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Valorization of Aquaculture Waste Through Conversion into Algal Biomass and Biomethane

Elvira E. Ziganshina, Svetlana S. Bulynina and Ayrat M. Ziganshin *

Department of Microbiology, Institute of Fundamental Medicine and Biology, Kazan (Volga Region) Federal University, 420008 Kazan, Republic of Tatarstan, Russia; elvira.ziganshina@kpfu.ru (E.E.Z.)
* Correspondence: ayrat.ziganshin@kpfu.ru

Abstract

Uneaten feed and feces are major sources of waste in aquaculture systems, which lead to the eutrophication of water bodies. This study proposes a multidisciplinary approach to the valorization of two types of aquaculture waste. The first component of the strategy is the cultivation of microalgae in raw wastewater. The wastewater from a recirculation aquaculture system (RAS) for sturgeon was tested as an alternative growth medium for eight microalgae species. The highest biomass concentration was achieved when cultivating *Tetradesmus obliquus*, reaching 1.34 ± 0.04 g L^{-1}. Anaerobic digestion (AD) of sludge from the RAS and mixed algal biomass harvested from wastewater treatment was proposed as a secondary component of a waste valorization strategy. Granular activated carbon (GAC) was selected as the conductive material to enhance methane production from tested substrates. The specific methane production (SMP) during AD of the sludge ranged from 94 to 99 NmL $g^{-1}_{VS\ (volatile\ solids)}$. In contrast, the SMP for treatments containing algae reached 321–327 NmL g^{-1}_{VS}. The addition of GAC to the anaerobic reactors was an effective method for enhancing methane formation from algal biomass. Finally, this study combines aquaculture water treatment and sludge management with the production of valuable algal biomass and biofuel.

Keywords: green algae; algal biomass; aquaculture wastewater; sludge; integrated biotechnologies; anaerobic digestion

1. Introduction

Aquaculture is a crucial sector of animal husbandry that significantly contributes to food systems. In response to the growing demand for aquatic animal products, which serve as a vital source of protein, production volumes within this sector are increasing annually. Modern aquaculture is developing primarily through intensification, which poses environmental risks due to the generation of substantial amounts of waste, the accumulation of pollutants in the water used, and the production of feed. Aquaculture by-products include semi-solid waste—aquaculture sludge—and liquid waste containing various dissolved substances. Failure to promptly remove solid waste from aquatic animal farming systems results in increased concentrations of dissolved compounds. The amount of waste generated and the composition of water used in aquaculture depend on the conversion efficiency and composition of feed, as well as the metabolic characteristics of the farmed aquatic animals [1,2]. It is worth noting that the wastewater may also contain other contaminants from anthropogenic activities, such as microorganisms, including pathogenic

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ones, antibiotics, microplastics, and heavy metals, which pose a threat to consumers and the environment [3].

To reduce environmental impacts, various technologies are being developed to improve the sustainability of the aquaculture sector while ensuring productivity and product quality [4]. Primarily, these technologies aim to overcome the limitations of intensive aquaculture. Currently, the most technologically advanced and environmentally sustainable systems used for growing fish and other aquatic animals are recirculating aquaculture systems (RAS). The advantages of RAS include resource efficiency, control of growing conditions, minimization of water consumption, and the use of an integrated approach to wastewater treatment that combines physical and biological treatment methods [4–6]. Despite the advantages of this cultivation system, the high-density rearing of aquatic organisms results in elevated nutrient levels in the water. This situation requires additional treatment before the water can be recycled or discharged into receiving water bodies. Water exchange, maintenance of active denitrification, and phytoremediation are strategies used to address this issue. The use of micro- and macroalgae, agents of phytoremediation, allows active removal of nutrients from wastewater and decrease in the carbon footprint as well as production of valuable biomass [7–9].

The role of microalgae in wastewater treatment from various sources is to improve water quality, such as reducing the level of total nitrogen (TN), total ammonia nitrogen (TAN), total phosphorus (TP), chemical oxygen demand (COD), and total solids (TS) [10]. The bioremediation potential of microalgae varies by species, so for the most effective removal of pollutants, it is advisable to use species adapted to growth in wastewater [11]. The ability of microalgae to synthesize antimicrobial compounds and compete with other microorganisms for nutrients helps reduce the number of bacteria, including pathogenic ones, in wastewater [12]. The advantages of biological water treatment using microalgae include highly efficient removal of various pollutants, the absence of secondary pollution, low costs, and the accumulation of biomass, which can subsequently be converted into valuable bioproducts. The nutritional value of algal biomass is due to its content of proteins, fatty acids, polysaccharides, carotenoids, and vitamins. This biomass is considered a feed ingredient for aquatic animals, which reveals a positive aspect of the integration of microalgae in aquaculture [13]. Furthermore, microalgae fix CO_2 and saturate water with dissolved O_2 [10]. Among the limitations to the large-scale implementation of this technology, one can note the need for specialized equipment, as well as the costs associated with microalgae cultivation and the harvesting and processing of biomass. *Nannochloropsis oculata*, *Pavlova gyrans*, *Tetraselmis suecica*, *Phaeodactylum tricornutum*, and their consortium were assessed as microorganisms that can produce valuable biomass and effectively remove nutrients from RAS water with low concentrations of nitrate and phosphate [8]. The authors emphasized the importance of the composition of this alternative culture medium for the growth efficiency of marine microalgae.

Aquaculture sludge—a semi-solid waste consisting of excrement, uneaten feed, and organic residues—also requires careful treatment. One promising yet under-researched approach involves anaerobic digestion (AD) of this substrate to generate biogas [2,14]. As a result of AD, complex organic compounds are converted into simple compounds, which serve as a substrate for the formation of organic acids, H_2, and CO_2. In the final stage, the intermediate products serve as substrates for the CH_4 and CO_2 formation (methanogenesis stage). Microbial communities, primarily composed of bacteria and archaea, play a crucial role in the complex interdependent anaerobic biochemical processes. The effectiveness of AD relies on the syntrophic relationships among microbial community members. The abundance and activity of these microbes are influenced by various factors, including the substrate-to-inoculum ratio, temperature, pH, organic loading rate, hydraulic retention

time, and the presence of inhibiting compounds. One way to optimize AD is through the conductive interspecies electron transfer (CIET) between syntrophic microbial partners, which is achieved by adding conductive materials into anaerobic digesters [15,16]. A number of studies have described the influence of iron-based materials (magnetite nanoparticles and nano particles of other iron oxides and hydroxides) and carbon-based materials (granular activated carbon (GAC) and carbon nanotubes) on the efficiency of methane production from various substrates. It has been observed that, at specific concentrations, these agents enhance biogas production, including through the distribution of microorganisms that participate in interspecies electron transfer [17–19]. GAC, as a highly effective, low-cost stimulator of methanogenesis, also reduces the inhibitory effects of substrate components and microbial metabolites in anaerobic systems [20].

Sludge generated on fish farms is a poorly studied substrate for AD. The sludge composition depends on the feed composition and the degree of aquaculture intensification, which will affect the efficiency of AD [1,14]. Another characteristic of aquaculture sludge is its low dry matter content, which requires pre-concentration of the substrate [21]. While aquaculture sludge has significant potential for the production of biogas, the widespread adoption of AD technology for processing this type of waste is constrained by its compositional characteristics and the labor-intensive requirements of preliminary preparation [14]. Additionally, biomass generated during cultivation of microalgae in liquid aquaculture waste can be used as a substrate for AD. The combination of microalgae-based wastewater treatment and AD of sludge and nutrient-rich algal biomass offers several benefits, including water purification, the recovery of valuable components from waste to produce useful bioproducts, and the development of the aquaculture industry toward cyclical processes.

This study proposes a comprehensive approach to treating liquid and solid aquaculture waste while producing valuable algal biomass and biofuel. The main objective of this study was to select microalgal strains of the genera *Chlorella*, *Micractinium*, *Desmodesmus*, *Tetradesmus*, and *Neochloris* for wastewater treatment, as well as to evaluate their potential for integration into the aquaculture sector. AD of aquaculture sludge and microalgal biomass harvested from wastewater treatment was proposed as a secondary component of a waste valorization strategy.

2. Materials and Methods

2.1. Aquaculture Waste Collection and Analysis

The experimental design of this study is depicted in Figure 1.

