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Hydrothermal but Not Mechanical Pretreatment of Wastewater Algae Enhanced Anaerobic Digestion Energy Balance due to Improved Biomass Disintegration and Methane Production Kinetics

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Abstract: This study used pilot-scale high-rate algae ponds to assess algal–bacteria biomass productivity and wastewater nutrient removal as well as the impact of mechanical and hydrothermal pretreatments on biomass disintegration, methane production kinetics, and anaerobic digestion (AD) energy balance. Mechanical pretreatment had a minor effect on biomass disintegration and methane production. By contrast, hydrothermal pretreatment significantly reduced particle size and increased the solubilized organic matter content by 3.5 times. The methane yield and production rate increased by 20–55% and 20–85%, respectively, with the highest values achieved after pretreatment at 121 °C for 60 min. While the 1st-order and pseudo-1st-order reaction equation models fitted methane production from untreated biomass best ($R^2 > 0.993$), the modified Gompertz sigmoidal-type model provided a superior fit for hydrothermally pretreated algae ($R^2 \geq 0.99$). The AD energy balance revealed that hydrothermal pretreatment improved the total energy output by 25–40%, with the highest values for volume-specific and mass-specific total energy outputs reaching 0.23 kW per digester m^3 and 2.3 MW per ton of biomass volatile solids. Additionally, net energy recovery (energy output per biomass HHV) increased from 20% for untreated algae to 32–34% for hydrothermally pretreated algae, resulting in net energy ratio and net energy efficiency of 2.14 and 68%, respectively.

Keywords: wastewater-grown algae; biomass productivity and settleability; anaerobic digestion; mechanical and hydrothermal pretreatment; methane production kinetic modelling; AD system energy balance

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1. Introduction

By utilizing waste nutrients and low-cost fresh water resources, algae cultivation in wastewater represents a potentially sustainable process for producing biofuel feedstock [1]. A substantial amount of effort has been devoted to screening and isolating algal strains capable of growing prolifically in wastewater, as well as developing cultivation methods and crop protection techniques to achieve stable and productive monocultures of these organisms [2–7]. Recently, it has been demonstrated that the application of engineered binary cultures or even polycultures using selected algal species provides significant advantages: improved growth and treatment efficiency can be achieved as a result of in situ

oxygen exchange [8]; removal of complex organic matter and toxic compounds inhibiting algae in monocultures [9,10]; and the possibility for synergistic exchange of metabolites (e.g., vitamins) resulting in higher biomass productivity and stress resistance [11,12]. An alternative approach that might be more straightforward is to use the power and robustness of the local algal–bacterial community by growing and harvesting such natural polycultures of organisms that inhabit wastewater [13,14]. Due to their diverse and flexible metabolic capabilities, such communities are more resilient to fluctuating environmental conditions, grazers, pathogens, and can remove wastewater contaminants more effectively. However, biomass derived from the mixture of indigenous algae and bacteria species typically contains low levels of energy-rich lipids [15]. Energy can only be recovered from lipid-poor biomass by applying technology that converts all other cellular components, including proteins and carbohydrates. One example of such a technology is anaerobic digestion (AD); however, improvements are still needed to maximize energy recovery from algae–bacterial biomass.

The rigidity of algal cell walls and/or other components, which results in low methane yields, prevents algal biomass AD from becoming more widely accepted. The methane yields from algal biomass grown in wastewater rarely exceed 0.1–0.17 L of CH_4 per gVS, which corresponds to the conversion of only 30% of organic matter [16–20]. These values are significantly lower than the methane yields reported for sewage sludge of 0.4–0.75 L CH_4 per gVS (VS reduction up to 50–70%), municipal solid waste of 0.3–0.4 L CH_4 per gVS (VS reduction 50–70%), and food waste of 0.4–0.6 L CH_4 per gVS (VS reduction 70–90%) [21–23]. Several studies, however, show that hydrothermal pretreatment can increase the methane yield by 1.5–3 fold from recalcitrant organic materials [24–30]. Based on these studies, thermal pretreatment is advantageous for a number of reasons, including high biomass hydrolysis efficiency, short treatment duration, lack of chemical agents, and the possibility of using waste heat. A significant advancement in the biodegradability and digestibility of wastewater-grown algae–bacteria biomass would improve the economics and technology of integrated wastewater treatment and biofuel production.

Studies have shown that hydrothermal pretreatment can increase the methane yield from algal biomass by up to 70–150% [31–38]. However, these studies have focused on the pretreatment of biomass obtained from algal monocultures grown on defined growth media in highly controlled photobioreactors or ponds. Limited research has been conducted on the hydrothermal pretreatment of algae grown in wastewater with somewhat inconsistent results. In spite of the fact that pretreatment enhanced methane yields by 20–70% [19,20,39], this corresponded to improved yields of only 0.12–0.18 L CH4 per gVS (after pretreatment). Despite pretreatment, methane yield values from pretreated wastewater algae polyculture are still substantially lower than yields obtained from algae biomass grown as single strains in defined media or from other organic feedstocks examined previously. Additionally, since thermal treatment consumes a substantial amount of energy, it is imperative to evaluate how pretreatment impacts AD performance and energy balance, particularly before implementing pilot-scale or large-scale systems.

Identifying the optimal pretreatment conditions based on the AD system energy balance requires the development of mathematical models predicting the methane yield and energy balance parameters (input, output, net energy ratio, etc.) for different pretreatment conditions and hydraulic retention times. Anaerobic digestion model no. 1 (ADM1) [40] is frequently used to describe methane production in continuous AD systems. Despite its complexity, the ADM1 model does not take into account dynamics in the microbial population, mixing and heat gradients in the digester, physicochemical reactions for some essential elements (phosphate precipitation or sulfur reduction/oxidation), changes in biodegradability of substrates (for instance as a result of pretreatment) with the same composition of proteins, carbohydrates and lipids [41,42]. Although recent advances have been made in the modeling of AD computational fluid dynamics (CFD) [43], reactions associated with P, S, and Fe [44], and dynamics of the microbial population [45,46], the integrated model would be restrictively complicated for the assessment of pretreatment techniques and forecasting of AD system performance on a high-throughput basis. As an

alternative, the methane production data from the high-throughput biomethane potential tests (BMP) [47] may be described using more simplistic kinetic models [48–50] and used to predict the effect of different pretreatment approaches on AD performance. It has been demonstrated in several studies that BMP results correlate well with methane production in continuously operated AD systems despite the presence of scaling effects and fluctuations in methane production [51–54].

As a first objective, this study will evaluate parameters for outdoor growth of indigenous algal–bacterial communities in primary wastewater, including biomass productivity, settleability, and composition, as well as assess the efficacy of wastewater treatment. As a secondary objective, we aim to determine the impact of mechanical and hydrothermal pretreatment on methane yields from harvested biomass. As a third objective, several kinetics models will be examined, including the 1st-order reaction equation, pseudo-1st-order reaction equation, and modified Gompertz sigmoidal-type equation, to determine if they can accurately describe methane production. Lastly, the developed kinetic model for methane production will be used to estimate the energy balance for a scaled AD system to determine the feasibility of the hydrothermal pretreatment strategy.

2. Materials and Methods

2.1. High-Rate Raceway Ponds for Algae Cultivation in Wastewater

The algal cultivation facilities were described previously [14]. In summary, the 30 cm deep paddlewheel-driven open raceway ponds were located in San Luis Obispo, CA, USA and had a total area of 32.3 m^2. The ponds were continuously fed with wastewater after the primary clarifier at the 6 MGD San Luis Obispo Water Resource Recovery Facility to maintain a channel velocity of 27 cm/s and hydraulic residence time of 2 days. Compressed 99.5% CO_2 (Airgas, Radnor, PA, USA) was supplied into ponds as needed to maintain a stable pH of 8.3–8.5. Algae was harvested periodically using a custom-made tube settler with a cross-section area of 0.085 m^2 and 60° up-flow of 2.25 L/min. The efficiency of wastewater treatment, productivity and characteristics of algae biomass were measured from 20 June to 27 July. The batch of algal slurry for the anaerobic digestion study was collected from the algae up-flow settler on July 13 and stored at −20 °C.

