, mussels fed aquaculture-derived microalgae reached mean final weights of 6.45 ± 0.57 g (To-Aq.), 5.00 ± 0.29 g (Ms-Aq.), and 5.18 ± 0.37 g (To + Ms - Aq.), corresponding to weight gains of 2.47, 2.56, and 2.56%, respectively. In contrast, mussels fed synthetic-grown microalgae reached 5.25 ± 0.28 g (To-Sy.), 5.27 ± 0.44 g (Ms-Sy.), and 5.15 ± 0.34 g (To + Ms - Sy.), with weight gains of 1.76, 1.95, and 1.15%, respectively. Percentage weight gain was used for statistical comparison due to the intermediate sampling at day 30. Overall, mussels fed aquaculture-derived microalgae exhibited significantly higher weight gain ($p < 0.05$) than those fed synthetic-grown microalgae. Concerning shell length, no significant differences were observed among pure culture diets (To-Aq., Ms-Aq., To-Sy., and Ms-Sy.). However, mixed diets resulted in significantly lower growth rates.

According to previous studies, mussels fed with diets of more than one microalgal species tend to grow more because this increases nutritional diversity. However, in a similar study carried out by Chen et al. [1], the authors



obtained similar results to those of the present trial, where mussels grew more with non-mixed feeds. The diet with *T. obliquus* gave better results, suggesting that tightly packed multicellular microalgae can be more easily absorbed.

The better performance obtained with *T. obliquus* may be related to intrinsic morphological differences between the two species. *T. obliquus* cells are substantially larger than those of *M. salina* and typically form tightly associated groups of 2–8 cells (coenobia), resulting in larger and more compact particles (~10–20 µm). In contrast, *M. salina* is unicellular and considerably smaller (~2–5 µm). In bivalves, feeding efficiency depends on particle size relative to gill filtration capacity, with particles within an optimal size range being retained and ingested more efficiently, whereas very small or excessively large particles may be poorly captured or rejected [59]. Differences in particle size can therefore strongly influence filtration and ingestion efficiency in mussels. The size range of *T. obliquus* coenobia may fall closer to the optimal particle size for mussel retention, potentially contributing to the higher growth observed in this treatment [60]. Although we did not assess changes in morphology after freezing and thawing, the natural size and structural configuration of *T. obliquus* may have played a role in the observed performance differences.

3.3.2. Mussels' Condition Index

Concerning the CI, it was found that the average of the individuals from t_0 was 9.7 ± 1.6 (ranging from 7.6 to 12.7). The CI at day 30 (6.45–8.22) and day 45 (6.6–8.6) is shown in Figure 9 for all the different diets.

The CI variation throughout the year in the wild is natural and is due to several factors such as temperature, salinity, oxygen concentrations, food

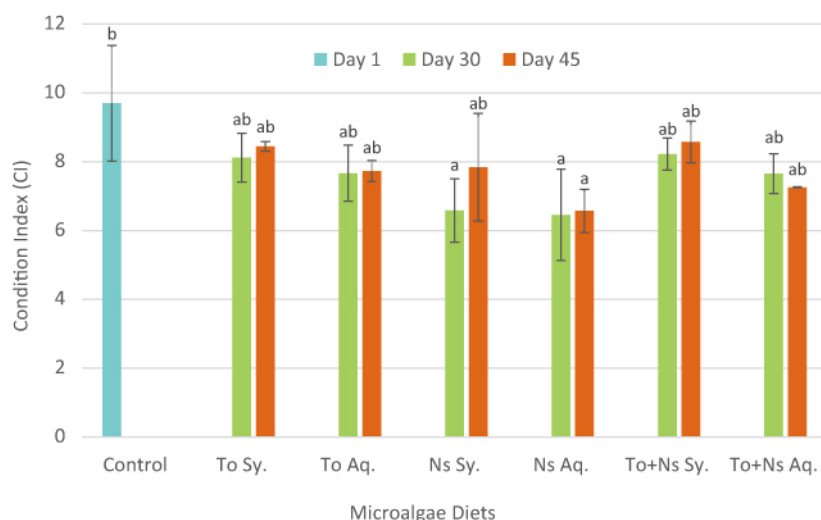


Figure 9. Average condition index (CI) of mussels at days 0, 30, and 45, for all the different diets (mean ± SD; $n = 2$; To, *Tetrademus obliquus*; Ms, *Microchloropsis salina*; Aq., aquaculture effluent; Sy., synthetic medium). Values with different index letters show significant differences with $p < 0.05$.



availability, and changes in the reproductive cycles [25]. CI tends to be higher at the end of winter, presenting its lowest value in August and September when most individuals are at the resting/inactive stage to cope with higher temperatures in the summer [25].

