

Article

A Standardised Protocol for Assessing Aquaculture Effluent Suitability for Microalgae Cultivation: Validation Using *Phaeodactylum tricornutum* and Biofloc Shrimp Effluent

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Abstract

There is a notable lack of standardised and validated protocols for evaluating the potential of aquaculture effluents as culture media for microalgae. This study addresses this gap by developing and validating a comprehensive protocol for assessing the suitability of aquaculture effluents for microalgal production. The protocol was designed and standardised based on effluent characterisation, effluent pre-treatment, standardised inoculation, assessment of microalgal growth performance, and evaluation of nutrient removal. Validation was conducted through the cultivation of *Phaeodactylum tricornutum* in biofloc technology (BFT) shrimp (*Penaeus vannamei*) effluent, demonstrating the protocol's reliability, robustness, and adaptability across media with varying nutrient profiles. The approach effectively identified nitrate and orthophosphate removal patterns of up to $97.34 \pm 2.15\%$ and $87.88 \pm 1.61\%$, respectively, in culture media containing 100% shrimp effluent, while providing consistent and accurate growth performance data across different treatments. The treatment containing 100% shrimp effluent achieved a maximum cell density of 8488 ± 772 ($\times 10^4$ cells mL^{-1}). As the proposed framework is based on broadly applicable performance indicators, it provides a methodological basis that can be adapted for evaluating other aquaculture effluents, microalgal species, and cultivation systems. However, additional validation under these conditions is required before broader application can be confirmed.

Keywords: alternative medium; aquaculture; standardisation; validation



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1. Introduction

Aquaculture has become one of the fastest-growing food production sectors worldwide and plays a crucial role in global food security [1]. The continued expansion and intensification of aquaculture systems have increased production efficiency but have also given rise to significant environmental challenges associated with nutrient-rich effluents [2]. Biofloc Technology (BFT) represents one of the most widely adopted approaches for the sustainable intensification of aquaculture [3]. By promoting the growth of microbial communities that recycle organic matter and dissolved nutrients, BFT systems reduce water-exchange requirements and improve nutrient-utilisation efficiency [4]. Nevertheless, despite the substantial nutrient retention achieved within these systems, residual concentrations of

various compounds remain in the culture water and may represent valuable resources for secondary biomass production processes. These effluents often contain elevated concentrations of nitrogen and phosphorus compounds originating from uneaten feed, faecal matter, and microbial transformations within the culture environment. If not properly managed, their discharge may contribute to eutrophication and the degradation of receiving aquatic ecosystems [2,5].

Among the strategies proposed to mitigate these impacts, the cultivation of microalgae using aquaculture effluents has attracted increasing attention [6,7]. Microalgae are capable of assimilating dissolved nutrients while simultaneously producing biomass that can be utilised in aquaculture, biotechnology, biofuel production, and the manufacture of high-value bioproducts [8]. Consequently, the integration of microalgal cultivation with aquaculture wastewater treatment has emerged as a promising strategy for nutrient recovery and the development of a circular bioeconomy [7].

Several studies have demonstrated the feasibility of cultivating microalgae in aquaculture effluents [7,9–11], including effluents derived from shrimp farming systems [8,11,12]. Positive results have been reported for species such as *Nannochloropsis oculata*, *Tetraselmis chuii*, and *Chaetoceros muelleri*, highlighting the potential of these waste streams as alternative nutrient sources for microalgal cultivation [8]. However, direct comparisons among studies remain challenging because experimental methodologies vary considerably with regard to effluent preparation, inoculation procedures, cultivation conditions, performance indicators, and nutrient-removal assessments [11]. This methodological heterogeneity represents a significant limitation to the advancement of integrated aquaculture–microalgae systems. Currently, there is no widely applicable and standardised framework that enables researchers and industry practitioners to consistently evaluate the suitability of aquaculture effluents as alternative culture media for microalgae. As a result, the interpretation, comparison, and transferability of findings across different studies and production systems remain challenging. To address this gap, there is a need for reproducible and transferable evaluation protocols that integrate biological performance indicators with nutrient recovery metrics. Such protocols should facilitate the systematic assessment of effluent suitability while generating data that can be compared across different aquaculture systems, microalgal species, and environmental conditions.

Standardised methodologies are particularly important for supporting decision-making processes related to wastewater valorisation, resource recovery, and sustainable aquaculture management [13–18]. In environmental biotechnology, the absence of harmonised assessment methodologies frequently limits the comparison of treatment technologies across studies and geographical regions [13,14,16]. This challenge is particularly evident in nutrient recovery research, where differences in cultivation conditions, analytical procedures, and performance metrics often hinder objective evaluations of process efficiency [11]. Consequently, the development of standardised protocols has become increasingly important for supporting technology benchmarking, resource-recovery assessments, and evidence-based decision-making.

Therefore, the objective of this study was to establish and evaluate a standardised assessment framework for determining the suitability of aquaculture effluents as alternative culture media for microalgae cultivation. To evaluate the performance of the proposed framework, the protocol was applied to the marine diatom *Phaeodactylum tricornutum* cultivated in effluent obtained from a super-intensive shrimp (*Penaeus vannamei*) farming system operated under Biofloc Technology (BFT). The diatom *P. tricornutum* was selected as the model organism because it is one of the best-characterised marine microalgae, exhibiting rapid growth, stable laboratory cultivation, and high sensitivity to variations in environmental conditions. Owing to these characteristics, it has been widely employed in

physiological, ecotoxicological, and wastewater-related studies, including the ISO 10253 standard for marine algal growth inhibition tests [19]. These attributes make *P. triornutum* particularly suitable for validating standardised methodological approaches.

In addition to biological performance and nutrient recovery assessments, protocol performance was evaluated, providing evidence of its repeatability, robustness, and applicability under the experimental conditions evaluated. The resulting framework is intended to provide a transferable methodological basis for future validation using other microalgal species and aquaculture effluents.

2. Materials and Methods

2.1. Protocol Design

The proposed framework was designed to provide a reproducible and transferable methodology that can be applied across different aquaculture systems and microalgal species (Figure 1 and Table 1). The framework consists of seven main sequential stages: (i) effluent collection and characterisation, in which aquaculture effluents are characterised and (ii) pre-treated to ensure consistent starting conditions across experimental units; (iii) preparation of experimental culture media, in which standardised cultivation procedures are implemented to minimise methodological variability and facilitate comparisons among treatments; (iv) standardised microalgal cultivation and growth performance assessment; (v) nutrient recovery assessment, in which growth performance is evaluated using a set of predefined indicators, including maximum cell density, maximum biomass production, cultivation duration, and volumetric productivity, while nutrient recovery performance is assessed through nitrate- and orthophosphate removal efficiencies, which serve as (vi) indicators of resource recovery and bioremediation potential; and (vii) validation.