2.2. Algae Biomass Pretreatment, Experimental and Theoretical Methane Potential

By using experimental biomethane potential tests as well as theoretical calculations, the biomethane potential of an algae–bacteria polyculture grown in wastewater was evaluated. In addition to testing raw (untreated) biomass, mechanical and hydrothermal pretreatment methods were examined to assess their effect on biomethane production.

2.2.1. Mechanical and Hydrothermal Pretreatments

The mechanical pretreatment was performed using the TissueLyser II Bead Mill (Retsch GmbH & Co. KG, Haan, Germany) equipped with a stainless steel grinding jar. In order to identify optimal milling conditions, biomass beads were milled at a radial oscillation speed of 10 Hz by several means: with a ceramic ball (CB, 0.25″) for either 2 or 20 min, and with stainless steel balls (SSB, 2.4 mm) for either 2 or 20 min. A 400LS/500LS Series Steam Sterilizer (Getinge USA, Inc., Wayne, NJ, USA) was used for hydrothermal pretreatment of algae samples at 100 °C (0 min or no prolonged exposure) or 121 °C (exposure 0, 10, 30 and 60 min). A one-hour cooling period was followed by hydrothermal treatment of the samples. A comparison of particle-size distributions and fractions of soluble organic matter in raw biomass (untreated control) and pretreated biomass, as well as the experimentally determined yield of biogas and methane, was undertaken to assess pretreatment effectiveness.

2.2.2. Solubilization of Organic Matter

As a measure of the solubilization of organic matter after pretreatment, the rate of soluble chemical oxygen demand (*sCOD*, Equation (1)) was compared with particulate chemical oxygen demand (*pCOD*, Equation (2)). *sCOD* was measured as COD in the

suspension (supernatant phase) after centrifuging algae samples at 6500 rpm for 10 min at 4 °C. Particulate COD (*pCOD*) was measured as COD in biomass pellets (precipitate phase) after centrifugation. Total COD represents the carefully mixed sample's COD.

$$sCOD\ (\%) = \frac{sCOD}{COD^{total}} \times 100\ \% \quad (1)$$

$$pCOD\ (\%) = \frac{COD^{total} - sCOD}{tCOD^{total}} \times 100\ \% \quad (2)$$

2.2.3. Particle-Size Distribution

The particle size distribution in biomass prior to and after pretreatment was assessed using FlowCam® 8000 (Fluid Imaging Technologies, Scarborough, ME, USA) equipped with a high-resolution (1920 × 1200 pixels) CMOS camera and excitation laser (488 nm, 532 nm, 633 nm) with 2-channel fluorescence detection. It allows relating the number of biomass particles of a certain size to their volume by accurately enumerating and tracking the size of biomass particles. All samples were diluted accordingly in water to ensure that the particles per used image did not exceed the suggested optimum value of 1.10 [55]. The particles were measured by using the FOV300 flowcell with 10× objective (100× magnification) and with 4× objective (40× magnification) to analyze particles up to 90 um size class and >90–210 um size class, respectively [55], in auto-trigger mode. Then, the particles were classified into the following size fractions: 0–30, 30–60, 60–90, 90–120, 120–150, 150–180, and 180–210 µm. Finally, the volume of particles for each size fraction was calculated from the measured Equivalent Spherical Diameter of particles and normalized to the total volume of all particles.

2.2.4. Experimental Biomethane Potential

The experimental biomethane potential (BMP) of wastewater algae was assessed using the procedure suggested by Owen [47]. A few modifications to the BMP protocol, gas analysis and data analysis were previously discussed [34]. Briefly, BMP tests were carried out in serum bottles of 150 mL. Biomass organic load of 2.0 g algae vs. per L of final medium and a substrate to inoculum (S/I) ratio of 1.0 were used. As an inoculum, anaerobic sludge from the 3.0 MG egg-shaped mesophilic (35 °C) digesters treating a mixture of primary and secondary sludge at the Back River Wastewater Treatment Plant, Baltimore, MD, was employed. The BMP test samples were incubated at 35 ± 0.5 °C. Biogas generation was determined volumetrically by spiking an empty gas-tight syringe (5 or 20 mL) into the serum bottle and equilibrating to atmospheric pressure to quantify the amount of produced biogas. A Hamilton SampleLock syringe was used to collect 250 L of biogas and analyze it instantly for CH_4 and CO_2 contents on a Shimadzu GC-8AIT (#221-22274-92; Shimadzu Scientific Instruments, Columbia, MD, USA) with a thermal conductivity detector (TCD) operated at 130 °C and 80 mA current. Biogas fractionation was performed on a Hayes Q 80/100 mesh 1/8 stainless 2.0 m column (part 220-94715-20; Shimadzu Scientific Instruments, USA) with helium at 2 bar pressure as a carrier gas. The GC instrument was re-calibrated prior to and after every analysis by injecting analytical standard grade (Supelco, St. Louis, MO, USA) nitrogen, methane and carbon dioxide gases at amounts of 50 µL, 150 µL and 250 µL.

2.2.5. Theoretical Biomethane Potential

The methodology proposed by Buswell and Müller [56] (Equation (3)) was used to predict the theoretical maximum of biogas and methane yields:

$$C_aH_bO_cN_d + \left[a - \frac{b}{4} - \frac{c}{2} - \frac{3d}{4}\right]H_2O \rightarrow \left[\frac{a}{2} + \frac{b}{8} - \frac{c}{4} - \frac{3d}{8}\right]CH_4 + \left[\frac{a}{2} - \frac{b}{8} - \frac{c}{4} + \frac{3d}{8}\right]CO_2 + d(NH_3) \quad (3)$$

2.3. Modelling Gas Production Kinetics and Analyzing Statistics

Three different kinetic models, including 1st-order reaction equation (Equation (4)), pseudo-1st-order reaction equation (Equation (5)), and modified Gompertz sigmoidal-type equation (Equation (6)), were tested to fit the biogas and methane production experimental data:

$$Y_{model.i} = Y_m \left(1 - e^{-k\ t_i}\right) \tag{4}$$

$$Y_{model.i} = Y_{theor.m}\left(1 - P_{bd}\ e^{-k_{bd}\ t_i} - P_{rs}\ e^{-k_{rs}\ t_i}\right) \tag{5}$$

$$Y_{model.i} = Y_m \exp\left(-exp\left(\frac{K\ (\lambda - t_i)\ e^1}{Y_m}\right)\right) \tag{6}$$

where: Y_m is the total yield for biogas or methane (mL gVS^{-1}); $Y_{theor.m}$ is the theoretical maximum total yield for biogas or methane calculated using Buswell and Müller (Equation (3)) (mL gVS^{-1}); k is the constant for the 1st-order reaction equation (day^{-1}); k_{bd} and k_{rs} are the constants for easily biologically degradable and resistant parts of biomass for the pseudo-1st-order reaction equation (day^{-1}); K is the specific rate constant for modified Gompertz equation (mL gVS^{-1} d^{-1}); P_{bd} and P_{rs} are the ratios of easily biologically degradable and resistant parts in biomass with $P_{rs} = 1 - P_{bd}$; λ is the lag phase time constant (day); and t is the incubation time (day).