In the present experiment, only one mussel died during the entire assay, in the period between 30 and 45 days, in the To + Ms synthetic diet. Individuals at t_0 showed a significantly higher CI (9.7; group 'b') compared with all experimental diets at t_{30} and t_{45} . At both time points, most treatments clustered within groups 'ab' or 'a', indicating only moderate differences among them. The lowest CI values were consistently observed in mussels fed with *M. salina*, particularly in the aquaculture medium (group 'a'). By contrast, diets based on *T. obliquus*, either alone or combined with *M. salina*, often showed intermediate values (group 'ab'). Although the CI tended to increase from days 30 to 45 in most treatments, these differences were not statistically significant. Overall, mussels fed on algae grown in aquaculture effluent displayed slightly lower CI values than those fed on algae grown in synthetic medium, which may result from a higher mineral load in the aquaculture-grown biomass, potentially increasing shell weight. These results may also be explained by differences in cell size, cell wall thickness, digestibility, toxic metabolites, fatty acid content, nutrient composition, and trace elements among the diets [33]. In particular, *M. salina* has a smaller cell size compared with *T. obliquus*, which could influence filtration/absorption efficiency. Additionally, *M. salina* biomass presented a higher ash content and lower levels of carbohydrates and fatty acids compared with *T. obliquus*. Wyatt et al. [26] also evaluated mussels in terms of CI. For freshly caught mussels, the CI was 16.86 ± 4.81 , but those individuals had a commercial length and were much larger (58.83 ± 5.35 mm) than in the present study (37.8 ± 1.1 mm) and were harvested in June. Moreover, their experiment focused on 2 months of storage in wet-holding facilities, which caused stress and a decline in CI to 5.89 ± 1.46 [26].

3.3.3. Mussels' Nutritional Composition

The edible tissue of mussels was used to analyse their nutritional composition (including protein, carbohydrate, lipid, fatty acid profile, and ash content). Results for the six treatments at t_0 , t_{15} , t_{30} , and t_{45} are presented in Table 3.

The nutritional composition of mussels at 30 and 45 days of exposure to diets presented significant differences relative to t_0 organisms, mainly in the amount of ash, carbohydrates, and lipids. Over time, there was a decrease in mussels' carbohydrates and an increase in lipids and ash content in their biomass. The protein content of mussels grown on synthetic diets is slightly higher than that of mussels on diets with microalgae from aquaculture effluent. The opposite is true for carbohydrates and ash content. The increase in protein and lipids from days 30 to 45 revealed a greater metabolic adaptation to the given diet. The ash in the mussel biomass practically



Table 3. Nutritional composition (% dried biomass) of mussel biomass by type of microalgae diet at day 0 (t_0), 30 (t_{30}), and 45 (t_{45}) (mean $\pm$ SD).

Biomass component	Sampling day	Mussel biomass samples					
		To – Sy.	To – Aq.	Ms – Sy.	Ms – Aq.	To + Ms – Sy.	To + Ms – Aq.
Protein	t_0			55.7 $\pm$ 4.9 ^a			
	t_{30}	54.4 $\pm$ 3.3 ^a	51.7 $\pm$ 0.3 ^a	57.9 $\pm$ 0.7 ^a	53.9 $\pm$ 5.8 ^a	53.1 $\pm$ 2.7 ^a	48.3 $\pm$ 0.1 ^a
	t_{45}	58.3 $\pm$ 0.8 ^a	54.7 $\pm$ 0.1 ^a	56.6 $\pm$ 1.8 ^a	55.5 $\pm$ 1.4 ^a	58.2 $\pm$ 2.3 ^a	54.3 $\pm$ 0.7 ^a
	t_0			2.1 $\pm$ 0.6 ^a			
Lipid	t_{30}	3.3 $\pm$ 0.2 ^{abc}	3.6 $\pm$ 0.2 ^{abcd}	3.0 $\pm$ 0.3 ^{ab}	3.1 $\pm$ 0.1 ^{ab}	3.1 $\pm$ 0.5 ^{abc}	3.8 $\pm$ 0.1 ^{abcd}
	t_{45}	5.2 $\pm$ 0.1 ^d	4.1 $\pm$ 0.1 ^{bcd}	5.0 $\pm$ 0.4 ^{cd}	4.9 $\pm$ 1.0 ^{bcd}	4.3 $\pm$ 0.2 ^{bcd}	4.7 $\pm$ 0.5 ^{bcd}
Carbo-hydrate	t_0			30.7 $\pm$ 3.1 ^c			
	t_{30}	23.0 $\pm$ 1.8 ^{abc}	24.9 $\pm$ 0.5 ^{abc}	18.7 $\pm$ 2.1 ^{ab}	22.5 $\pm$ 2.8 ^{abc}	22.0 $\pm$ 0.6 ^{ab}	25.5 $\pm$ 0.3 ^{bc}
	t_{45}	16.4 $\pm$ 1.1 ^a	19.5 $\pm$ 1.1 ^{ab}	19.5 $\pm$ 1.4 ^{ab}	20.6 $\pm$ 0.6 ^{ab}	18.8 $\pm$ 2.2 ^{ab}	21.3 $\pm$ 0.1 ^{ab}
Ash	t_0			11.5 $\pm$ 1.2 ^a			
	t_{30}	19.3 $\pm$ 1.6 ^b	19.8 $\pm$ 0.4 ^b	20.5 $\pm$ 1.1 ^b	20.5 $\pm$ 2.9 ^b	21.8 $\pm$ 2.8 ^b	22.5 $\pm$ 0.1 ^b
	t_{45}	20.1 $\pm$ 1.7 ^b	21.7 $\pm$ 1.2 ^b	18.9 $\pm$ 0.8 ^b	19.1 $\pm$ 0.2 ^b	18.7 $\pm$ 0.3 ^b	19.7 $\pm$ 1.2 ^b