Samples of aquaculture waste were collected in February 2025 at a specialized fish farm (Laishevsky District, Republic of Tatarstan, Russia) from the RAS designed for sturgeon farming. In this system, water from the fish tanks passes through a mechanical drum filter that removes large particles. The water then flows through a biofilter, which reduces the levels of toxic compounds harmful to fish. Next, the water is directed to a settling tank, where it undergoes treatment with ozonation and ultraviolet light before being returned to the fish farming systems. Water and sludge samples for the experiments were collected from the waste collection system and were delivered to the laboratory within an hour. The water was then filtered through multi-layer gauze to separate solid particles, which is necessary for the subsequent cultivation of algae. Containers containing sludge were left in a refrigerator at 4 °C for 24 h to settle. Raw wastewater was immediately used as a growth medium for microalgae, and concentrated sludge was stored in sterile borosilicate glass bottles in a refrigerator at 2 °C until use. The concentrated aquaculture sludge (mainly presented by excrements and feed residues) was tested for biomethane yield.

The analysis of the aquaculture wastewater included determination of pH, concentrations of chloride, nitrate, nitrite, phosphate, sulfate, ammonium, urea, iron, and total

microbial count (TMC). pH was determined using a Starter ST300 portable pH meter (OHAUS Corporation, Shanghai, China). Concentrations of inorganic anions were determined using a Dionex ICS-900 ion chromatography system (Thermo Fisher Scientific, Wilmington, DE, USA) equipped with an IonPac AG22 guard column (4 × 50 mm) and an IonPac AS22 analytical column (4 × 250 mm). The mobile phase consisted of 4.5 mM Na_2CO_3 and 1.4 mM $NaHCO_3$ at a flow rate of 1.2 mL min^{-1}. Separation was performed at room temperature. Total ammonia nitrogen (TAN) concentrations were determined photometrically using a Nessler's reagent (Sigma-Aldrich, St. Louis, MO, USA). The urea level was determined using a commercial kit, Urea-1-Olvex (Olvex Diagnosticum, St. Petersburg, Russia). Dissolved iron concentrations were determined with a Hanna kit (Hanna Instruments, Nusfalau, Romania). TMC was estimated by colony counting using a ColonyDoc-It imaging system (UVP, Cambridge, UK) after 72 h of cultivation on meat-peptone agar at 30 °C in an RI 53 Red Line incubator (Binder, Tuttlingen, Germany).

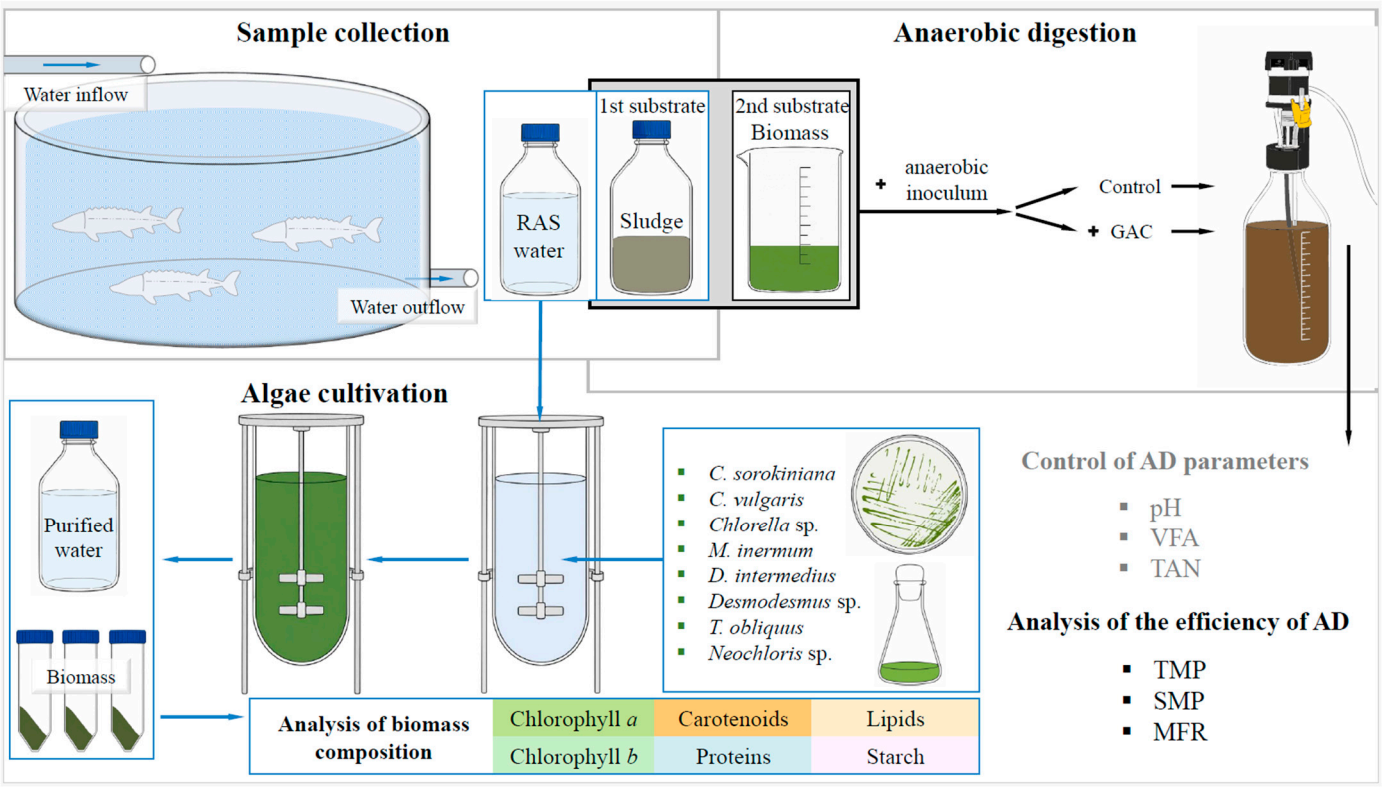


Figure 1. A schematic diagram summarizing the experimental design of the present study.

2.2. Tested Algal Strains

The strains *Chlorella sorokiniana* EZ-07, *Chlorella vulgaris* SB-M4, *Chlorella* sp. EE-P5, *Micractinium inermum* EE-M2, *Tetradesmus obliquus* EZ-B11, *Desmodesmus* sp. EE-M8, and *Neochloris* sp. EE-K3 were selected from our existing collection. The strain *Desmodesmus intermedius* EE-M11 was newly isolated from a freshwater river (Republic of Tatarstan, Russia). Water samples were collected in sterile 0.5 L Duran bottles and delivered to the laboratory within 2–3 h. A synthetic nutrient medium—standard Bold's Basal Medium (BBM)—was used to obtain enrichment cultures. The flasks with inoculated media were incubated for one week in an IKA KS 4000 IC control shaker-incubator (IKA, Staufen, Germany) at a speed of 120 rpm and a temperature of 30 °C. Constant illumination was provided, with an average intensity of 250 µmol photons m^{-2} s^{-1}. Each enriched culture sample was then plated on sterile Petri dishes with agarized standard BBM with antibiotics (10 µg of ampicillin and 50 µg of kanamycin per 1 mL of medium) and incubated at room

temperature under continuous illumination with a PPFD of 250 µmol photons m^{-2} s^{-1}. The subculture procedure was performed multiple times to isolate axenic cultures. Using a light microscope, phenotypic differences among the cells in individual colonies were examined.

Pure cultures were incubated in liquid BBM for 7 days under the previously described conditions. Algal DNA was isolated from pelleted biomass samples using a FastDNA spin kit (MP Biomedical, Solon, OH, USA). DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, DE, USA). Microalgae were then identified using two molecular gene markers: *rbcL* and *tufA* genes. DNA amplification was performed in a T100 thermal cycler (Bio-Rad, Hercules, CA, USA). Amplicons were then purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). DNA sequencing was performed on an ABI 3730 DNA Analyzer (Thermo Fisher Scientific, CA, USA). The basic local alignment search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 21 October 2026) was then used for received data analysis.

2.3. Cultivation Conditions of Microalgae

Inoculum cultures were prepared by adding individual colonies to liquid BBM and were cultured under the previously described conditions. The algal biomass was concentrated by centrifugation in 50 mL Falcon tubes at 3000× *g* for 3 min and washed twice with a sterile 0.9% solution of NaCl. The initial optical density measured at 750 nm (OD_{750nm}) was 0.05 in all experiments. Microalgae were cultivated in autoclavable bioreactors with a working volume of 2.0 L (BIOSTAT A-plus, Sartorius, Germany) under controlled parameters: 30 °C, agitation at 100 rpm, continuous illumination (800 µmol photons m^{-2} s^{-1}), aeration (1.2 L min^{-1}), and a continuous supply of 1.5% CO_2 (*v*/*v*). Uniform illumination of the bioreactor surfaces was achieved using four LED phytolamps (ULI-P10-18W/SPFR, Uniel Lighting Co., Hangzhou, China), which were positioned on stands on both sides of the bioreactors. SmartTrak 50 thermal mass flow meters (Sierra Instruments, Monterey, CA, USA) were used to deliver CO_2. Air and CO_2 were mixed and then added to the media by bubbling through Midisart 2000 filters. O_2 and CO_2 concentrations were analyzed using a gas analyzer and a Labfors 5 Lux photobioreactor data display system (Infors HT, Bottmingen, Switzerland). The pH of the growth media was measured using EasyFerm Plus PHI K8 200 electrodes (Hamilton, OH, USA). Foam formation was prevented by adding a sterile 2.0% solution of Antifoam B (Sigma-Aldrich, MO, USA).

