**Anaerobic Digestion of Wastewater Generated from the Hydrothermal
Liquefaction of *Spirulina*: Toxicity Assessment and Minimization**

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Abstract: Previous studies demonstrate anaerobic digestion of hydrothermal liquefaction wastewater (HTL-WW) is significant to the sustainability of algal biofuel development for nutrient reuse and residual energy recovery. HTL-WW contains substantial amounts of residual energy but is toxic to anaerobes. With 6% HTL-WW converted from cyanobacteria (e.g. *Spirulina*), anaerobes were 50% inhibited. In this study, zeolite, granular activated carbon (GAC), and polyurethane matrices (PM) were used during a two-round anaerobic batch test with HTL-WW, and in the presence of each material, the total methane yields were 136 mL/g COD, 169 mL/g COD, and 168 mL/g COD, respectively, being 11%, 37% and 36% higher than the control. GAC was considered promising due to its highest methane yield of 124 mL/g COD at the second feeding, indicating a good recovery of adsorption capacity. The observed low methane production rates indicated the necessity for anaerobic process optimization. The physicochemical analysis of the digestates demonstrated that most of the compounds identified in the HTL-WW were degraded.

Keywords: biogas; hydrothermal liquefaction wastewater; adsorbents; biofilm; detoxification; algae

40 **Abbreviations**

ATA	anaerobic toxicity assay
COD	chemical oxygen demand
C/N ratio	carbon/nitrogen ratio
E2-Energy system	Environment-Enhancing Energy system
GAC	granular activated carbon
HTL	hydrothermal liquefaction
HTL-WW	hydrothermal liquefaction wastewater
I	percent inhibition
NH ₄ ⁺ -N	ammonia nitrogen
PM	polyurethane matrices
RPA	total relative peak area
SCOD	soluble chemical oxygen demand
TCD	thermal conductivity detector
TN	total nitrogen
TOC	total organic carbon
TP	total phosphorus
TS	total solid
VFA	volatile fatty acid
VS	volatile solid
VSS	volatile suspended solid

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1. Introduction

Hydrothermal liquefaction (HTL) is a thermochemical process that converts organic compounds of the feedstock into biocrude oil, solid residues, aqueous products and gas products at 250–350°C and 5–25 MPa [1]. The process is specially appropriated to convert wet substrates, such as algae (moisture content >90 wt%). Wet feedstocks can be treated directly by HTL without a drying process, and energy-dense oil products can self-separate from the aqueous products after HTL treatment [2].

Earlier studies reported that during the HTL process, only approximately 40% of carbon and 35% of hydrogen in algal feedstocks were converted into oil [3]. From 10% to 45% of the organic compounds present in the feedstock was converted into aqueous products [3, 4]. In addition, Yu et al. (2011) [5] found that 65–70% of nitrogen in the algal feedstock was converted into water-soluble compounds. The extraction and recovery of carbon and nutrients from the aqueous products is paramount to improve the overall economic viability of the HTL process [6]. As previous studies have demonstrated, the Environment-Enhancing Energy (E2-Energy) system is an integration of algal wastewater treatment with HTL of algal biomass for bioenergy production, which provides synergistic recycling of carbon dioxide from the HTL gaseous product and the nutrients from the HTL aqueous product to support multiple rounds of algae cultivation [2, 5, 7].

The HTL aqueous product, henceforth called HTL wastewater (HTL-WW), is a high-strength wastewater with high concentrations of chemical oxygen demand (COD, 0.42–104 g/L), high concentrations of ammonia (1.86–7.07 g/L), and a wide variety of organic compounds. These compounds include sugars, alcohols, ketones, cyclic hydrocarbons, and various nitrogen-containing compounds [2, 8, 9, 10].

Anaerobic digestion is a natural choice for the treatment of highly concentrated effluents, which proved feasible for the treatment of HTL-WW [9, 11]. According to Ward et al. (2014) [12], anaerobic digestion produces a nutrient-rich digestate that is ideal for the cultivation of algal species. Thus, a closed loop for an integration system, such as the E2-Energy paradigm, can be realized based on the nutrients cycle in which algae can be cultivated in anaerobically treated HTL-WW, and then harvested and converted to produce more algal bio-crude oil. This system is anticipated to enhance the cost-effectiveness of the biocrude oil production and improve the feasibility and sustainability of algal biofuel production.