Note: To, *Tetradesmus obliquus*; Ms, *Microchloropsis salina*; Aq., aquaculture effluent; Sy., synthetic medium. The results shown in bold correspond to the highest values. Values with different index letters show significant differences at $p < 0.05$.



doubled compared to the initial value at t_0 , even in the case of mussels fed with the microalga To grown in synthetic media (which had a considerably lower amount of ash in their biomass compared with the others).

Figure 10 shows the evolution over time of the average concentration of mussel constituents for the groups of diets.

According to the analysis of Fidalgo et al. [33], the composition of wild mussels was 52.9% protein, 11.7% carbohydrates, 8.3% lipids, and 14.6% ash. According to the Instituto Nacional de Saúde Doutor Ricardo Jorge, raw mussels are composed of 69.1% protein, 11.4% carbohydrates, 8.6% lipids, and 10.9% ash [61]. It is conceivable that the amount of lipids found in the mussels in the present study is low since they are younger animals that have not yet reached sexual maturity, so their lipid content is naturally lower [28]. According to Gallardi et al. [24], mussels take between 12 and 24 months to reach the appropriate size (between 50 and 75 mm) to be captured and traded.

Regarding the fatty acid profile, there was a slight increase in the amount of palmitic acid and a decrease in the palmitoleic acid in the various diets, compared to t_0 profile group. The fatty acids present in mussels grown on different diets are given in Figure 11.

The fatty acid profile showed a predominance of palmitic acid (C16:0) with $47.2 \pm 1.2\%$, followed by palmitoleic acid (C16:1) with $12.9 \pm 0.3\%$ and stearic acid (C18:0) with $10.6 \pm 0.2\%$. There were no significant differences between the various diets. Regarding the fatty acid profile of mussels, other authors also mention the predominance of these fatty acids for mussels fed with microalgae [28, 62].

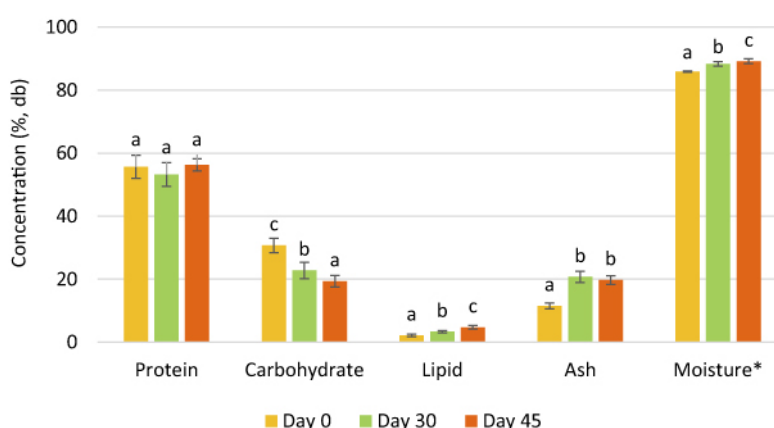


Figure 10. Mussels' average concentration of protein, carbohydrate, lipid, and ash content (in db) and moisture content (*as received) at days 0, 30, and 45 (mean $\pm$ SD; $N = 3$). Values with different index letters show significant differences with $p < 0.05$.