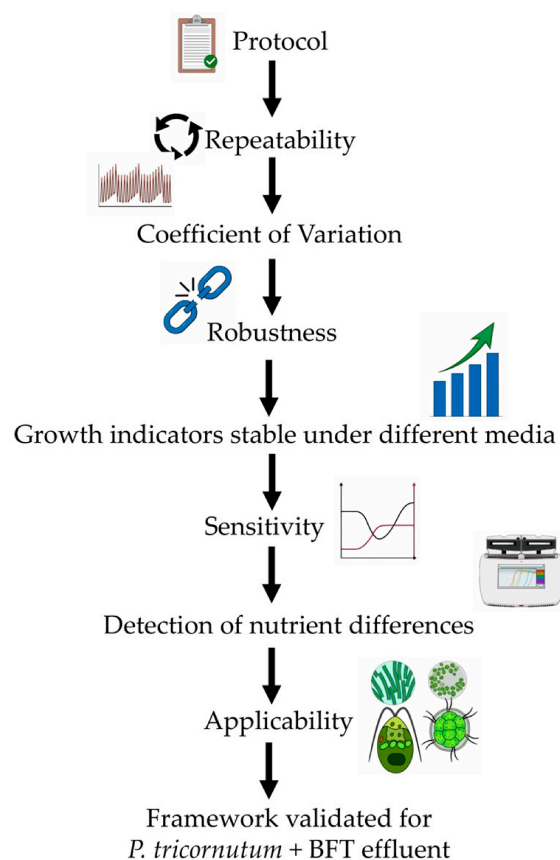


Figure 1. Validation workflow of the proposed protocol for microalgae cultivation using *Phaeodactylum triornutum* and biofloc shrimp effluent.

Table 1. Proposed framework was designed to provide a reproducible and transferable methodology that can be applied across different aquaculture systems and microalgal species.

Indicator	Purpose	Unit	Interpretation
Maximum Cell Density (CD _{max})	Growth capacity	cells mL ⁻¹	Higher values indicate greater adaptation
Maximum Biomass (B _{max})	Biomass generation potential	g L ⁻¹	Higher values indicate greater production
Volumetric Productivity (VP)	Production efficiency	g L ⁻¹ day ⁻¹	Higher values indicate improved productivity
Nitrate Removal Efficiency (NRE)	Nutrient recovery	%	Higher values indicate better nutrient utilisation
Orthophosphate Removal Efficiency (PRE)	Nutrient recovery	%	Higher values indicate better phosphorus uptake
Cultivation Duration (CD)	Operational feasibility	days	Lower values indicate faster production cycles

2.2. Effluent Characterisation and Pre-Treatment

Effluent was obtained from a laboratory-scale super-intensive shrimp farming system operated under Biofloc Technology at the Marine Shrimp Laboratory (LCM) of the Federal University of Santa Catarina (UFSC). The collection was carried out in the morning. Approximately 15 L of effluent from the biofloc system were collected and subsequently used in the preparation of the experimental treatments. After collection, the material was immediately transported to the Laboratory of Algae Cultivation (LCA) for sedimentation, filtration, and use in the experimental cultures.

Prior to its use as a culture medium, the effluent was characterised through measurements of nitrate, nitrite, orthophosphate, and pH. The physicochemical parameters of the water were monitored regularly. Daily measurements included temperature, dissolved oxygen (YSI® Ecosense DO200A, Valinhos, SP, Brazil), pH (Tecnal® Tec-5, Piracicaba, SP, Brazil), and salinity (YSI30® Digital Salinity Meter, Valinhos, SP, Brazil). Weekly measurements included alkalinity (CaCO₃, mg L⁻¹), orthophosphate phosphorus (PO₄³⁻-P, mg L⁻¹), and total suspended solids (TSS, mg L⁻¹). Monitoring of nitrogen compounds was conducted to quantify total ammoniacal nitrogen (TAN = NH₄-N + NH₃-N, mg L⁻¹) and nitrite nitrogen (NO₂⁻-N, mg L⁻¹), measured daily, and nitrate nitrogen (NO₃⁻-N, mg L⁻¹), assessed weekly. Analyses of TAN, nitrite, nitrate, and orthophosphate were performed using colorimetric methods, following previously described procedures [20]. At the time of collection, the effluent presented a TAN concentration of 0.23 mg L⁻¹, a nitrite concentration of 0.10 mg L⁻¹, an alkalinity of 184 mg L⁻¹ CaCO₃, a salinity of 34.7‰, and a TSS concentration of 604 mg L⁻¹. During system management, 550 g of lime were added to maintain alkalinity. Physicochemical determinations were performed by filtering 100 mL of water from the system, according to the monitoring protocol adopted by the LCM.

To ensure experimental consistency and minimise interference from suspended particles, the effluent was subjected to a pre-treatment procedure prior to inoculation. Initially, solids present in the effluent were allowed to settle to avoid light attenuation caused by particulate matter in BFT systems. For this purpose, the effluent was left to stand for 15 min. Subsequently, the supernatant was filtered using a cartridge filter system (10 µm, INDFILTROS®, Mogi das Cruzes, SP, Brazil). The filtration step aimed to remove the remaining particles after sedimentation and standardise the physical characteristics of the medium used in the experiments. The resulting effluent was then chlorinated for 30 min

(calcium hypochlorite, 12.5%), at a dosage of 1.0 mL per litre, for disinfection. This step was performed to reduce the presence of potential contaminating microorganisms from the BFT system, thereby preventing interference with the growth of microalgal cultures. At the end of the chlorination period, residual chlorine was neutralised using a sodium thiosulfate solution (10%, Quimlab[®], Jacareí, SP, Brazil), at a dosage of 2.0 mL per litre. After neutralisation, the media were left to stand until the inoculation stage. The seawater used for the preparation of the LCA-AM medium did not undergo sedimentation or filtration due to the low level of particulate matter present, and was sterilised in an autoclave for 30 min at 125 °C.

The resulting effluent was subsequently used to formulate the experimental media evaluated by the protocol. Three media were prepared: LCA-AM (control), conventional culture medium used as the reference treatment; BFT50, medium containing 50% BFT effluent and 50% LCA-AM medium; and BFT100, medium consisting exclusively of BFT effluent.