However, HTL-WW could be inhibitory or toxic to anaerobes due to its high ammonia concentrations or recalcitrant organic compounds (e.g., phenols), which limits the digestibility and biogas production [9]. Potential detoxification methods include adsorption, ozonation, and biological treatment with adapted or specialized microbes. Zeolite and activated carbon have a high degree of porosity, showing a great capacity for pollutant adsorption [13, 14]. Moreover, Zhou et al. (2015) observed the positive effects of activated carbon in the anaerobic stabilization of HTL-WW converted from swine manure. On the other hand, microorganisms organized in biofilms showed better responses than suspended biomass when used in tests for acute and chronic toxicity [15]. Reticular polyurethane foam has a high specific surface area that can reach 2400 m²/m³ and a porosity of 97% [16]. Polyurethane foam has been widely studied as a support matrix to immobilize anaerobic biomass and as an isolation material to prevent the release of hazardous materials into the environment [17, 18]. This material has been primarily used in fixed-bed reactors and provides adequate environmental conditions for biomass growth and retention.

This study focuses on the characterization and quantification of the HTL-WW composition, with an emphasis on the toxicity to anaerobes, and explores methods for its detoxification. Thus, an anaerobic toxicity assay (ATA) was conducted to determine the level at which HTL-WW causes an adverse effect on the anaerobic digestion process. Two types of adsorbents, zeolite and activated carbon, were used to evaluate the toxicity reduction towards a microbial consortium during the anaerobic treatment of HTL-WW. In addition, polyurethane matrixes (PM) were also used to provide a suitable environment for the biofilm formation during the anaerobic digestion process of highly toxic HTL-WW.

2. Materials and methods

2.1 HTL process

The HTL process using *Spirulina* powder (from Cyanotech, Kailua-Kona, Hawaii, USA) as feedstock was conducted in triplicate in a 2 L stirred bench scale reactor (Parr Instrument Co., Moline, USA), according to the method by Pham *et al.* (2013)[13]. The reactor was filled with 600 g of feedstock at 20% solid content (80% water), sealed, and then purged three times with nitrogen to build up an initial pressure of 607–635 kPa to prevent water from boiling. The temperature was raised to 300°C, maintained for 30 minutes and then rapidly cooled to room temperature (25-30°C) using circulated tap water. The pressure during the process increased from 0.7 MPa to 8-9 MPa, and at the end, the pressure was 0.9-1 MPa. A typical temperature-pressure diagram under the HTL process is also available in supplementary data (Fig S1). The gaseous product was vented, and the crude oil, solid residue, and wastewater were collected into sampling cups for separation. HTL-WW was separated from the crude oil and solid residue using a 1.0 µm pore size glass fiber filter. Filtered HTL-WW was used for the following analysis and experiments.

2.2 Anaerobic toxicity assay (ATA) of HTL-WW

An ATA was conducted to determine the potential toxicity of HTL-WW. The ATA was primarily based on the method and feedstock preparation proposed by Moody et al. (2011) [19] with one modification: acetate (1 mol/L) was utilized instead of glucose, according to Lisboa and Lansing (2013)[20]. They determined that compared with glucose, acetate as the standard feedstock produced more methane in the control bottles, facilitating the discernibility of the potential inhibition from the ATA. An inoculum and a standard feedstock were assayed without HTL-WW as controls and in combination with varying percentages of the HTL-WW as potential toxicants, as shown in Table 1. The inclusion ratios of HTL-WW were performed according to previous results obtained by Zhou et al., (2015) [21]. The inoculum from an anaerobic reactor treating secondary sludge was provided by the Urbana-Champaign Sanitary District (IL, USA) with total solids (TS) of 2.61%, volatile solids (VS) of 1.92%, and volatile suspended solids (VSS) of 18.6 g/L. Sludge addition was performed according to Angelidaki et al., (2009) [22]. The inoculum was degassed for 2-5 days until there was no significant methane production. Deionized (DI) water was added to the substrate to maintain a constant headspace volume in each bottle, while the headspace was sampled every day during the short testing period (6 days).