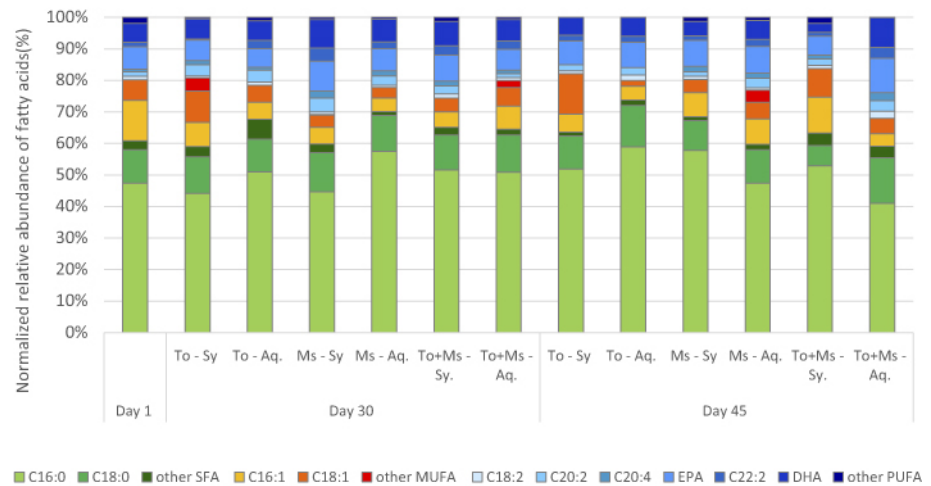


Figure 11. Characterization of fatty acids of mussels at day 1 and fed with different microalgae diets at days 30 and 45 (To, *Tetrademus obliquus*; Ms, *Microchloropsis salina*; Aq., aquaculture effluent; Sy., synthetic medium). SFA, saturated fatty acids; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Pettersen et al. [28] investigated the feeding of *Mytilus galloprovincialis* larvae and showed that diets including the diatom *C. calcitrans* improved larval survival. Although the mechanisms were not fully clarified, the authors suggested that the nutritional profile of the microalgae—particularly their fatty acid composition—played an important role. Fatty acids, such as DHA, EPA, and ARA, are considered essential for bivalve larvae, as their relative balance influences successful settlement and metamorphosis. However, the specific fatty acid requirements of mussel larvae remain only partially understood, and different studies report variable optimal profiles depending on the species and developmental stage. In this context, the present study contributes additional data on the fatty acid composition of the tested microalgae, though further research is needed to fully establish how differences in microalgal lipid profiles translate into physiological benefits for mussels.

Another study with juvenile mussels of the same species fed with three different algae (*Tetraselmis suecica*, *Dunaliella tertiolecta*, and *Phaeodactylum tricornutum*) or in a two-by-two mixture concluded that the mixture *T. suecica* + *P. tricornutum* led to the highest growth rates, followed by *T. suecica*. However, Chen et al. [1] tested the feeding of oysters (*S. cumingii*) fed with *S. dimorphus* and showed a high growth rate. In the present study, the results were similar for feeding mussels with To Aq. and To + Ms Aq. mixtures in terms of weight gain; however, the To-Aq. diet had better results for increasing shell length.

3.3.4. Mussels' Mineral Composition

The mineral composition of mussels' biomass was analysed and is shown in Figure 12.



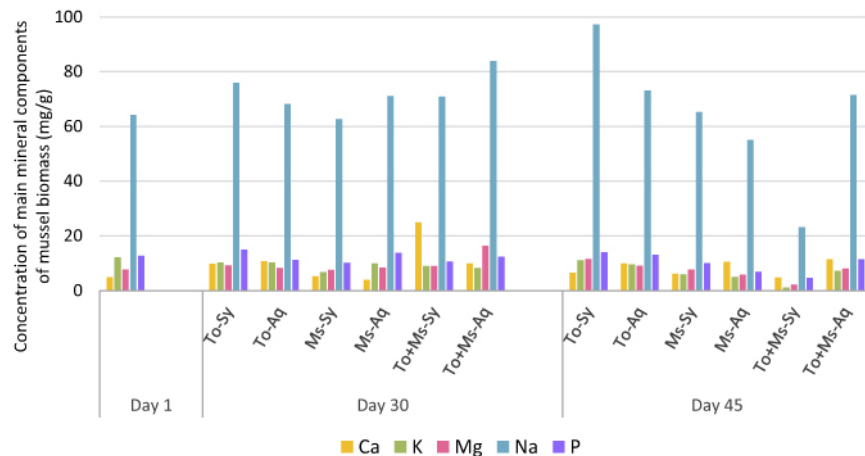


Figure 12. Composition of the mineral fraction from mussel biomass (mg/g), days 0, 30, and 45 for different microalgae diets (To, *Tetrademus obliquus*; Ms, *Microchloropsis salina*; Aq, aquaculture effluent; Sy, synthetic medium). Undetected elements: Ag, As, Bi, Cd, Co, Cr, Hg, Li, Mo, Pb, Sb, Se, Sn, Tl, V, W, and Zr.