All treatments were established using the same inoculum source and initial biomass concentration to ensure comparability among experimental units. Each treatment was conducted in quadruplicate. Environmental conditions, including temperature, aeration, photoperiod, and pH management, were maintained according to routine cultivation procedures adopted by the LCA for marine microalgal production. This design enabled the protocol to be tested across media with contrasting nutrient compositions and allowed the assessment of methodological robustness.

2.3. Standardised Cultivation Procedure

In the present study, a strain of the diatom *P. tricornutum* (CCAP1055/1) was used. This strain has been maintained in the strain bank at the Laboratory of Algae Cultivation (LCA), Federal University of Santa Catarina (UFSC), Florianópolis, SC, Brazil, in a germination chamber under controlled conditions, at a constant temperature of 20 °C and a photoperiod of 8:16 (light:dark). The experimental cultures were grown in 2.0 L borosilicate Schott flasks containing LCA-AM medium (seawater, 35‰), consisting of nitrogen 255 mg L⁻¹, phosphorus 27 mg L⁻¹, nitrate 2 mL of stock solution (SS) (SS = 510.0 g L⁻¹ NaNO₃, 8.0 g L⁻¹ FeCl₃·6H₂O, 10.0 g L⁻¹ EDTA Na₂) phosphate 2 mL of stock solution (SS = 48.0 g L⁻¹ NaH₂PO₄), trace metals 1 mL of stock solution (SS = 8.82 g L⁻¹ ZnSO₄·7H₂O, 1.44 MnCl₂·4H₂O, 6.61 g L⁻¹ (NH₄)₆Mo₇O₂₄·4H₂O, 1.57 g L⁻¹ CuSO₄·5H₂O, 0.40 g L⁻¹ CoCl₂·6H₂O), and 1.0 mL vitamins (Citoneurin, 5000 mg) [18]. The cultures were maintained at a constant temperature of 24.0 ± 1.0 °C, under constant agitation provided by aeration with air enriched with 0.5% CO₂ (v/v), under an irradiance of 250 µmol photons m⁻² s⁻¹, and under a continuous 24 h photoperiod provided by 75 W light-emitting diode (LED) luminaires (colour temperature: 6500 K, Taschibra[®], Indaial, SC, Brazil). The pH was maintained between 7.5 and 8.5 using an automated system (Neptune Systems[®], Morgan Hill, CA, USA).

Cultures were monitored until the stationary growth phase was reached. The cultivation period was defined as the number of days between inoculation and the attainment of maximum cell density, thereby ensuring a standardised endpoint for protocol evaluation. Prior to inoculation, all materials used in the experiment, including Schott flasks, aeration hoses, pipette tips, and handling utensils, were cleaned and sterilised to minimise the risk of contamination by unwanted microorganisms. Before commencing the procedures, the work surface and materials were disinfected with 70% alcohol, reducing the possibility of contamination by environmental microorganisms.

After preparation of the culture media, a *P. tricornutum* inoculum, previously maintained in the exponential growth phase, was added. The volume of inoculum added to

each experimental unit was calculated using a previously established regression equation between biomass and turbidity for *P. tricornutum*, with the aim of obtaining an initial biomass of approximately 0.20 g L^{-1} in a final culture volume of 2.0 L per experimental unit. Following inoculation and determination of the initial biomass, the effective concentration obtained was approximately 0.16 g L^{-1} .

After the addition of the inoculum, the cultures were manually homogenised to ensure uniform distribution of microalgal cells throughout the culture volume. During inoculation, the containers were kept open only for the time necessary for inoculum transfer, minimising exposure to the external environment. The entire inoculation procedure was performed under aseptic conditions, within a laminar flow hood and near a Bunsen burner flame, reducing the risk of contamination. After inoculation, the flasks were immediately closed, labelled, and connected to the aeration system. The experimental units were then transferred to the culture room, where they remained under controlled conditions of temperature and lighting, with a continuous supply of atmospheric air enriched with CO_2 throughout the experimental period.

2.4. Growth Performance Assessment

The quality parameters of the cultures analysed were cell density (CD) and biomass (B). For comparison, the following parameters were determined: maximum cell density (CDmax), cultivation time, and volumetric productivity (VP). Maximum biomass (Bmax) was estimated. Growth performance was assessed through daily monitoring of microalgal cultures throughout the cultivation period. Cell density measurements were performed using a Neubauer's chamber following sample homogenisation. Manual cell counts were conducted under an optical microscope (Leica Microsystems®, Barra Funda, SP, Brazil), and mean values were calculated from four independent replicates. Growth curves were generated from daily cell density measurements to identify the different cultivation phases and determine the onset of the stationary phase. Maximum cell density (CDmax) was defined as the highest cell concentration recorded during cultivation and was used as the primary indicator of growth performance and as the reference point for subsequent biomass calculations.

Biomass concentration was estimated indirectly using a previously established turbidity–biomass regression curve obtained in previous experimental cultures (Supplementary File). Turbidity measurements were performed daily using a HANNA HI 98703 turbidimeter (HANNA® Instruments, Tamboré, Barueri, SP, Brazil). Ten millilitres of culture from all experimental units were analysed daily. When turbidity values exceeded 600 NTU, samples were diluted to ensure reliable instrument readings. Background turbidity originating from the culture media was measured prior to inoculation and subsequently accounted for in biomass estimation.

To avoid analytical errors, the turbidity of the BFT100, BFT50, and LCA-AM (control) media was measured and subtracted from the sample readings to improve the accuracy of the turbidity–biomass correlation. The BFT100 and BFT50 media presented turbidity values of 18.20 and 9.51 NTU, respectively, whereas the control medium showed no initial turbidity at the start of the experiment. Regression analysis was used to establish the relationship between the variables. Biomass values were estimated by applying Equation (1).

$$\text{Biomass}(\text{g L}^{-1}) = \text{Turbidity}(\text{NTU}) \times 0.0017; R^2 = 0.9903 \quad (1)$$

The reliability of the turbidity-based biomass estimation was verified through gravimetric dry-weight determinations performed at the beginning (T0) and end (T10) of cultivation. Samples were filtered through glass-fibre filters, dried at 50°C for 24 h, and weighed

to determine initial dry biomass, final dry biomass, and total dry biomass. Maximum biomass ($B_{\max}$) was defined as the biomass concentration corresponding to the day on which $CD_{\max}$ was observed. Volumetric productivity (VP) was calculated according to Equation (2).

$$VP = \frac{(B_{\max} - BI)}{C_t} \quad (2)$$

where VP represents volumetric productivity ($\text{g L}^{-1} \text{ day}^{-1}$), $B_{\max}$ represents maximum biomass concentration (g L^{-1}), BI represents initial biomass concentration (g L^{-1}), and C_t represents cultivation duration (days required to reach $CD_{\max}$). These indicators were selected because they provide complementary information regarding culture performance, production efficiency, and operational feasibility.