2.4. Growth Characteristics of Algal Cultures and Bioremediation Potential

Since the RAS water was not sterile, other microorganisms—specifically bacteria—were present alongside the microalgae. However, their proportion was negligible compared to that of the algae, which is why the term "algal biomass" is used hereafter. To assess the growth dynamics of the cultures, algal suspension samples were taken daily, and the optical density was measured at 750 nm using quartz cuvettes with a 1 cm optical pathlength and a Lambda 35 UV/visible spectrophotometer (PerkinElmer, Singapore). The accumulated biomass was collected after the cultures reached the stationary growth phase, which, under these growth conditions, occurred on average after 4 days. The biomass was concentrated by centrifugation at 3000× *g* for 3 min in 50 mL Falcon tubes. The collected biomass was washed twice with distilled water to remove residual components of the nutrient medium. Next, one part of the biomass was dried at 60 °C for 24 h for the subsequent biochemical analysis, and the other part was dried at 105 °C for 16 h and subsequently burned at 550 °C for 2 h to determine dry matter and organic matter, respectively. Biomass productivity was calculated using a known equation (on a batch basis). To determine the microalgae's bioremediation potential, supernatant samples were collected after cultivation. Water analysis included determination of TAN, phosphate, and sulfate concentrations.

2.5. Determination of the Biochemical Composition of Algal Biomass

Upon completion of cultivation, samples (1 mL of algal suspension) were collected in 1.5 mL test tubes to determine the pigment concentration (chlorophyll *a*, chlorophyll *b*, and total carotenoids) [22]. The biomass was concentrated by centrifugation at 10,000× *g* for 5 min, after which dimethyl sulfoxide (DMSO), preheated to 60 °C, was added to the pellet. The contents of the test tubes were mixed using a vortex and incubated at 60 °C for 5 min. The extract was then separated from the cells by centrifugation at 10,000× *g* for 5 min, and the optical density was measured using the Lambda 35 spectrophotometer. Pigment concentration (mg L^{-1}) was calculated using the equations presented by Wellburn [23]. Determination of protein, lipid, and starch concentrations in the final dried algal biomass was described in detail in our previous studies [24,25]. Briefly, for protein content determination, the dried biomass (20 mg) was combined with 1 mL of 0.5 M NaOH and beads, then homogenized using a FastPrep-24 homogenizer (MP Biomedicals, Solon, OH, USA) in 2 mL metal lysing tubes for two 30 s pulses at a speed of 6 m s^{-1}. Subsequently, the samples were incubated at 80 °C and 500 rpm for 10 min in a TS-100C thermoshaker (BioSan, Riga, Latvia). The mixture was then cooled and transferred to clean microcentrifuge tubes. The samples were centrifuged at 2000× *g* for 5 min, and supernatants were analyzed using a protein assay kit (Bio-Rad, Munich, Germany) in accordance with the manufacturer's instructions. Lipid content was determined using a modified Folch extraction protocol. The dried biomass (30 mg) was combined with chloroform, methanol, and beads, then homogenized using the FastPrep-24 homogenizer in 2 mL metal lysing tubes for four 30 s pulses at a speed of 6 m s^{-1} following centrifugation at 4600× *g* for 5 min. The supernatants were transferred to new glass tubes, centrifuged again, and subsequently transferred to new glass tubes. Then, 0.9% NaCl (20% of the volume) was added, and the resulting mixtures were vortexed. After a 10 min incubation, the lower phase containing lipids was carefully collected into clean glass tubes. The lipid extracts were weighed after solvent removal. The concentration of pigments also extracted by this method was subtracted from the total lipid content. For starch content determination, the dried biomass (50 mg), 0.2 mL of 80% (*v*/*v*) aqueous ethanol, 0.8 mL of 1.7 M NaOH, and beads were added to 2 mL metal lysing tubes. Homogenization was performed with the FastPrep-24 homogenizer at a speed of 6.0 m s^{-1} for 30 s pulses twice. Finally, starch content was analyzed using a K-TSTA starch assay kit (Megazyme, Wicklow, Ireland) in accordance with the manufacturer's instructions. All these analyses were performed in triplicate.

2.6. Anaerobic Digestion of Aquaculture Sludge and Microalgal Biomass

The biochemical methane potential of two substrates, aquaculture sludge and algal biomass, was estimated using automatic methane potential test systems (AMPTS II Light, Bioprocess Control, Lund, Sweden) in a mesophilic mode (38 °C) with a stirring speed of 60 rpm for 1 min followed by a 2 min rest. The resulting biomethane was purified of gas impurities (CO_2, H_2S) by passing it through a 3 M NaOH solution and then directed to a methane gas volume measuring device. To prepare the inoculum, AD of diluted cow manure was conducted until methane emissions were minimized. The total solids (TS) and volatile solids (VS) in both substrate and inoculum samples were subsequently determined. The TS and VS of the inoculum were found to be 3.83 ± 0.04% and 2.86 ± 0.05%, respectively. In the first round of experiments, aquaculture sludge was tested as a mono-substrate. The TS and VS of the concentrated aquaculture sludge were found to be 5.14 ± 0.03% and 2.80 ± 0.04%, respectively. Each anaerobic bioreactor received 745 g of sludge and 500 g of inoculum, with the total volume adjusted to 1500 mL using water. The inoculum-to-substrate ratio was 14.3/20.9 g of VS for the first round. In the second round of experiments, all algal biomass obtained in these experiments, as well as in some others, was dried,

combined, and then used as a substrate for AD. Before starting the second batch, the reactors were opened for a very short period, and algal biomass was added. The TS and VS of the algal biomass were found to be 94.5 ± 0.51% and 88.7 ± 0.62%, respectively. Each reactor was supplemented with 37 g of mixed algal biomass as a substrate and water to bring the total volume to 1600 mL. Thus, during the second round, biomass of microalgae of the genera *Chlorella*, *Micractinium*, *Desmodesmus*, *Tetradesmus*, and *Neochloris* was added to biogas reactors. This substrate was supplemented with algal biomass of the same species obtained in other experiments because the biomass yielded from growth in RAS water was insufficient.

Granular activated carbon (GAC) was added to the experimental reactors at the start-up stage (first round) at concentrations of 3.0 and 6.0 g L^{-1} to observe its effect on AD efficiency. GAC was purchased from Silcarbon Aktivkohle Gmb (Kirchhundem, Germany). Control reactors were operated without GAC. Blank reactors were also used to compensate for the amount of biogas produced by the inoculums itself. Experiments were designated as Sl_C, Al_C (control reactors containing sludge and algae, respectively), Sl_GAC_3, Sl_GAC_6 (experimental reactors with sludge after adding 3.0 and 6.0 g of GAC per 1 L, respectively), Al_GAC_3, and Al_GAC_6 (experimental reactors with algal biomass in the presence of 3.0 and 6.0 g of GAC per 1 L, respectively).

2.7. Analysis of the Efficiency of AD

The biochemical methane potential was calculated after normalizing the methane values reported by the AMPTS II Light devices to 1.0 standard atmospheric pressure, 0 °C and zero moisture content. The efficiency of AD in terms of methane production was estimated using several parameters: total methane production (TMP; NmL), specific methane production (SMP; NmL $g^{-1}{}_{VS}$), and methane flow rate (MFR; NmL day^{-1}). During AD, gas samples were periodically collected to determine the composition of the outgoing biogas, and digestate samples were collected to determine volatile fatty acids (VFAs) and TAN concentrations, as well as pH level. Gas composition was monitored using a Clarus 580 gas chromatograph (PerkinElmer, Shelton, CT, USA) to evaluate the efficiency of methane purification from gas impurities. Organic acid concentrations (acetic, propionic, n-butyric, and isobutyric acids) in digestates were determined by HPLC using an UltiMate 3000 HPLC system (Thermo Fisher Scientific, Hamburg, Germany) equipped with an injector valve, pumps, a variable wavelength detector, a temperature-controlled column compartment, a precolumn, and a Rezex^{TM} ROA-Organic Acid H^+ (8%) column (Phenomenex, Torrance, CA, USA). A 0.005 N H_2SO_4 solution was prepared as the mobile phase. Separation was performed at 40 °C. The concentration of TAN was determined using the Nessler's reagent (Sigma-Aldrich, St. Louis, MO, USA). To determine pH, samples were analyzed using the Starter ST300 portable pH meter (OHAUS Corporation, Shanghai, China).