All ATA tests were performed in triplicate, with 50 mL of total liquid volume and 72 mL of headspace purged with nitrogen. Biogas production and methane content were measured daily. The volume and removal of biogas during the tests were measured and performed with glass syringes equipped with a 20-gauge needle. The methane content was determined by direct injection into the gas chromatography equipped with a

141 thermal conductivity detector (GC-TCD) described in Section 2.4. The results were
142 used to calculate the percent inhibition (I) of methane production for each substrate
143 inclusion ratio using Equation 1:

$$144 \quad I = \left(1 - \frac{V_{CH_4 test}}{V_{CH_4 Fed control}} \right) * 100 \quad (1)$$

145 where $V_{CH_4 test}$ is the methane volume produced at the selected time for each
146 potential toxicant percentage inclusion, and $V_{CH_4 Fed control}$ is the volume of methane
147 produced at the selected time for the feed control. A negative value of “I” implies that
148 the substrate enhances the methane production.

Table 1 Experimental design for anaerobic toxicity assays (ATA) of HTL-WW generated from hydrothermal liquefaction of *Spirulina* (300°C with a retention time of 30 minutes) with nine inclusion ratios (2 to 24% inclusions).

	Control	1	2	3	4	5	6	7	8	9
% Inclusion	/	2%	3%	4%	5%	6%	12%	15%	18%	24%
Inoculum sludge (mL)	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0
HTL-WW(mL)	/	1.0	1.5	2.0	2.5	3.0	6.0	7.5	9.0	12.0
Deionized water (mL)	23.0	22.0	21.5	21.0	20.5	20.0	17.0	15.5	14.0	11.0
Standard feedstock* (mL)	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
COD inclusion from HTL-WW(mg/L)	/	1781	2671	3562	4452	5342	10685	13356	16027	21369
Total volume (mL)	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0

*including 1 mol/L acetate; COD of standard feedstock is 2561 mg COD/L

2.3 Effect of adsorption and biofilm on methane production enhancement

Anaerobic batch tests were assembled in 246 mL serum bottles with 100 mL of working volume and were performed in a 37°C shaking incubation chamber. Each flask was initially supplied with 50 mL of inoculum sludge, 6.5 mL of HTL-WW and 43.5 mL basal medium (Angelidaki et al., 2009) [22]. Three types of conditions were tested, as shown in Table 2: the addition of 2 g/L zeolite, 2 g/L granular activated carbon (GAC, Calgon F-400), and 2 g polyurethane matrix (PM) [23, 24]. The zeolites used were obtained from St. Cloud Mine (St. Cloud Zeolite, Winston, NM) and were preprocessed into a standard mesh size of 14×40. The materials were rinsed thoroughly with DI water until the turbidity was low; they were then soaked in 10% saltwater overnight and rinsed once more with DI water. Zeolites and GAC were added according to recommendations of Montalvo et al., (2005) [25] and Zhou et al (2015) [21], respectively. The PM used was black and was cut into cubes with side lengths of approximately 0.5 cm. Because the main effect of the PM was to provide an environment for biofilm formation, its dosage is different from the other two adsorbents. Therefore, the amount of polyurethane matrices was to be enough to fulfill the liquid volume in the reactors. The test without any adsorbent or biofilm promoter was set as the control. The pH was adjusted to 7.4 with the addition of 1 mL of 10% HCl. The inoculum used was the same as that used for the ATA described in Section 2.2. The average initial soluble COD (SCOD) in the test bottles was 6940 ± 399 mg/L. The HTL-WW addition was based on the results obtained in the ATA. Biogas-volume sampling and removal during the tests were performed with glass syringes equipped with a 20-gauge needle. When virtually no biogas was produced at the end of the

digestion, the appropriate volumes of liquids were sampled for the residual volatile fatty acids (VFAs), COD and ammonia concentration analyses.