2.5. Nutrient Recovery Assessment and Standardised Performance Indicators

Nutrient recovery performance was evaluated through monitoring of dissolved nitrogen and phosphorus compounds throughout the cultivation period. Nitrate (NO_3^- -N), nitrite (NO_2^- -N), and orthophosphate (PO_4^{3-}) concentrations were measured using spectrophotometric methods routinely employed in the laboratory. For all analyses, 25 mL samples were collected from each experimental unit and filtered through glass-fibre filters prior to chemical determination. Sampling was performed at predefined cultivation times (T0, T1, T2, T3, T6, T10, T12, and T16) to characterise nutrient dynamics during both the exponential and stationary growth phases.

Nitrate concentrations were determined using commercial analytical kits (Alfakit®, Florianópolis, SC, Brazil). After the addition of all reagents, a reaction time of 15 min at room temperature was allowed. The absorbance was then measured using a spectrophotometer at 543 nm. Nitrite concentrations were determined following colorimetric reactions involving sulphanilic acid and N-(1-naphthyl)ethylenediamine reagents, with absorbance likewise measured at 543 nm. Orthophosphate concentrations were determined according to the molybdenum blue method, with spectrophotometric readings taken at 885 nm [21]. For protocol standardisation purposes, nutrient-removal performance was quantified using three indicators: initial concentration (C_i), final concentration (C_f), and removal efficiency (RE). Removal efficiency was calculated according to Equation (3).

$$RE(\%) = \left[\frac{C_i - C_f}{C_i} \right] \times 100 \quad (3)$$

where C_i represents the initial nutrient concentration and C_f represents the final nutrient concentration.

Among the evaluated parameters, nitrate removal efficiency (NRE) and orthophosphate removal efficiency (PRE) were selected as the primary nutrient recovery indicators because they directly reflect the capacity of the cultivation system to recover dissolved nutrients and contribute to effluent remediation. In addition to nutrient analyses, pH was monitored throughout cultivation as a process-control parameter. Measurements were performed using a pH meter (HANNA® Instruments, Tamboré, Barueri, SP, Brazil), and carbon dioxide supplementation was adjusted whenever necessary to maintain pH within the optimal range for marine microalgal cultivation. Approximately 20 mL of sample was collected from each experimental unit to allow probe immersion and pH measurement. When pH increased, CO_2 injection was intensified to reduce pH, maintaining levels within the desired range (8.2–9.5) for marine microalgae [22].

2.6. Protocol Validation Strategy

The performance of the proposed assessment framework was evaluated according to three validation attributes: repeatability, robustness, and applicability. Repeatability was assessed through analysis of variability among the four independent replicates of each treatment. Coefficients of variation (CVs) were calculated for all primary performance indicators. Robustness was evaluated by applying the protocol to culture media with contrasting nutrient compositions (LCA-AM, BFT50, and BFT100), thereby assessing the stability of the methodology under different cultivation conditions. Applicability was assessed by examining whether the selected indicators adequately characterised both biological performance and nutrient recovery efficiency. The transferability of the protocol to other aquaculture effluents and microalgal species was also considered. Together, these validation attributes provide evidence of the suitability of the proposed framework as a standardised methodology for evaluating aquaculture effluents as alternative culture media for microalgal cultivation.

2.7. Data Analysis

The experimental data for CD_{max}, B_{max}, cultivation time, and productivity underwent comprehensive statistical analysis to assess significant differences among treatments. Initially, tests of normality and homoscedasticity were conducted to verify that the data followed an approximately normal distribution and that variances across groups were equal. Following these preliminary checks, analysis of variance (ANOVA) was performed at a 5% significance level ($p < 0.05$) to determine whether statistically significant differences existed among treatment means. When ANOVA indicated significant differences, Tukey's mean comparison test was applied to identify specific groups with differing outcomes. This methodological approach provided an in-depth understanding of treatment effects on the evaluated parameters, ensuring reliable results and enabling robust statistical interpretation.

3. Results

3.1. Protocol Validation

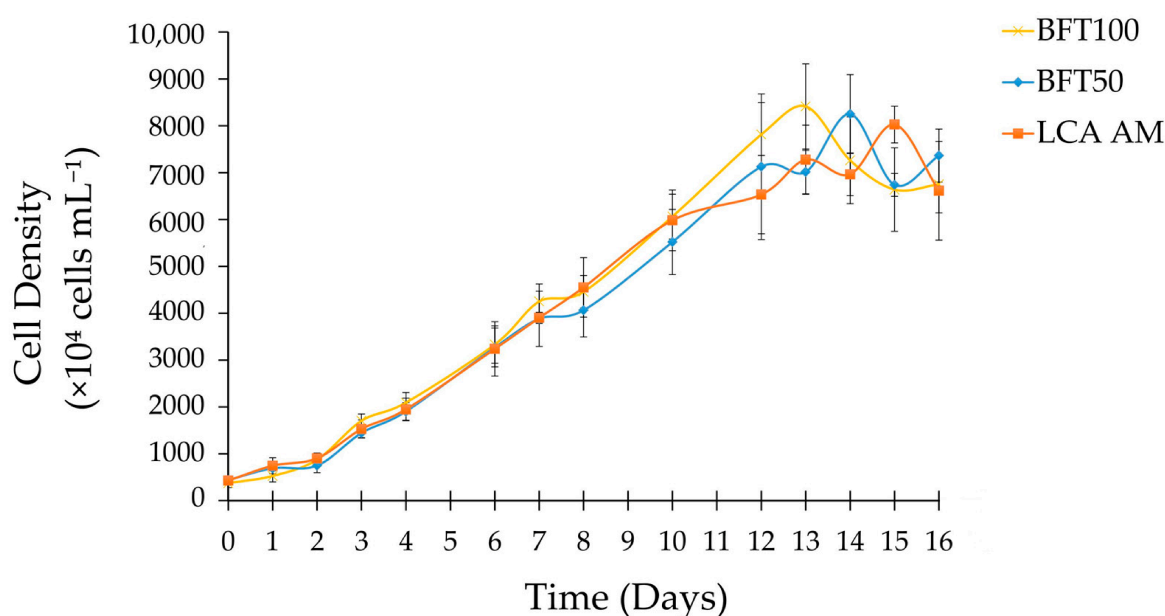
The proposed protocol was validated according to three methodological attributes: repeatability, robustness, and applicability. Repeatability was assessed through the use of four independent replicates per treatment and the calculation of descriptive variability metrics for all primary performance indicators (Table 2). The low variability observed for maximum cell density among treatments demonstrated satisfactory experimental consistency and reproducibility.