2.8. Data Analysis

All experiments and analytical measurements were performed in triplicate. Data are presented as mean values ± one standard deviation. The Shapiro–Wilk test for normality and the Levene's test for homogeneity of variance were initially used. Data comparisons were then performed using the Tukey method and 95% confidence intervals. Minitab statistical software (version 22.4.0.0, Minitab, LLC, State College, PA, USA) was used for the statistical analyses.

3. Results

3.1. Efficiency of Microalgae Cultivation in the Water of a Recirculating Aquaculture System

Water samples from the recirculating aquaculture system were analyzed for several parameters, with the results presented in Table 1. After the start of cultivation, the initial pH of the medium decreased from an average of 7.6 to 7.3 within an hour due to the supply of CO_2 for algal growth. After the addition of algal cells, the pH of the medium decreased slightly, reaching final values between 7.0 and 7.1. The available nitrogen in the studied water was primarily in the form of ammonium ions, while phosphate and sulfate ions were present in lower concentrations. At the end of cultivation, the concentrations of NH_4^+–N, PO_4^{3-}–P, and SO_4^{2-}–S were also determined. Thus, all studied algal strains completely removed these compounds from the growth medium. While the total microbial content (TMC) in water before treatment in the RAS was 3.3–3.7 log CFU mL^{-1}, after algae cultivation the TMC values slightly decreased to 2.9–3.2 log CFU mL^{-1}.

Table 1. Characteristics of aquaculture wastewater.

Parameter	Unit	Value
pH		7.6 ± 0.1
TS	%	0.19 ± 0.03
NH_4^+–N	mg L^{-1}	31.4 ± 1.4
Urea	mg L^{-1}	trace amounts
NO_2^-–N	mg L^{-1}	trace amounts
NO_3^-–N	mg L^{-1}	trace amounts
PO_4^{3-}–P	mg L^{-1}	4.3 ± 0.5
SO_4^{2-}–S	mg L^{-1}	7.6 ± 0.6
Cl^-	mg L^{-1}	115.0 ± 8.6
Fe^{2+}/Fe^{3+}	mg L^{-1}	<1
TMC	log CFU mL^{-1}	3.3–3.7

The efficiency of biomass accumulation among the studied strains varied significantly. Figure 2a demonstrates the biomass yield at the end of cultivation, calculated based on dry matter (dry weight, DW) and organic matter (ash-free dry weight, AFDW). The resulting biomass was characterized by a high organic matter content. The maximum biomass yield, recorded during the cultivation of *T. obliquus* EZ-B11, was 1.34 ± 0.04 g L^{-1}. The lowest biomass yield, recorded during the cultivation of *Neochloris* sp. EE-K3, was 0.60 ± 0.08 g L^{-1}. The average biomass yield of the remaining six algal strains did not show statistically significant differences and ranged from 0.86 to 1.12 g L^{-1}. The average biomass productivity across all eight microalgal strains was between 0.15 and 0.34 g L^{-1} day^{-1}, respectively.

Figure 2b illustrates the total chlorophyll and total carotenoid contents of the microalgal cultures, which were evaluated at the end of the experimental period to assess the photosynthetic potential of each culture. The final chlorophyll concentrations varied between 3.4 and 8.3 mg L^{-1}, depending on the algal strain used. The lowest chlorophyll concentrations were recorded in experiments involving *Neochloris* sp. EE-K3 and *Desmodesmus* sp. EE-M8 (3.4 ± 0.7 and 4.4 ± 0.4 mg L^{-1}, respectively). Other strains accumulated chlorophyll *a* and chlorophyll *b* more efficiently. *Neochloris* sp. EE-K3 was also identified as a less efficient producer of total carotenoids, yielding a concentration of 0.7 ± 0.2 mg L^{-1}. In contrast, the concentration of detected total carotenoids in the other experiments varied from 1.4 to 2.5 mg L^{-1}. It is important to note that the pigment concentrations in these experiments were low, as were the final biomass yields; this is attributed to the low initial concentrations of nitrogen, phosphorus, and sulfur, as well as other chemical elements, in the water of a recirculating aquaculture system.

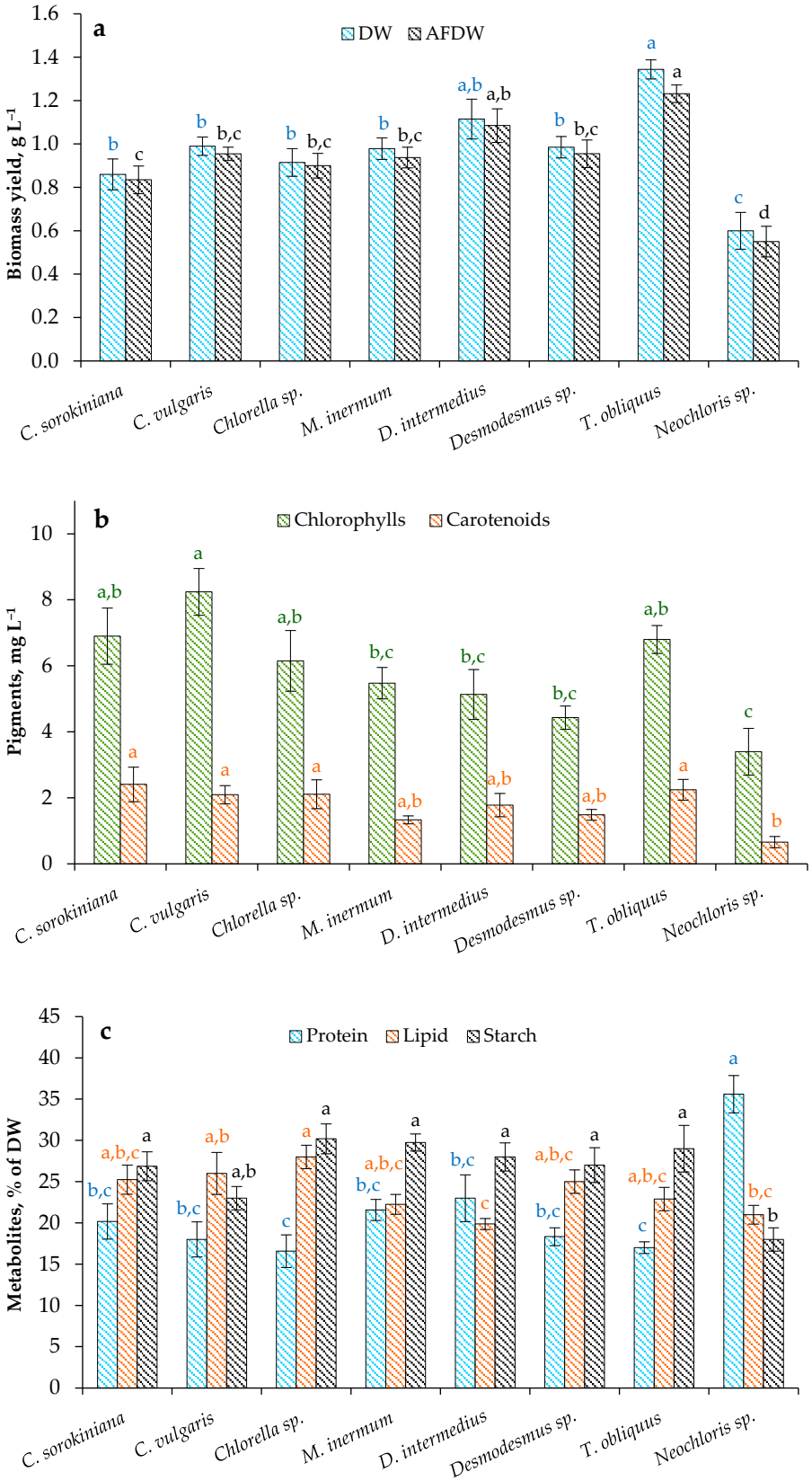


Figure 2. (**a**) Biomass yield and (**b**) pigment concentration, as well as (**c**) protein, lipid, and starch content of biomass obtained during growth of cultures *C. sorokiniana* EZ-07, *C. vulgaris* SB-M4, *Chlorella* sp. EE-P5, *M. inermum* EE-M2, *D. intermedius* EE-M11, *Desmodesmus* sp. EE-M8, *T. obliquus* EZ-B11, and *Neochloris* sp. EE-K3 in aquaculture wastewater. Error bars represent one standard deviation ($n = 3$). Arithmetic means (averages) that do not share a letter are statistically significantly different from each other according to the Tukey method and 95% confidence.