The constants for all gas production models were estimated by using the MS Excel optimization tool, "Solver", by optimizing Root Mean Square Deviation (RMSD) (Equation (7)) to a minimal value. Supplemental Information Table S1 contains more details concerning the calculation methodology. Then, RMSD and R^2 (Equation (8)) for observed data and model predictions were used to assess fitting accuracy of all models:

$$RMSD = \sqrt{\frac{\sum_{i=1}^{N}\left(Y_{model.i} - Y_{exper.i}\right)^2}{N}} \tag{7}$$

$$R^2 = \left[\frac{N\sum_{i=1}^{N}\left(Y_{model.i}\ Y_{exper.i}\right) - \sum_{i=1}^{N} Y_{model.i} \sum_{i=1}^{N} Y_{exper.i}}{\sqrt{N\left(\sum_{i=1}^{N} Y_{model.i}^2\right) - \left(\sum_{i=1}^{N} Y_{model.i}\right)^2}\ \sqrt{N\left(\sum_{i=1}^{N} Y_{exper.i}^2\right) - \left(\sum_{i=1}^{N} Y_{exper.i}\right)^2}}\right]^2 \tag{8}$$

where: $Y_{model.i}$ and $Y_{exper.i}$ are the gas production from the model or the experimental data at the time i. N is the number of experimental measurements for gas production.

2.4. Simulation of the Impact of Pretreatment on the Energy Balance Parameters of a Scaled Anaerobic Digestion (AD) System

The kinetic models for biogas and methane production were used to evaluate the effects of pretreatment of algae biomass on the energy balance of a full-scale AD system with completely mixed continuous (CSTR) reactors using the methodology described earlier [48]. A summary of the methodology for calculating energy performance metrics for a scaled CSTR AD system is presented in Table 1. Biogas and methane production at various HRTs was predicted for a scaled system using the modified Gompertz equation (identified as the most reliable kinetic model), and residence time distribution for the CSTR AD reactor was calculated using the segregation model [57] and numerical integration using the trapezoidal rule (see Equations (9)–(11)). Biogas generated by this plant is assumed to be used onsite as part of a combined heat and power (CHP) system utilizing a boiler and steam turbine. To estimate the energy input for running the scaled CSTR AD system, Equations (12)–(14) were used, and Equations (15)–(19) were used to estimate the system's energy output. Input and output of heat, electricity, and total energy were calculated in watts. Finally, Equations (20)–(25) were used for the calculation of AD energy balance metrics. The AD system size and performance were simulated for an algae feedstock supply of about

8.6 tons of AFDW per day generated on an algal cultivation facility processing municipal wastewater (see Table S2 of Supplemental Information for calculation details).

Table 1. Scaled CSTR AD system energy balance calculation and evaluation metrics.

AD Parameter	Equation	#
Estimation of Biogas and Methane Production by Scaled AD (Equations (9)–(11))		
Gas production	$Y_{CSTR} = \int_0^\infty Y_{model}(t)\ E(t)\ dt$	(9)
Biomass residence time distribution fxn	$E(t) = \frac{1}{\tau} e^{\frac{-t}{\tau}}$	(10)
Numerical solution for gas production	$Y_{CSTR} = \sum_{n=0}^{N-1} \frac{1}{2}(Y_{model.n}\ E_n + Y_{model.n+1}\ E_{n+1})(\Delta t)_n$	(11)
where : $Y_{model}(t)$-kinetic equation for gas production the; τ-reactor HRT, d; t—biomass SRT, d.		
Estimation of Energy Input for Scaled AD (Equations (12)–(14))		
Total energy input	$E_{Input}^{Total} = E_{Input}^{Heat} + E_{Input}^{Electricity}$	(12)
heat input	$E_{Input}^{Heat} = m\gamma(T_d - T_a)/(24 \times 3600) + k_i A_i (T_d - T_a)$	(13)
electricity input	$E_{Input}^{Electr} = E_{Input}^{pump} + E_{Input}^{mix} + E_{Input}^{recycl.} + E_{Input}^{CHP} + E_{Input}^{other}$	(14)
where : m—wet mass; γ—specific heat, 4.19 kJ kg^{-1} °C^{-1}; T_a & T_d—ambient (10 °C) and digestion (35 °C) T; k_i—heat transfer coeff., W m^{-2} °C^{-1}; A_i—digester surface area, m^2; E_{Input}^{pump}-head losses for pumping; E_{Input}^{mixing}—mixing energy as 3.8 W per digester m^3 [58]; $E_{Input}^{recycling}$-biomass circulation for heating, 2.4 W m$-^3$; E_{Input}^{other}-other electricity (e.g., lighting), 3.6 W m$-^3$; E_{Input}^{CHP}—CHP unit operation, 74 W per methane m^3 [59]		
Estimation of Energy Output from Scaled AD (Equations (15)–(19))		
Total energy production	$E_{Production}^{Total} = E_{Production}^{Heat} + E_{Production}^{Electricity}$	(15)
heat production	$E_{Production}^{Heat} = E_{Boiler}^{Heat} + E_{CHP}^{Heat}$	(16)
heat from boiler	$E_{Boiler}^{Heat} = 0.05\ Y_{CSTR}\ \xi\ \eta_{boiler}^{heat}$	(17)
heat from CHP	$E_{CHP}^{Heat} = 0.9\ Y_{CSTR}\ \xi\ \eta_{CHP}^{heat}$	(18)
electricity production	$E_{Production}^{Electricity} = 0.9\ Y_{CSTR}\ \xi\ \eta_{CHP}^{electricity}$	(19)
where: 0.05 and 0.9 corresponding to utilization of 5% of biogas in boiler and 90% in CHP (remaining 5% is flared); Y_{CSTR}—gas yieled, m^{-3}; ξ—methane LHV of, 36.6 MJ m^{-3}; η_{boiler}^{heat}, η_{CHP}^{heat} and $\eta_{CHP}^{electricity}$—energy conversion efficiencies 85%, 55% and 30%, respectively.		
Scaled AD System Evaluation Metrics (Equations (20)–(25))		
Net Energy Output	$NE_{Output} = E_{Production}^{Total} - E_{Input}^{Total}$	(20)
Volume-specific Net Energy Output	$NE_{Output}^{mass} = E_{Output}\ /\ m_{VS}$	(21)
Mass-specific Net Energy Output	$NE_{Output}^{volume} = E_{Output}/\ V_{AD}$	(22)
Net Energy Ratio	$E_{Output}/E_{Input}^{Total}$	(23)
Net Energy Efficiency	$NEE = \left[E_{Output}\ /\ E_{Production}^{Total}\right] \times 100\%$	(24)
Net Energy Recovery	$NERec = \left[E_{Output}\ /\ HHV_{biomass}\right] \times 100\%$	(25)
where : m_{AFDW}—digested biomass in ton of ash-free dry weight (organic matter); V_{AD}—volume of scaled AD, m^3; $HHV_{biomass}$ —algae biomass high heating value.		

2.5. Analytical Techniques

Data were collected weekly on algae biomass productivity, settleability, and influent and effluent water quality during the experimental period. The biomass areal productivity was determined in g of algae ash-free dry weight per pond m^2 per day (AFDW/m^2-d) according to methods 2540 D and 2540 E from APHA's Standard Methods for the Examination of Water and Wastewater [60]. Pond solids content was used to calculate gross biomass productivity.

By subtracting wastewater influent solids from pond solids, net productivity was estimated. An assessment of biomass settleability was conducted using gravity settling in Imhoff cones and measuring supernatant AFDW after 2 and 24 h of settling. An analysis of ammonical nitrogen, nitrate nitrogen, nitrite nitrogen, total Kjeldahl nitrogen (TKN) and biochemical oxygen demand (BOD) was conducted using the standard method described In A"HA [60]. Temperature, dissolved O_2, and pH of wastewater in ponds were logged and stored hourly in data loggers (Apex, Neptune Systems, San Jose, CA, USA). For algal species identification, an Olympus Model CX41RF phase contrast microscope (Evident Corporation, Tokyo, Japan) and Infinity 2-1C cameras (Teledyne Lumenera, Ottawa, ON, Canada) were used with InfinityAnalyze 2.0 software.