Table 2 Experimental design of the two-round anaerobic batch test of detoxified HTL-WW generated from the hydrothermal liquefaction of *Spirulina* (300°C with a retention time of 30 minutes) using different adsorbents

	Zeolite (g)	Active carbon (g)	Polyurethane matrix (g)	Sludge (mL)	HTL- WW(1 st feeding) (mL)	HTL- WW(2 nd feeding) (mL)	Total volume (mL)
Control	/	/	/	50.0	6.5	13.0	100
PM*	/	/	2.0	50.0	6.5	13.0	100
GAC	/	0.2	/	50.0	6.5	13.0	100
Zeolite	0.2	/	/	50.0	6.5	13.0	100

*PM, polyurethane matrices; GAC, granular activated carbon.

To investigate the response of the anaerobic microorganisms under stress conditions (high COD concentration), the same flasks received a second feeding of substrate, which included 13 mL of HTL-WW. This second feeding also represented an inclusion of 12.4 % (Table 2). Basal medium was added proportionally, and the pH was again adjusted to 7.4 with the addition of 2 mL of 10% HCl.

The cumulative methane production curves were fitted by a modified Gompertz equation (Equation 2), according to Chen et al. (2006) [26]:

$$P_{CH_4}(t) = P_{CH_4} \exp \left\{ -\exp \left[\frac{k \cdot e}{P_{CH_4}} (\lambda - t) + 1 \right] \right\}, \quad (2)$$

where $P_{CH_4}(t)$ represents the cumulative methane production (mL) at the incubation time t (day), P_{CH_4} is the specific methane production potential (mL), k is the maximum methane production rate (mL/d), λ is the lag phase time (day), and e is Euler's number, which is the mathematical constant (2.71828). The model was fitted using the Levenberg-Marquardt method (Origin software version 9.0).

2.4 Analytical methods

The chemical compositions of the HTL-WW were analyzed as described by using a GC-MS (Agilent Technologies, Santa Clara, CA, USA). A 2 μ L sample was injected in split mode (7:1) into the GC-MS system consisting of an Agilent 6890 (Agilent Inc., Palo Alto, CA) gas chromatograph, an Agilent 5973 mass selective detector, and an Agilent 7683B autosampler. Gas chromatography was performed on a 15 m ZB-FFAP column with a 0.25 mm inner diameter and a 0.25 μ m film thickness (Phenomenex, Torrance, CA), an injection temperature of 250°C, and a Mass Selective Detector (MSD) transfer line of 250°C; the ion source was adjusted to 230°C. The helium carrier gas was set to a constant flow rate of 1.6 mL/min. The temperature program was 5 minutes at 50°C, followed by an oven temperature ramp of 5°C/min to 250°C for the final 20 minutes. The mass spectrometer was operated in positive electron impact mode at 69.9 eV ionization energy in the m/z 30-800 scan range. The spectra of all chromatogram peaks were evaluated using the HP Chem Station (Agilent, Palo Alto, CA) and AMDIS (NIST, Gaithersburg, MD) programs. The spectra of all chromatogram peaks were compared with an electron impact mass spectrum from the NIST Mass Spectral Database (NIST08) and the W8N08 library (John Wiley & Sons, Inc., Hoboken, NJ). To allow comparison between samples, all data were normalized to the internal standard, isopentanoic acid (0.1 μ M). In other words, the normalized peak area (N_i) is defined as the peak area of target compounds (X_i) divided by that of internal standard (X_{IS}) (i.e., $N_i = X_i / X_{IS}$). The instrument variability was within the standard acceptance limit (5%). More details can be found in previous studies [9, 10, 27].

The concentrations of Na, K, Al, B, Ca, Cu, Fe, Mg, Mn, Se and Zn were measured using an inductively coupled plasma optical emission spectrometer (Perkin Elmer, 2000DV, ICP-OES, Norwalk, CT, USA). The methane content of the biogas was

analyzed by a Varian CP-3600 gas chromatograph (Palo Alto, CA, USA) coupled with a thermal conductivity detector. The GC analysis used a Haysep D 100/120 column (20-ft, 1/8-in diameter), with an injection temperature of 120°C and a filament temperature of 140°C. The carrier gas was helium at 30 mL/min. The methane volume was then converted to a volume of gas at standard temperature and pressure using the ideal gas law.