Although the total ash content of mussels showed significant differences ($p < 0.05$) between days 0, 30, and 45 (Table 3), the qualitative composition of the mineral fraction did not exhibit a clear trend. This lack of variation was independent of the sampling day, the type of microalgae fed to the mussels, or the culture medium used to produce the microalgae. There is a predominance of sodium in the ash of the different mussel biomass, coming from the mineral components of the seawater where the mussels grew, which is why the mussels were not very sensitive to the different ash contents of the microalgae themselves [63]. These results may explain why mussels fed low ash algae still showed an increase in ash content from day 0 to days 30 and 45. According to Santhakumaran et al. [58], sodium (9.87 mg g^{-1}), potassium (6.43 mg g^{-1}), magnesium (1.62 mg g^{-1}), calcium (1.39 mg g^{-1}), and phosphorus (0.99 mg g^{-1}) are the primary mineral constituents of *T. obliquus*, matching those measured in the analysed mussel biomass. Also, the microalga *M. salina* shares the same main minerals with the mussel biomass, except for sulphur.

It can be concluded that all algae diets led to the growth of mussels without relevant mortality. Additionally, it is important to verify whether microalgal diets resulted in increases in mussel weight and shell length, with a higher increase observed in mussels with microalgae-fed diets cultured in aquaculture effluent. Thus, the advantage of feeding mussels with microalgae that have already been treated with aquaculture effluents becomes clear, representing a great potential in feed supplementation for aquaculture mussels.

Future work could be strengthened by selecting more homogeneous individuals (with initial weight and size more identical) at the beginning of the trial, which would further improve the robustness of the results. In addition,



extending the duration of the experiment (e.g., to several months) could provide insights into long-term responses, although such trials are often constrained by logistical factors.

It is important to mention that effective effluent management not only reduces pollutants released into natural water bodies but also contributes to the sustainable reuse of vital resources, such as aquaculture water. By improving the quality of treated effluents, industries can promote water conservation and support circular economy principles, aligning with global efforts toward more sustainable practices and the reduction of environmental degradation.

4. Conclusions

This study demonstrates that aquaculture transport effluent can be effectively valorized through microalgal cultivation, achieving high nutrient removal efficiencies while producing biomass suitable for mussel feeding. The microalgae *T. obliquus* and *M. salina* grew successfully in the effluent, yielding biomass with balanced biochemical composition comparable to that obtained in synthetic media.

Mussels fed microalgae produced in aquaculture effluent exhibited significant weight gain and normal shell growth, indicating that effluent-derived biomass does not impair performance and can support mussel development. Although the CI declined over the experimental period, this trend occurred across treatments and did not translate into compromised growth or altered biochemical composition. The slightly higher CI observed in mussels fed synthetic-grown microalgae suggests that further optimization of effluent-based production conditions may enhance physiological performance.

Overall, the integration of aquaculture effluent remediation with microalgal biomass production and subsequent mussel feeding represents a promising circular approach within integrated multitrophic aquaculture systems. These promising results open new horizons both in the treatment of waste effluents and enhancing marine species feeding, but also for mitigating pollution caused by seafood production industries, aligning with a broader commitment to environmental sustainability and safeguarding ecosystems for future generations.

Author Contributions

Catarina Viegas: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing; **Marta Martins:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Validation, Writing – review & editing; **Joana Neves:** Data curation, Investigation, Resources, Writing –



review & editing; **Paula Guedes:** Data curation, Formal analysis, Resources, Software, Writing – review & editing; **Marco Silva:** Data curation, Formal analysis, Methodology, Resources, Software, Writing – review & editing; **Eduardo Mateus:** Data curation, Funding acquisition, Methodology, Resources, Writing – review & editing; **Alice Ferreira:** Formal analysis, Investigation, Writing – review & editing; **Luísa Gouveia:** Formal analysis, Investigation, Writing – review & editing; **Margarida Gonçalves:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing – review & editing.

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Ethical Statement

This study did not require ethical approval in accordance with applicable national and European legislation, as it involved invertebrate species (bivalve molluscs).

Data Availability Statement

Source data available through the Figshare data repository: 10.5772/geet.deposit.c.8440492

Conflict of Interest

The authors declare no conflict of interest.

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