Prior to pretreatment and BMP testing, the algal biomass was assayed for: (i) total solids (TS) and volatile solids (VS) using APHA methodology [60]; (ii) COD with a HACH COD TNTplus kit and manufacturer instructions; (iii) crude lipids were extracted from the wet homogenized sample (bead milling with SSB for 20 min) according to Bligh and Dyer protocol with chloroform and methanol and determined [61]; and, fatty acid methyl esters (FAMEs) using the following protocol. The lipids in 30 mg of lyophilized sample were in situ transesterified in an extraction apparatus (round-bottom flask with Dimroth condenser) by adding 3 mL of methanol with 5% (v/v) of hydrochloric acid. As an internal standard, 1 mL of 0.5 mg/L heptadecanoic methyl ester was added to the sample, and the mixture was agitated for 90 min at 70 °C in a water bath. After cooling the sample, FAME were extracted by adding a mixture of water and hexane in a three-to-one ratio, centrifuging (6500 rpm for 10 min at 20 °C) and cautiously recovering the hexane phase. Shimadzu 2010 GC (Shimadzu Corporation, Kyoto, Japan) with Stabilwax-DA (30 m, 0.25 mm ID, 0.5 μm df) capillary column (Restek Corporation, Bellefonte, PA, USA) and FID detector. Each FAME peak was identified by matching its retention times to the FAME standards (Sigma-Aldrich, St. Louis, MO, USA) and quantified based on internal standards. An algae elemental composition was measured after lyophilization for 24 h at Micro Analysis, Inc. (Wilmington, DE, USA) using a %CHN Analyzer. The samples were analyzed in triplicate, with the average and standard deviation provided.

3. Results and Discussion

3.1. Algae Cultivation in Wastewater Ponds

For the project period from 20 June to 25 July, the average algal biomass gross productivity was 66 ± 15 g m^{-2} day^{-1}, and the net productivity was 57 ± 16 g m^{-2} day^{-1} (Figure 1A). Over this period, the average 24 h solar insolation and temperature were 313 ± 42 W m^{-2} and 15.7 ± 1.1 °C, respectively, contributing to the high productivity. In the wastewater open pond, a spontaneous algal–bacterial polyculture was dominated by a green alga *Micractinium* and a diatom *Cyclotella*, trailed by pennate diatoms and other green algae *Chlorococcum*, *Scenedesmus* and *Chlorella*. Over the six-week cultivation experiment, the algae gravity settleability was 92.3 ± 3.5% after two hours of settling and 97.0 ± 1.1% after 24 h, revealing strong bio-flocculation properties of the algal–bacterial polyculture (Figure 1B). The AFDW of clarified wastewater after 24 h of settling was 14 ± 8 mg of AFDW per L. In addition, recent studies have shown that chemical or natural flocculants can further decrease sedimentation time and residual biomass concentration in water [62,63].

In order to assess the efficiency of wastewater treatment in algae ponds, the removal of volatile suspended solids (VSSs) and carbonaceous soluble biochemical oxygen demand (csBOD5) were measured (Figure 1C), as well as the conversion of soluble nitrogen into mostly insoluble organic nitrogen by the algal–bacteria community and removable through bioflocculation and settling (Figure 1D). The total removal efficiencies of VSS and csBOD5 were 76 ± 17% and 98.0 ± 0.2%, respectively. As a result, the effluent VSS and csBOD were 14 ± 8 mg L^{-1} and 3.2 ± 0.5 mg csBOD5 L^{-1}, respectively. Ammoniacal nitrogen represented the primary nitrogen form in the wastewater influent (30 ± 7 mg NH_3/NH_4^+ L^{-1} as N). In contrast, organic form nitrogen dominated the effluent nitrogen composition

(46 ± 6 mg N_{org} L^{-1} as N). Nitrogen removal as the result of algae cultivation was evaluated using 24 h settling efficiency with an effluent total nitrogen concentration of 10.6 ± 8.5 mg N L^{-1}. Further improvement of nitrate and nitrite removal can be achieved by applying standard denitrification techniques or a tertiary algae–based polishing step.

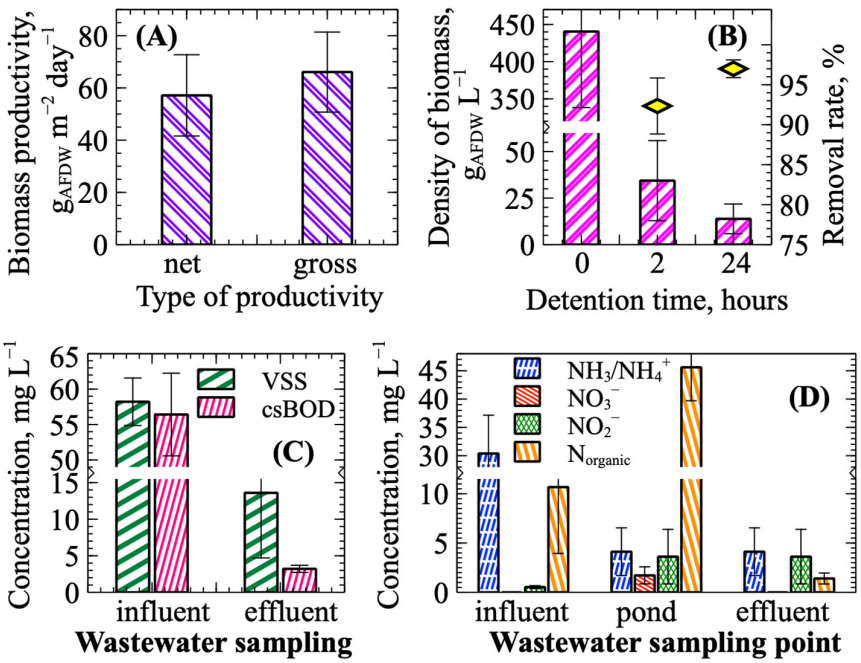

Figure 1. Average biomass productivity (**A**), biomass settling characteristics (yellow rhombus represents biomass removal rate) (**B**), and wastewater quality (**C**,**D**) throughout the cultivation study.

3.2. Enhancing of Biogas and Methane Yields through Algal Biomass Disintegration by Mechanical Pretreatment

The composition of algal biomass harvested from wastewater ponds is shown in Table 2, as well as the theoretical biogas and methane yields predicted from its use. The methane production from raw algae (no treatment control) after 60 days of fermentation was 0.323 ± 0.005 L_{CH4} per g of VS, an amount equivalent to <50% of theoretical yield and indicative of limited bioconversion due to the recalcitrance of algae biomass. The pretreatment of algal biomass with ceramic or stainless steel balls for 2 and 20 min did not result in a significant reduction in the particle size of the biomass or an increase in the soluble chemical oxygen demand (COD). This resulted in milling having marginal positive effects on biogas and methane yields of 3–7% and 3–9%, respectively (Figure 2). Among the kinetic models tested for fitting biogas and methane production, all show consistency with the experimental results. In this case, the 1st order rate and modified Gompertz equation demonstrated a slightly better fit to the experimental data, with a majority of R^2 values exceeding 0.99 in both cases. It is important to note, however, that while milling increased the ultimate amount of biogas and methane produced, it had an adverse influence on their production rates. The kinetic constants obtained from all three models have been somewhat reduced as a result of mechanical pretreatment. A summary of the estimated values for model parameters is provided in Table 3. In general, milling of algal biomass was found to be ineffective in enhancing biogas and methane production.

Table 2. Composition and theoretical methane yield of harvested algal–bacteria biomass.

Parameter	Relative Content (% dw [A])
Ash	19.9 ± 0.7
Volatile solids	80.1 ± 0.5
Crude protein [B]	51.6
Total lipids	23 ± 0.4
Fatty acid methyl esters	9.9 ± 0.6
Carbon	46.9
Nitrogen	8.26
Hydrogen	7.06
Biomass formula	$C_{6.62}H_{12.0}O_{1.93}N$
Theoretical biogas yield, L_{biogas} gVS^{-1}	1.09
Theoretical methane yield, L_{CH4} gVS^{-1}	0.65
Biogas methane content, %	60

[A]—percent of dry weight unless indicated; [B]—estimated as N × 6.25.