Regarding cell density, no significant differences ($p = 0.5231$) were observed among the treatments throughout the cultivation period. Analysis of the growth curves revealed a similar pattern among treatments, with cultures initiating the exponential growth phase without an apparent adaptation (lag) phase. The stationary phase was reached approximately on day 13 of cultivation. Visually, growth in media containing effluent (BFT50 and BFT100) was comparable to that in the control medium (LCA-AM), suggesting that the effluent did not exert negative effects on microalgal growth in BFT effluent (Figure 2).

Robustness was evaluated by applying the protocol to three culture media with contrasting nutrient compositions: a conventional synthetic medium (LCA-AM), a mixed medium containing 50% aquaculture effluent (BFT50), and an undiluted aquaculture effluent medium (BFT100). The protocol successfully generated comparable growth curves and performance indicators across these distinct nutrient environments, demonstrating methodological stability.

Table 2. Repeatability assessment of the proposed protocol for standardising the evaluation of nutrient recovery and biomass production using aquaculture effluent for microalgae cultivation.

Indicator	Treatment	Mean $\pm$ SD	CV (%)	Interpretation
Maximum cell density (CD _{max} $\times 10^4$ cell mL ⁻¹)	LCA-AM	8025 $\pm$ 393	4.9	Excellent
	BFT50	8450 $\pm$ 622	7.4	Excellent
	BFT100	8488 $\pm$ 772	9.1	Excellent
Maximum biomass (B _{max} g L ⁻¹)	LCA-AM	3.8 $\pm$ 0.8	21.1	Acceptable
	BFT50	4.5 $\pm$ 0.5	11.1	Good
	BFT100	4.8 $\pm$ 1.3	27.1	Acceptable
Volumetric productivity (g L ⁻¹ d ⁻¹)	LCA-AM	0.24 $\pm$ 0.05	20.8	Acceptable
	BFT50	0.27 $\pm$ 0.03	11.1	Good
	BFT100	0.30 $\pm$ 0.07	23.3	Acceptable
Nitrate removal efficiency (%)	LCA-AM	97.36 $\pm$ 1.10	1.13	Excellent
	BFT50	98.34 $\pm$ 0.31	0.32	Excellent
	BFT100	97.34 $\pm$ 2.15	2.21	Excellent
Orthophosphate removal efficiency (%)	LCA-AM	99.72 $\pm$ 0.09	0.09	Excellent
	BFT50	88.03 $\pm$ 0.70	0.80	Excellent
	BFT100	87.88 $\pm$ 1.61	1.83	Excellent

**Figure 2.** Growth curves (cell density) (means $\pm$ standard deviation) of *Phaeodactylum tricornutum* cultures using traditional culture medium (LCA-AM) and shrimp farming effluent from a biofloc system.

Statistical analysis of the data indicated that there were no significant differences ($p > 0.05$) among treatments for any of the evaluated parameters (Table 3). This is a key result of the study, as it demonstrates that effluent from the BFT system can fully replace the LCA-AM medium without compromising the growth and productivity of *P. tricornutum*. Although biomass and productivity values were numerically higher in the BFT100 treatment, the high variability in the data, particularly for B_{max}, prevented the detection of statistical differences. This variability is a known limitation of biomass estimation using turbidity, which can introduce inaccuracies, especially at high cell densities requiring dilution.

Table 3. Growth parameters (means $\pm$ standard deviation) of *Phaeodactylum tricornutum* using effluent-shrimp as the culture medium and control culture medium (LCA-AM).

Treatments	CDmax ($\times 10^4$ cells mL $^{-1}$)	Bmax (g L $^{-1}$)	Time (Days)	Productivity (g L $^{-1}$ d $^{-1}$)
LCA AM	8025 $\pm$ 393 ^a	3.8 $\pm$ 0.8 ^a	15.3 $\pm$ 1.0 ^a	0.24 $\pm$ 0.05 ^a
BFT50	8450 $\pm$ 622 ^a	4.5 $\pm$ 0.5 ^a	15.8 $\pm$ 0.5 ^a	0.27 $\pm$ 0.03 ^a
BFT100	8488 $\pm$ 772 ^a	4.8 $\pm$ 1.3 ^a	15.3 $\pm$ 1.0 ^a	0.30 $\pm$ 0.07 ^a
<i>p</i> -value	0.3175	0.4053	0.3395	0.6337

Note: CDmax: Maximum cell density achieved. Bmax: Maximum biomass achieved. Time: Cultivation time until Bmax. Productivity: $B_{\text{max}} - B_{\text{initial}}/\text{time}$. Different letters in the same row indicate significant differences ($p < 0.05$) between treatments.

Although biomass was estimated indirectly using a turbidity-based regression, this approach was supported by a calibration model developed from experimental cultures of *P. tricornutum* maintained in our laboratory over several years. The corresponding calibration data are provided as Supplementary Material. Indirect biomass estimation may introduce additional uncertainty compared with direct gravimetric measurements, particularly when changes in cell morphology, pigment content, or physiological status modify the optical properties of the culture. Nevertheless, because the same calibration model and measurement procedure were consistently applied to all treatments, this potential source of systematic error remained constant throughout the experimental validation. Consequently, the use of turbidity is unlikely to compromise the comparative assessment of repeatability, robustness, and sensitivity proposed in the present protocol. Moreover, the independent determination of maximum cell density exhibited low coefficients of variation among replicates, providing additional evidence that the observed treatment differences reflected biological responses rather than measurement artefacts. Although direct dry-weight determination remains the reference method for biomass quantification, turbidity-based estimation represents a rapid, non-destructive, and operationally practical alternative for routine protocol implementation, provided that an appropriate species-specific calibration is available.

The results observed in the present study are both consistent with and divergent from previous reports in the literature. Holanda et al. [23], who cultivated *Arthrospira platensis* in shrimp farming biofloc effluent (1, 10, and 100%), observed that the 100% biofloc-effluent treatment exhibited significantly higher cell density than the treatment with a laboratory-prepared fertilising medium (control group). Conversely, Magnotti et al. [8] reported that the cultivation of the marine microalgae *N. oculata* and *T. chuii* in effluent from super-intensive shrimp farming in biofloc systems also yielded positive outcomes, achieving biomass gains equivalent to those obtained with a conventional synthetic medium.