The protein, lipid, and starch contents of the algal biomass were expressed as a percentage of dry weight (Figure 2c), and the metabolite concentration was additionally calculated in mg L^{-1}. The final biomass exhibited protein, lipid, and starch contents ranging from 16.6% to 35.6%, 19.9% to 28.0%, and 18.1% to 30.2%, respectively. Experiments with *D. intermedius* yielded the highest protein, reaching 256 ± 21 mg L^{-1}. In contrast, *T. obliquus* demonstrated greater efficiency in lipid and starch production compared to other cultures (308 ± 10 and 390 ± 13 mg L^{-1}, respectively).

3.2. Potential for Using Aquaculture Sludge and Algal Biomass as Substrates for Anaerobic Digestion

The two test substrates were subjected to AD for 30–32 days, with the experiments ending when methane production was negligible (<1% of cumulative methane production) [26]. Figure 3 shows total methane production and methane flow rate during AD of aquaculture sludge in the absence and presence of different concentrations of GAC.

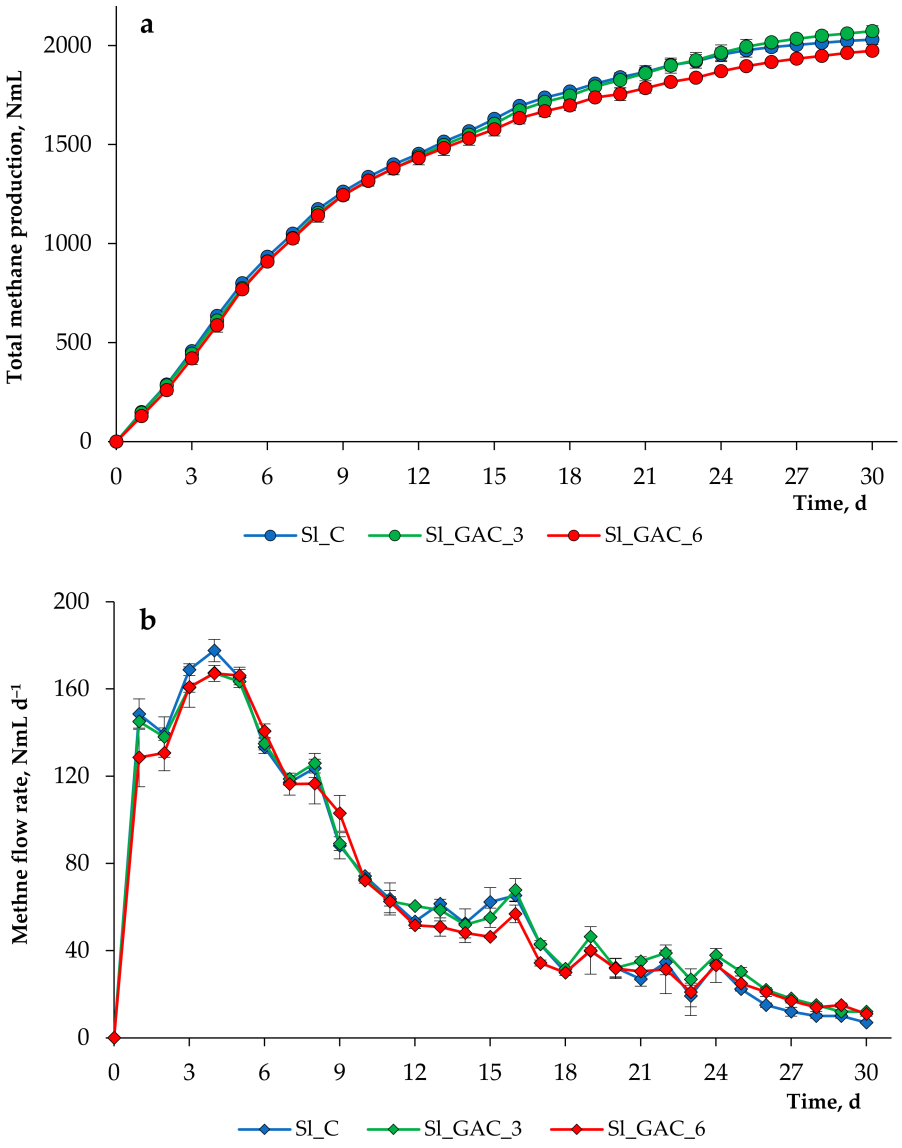


Figure 3. Influence of GAC on (**a**) total methane production and (**b**) methane flow rate during AD of aquaculture sludge. Various concentrations of GAC were added to the reactors, specifically 3.0 g L^{-1} (Sl_GAC_3) and 6.0 g L^{-1} (Sl_GAC_6). The reactors Sl_C operated in the absence of GAC. Error bars represent one standard deviation (n = 3).

Throughout the AD period, the TMP in both the control treatment (Sl_C) and the treatment with the addition of 3 g L^{-1} GAC (Sl_GAC_3) exhibited similar trends. After 30 days, the TMP in the Sl_C and Sl_GAC_3 treatments reached 2030 ± 25 NmL and 2073 ± 28 NmL, respectively. In the treatment with the addition of 6 g L^{-1} GAC (Sl_GAC_6), the TMP reached only 1973 ± 15 NmL. The MFR increased over a period of 4 days, reaching peak values of 178 ± 5 NmL d^{-1} for Sl_C, 168 ± 2 NmL d^{-1} for Sl_GAC_3, and 167 ± 4 NmL d^{-1} for Sl_GAC_6. The final MFRs were 7 ± 1 NmL d^{-1} for Sl_C, 12 ± 2 NmL d^{-1} for Sl_GAC_3, and 11 ± 2 NmL d^{-1} for Sl_GAC_6. Therefore, the addition of GAC did not significantly influence methane yield in these experiments; in fact, with the addition of 6 g L^{-1} GAC, a lower methane yield was noted. However, in line with a limited number of studies in this field [27,28], the results obtained indicate the possibility of producing methane from waste of this type. Lastly, the SMP values ranged from 94 to 99 NmL g^{-1}_{VS}. The absence of any effect of GAC on AD of sludge may be attributed to a potentially longer adaptation period needed for microorganisms to acclimate to the substrate, as well as the low concentration of the substrate used for AD.

Figure 4 demonstrates the effect of GAC addition on the concentrations of acetate, propionate, n-butyrate, and isobutyrate, as well as the concentration of TAN during AD of aquaculture sludge. A key indicator of the stability of the AD process is the dynamics of VFAs. The peak accumulation of VFAs across all studied conditions occurred on day 2. On this day, the concentrations of acetate, propionate, n-butyrate, and isobutyrate in all reactors were nearly identical, falling within the ranges of 598–664 mg L^{-1}, 175–223 mg L^{-1}, 45–75 mg L^{-1}, and 5–15 mg L^{-1}, accordingly. Ultimately, all VFAs were effectively consumed by microorganisms during the AD process. The dynamics of TAN accumulation across all studied conditions indicated the stability of the AD process. The initial TAN concentration, which fluctuated from 495 to 528 mg L^{-1}, increased to 723 ± 45 mg L^{-1}, 698 ± 34 mg L^{-1}, and 649 ± 37 mg L^{-1} after 25 days in the Sl_C, Sl_GAC_3, and Sl_GAC_6 treatments, respectively. Based on the TAN dynamics, conditions involving the addition of GAC appear to be the most optimal, ensuring a balance between TAN accumulation and its consumption. The enhanced formation of TAN during the initial stage of AD in the Sl_GAC_3 and Sl_GAC_6 treatments is attributed to the stimulation of metabolic processes within the system, facilitated by the coordinated work of the microbial community. In all conditions tested, the maximum concentration of TAN remained relatively low, suggesting a limited nutritional value of the substrate for anaerobic microorganisms in the AD system.