3.3. Enhancing Biogas and Methane Yields through Algal Biomass Disintegration by Hydrothermal Pretreatment

As a result of hydrothermal pretreatment, the particles and aggregates present in algal biomass were significantly disintegrated and solubilized. The particle-size distribution (PSD) normalized to the total volume of all particles in algal biomass is shown in Figure 3A. In untreated biomass, large particles and aggregates with a size of 100 mm or above make up more than 50% of the total particle volume. The fraction of large particles was reduced to nearly 40% or 30% following hydrothermal treatments at 100 °C or 121 °C, respectively. Moreover, extended treatment at 121 °C for 60 min resulted in the complete elimination of particles larger than 180 mm. In contrast, thermal hydrolysis increased the fraction of small particles (below 30 mm) from about 15% to 20–25%. In addition, a substantial portion of the algal biomass particles was hydrolyzed and transformed into soluble organic matter estimated as chemical oxygen demand (sCOD). As treatment temperature and duration were increased, the fraction of sCOD became more prominent, reaching up to 35% of total COD (for 60 min pretreatment at 121 °C) in comparison to only 10% in the original biomass (Figure 3B). Particle size reduction and an increase in sCOD are likely to be caused by fragmentation of cell aggregates, disruption of the cell wall, and partial disintegration of internal cell structures and biopolymers. Indeed, it has been reported that thermal pretreatment can result in the rupture of the cell wall and organelles in wastewater-grown algae [39,64]. Additionally, several studies have reported an increase in sCOD after thermal pretreatment of other biomass types as a result of the solubilization and partial hydrolysis of carbohydrates, proteins, and lipids [65,66]. However, to achieve more extensive hydrolysis of these biopolymers, hydrothermal pretreatment at a higher temperature than that applied in the current study is required [67,68].

A summary of the cumulative experimentally measured levels of biogas and methane production from hydrothermally pretreated algae, as well as predictions based on three different kinetic models, are presented in Figure 4. As compared to untreated algae, the ultimate yields of biogas and methane from the pretreated biomass increased by 20–55%. As expected, the yield improved with an increase in treatment temperature and duration. A 60 min pretreatment of algae at 121 °C produced the highest yields of biogas and methane, approximately 670 mL and 450 mL per gVS of biomass, respectively. Importantly, the observed methane yield increased from less than 50% to nearly 70% of the theoretical maximum. In accordance with experimental data, the pseudo-1st-order reaction equation model predicted an increase in the algal biomass biodegradable fraction from 46.5% to 71%.

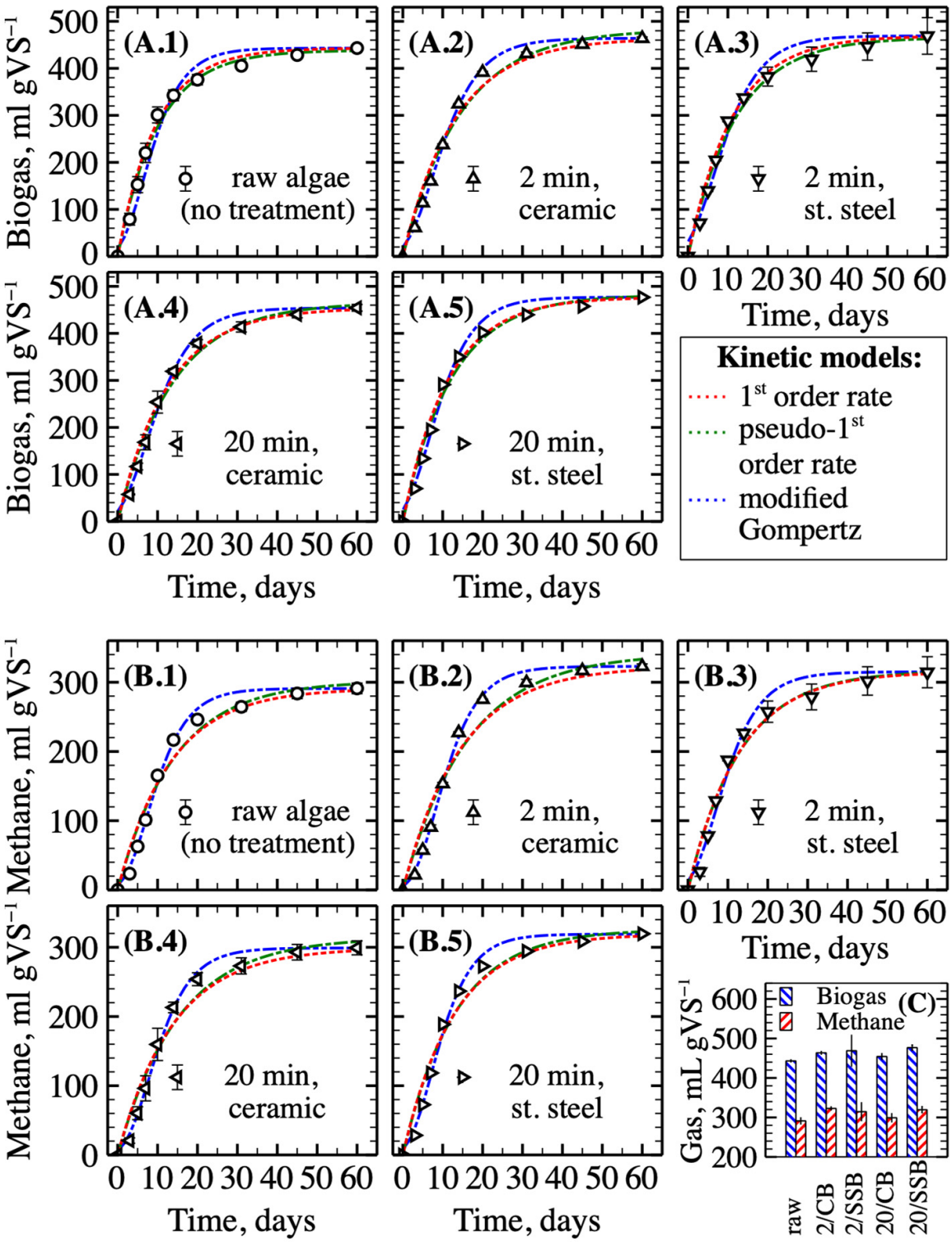

Figure 2. Experimental (markers) and model predicted (lines) production of biogas (**A.1**–**A.5**) and methane (**B.1**–**B.5**) from raw algal–bacteria biomass and biomass milled with either ceramic ball (CB) or stainless steel balls (SSBs) for either 2 or 20 min. Model yields calculated using the 1st-order equation (dotted line), pseudo-1st-order equation (dash-dot line) and modified Gompertz equation (dash-dot-dot line). Ultimate yields for biogas and methane are compared on panel (**C**).

Table 3. Parameters for fitting experimental data of biogas and methane production from mechanically pretreated algal biomass into various kinetic models.