Applicability was assessed through the integration of growth performance metrics and nutrient-removal indicators. The protocol was capable of detecting differences in nutrient dynamics while maintaining consistent growth assessments, indicating its suitability as a general framework for evaluating alternative nutrient sources in microalgal cultivation systems. Although the present validation was performed using *P. tricornutum* cultivated in shrimp biofloc effluent, the protocol was intentionally structured around performance indicators that are not species-specific. Consequently, the methodological framework may serve as a basis for future applications involving other microalgal taxa and aquaculture effluents. Nevertheless, additional validation studies will be required to confirm its performance under different biological and operational conditions.

3.2. Repeatability and Precision Assessment

The repeatability of the proposed protocol was evaluated using the coefficient of variation (CV) obtained from four independent replicates for each treatment. Maximum

cell density (CD_{max}) exhibited low variability, with CV values ranging from 4.9% to 9.1%, indicating excellent repeatability across treatments. Biomass and volumetric productivity (VP) showed higher variability, with CV values ranging from 11.1% to 20.8%, which is expected for biological systems relying on indirect biomass estimation.

Nutrient-removal indicators demonstrated particularly high precision, with orthophosphate removal differing significantly ($p < 0.001$) among the treatments (Table 4). Nitrate removal efficiency presented CV values between 0.3% and 2.2%, whereas orthophosphate removal efficiency showed CV values between 0.1% and 1.8%. These results indicate that the protocol generates highly reproducible measurements for nutrient recovery assessments. Overall, the low variability observed in the primary growth and nutrient-removal indicators supports the repeatability and analytical precision of the proposed standardised testing framework.

Table 4. Nutrient concentrations and removal rates in experimental cultures of the diatom *Phaeodactylum tricornutum* using effluent-shrimp as the culture medium.

Nitrate	Initial Concentration (mg L ⁻¹)	Final Concentration (mg L ⁻¹)	Removal (%)
Treatments			
LCA AM	67.06 ± 12.72 ^c	1.74 ± 0.77 ^a	97.36 ± 1.10 ^a
BFT50	85.95 ± 9.34 ^b	1.41 ± 0.16 ^a	98.34 ± 0.31 ^a
BFT100	96.27 ± 13.95 ^a	2.73 ± 2.62 ^a	97.34 ± 2.15 ^a
<i>p</i> -value	0.0227	0.4948	0.5398
Orthophosphate	Initial Concentration (mg L ⁻¹)	Final concentration (mg L ⁻¹)	Removal (%)
Treatments			
LCA AM	15.99 ± 6.83 ^a	0.035 ± 0.0 ^a	99.72% ± 0.09% ^a
BFT50	0.34 ± 0.02 ^b	0.035 ± 0.0 ^a	88.03% ± 0.70% ^b
BFT100	0.34 ± 0.05 ^b	0.035 ± 0.0 ^a	87.88% ± 1.61% ^b
<i>p</i> -value	0.0004	0.9821	<0.0001

Note: Different letters in the same column indicate significant differences (Tukey test, $p < 0.05$).

In the present study, it was observed that the cultures started with different nitrate concentrations, with media prepared using effluent exhibiting higher concentrations than the control group. This is possibly due to the fact that, in BFT systems, water renewal is minimal, leading to greater accumulation of faeces and uneaten feed, and consequently to the accumulation of this nutrient [3,4]. After 16 days, significant removal was observed in all treatments, with removal percentages exceeding 97%, indicating efficient nutrient uptake by the microalgae. According to Magnotti et al. [8], the microalgae *T. chuii* and *N. oculata* achieved 83% nitrate removal in water over 10 days, which can be considered substantially lower than that achieved in the present protocol. In the present study, although there was no statistically significant difference in CD_{max}, the treatment with the highest nitrate Ci (BFT100) showed the highest numerical values for biomass and productivity. Greater nitrogen availability may have supported increased biomass accumulation per cell, even though cell numbers were similar to those in the control.

Regarding nitrite concentration (Figure 3), net accumulation of nitrite was observed in all treatments. However, a high standard deviation was recorded, suggesting possible imprecision in the drop-based colorimetric analytical method, which is sensitive to small variations in execution. Alternatively, one possible explanation is the metabolism of the microalga itself, which may reduce nitrate (Figure 4) to nitrite more rapidly than it is assimilated [24,25]. Additionally, microbial communities present in the environment can influence the nitrogen cycle in aquaculture effluents, potentially leading to the buildup of intermediates like nitrite [26].

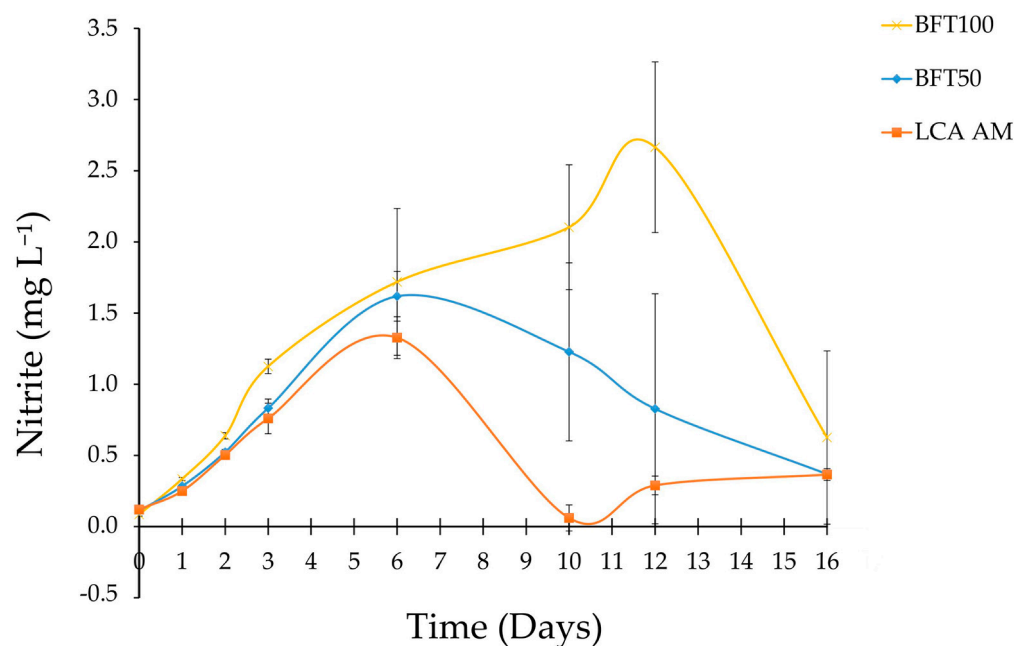


Figure 3. Temporal kinetics of nitrite concentrations in experimental cultures of the diatom *Phaeodactylum tricornutum* using traditional culture medium (LCA-AM) and shrimp farming effluent from a biofloc system.