Samples for VFAs concentration analysis were obtained at the end of the first run, centrifuged at 5,000 rpm for 10 minutes and filtered using 0.25 µm pore size Nylon filter). VFAs were quantitatively analyzed using a high-performance liquid chromatograph (Shimadzu Scientific Instruments, MD, USA) equipped with an SPD-M20A UV detector and an Aminex HPX-87H column (300 mm × 7.8 mm and 9 µm particle size) (Bio-RAD, California, USA). More details can be found in previous studies [9].

All laboratory analyses, such as COD, ammonia nitrogen ($\text{NH}_4^+\text{-N}$), total nitrogen (TN), total phosphorus (TP), total organic carbon (TOC), TS, VS and VSS, were performed according to the Standard Methods [28]. All the gas volume results are presented under standard conditions (0°C, 101.3 kPa). Each analytical datum was the mean value of at least three measurements.

3. Results and discussion

3.1 Characteristics of HTL-WW

The physical-chemical characterization of the HTL-WW is presented in Table 3. The concentration of COD had the same magnitude as the values observed by Tommaso et al. (2015) [9] in the HTL-WW from the conversion of mixed-culture wastewater algae and those from Gai et al. (2015) [10] in the HTL-WW from the conversion of *Chlorella pyrenoidosa*. A high concentration of COD indicates that a large amount of

energy can be recovered; however, depending on its complexity, high strength wastewater could result in long lag phases and slow rates of biogas production [29]. Among the possible inhibitors to an anaerobic consortium, free ammonia (NH_3) and the ammonium ion (NH_4^+) are considered the most significant. Anaerobic process inhibition is related to particular characteristics of the substrate, such as pH, process temperature, type of seed sludge, and the concentrations of ammonium and ammonia [30]. Researchers reported that methane production inhibition occurred with the concentrations of NH_4^+ -N above 4920 mg/L and with 100% inhibition above 8000 mg/L [31, 32]. Moreover, a very low carbon/nitrogen (C/N) ratio of 2.4 was observed. When the C/N ratio is below 20, there is an imbalance between carbon and nitrogen requirements for the anaerobic consortia [12]. This imbalance leads to nitrogen release in the form of ammonia during digestion, which can further increase the concentration of ammonia. However, given the high concentration of COD, further dilutions are required to circumvent all potential problems related to microbial inhibition.

Table 3 Physicochemical characterizations of HTL-WW generated from the hydrothermal liquefaction of *Spirulina* (300°C with a retention time of 30 minutes) (mean $\pm$ SE).

Parameters	pH	COD		TN		NH_4^+ -N		TP		C/N ratio		
Concentration (mg/L)	7.8 $\pm$ 0.3	89039 $\pm$ 3321		22981 $\pm$ 2482		10117 $\pm$ 1167		4400 $\pm$ 198		2.4		
Metals	Al	B	Ca	Zn	Cu	Fe	Mn	Se	Mg	Co	K	Na
Concentration (mg/L)	3.9	22.5	13.5	1.3	0.6	4.7	0.3	11.7	1.4	0.9	3400.0	2254.0

The measured minerals in the HTL-WW, excluding potassium and sodium, were in concentrations below the requirement for anaerobic consortia treating wastewaters [33]. In addition, there was no nickel (Ni) or molybdenum (Mo) detected in the HTL-WW. The concentration of sodium was 2254 mg/L, which was ten times higher than the value

considered as stimulatory. The potassium concentration (3400 mg/L) was at the levels considered as moderately inhibitory [34].

The major components identified by GC-MS of the HTL-WW are shown in Table 4. Identified compounds were categorized into several groups such as short chain organic acid (C2-C4) and straight amide derivatives. GC-MS spectrum of the HTL-WW is available in Figure 5. It should be pointed out that only a fraction of the HTL-WW is identifiable by GC-MS. This is due to that the HTL-WW may contain compounds with high molecular weights and boiling points and some chemicals are not volatilable under GC-MS [9, 10, 27].