Sample	1st-Order Equation Model			Pseudo-1st-Order Equation Model					Modified Gompertz Model			
	k, d^{-1}	RMSE	R^2	k_1, d^{-1}	k_2, d^{-1}	P_{biodeg}	RMSE	R^2	k, mL gVS^{-1} d^{-1}	λ, d	RMSE	R^2
Biogas production												
Raw algae	0.104	18.3	0.990	0.099	0.000	0.403	15.9	0.989	29.6	0.00	20.8	0.985
2 min CB	0.076	21.7	0.989	0.069	0.000	0.444	20.1	0.987	26.1	0.94	26.1	0.997
2 min SSB	0.090	18.6	0.991	0.084	0.000	0.428	15.9	0.991	29.3	0.75	29.3	0.984
20 min CB	0.080	19.5	0.990	0.073	0.000	0.428	18.2	0.989	25.8	0.70	25.8	0.993
20 min SSB	0.088	20.3	0.990	0.082	0.000	0.443	18.7	0.989	28.9	0.51	28.9	0.991
Methane production												
Raw algae	0.076	19.1	0.976	0.071	0.000	0.465	18.6	0.974	19.1	1.79	10.2	0.993
2 min CB	0.069	24.9	0.974	0.062	0.000	0.526	23.4	0.971	20.3	2.49	6.7	0.998
2 min SSB	0.078	17.0	0.980	0.077	0.000	0.488	16.9	0.980	19.1	0.97	15.9	0.984
20 min CB	0.072	20.3	0.978	0.066	0.000	0.484	19.4	0.975	18.9	1.98	8.9	0.995
20 min SSB	0.079	19.8	0.978	0.076	0.000	0.502	19.5	0.977	21.1	1.60	11.0	0.993

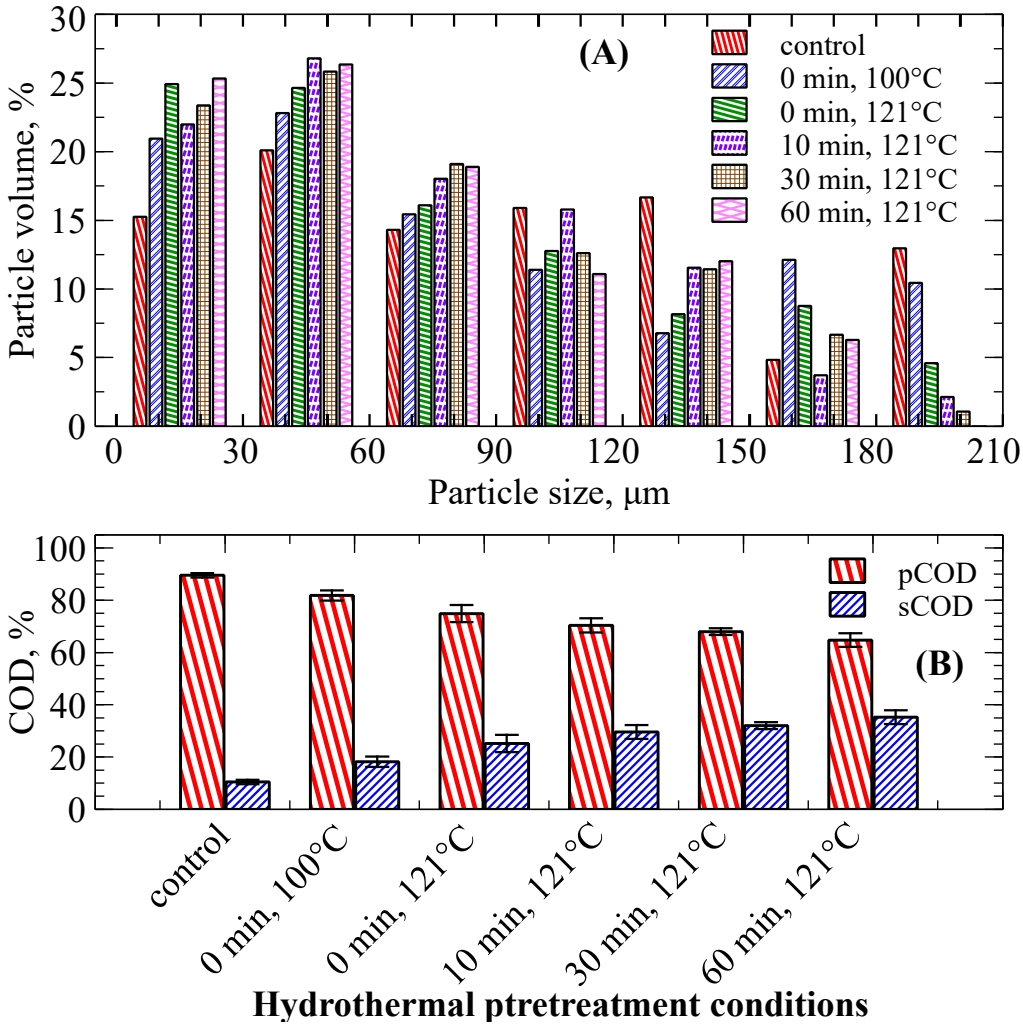

Figure 3. Distribution in algal biomass particle size (PSD) (**A**), and particulate vs. soluble chemical oxygen demand (pCOD and sCOD, respectively) (**B**). The particle size is reported as a volume equivalent sphere with diameter ranges of 0–30 μm, 30–60 μm, 60–90 μm, 90–120 μm, 120–150 μm, 150–180 μm, 180–210 μm.

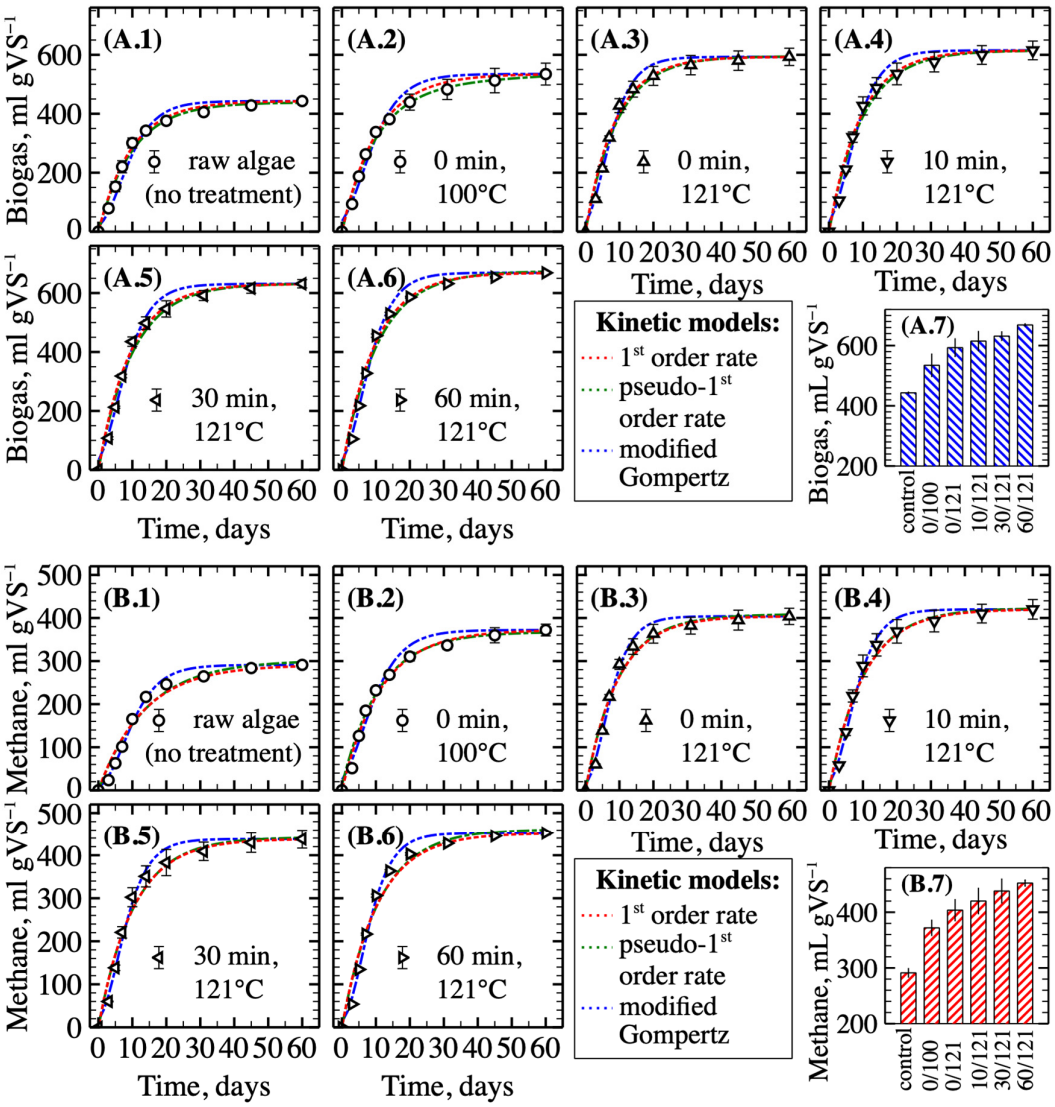

Figure 4. Experimental (markers) and model predicted (lines) production of biogas (**A.1**–**A.6**) and methane (**B.1**–**B.6**) from raw and thermally pretreated algal–bacteria biomass. Model yields calculated using the 1st-order equation (dotted line), pseudo-1st-order equation (dash-dot line) and modified Gompertz equation (dash-dot-dot line). Ultimate yields for biogas and methane are compared on panel (**A.7**) and (**B.7**), respectively.