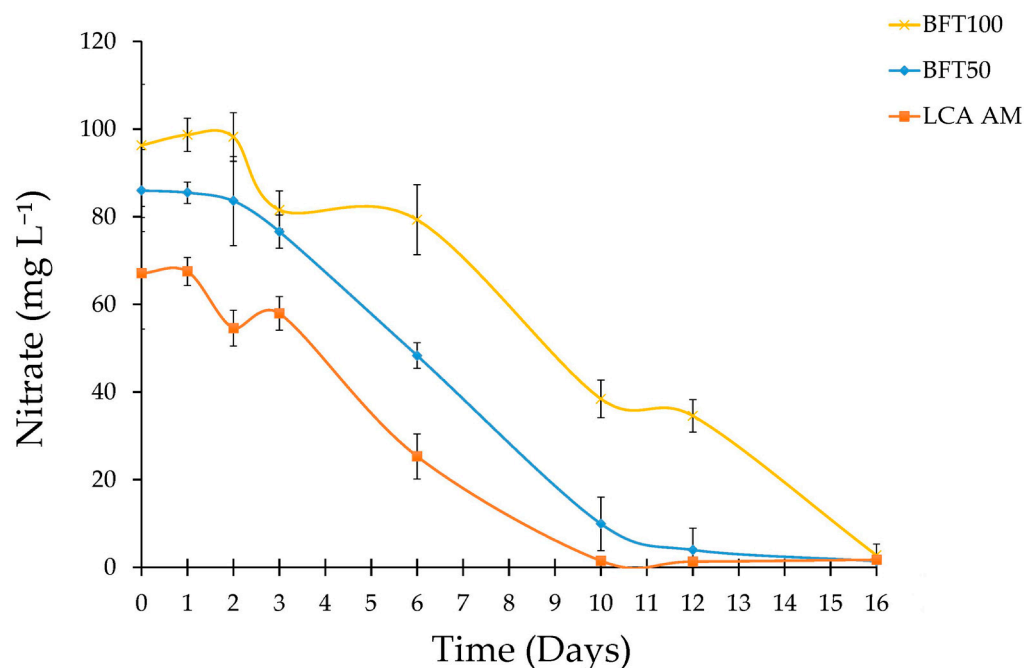


Figure 4. Temporal kinetics of nitrate concentrations in experimental cultures of the diatom *Phaeodactylum tricornutum* using traditional culture medium (LCA-AM) and shrimp farming effluent from a biofloc system.

Despite substantial differences in the initial nitrate (Figure 4) and phosphate concentrations (Figure 5) among treatments, the protocol generated consistent growth performance measurements. Maximum cell density, maximum biomass, cultivation time, and volumetric productivity did not differ significantly among treatments ($p > 0.05$), indicating that the methodology remained stable under contrasting nutrient conditions. Furthermore, the protocol successfully quantified nutrient-removal dynamics in all treatments, demonstrating its ability to operate across a broad range of nutrient concentrations with-

out requiring modifications to the analytical workflow. These findings indicate that the proposed protocol is robust and can be applied to aquaculture effluents with variable physicochemical characteristics.

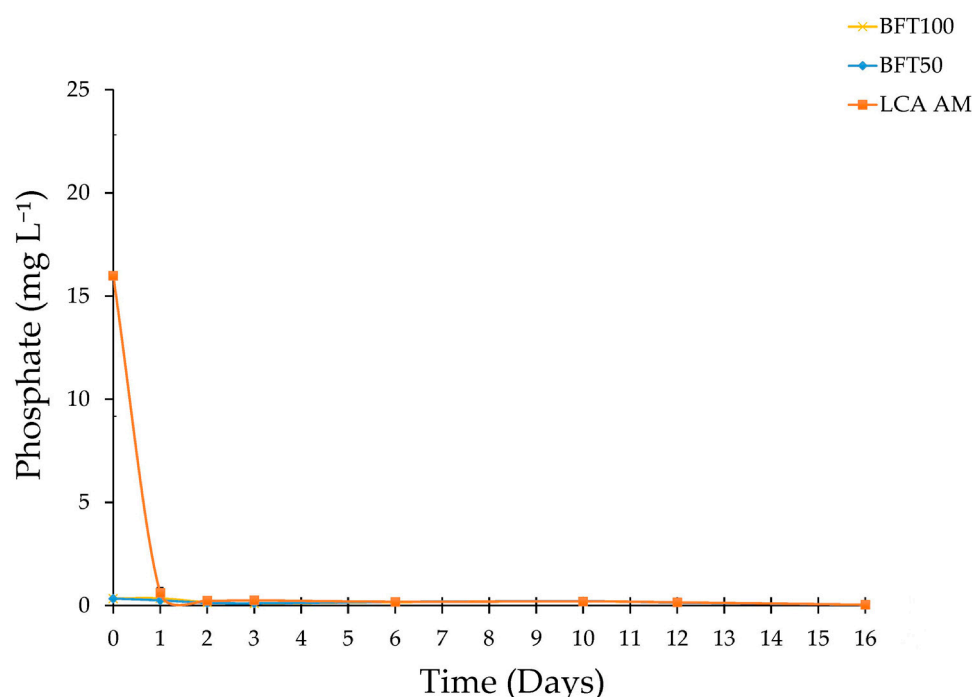


Figure 5. Temporal kinetics of orthophosphate concentrations in experimental cultures of the diatom *Phaeodactylum tricornutum* using traditional culture medium (LCA-AM) and shrimp farming effluent from a biofloc system.