Figure 5 illustrates the TMP and MFR values obtained during AD of algal biomass in the absence of GAC (Al_C) and in the presence of GAC (Al_GAC_3 and Al_GAC_6). Although no significant difference in the TMP was observed at the end of the AD process (10,697 ± 19 NmL, 10,721 ± 53 NmL, and 10,544 ± 65 NmL in the Al_C, Al_GAC_3, and Al_GAC_6 treatments, respectively), the addition of GAC substantially increased the methane yield during the periods from 3 to 10 days and from 13 to 16 days. Throughout the entire duration from 17 to 32 days, methane production was greater in the Al_GAC_3 treatment. During AD of algal biomass, four peaks in methane release were observed. The Al_GAC_3 treatment demonstrated a positive effect on days 7, 10, and 16. The Al_GAC_6 treatment demonstrated a pronounced positive effect on days 7, 10, and 15. At the end of the AD process (days 27–32), the MFR values were higher in the Al_C treatment, attributed to the delayed methane formation from the remaining substrate. Consequently, the addition of GAC substantially increased the MFR during AD of algal biomass. As a result, the SMP values ranged from 321 to 327 NmL g^{-1}_{VS}, which were significantly higher than the SMP values observed during AD of aquaculture sludge.

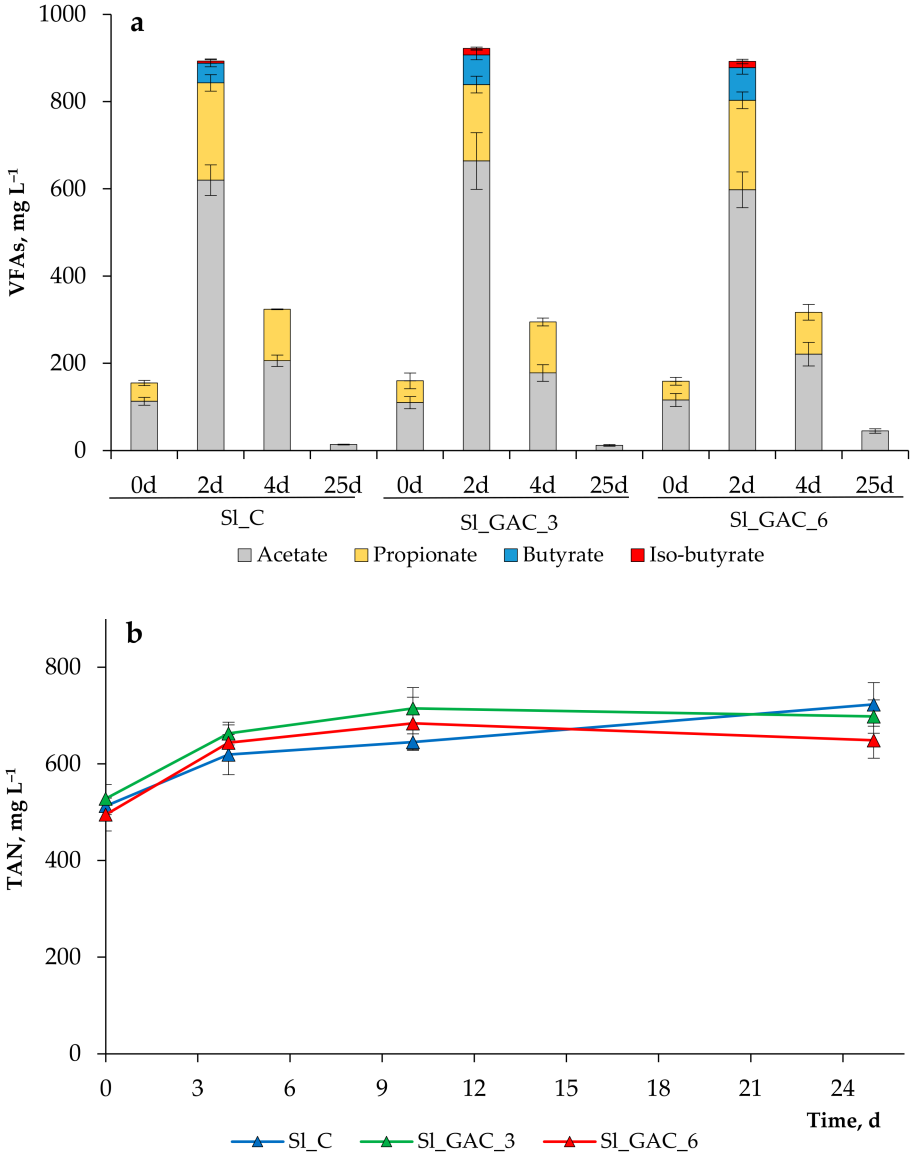


Figure 4. Influence of GAC on (**a**) individual organic acids and (**b**) total ammonia nitrogen concentrations during AD of aquaculture sludge. Various concentrations of GAC were added to the reactors, specifically 3.0 g L^{-1} (Sl_GAC_3) and 6.0 g L^{-1} (Sl_GAC_6). The reactors Sl_C operated in the absence of GAC. Error bars represent one standard deviation (n = 3).

Figure 6 demonstrates the effect of GAC addition on acetate, propionate, n-butyrate, and isobutyrate concentrations, as well as TAN concentration during AD of algal biomass. The peak accumulation of VFAs across all studied conditions also occurred on day 2, while the propionate-to-acetate ratio remained below levels that negatively affect methanogenesis [29]. On day 7 of the experiment, the concentration of n-butyrate was higher in the Al_C treatment. The addition of GAC contributed to the active involvement of analyzed VFAs in methanogenesis, which can also be explained by the activation of the CIET mechanism [20]. On day 11, isobutyrate (26 ± 5 mg/L) was still detectable in the Al_C treatment. By day 11, the VFA concentrations decreased more than 2-fold across all treatments, dropping from 4691–4978 mg L^{-1}, which were the levels achieved on day 2, to 1796–2036 mg L^{-1}. On day 14, a delay in the consumption of VFAs was noted in the Al_C and Al_GAC_3 treatments, whereas in the Al_GAC_6 treatment, the VFA concentration continued to decline. While the concentrations of acetate and propionate on day 14 in the control treatment were 703 ± 63 and 1494 ± 105 mg L^{-1}, respectively, in the Al_GAC_6 treatment, the concentra-

tions of these VFAs were substantially lower, amounting to 412 ± 69 and 1121 ± 96 mg L^{-1}, respectively. On day 32, VFA concentrations reached a minimum in all treatments; however, in the presence of GAC, the decrease in VFA concentration was more pronounced. It should be mentioned that propionate consumption by microorganisms occurred only after the complete consumption of n-butyrate and isobutyrate, as well as after the concentration of acetate decreased to a minimum. By day 14, TAN values increased to 1332–1402 mg L^{-1}. On day 7, a sharp decrease in TAN was noted in the Al_C and Al_GAC_3 treatments, while in the Al_GAC_6 treatment, the decrease in TAN was less pronounced. The addition of GAC resulted in more effective TAN removal between days 14 and 32, which is advantageous for managing high TAN concentrations in the anaerobic digestion system. In various experiments, the final pH values ranged from 7.4 to 7.5.