The complex polymeric structure of algae–bacteria biomass is a major obstacle to the effective conversion of algae–bacteria biomass into methane. As a consequence of this complexity, substrate accessibility is limited to hydrolytic enzymes, causing the initial hydrolysis step to become a bottleneck in the multistep anaerobic digestion process. By disintegrating and partially dissolving biomass particles, hydrothermal treatment may improve organic polymers' accessibility to hydrolytic enzymes. As the bottleneck hydrolysis stage is accelerated, methane production rate and ultimate yield are increased. Improvements in biogas and methane yields obtained in this study are either superior or comparable to those observed earlier in other studies on the pretreatment of algal biomass. For instance, thermal pretreatment of wastewater-grown algae at 130 °C for 15 min improved methane yield from 120 to 170 mL per gVS [64]; treatment of *Chlorella* sp. at 121 °C for 20 min increased yield from 155 to 320 mL per gVS [69]; *C. vulgaris* at 121 °C for 40 min increased yield from 150 to 225 mL per gVS [36]; *Chlorella* sp. 120 °C for 30 min from 335 to 405 mL per gVS [33]; and, a 15 min treatment of a mixture of green microalgae at 110 °C,

130 °C and 140 °C resulted in enhancements of methane production from 170 mL to 320, 360 and 400 mL [70]. Variations in the phylogenetic and biochemical composition of algae could contribute to differences in methane yield from untreated biomass and response to thermal pretreatment. It has been shown, for example, that adding CO_2 during algae-based wastewater treatment can increase lipid content in algae–bacteria biomass [71], which can lead to an increase in methane yield during anaerobic digestion [72].

A good agreement was observed between all of the tested kinetic models and the experimental results (Figure 4). In contrast to mechanical pretreatment, hydrothermal pretreatment increased methane production kinetic rates by 40–75%. A major benefit of improved kinetics is that over 80% of ultimate methane yield was achieved within the first 14 days of digestion and over 90% within the first 20 days. Reducing digestion time is crucial to minimizing the bioreactor volume and improving technology economics. A modified Gompertz sigmoidal-type equation model provided the best fit for methane production with almost all R^2 values above 0.99. In contrast, the 1st-order and pseudo-1st-order reaction equation kinetic models provided a slightly better fit for biogas production with R^2 in the range of 0.986–0.994. A summary of all kinetic coefficients and performance parameters for all models can be found in Table 4.

Table 4. Parameters for fitting experimental data of biogas and methane production from thermally pretreated algal biomass into various kinetic models.

Sample	1st-Order Equation Model			Pseudo-1st-Order Equation Model					Modified Gompertz Model			
	k, d^{-1}	RMSE	R^2	k_1, d^{-1}	k_2, d^{-1}	P_{biodeg}	RMSE	R^2	k, mL gVS^{-1} d^{-1}	λ, d	RMSE	R^2
Biogas production												
Raw algae	0.104	18.3	0.990	0.099	0.000	0.403	15.9	0.989	29.6	0.09	20.8	0.985
0 min at 100 °C	0.096	18.0	0.994	0.099	0.001	0.454	14.1	0.994	32.6	0.00	29.4	0.978
0 min at 121 °C	0.114	25.6	0.988	0.107	0.000	0.547	24.1	0.987	46.5	0.51	21.3	0.992
10 min at 121 °C	0.108	27.2	0.988	0.101	0.000	0.565	25.0	0.987	44.9	0.43	25.5	0.989
30 min at 121 °C	0.106	27.2	0.989	0.099	0.000	0.581	25.2	0.988	45.1	0.42	25.6	0.989
60 min at 121 °C	0.104	30.9	0.987	0.096	0.000	0.620	29.1	0.986	48.6	0.66	23.8	0.992
Methane production												
Raw algae	0.076	19.1	0.976	0.071	0.000	0.465	18.6	0.974	19.1	1.8	10.2	0.993
0 min at 100 °C	0.088	13.6	0.989	0.091	0.000	0.564	13.5	0.989	22.6	0.0	19.9	0.979
0 min at 121 °C	0.106	21.7	0.980	0.106	0.000	0.623	21.7	0.979	33.9	1.0	14.4	0.992
10 min at 121 °C	0.099	21.7	0.981	0.098	0.000	0.651	21.6	0.981	32.4	0.9	17.1	0.990
30 min at 121 °C	0.098	22.8	0.981	0.096	0.000	0.681	22.7	0.981	33.5	0.9	17.5	0.990
60 min at 121 °C	0.096	26.2	0.979	0.092	0.000	0.712	25.8	0.978	35.3	1.3	14.7	0.994

According to the results presented above, all three tested models describe the experimental data from anaerobic digestion of raw or thermally pretreated wastewater-grown algal biomass adequately. A 1st-order equation model offers the most straightforward solution. In contrast, the pseudo-1st-order equation model allows a deeper and more mechanistic description of biomass conversion by estimating the easily biodegradable and recalcitrant fractions. The pseudo-1st-order equation model predicted that the biodegradable fraction of algal biomass increased from nearly 40% to 60–70% after thermal pretreatment, matching the ratio of experimentally observed to theoretically predicted biogas and methane production. The modified Gompertz sigmoidal-type equation model could provide a better fit for biomass with a substantial lag phase in gas production during the first few days of digestion. In the current study, wastewater-grown biomass did not exhibit an extended lag phase. The pseudo-1st-order equation model may, therefore, be the most appropriate approach to describe biogas and methane yields from pretreated algae.

3.4. Assessment of Energy Balance for Anaerobic Digestion of Raw and Hydrothermally Pretreated Biomass

In order to investigate whether hydrothermal pretreatment is feasible as an enhancement method for AD, we compared the process energy balance between raw (control) and

pretreated wastewater-grown algae. Specifically, AD kinetics data were used to simulate the performance and energy balance of a scaled AD system processing about 56×10^3 tons AFDW of algae–bacterial biomass per year generated using 56.8 acres of ponds treating domestic wastewater.

The effect of hydrothermal pretreatment on the energy balance of the scaled AD process carried out at different hydraulic retention times (HRTs) is shown in Figure 5. Processing of raw algae (control without pretreatment) resulted in 0.5 MW of heat, 0.25 MW of electricity, and 0.76 MW of combined heat and electricity (total) (Figure 5A). The application of hydrothermal pretreatment improved energy production by 25–40% (to up to 1.12 MW), with the highest improvements observed after treatment at 121 °C for extended periods of 30–60 min. Additionally, shorter HRTs of <30 days resulted in greater energy production gains. Importantly, algae pretreatment had a positive impact on energy output (production–input), indicating that energy production gains from pretreatment are greater than energy input during thermal hydrotreatment (Figure 5B). Furthermore, nearly half of the output energy was electricity, which could be readily used on-site for wastewater treatment processes or return power to the energy grid.