In the present study, a large disparity was observed in the initial concentrations of orthophosphate (Figure 4). The control treatment (LCA-AM) was rich in this nutrient, whereas the BFT50 and BFT100 treatments started with identical and extremely low orthophosphate concentrations, which would possibly not be sufficient to support the robust growth observed, since phosphorus is an essential macronutrient for the production of ATP, nucleic acids, and phospholipids [27]. Despite this apparent absence of phosphorus, the cultures in the BFT media grew similarly to the control, reaching high cell and biomass densities. This observation may be related to the analytical approach employed, which quantified only dissolved orthophosphate and may not fully represent the total bioavailable phosphorus in the culture medium. Moreover, it is plausible that growth was maintained through internal phosphorus reserves. As described by the concept of “luxury uptake,” microalgae can store phosphorus in the form of polyphosphates, enabling continued growth even in phosphorus-deficient environments [28]. Further research is imperative to explore whether *P. tricornutum* might have exploited alternative phosphorus forms present in the medium. Dyhrman [27] indicates that certain microalgae are capable of utilising non-traditional phosphorus forms, such as pyrophosphate, and converting them into biomass. Despite these uncertainties regarding phosphorus availability, the observed growth results remain valid, highlighting the potential of shrimp culture effluent as an alternative culture medium.

3.3. Sensitivity and Discriminatory Capacity

The sensitivity of the protocol was assessed by evaluating its capacity to detect treatment-dependent differences in nutrient availability and nutrient-removal dynamics. Although growth performance indicators remained statistically similar among treatments,

the protocol detected substantial differences in the initial concentrations and temporal behaviour of dissolved nutrients. Nitrate concentrations differed markedly among media, while orthophosphate dynamics exhibited distinct patterns throughout cultivation. The ability to identify nutrient-related differences while maintaining reproducible growth measurements demonstrates that the protocol possesses adequate discriminatory capacity. This characteristic is essential for a standardised evaluation framework, as it enables comparison of effluents with distinct nutrient profiles while generating biologically meaningful performance indicators. Therefore, the protocol is sufficiently sensitive to detect variations in nutrient availability and nutrient recovery efficiency, supporting its use as a comparative assessment tool for alternative culture media.

3.4. Applicability Domain

The protocol was designed to maximise methodological transferability by employing commonly used cultivation-performance and nutrient recovery indicators. Although these characteristics suggest potential applicability to other aquaculture systems and microalgal species, the present study validates the framework only for the cultivation of *P. tricornutum* in shrimp biofloc effluent. Future studies should evaluate the protocol under additional cultivation conditions to establish its broader applicability.

The results of the present study demonstrate that the proposed protocol possesses sufficient sensitivity to discriminate between alternative culture media, even when conventional growth indicators remain statistically similar. Although biomass production and growth rates did not differ significantly among treatments, consistent differences were observed in the initial nitrate concentrations and in the temporal dynamics of orthophosphate, highlighting the protocol's capacity to detect variations in nutrient availability and removal processes. This characteristic is particularly relevant in the context of standardised protocols, where the primary objective is not necessarily to maximise productive performance but rather to provide a robust and reliable framework for comparisons among different systems or treatments.

The importance of standardised methodologies in generating consistent and comparable assessments has been widely recognised across diverse areas of aquaculture. In the context of physiological assessments, Fazio [15] noted that the adoption of standardised methodologies in fish haematology is essential for ensuring the reliable interpretation of biological indicators and enabling meaningful comparisons among studies. Macfadyen et al. [13], for example, emphasised that structured methodological approaches are fundamental for objectively evaluating sector performance across different production contexts. Similarly, Palić et al. [14] highlighted that the standardisation of biosecurity protocols facilitates the consistent identification of risks and the harmonisation of procedures among aquaculture systems. More recently, Walsh et al. [16] demonstrated that standardised methodological frameworks provide the foundation for robust assessments of the conformity of fisheries and aquaculture management systems with international sustainability guidelines. Likewise, Moraes et al. [17] highlighted the value of model organisms and standardised experimental protocols for the comparative evaluation of biostimulants, reinforcing the need for sensitive and reproducible tools capable of discriminating among treatments.

Although several studies have demonstrated the feasibility of cultivating microalgae in aquaculture effluents, most investigations have focused primarily on biological performance indicators, such as biomass production, nutrient removal, or product yield, rather than on establishing a standardised assessment methodology. Consequently, direct comparisons among studies remain challenging because experimental designs, cultivation conditions, analytical procedures, and performance indicators often differ substantially. For

example, Magnotti et al. [8] successfully demonstrated the cultivation of marine microalgae using residual water from shrimp biofloc systems while evaluating biomass production and nutrient removal. Likewise, Holanda et al. [23] investigated the production of *A. platensis* in biofloc effluent, emphasising biomass productivity and wastewater treatment efficiency. Although both studies provide valuable evidence regarding the feasibility of effluent reuse, neither proposes a structured framework for assessing methodological repeatability, robustness, sensitivity, or applicability across different experimental conditions. Similarly, Chu et al. [10] evaluated microalgal growth and wastewater treatment performance under mixotrophic cultivation conditions. Their approach successfully demonstrated the influence of cultivation strategy on biomass production and nutrient removal; however, the evaluation remained centred on biological performance rather than on the validation of a transferable assessment protocol. The comprehensive review by Shitu et al. [11] further highlights the considerable diversity of methodologies currently employed for evaluating aquaculture wastewater as a culture medium for microalgae. Differences in pretreatment procedures, cultivation conditions, analytical methods, and performance indicators frequently limit comparisons among studies and hinder the development of harmonised evaluation criteria.

Within this context, the ability of the present protocol to detect variations in nutrient availability and nutrient recovery efficiency while maintaining the reproducibility of growth-related metrics, confirms its suitability as a comparative assessment tool for alternative culture media. Furthermore, these findings strengthen its potential application in future studies aimed at wastewater valorisation and the promotion of nutrient circularity within sustainable aquaculture systems.

4. Conclusions

This study proposed and experimentally validated a standardised protocol for assessing the suitability of aquaculture effluents as alternative culture media for microalgal cultivation. The framework integrates growth performance indicators, biomass-production metrics, nutrient-removal efficiency, and validation criteria into a reproducible methodological approach. Under the experimental conditions evaluated, the protocol demonstrated satisfactory repeatability, analytical precision, robustness, sensitivity, and applicability for assessing the cultivation of *P. tricornutum* using shrimp biofloc (BFT) effluent as an alternative culture medium. Although the protocol was intentionally designed using broadly applicable evaluation criteria, its performance has presently been validated only for the experimental system investigated. Consequently, further studies employing different microalgal species, aquaculture effluents, and cultivation systems are required to confirm its broader applicability. Nevertheless, the proposed framework provides a structured methodological basis for future comparative studies and may contribute to the harmonisation of evaluation procedures in research involving nutrient recovery and wastewater valorisation in sustainable aquaculture.

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