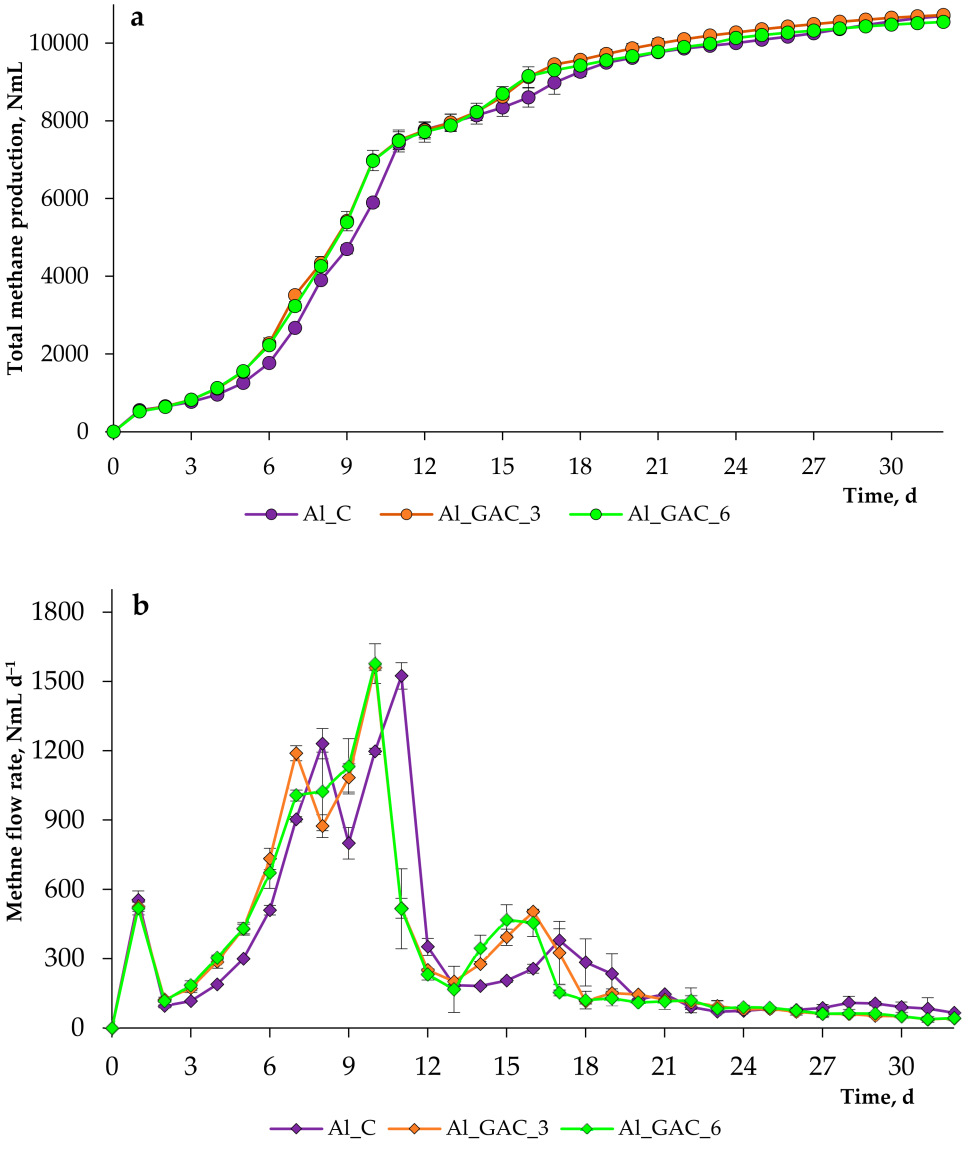


Figure 5. Influence of GAC on (**a**) total methane production and (**b**) methane flow rate during AD of algal biomass. Various concentrations of GAC were added to the reactors, specifically 3.0 g L^{-1} (Al_GAC_3) and 6.0 g L^{-1} (Al_GAC_6). The reactors Al_C operated in the absence of GAC. Error bars represent one standard deviation (n = 3).

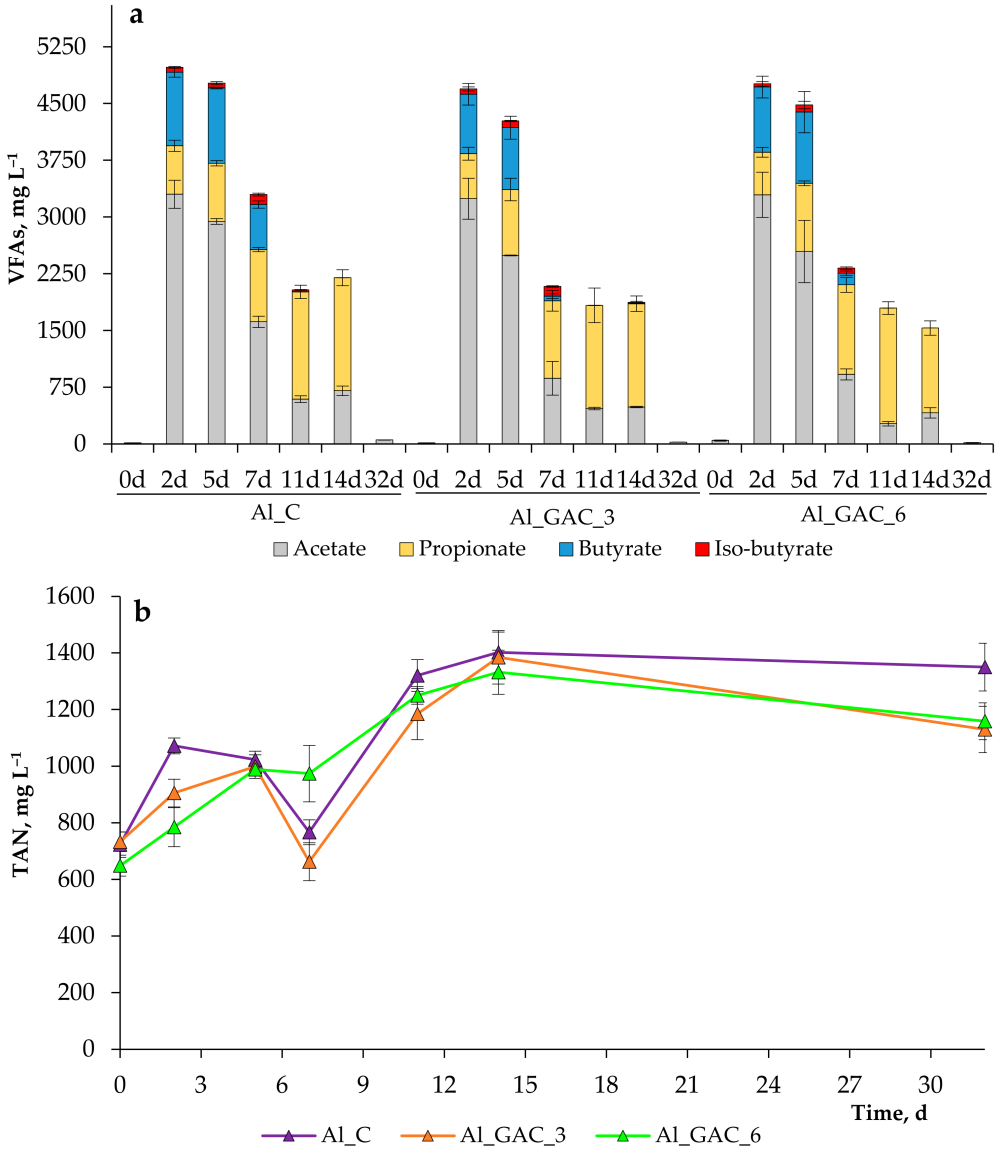


Figure 6. Influence of GAC on (**a**) individual organic acids and (**b**) total ammonia nitrogen concentrations during AD of algal biomass. Various concentrations of GAC were added to the reactors, specifically 3.0 g L^{-1} (Al_GAC_3) and 6.0 g L^{-1} (Al_GAC_6). The reactors Al_C operated in the absence of GAC. Error bars represent one standard deviation (n = 3).

According to the obtained results, aquaculture sludge is an accessible substrate for microorganisms that support the chain of metabolic processes leading to methane production. However, the efficiency of methane production from aquaculture sludge was limited because of an insufficient quantity of easily biodegradable organic components. The addition of GAC had no significant effect on the efficiency of AD in this case. Algal biomass has enormous potential as a substrate for methane production. In the case of algal biomass, a pronounced positive effect of GAC on AD was observed. It resulted in a higher rate of methanogenesis and contributed to a more stable process of VFAs formation and consumption, particularly observed in the Al_GAC_6 treatment.

4. Discussion

The metabolic flexibility of microalgae allows them to utilize a variety of inorganic and organic nitrogen sources, including NH_4^+, NO_3^-, NO_2^-, purines, urea, and amino acids [30]. It is worth clarifying that ammonium and nitrite ions pose the greatest threat

to the health of farmed aquatic animal species, so their accumulation in water negatively affects the efficiency of aquaculture systems [31]. In our systems, all microalgae were able to utilize ammonium, phosphate, and sulfate ions from the RAS water studied, indicating the potential for incorporation of the tested microalgal strains into water treatment systems. However, this type of wastewater is relatively limited in terms of key nutrients. Organic compounds present in the water of RAS could also have been processed by the microbial community into simpler compounds accessible to algal cells. Böpple et al. [32] also noted that RAS waters can be characterized as a nutrient-poor environment for microalgae.

The maximum values of biomass yield and productivity were observed during the cultivation of *T. obliquus* EZ-B11 and reached 1.34 ± 0.04 g L^{-1} and 0.34 ± 0.02 g L^{-1} day^{-1}, respectively. In another study, the productivity of *C. vulgaris* when grown in trout farm wastewater was estimated at 0.033 g L^{-1} day^{-1} [33], which is significantly lower than the productivity of most of the studied strains in our work. According to the results of the study performed by Calderini et al. [34], the biomass yield of *Haematococcus pluvialis*, *Monoraphidium griffithii*, and *Selenastrum* sp. during growth in raw and filtered RAS wastewater was in the range of 0.31–0.33 g L^{-1}. The productivity of algal cultures in aquaculture wastewater may be limited by nutrient deficiencies. Ansari et al. [35] showed that enrichment of such wastewater with nitrogen improved the cultivation efficiency. The biomass concentrations of several cultures, including *N. oculata*, *P. tricornutum*, *P. gyrans*, and *T. suecica*, cultivated in RAS wastewater achieved 0.41 g L^{-1}, 0.34 g L^{-1}, 0.30 g L^{-1}, and 0.16 g L^{-1}, respectively [8]. Consistently high biomass density was achieved through the addition of nitrate during continuous cultivation or by using membrane filtration during perfusion cultivation of *P. tricornutum* [32]. In the study performed by Lugo et al. [36], the biomass yield and productivity of *C. sorokiniana* cultured in RAS wastewater were 0.48 g L^{-1} and 0.14 g L^{-1} day^{-1}, respectively; these values are lower than the results obtained in our study for *C. sorokiniana* EZ-07 (0.86 ± 0.07 g L^{-1} and 0.22 ± 0.02 g L^{-1} day^{-1}).