As a result of algal hydrotreatment at 121 °C for 30–60 min, both volume-specific (Figure 5C) and mass-specific (Figure 5D) total energy outputs were enhanced by 38–42%. The highest specific energy output values amounted to 0.23 kW per m^3 of digester and 2.3 MW per ton of biomass volatile solids. The volume-specific energy output reached its maximum at an HRT of 14 days, while the mass-specific energy output peaked at HRT of 45 days. According to these data, the optimal HRT may be around 20–30 days, which is typical for scaled AD systems. Indeed, the net energy ratio (NER) and net energy efficiency (NEE) reached highest values of 2.14 (Figure 5E) and 68% (Figure 5F), respectively, at HRT of 31 days. However, optimal pretreatment conditions at 121 °C for 30–60 min resulted in NER and NEE values that were comparable to those obtained for untreated algae. By exposing algal biomass to a lower temperature and for shorter exposure times, thermal pretreatment of algal biomass resulted in a reduction in the values of NER and NEE compared to the AD of untreated algae. In contrast, all types of thermal treatment improved the net energy recovery (NERec), which is the energy output per HHV of processed algal biomass (Figure 5G). The NERec improved from a maximum value of about 20% for raw biomass to a maximum value of 32–34% for biomass pretreated at 121 °C for 30–60 min.

Algae cultivation in wastewater and processing through anaerobic digestion should be implemented through integration into existing wastewater treatment infrastructure. Moreover, several hydrothermal pretreatment technologies have been adopted by full-scale wastewater treatment facilities in recent years [73]. Thermal processing processes such as CAMBITM were introduced in 1995 [74], BioThelysTM in 2004 [75], TurboTecTM in 2012 [76], ExelysTM in 2014 [77], and LysoThermTM in 2016 [78], are commercialized and enable the production of biogas from sewage sludge to be increased by 20–50%. The results obtained in the current study suggest that methane yield and overall energy production from algae biomass grown in wastewater can be improved by either processing separately or co-processing with sewage sludge using available on-the-market hydrothermal technologies. This study also provides a framework for thermal pretreatment optimization by identifying pretreatment parameters that maximize energy output or volatile solid conversion through a biomethane potential test and energy modelling approach.

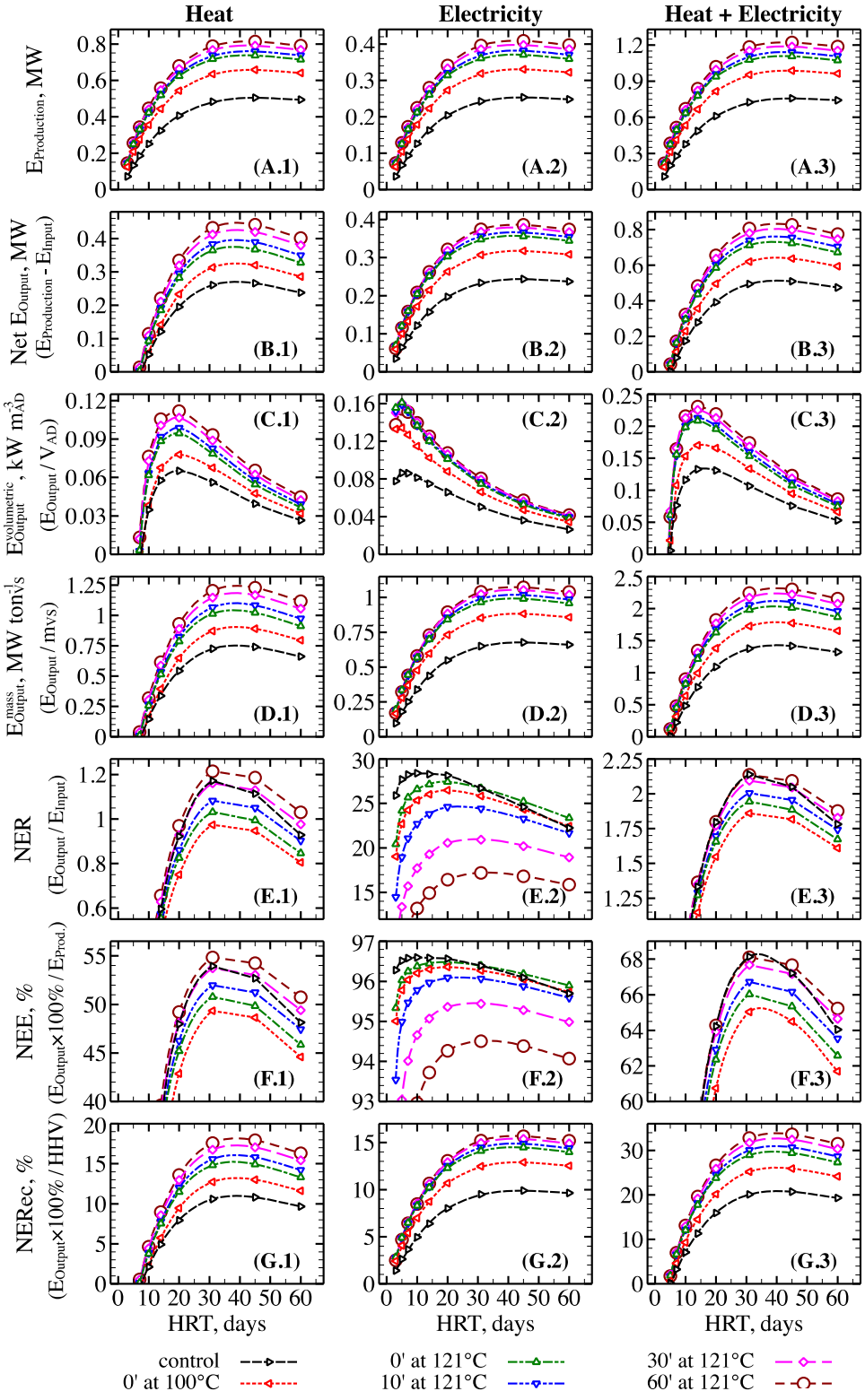

Figure 5. Predicted energy balance characteristics of a scaled anaerobic digestion system processing raw or hydrothermally pretreated wastewater-grown biomass. Energy production (**A.1**–**A.3**), net energy output (**B.1**–**B.3**), volumetric energy output (per m^3 of digester) (**C.1**–**C.3**), specific energy output (per ton of biomass vs. fed into digester) (**D.1**–**D.3**), net energy ratio of output to input (**E.1**–**E.3**), net energy efficiency as ratio of output to production (**F.1**–**F.3**), net energy recovery as ration of output per HHV of biomass fed into digester (**G.1**–**G.3**).

4. Conclusions

Municipal primary wastewater can be converted into bioenergy by cultivating and harvesting an indigenous algal–bacteria polyculture that has spontaneously established, which can then be processed into energy through the pretreatment-intensified anaerobic digestion process (AD). This study demonstrates that organic loading rates, in combination with algal–bacteria culture mutualism, allow for superior biomass productivity, bioflocculation, and nutrient removal efficiency. Further, hydrothermal treatment significantly increases the biodegradability of wastewater-grown algal biomass and methane production through AD, which leads to improved digester energy balance. In comparison to AD of raw biomass, pretreatment at 121 °C for 60 min resulted in the greatest increase in methane yield (55%) and production rate (85%), resulting in a significant increase in energy output despite the energy needed for pretreatment. A significant aspect of this study involves the demonstration of a practical biomass pretreatment evaluation tool combining high-throughput biomethane potential methodology, modeling of methane production kinetics, and simulation of the energy balance of the AD system. With this tool, it is possible to evaluate biomass pretreatment methods by predicting their impact on scaled AD system parameters, including total, volumetric- and mass-specific energy outputs, net energy ratio, efficiency and recovery.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/en16207146/s1, Table S1: Biog methane pretreat kinetic models; Table S2: AD Energy Balance Pretreat.

Author Contributions: Conceptualization, P.B. and T.J.L.; Methodology, P.B., D.P., R.E.S. and T.J.L.; Formal analysis, P.B.; Investigation, D.P. and R.E.S.; Resources, T.J.L.; Data curation, R.E.S.; Writing—original draft, P.B.; Writing—review & editing, P.B.; Visualization, P.B.; Project administration, P.B., R.E.S. and T.J.L.; Funding acquisition, P.B. and T.J.L. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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

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