This study also evaluated the concentrations of pigments, proteins, lipids, and starch in the final algal biomass. These metabolites determine the value of algal biomass for aquaculture as feed and various industrial applications (cosmetics, textile industries, and agriculture). During growth in RAS water, *C. vulgaris* SB-M4 exhibited the highest efficiency in accumulating total chlorophylls and total carotenoids, with concentrations of 8.3 ± 0.7 mg L^{-1} and 2.1 ± 0.3 mg L^{-1}, respectively. Additionally, another study examined the efficiency of total carotenoid production during the cultivation of *Scenedesmus armatus* in RAS water. Thus, the total carotenoid content achieved 3.09 mg g^{-1} [37]. The total chlorophyll and total carotenoid contents in the biomass of another *C. vulgaris* strain grown in trout farm wastewater were 0.99% and 0.21%, respectively [33].

The maximum protein, lipid, and starch contents in algal biomass achieved in this study were 35.6%, 28.0%, and 30.2%, respectively. This finding demonstrates the effectiveness of using RAS water for the production of these metabolites. Enwereuzoh et al. [38] determined the lipid and carbohydrate contents of the biomass of three microalgae species, including *T. obliquus*, *Heterochlorella luteoviridis*, and *Chlamydomonas reinhardtii*, in raw and diluted RAS waters. The lipid and carbohydrate contents of the biomass of the three species ranged from 36.4% to 44.1% and from 18.9% to 35.0%, respectively. In the study performed by Esteves et al. [33], the protein, lipid, and carbohydrate contents of *C. vulgaris* biomass cultured in trout farm wastewater were 17.9%, 15.8%, and 48.6%, respectively. The authors noted that the lower protein content observed with a nutrient-limited medium resulted from nutrient depletion during the final growth stage. The lipid content in *Nannochloropsis gaditana* biomass cultured in fish farm wastewater ranged from 7.8% to 27.2%, depending on the bioreactor type, photoperiod, and phosphorus content [39]. It is important to highlight that several algal strains examined in this study also exhibited the potential

for accumulating valuable compounds when cultivated in various types of wastewater, including that from poultry slaughterhouses [24], fish processing plants [40], and biogas reactor effluents [41].

The methanogenic potential of aquaculture sludge can vary significantly depending on the specific aquaculture system, particularly the diet of the aquatic organisms involved. The results of this study indicate that during AD, both in the absence and presence of GAC, the sludge serves as an accessible substrate for microbes involved in the AD process. However, it is not effective when used as the sole substrate. Moreover, the addition of GAC did not improve the process. In this study, SMP values during AD of sludge ranged from 94 to 99 NmL g^{-1}_{VS}. However, these values may differ considerably when sludge is obtained from other aquaculture systems. Choudhury et al. [27] evaluated the impact of altering fish diets on sludge composition and methanogenic potential. The authors found that a dietary change involving lipid enrichment of the feed increased the sludge's methanogenic potential from 279 to 346 mL g^{-1}_{VS}. Another study also evaluated the effect of fish diets on the methanogenic potential of aquaculture waste [28]. Methane production during AD of feces ranged from 85 to 261 mL g^{-1}_{VS}, depending on the composition of the fish feed. Also, similar studies have demonstrated the potential of aquaculture sludge as a co-substrate in the AD process [42,43]. The study performed by Brod et al. [44] noted that AD is a promising method for treating aquaculture sludge prior to its subsequent use as fertilizer.

The potential for using microalgae as a substrate for AD is closely linked to the composition of algal biomass. This biomass is abundant in organic matter, particularly carbohydrates, proteins, and lipids. Additionally, microalgae demonstrate significant potential in various integrated processes that promote efficient resource management [45]. AD of algal biomass represents a promising approach to receive biofuels from biomass that has accumulated during the bioremediation of contaminated waters, including those with toxic substances [46]. In this context, enhancing the efficiency of AD of algal biomass is an urgent scientific task aimed at increasing the role of algae in integrated processes. This study demonstrated that resulting biomass can be used as a substrate in the AD process to produce biogas, a valuable renewable energy source.

Previous studies have also focused on AD of algal biomass and residual algal biomass left after extracting valuable products for biomethane production [47–49]. For example, Cheenakula et al. [49] found that the SMP of algal-bacterial biomass using sewage sludge inoculum was 350 NmL per 1 g of VS. However, in our study, a strategy was developed to improve the efficiency of AD of algal biomass, which included the addition of GAC as a methanogenesis stimulator. According to the obtained results, the addition of GAC had a positive effect on the MFR during AD of algal biomass. In the present study, methane yield was higher than that reported in our earlier work [50], which may indicate, for example, a difference in the content of energy-rich lipids. The physical characteristics of GAC, which stimulate the formation of microbial biofilms on their surfaces and can increase the resistance of anaerobic microorganisms to various toxic compounds [20,51], and its role in CIET [18,51,52] are considered key characteristics of this methanogenesis-stimulating agent in various anaerobic systems. A study by Park et al. [52] demonstrated that the addition of GAC to anaerobic reactors contributed to an increase in both methane yield and the rate of methanogenesis during rural wastewater treatment. In addition, the authors demonstrated the effect of GAC on the composition of the microbial community: GAC stimulated the growth of methanogenic archaea. GAC supplementation, as shown in this study, is particularly effective during the early stages of AD. The CIET-mediated acceleration of electron transfer leads to a shortening of the lag phase, which enhances biogas yield and increases the rate of methanogenesis at this stage. Chen et al. [51] reported

beneficial effects of GAC supplementation on AD by supporting syntrophic metabolism, promoting the growth of electroactive bacteria and archaea of the genus *Methanothrix*, and reducing the inhibitory effect of sulfate on methanogenesis. In addition to GAC, other conductive materials, such as magnetite and carbon nanotubes, are also used to improve the long-term stability of anaerobic digestion processes [19]. Furthermore, we showed that the addition of GAC influenced the dynamics of acetate, propionate, butyrate, and isobutyrate. Excessive accumulation of VFAs during AD is a key factor in the inhibition of methanogenesis, and the dynamics of VFAs accumulation and consumption reflect the metabolic balance between microbial communities [53]. Thus, maintaining a balance between the accumulation and conversion of VFAs is an effective strategy for regulating AD. These results are consistent with the results of the study by Fountoulakis et al. [54], which indicated that the addition of GAC stimulates the consumption of VFAs and contributes to an increase in the rate of methane production.

5. Conclusions

This study presents the feasibility of a multidisciplinary approach to convert liquid and semi-solid aquaculture waste into valuable products. Cultivating green algae in the real wastewater enabled the recovery of nutrients (achieving 100% removal of N and P compounds) and the production of valuable biomass suitable for both fish farming and biomethane generation. Thus, *T. obliquus* was identified as the alga capable of accumulating the highest biomass concentration, 1.34 ± 0.04 g L^{-1}. The biomass of all studied microalgae is recognized as a resource rich in protein (up to 35.6% of DW), lipids (up to 28.0%), and starch (up to 30.2%). These biomass characteristics suggest its potential as an aquaculture feed additive. Additionally, aquaculture waste sludge and algal biomass have been evaluated as substrates for AD. Methane yield during the anaerobic conversion of the organic fraction of the sludge amounted to 94–99 NmL g^{-1}_{VS}, whereas microbial biomass obtained after wastewater treatment proved to be a more valuable substrate, with a methane yield of 321–327 NmL g^{-1}_{VS}. At the same time, in the algae-containing treatments supplemented with GAC, the methane flow rate increased. However, future research should evaluate the economic feasibility of this process. The study can also be supplemented by an assessment of microalgae growth in nutrient-enriched wastewater, as well as an evaluation of the co-digestion of aquaculture sludge and microalgal biomass. The results of this study will support the creation of environmentally sustainable biotechnologies for producing valuable bioproducts and renewable energy.

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