Table 4 Major components identified in the HTL-WW generated from the hydrothermal liquefaction of *Spirulina* (300°C with a retention time of 30 minutes) by GC-MS.

Chemical Groups	% of Total Relative Peak Area*
Short chain organic acid(C2-C4)	2.37%
Long chain organic acid (6>C>4)	2.86%
Benzene Derivatives	6.37%
Straight Amides Derivatives	13.2%
N&O-Heterocyclic compounds	45.8%
Phenol+Phytosterol Derivatives	0.40%
Fatty acid+Fatty alcohols	6.32%
Amino Acids	3.32%

* The total relative peak area (RPA) is defined as the normalized peak area (N_i) of the chemical groups divided by the total normalized peak area (i.e., $RPA = N_i$ of a chemical group/total normalized peak area) (Chet et al., 2016; Tommaso G. et al., 2014; Gai C. et al., 2015).

A wide variety of organic compounds were found in HTL-WW, including organic acids, alcohols, ketones, and various cyclic hydrocarbons, as well as many nitrogen-containing compounds, such as amides, azines, and pyrroles, as previously observed by Pham et al. (2013)[13]. Some of these compounds are easily degradable, such as sugars and organic acids, but most of them have been found to be inhibitory to the anaerobic process, such as phenol and benzene. For instance, the RPA of benzene derivatives is 6.37%. According to Johnson et al. (2003) [35], benzene is considered the most toxic and persistent of all petroleum components. Furthermore, N and O heterocyclic

compounds and straight amide derivatives are accounted for 45.8% and 13.2% of the RPA, respectively (Table 4). These compounds can complicate the biodegradation of HTL-WW. Tommaso et al. (2015) [9] characterized the aqueous products from the HTL of mixed-culture wastewater algae, verifying that the higher the HTL temperatures, the lower the RPAs associated with N and O heterocyclic compounds. Under these conditions, higher cumulative methane productions were observed. However, when the HTL was performed with lower temperatures (260 and 280 °C), lower cumulative methane productions were found with higher values of RPA of N and O heterocyclic compounds. In short, the identified complex organic compounds in HTL-WW could contribute significantly to the overall inhibition for microbes during the anaerobic digestion process [9, 21].

3.2 Anaerobic toxicity assay (ATA) of HTL-WW

An ATA was conducted to understand the potential anaerobic toxicity of HTL-WW. The ATA method was developed by Owen (1979) [36] as a screening method to determine the level at which a specific substrate causes an adverse effect on predominant methanogens when digesting a readily available substrate (acetate or glucose).

The cumulative methane production for each percent inclusion ratio and the feed control are shown in Fig. 1a. The near-linear sections of the curves in Fig. 1a were generally between days two and four, which was the middle stage of biogas production during the ATA tests. Therefore, using the cumulative methane production of days 2-4, percent inhibition (I) was calculated for the inclusion ratio using Equation 1 (Fig. 1b).

Fig. 1 Cumulative methane production in anaerobic toxicity assays (ATA) for HTL-WW (a) and percent inhibition for HTL-WW at each inclusion ratio (b).

As can be seen, methane productions for 2%, 3% and 4% of HTL-WW inclusion ratios were higher than those for the control but were lower when the inclusion ratios were higher than 5% (Fig. 1a). Accordingly, the percent inhibitions for the inclusion ratios of 2%, 3% and 4% (representing 1781, 2671, and 3562 mg/L COD inclusions, respectively) are -237%, -270% and -19%, respectively. The negative values of ATA indicated that the HTL-WW was considered non-toxic at such low inclusion ratios. Thus, if a suitable concentration of HTL-WW is introduced, an enhancement to the methane production can be obtained because it can provide easily degradable components (e.g. acetate) and necessary nutrients (e.g. N and P) for microorganisms [10, 37].

When the volumetric inclusion ratios were higher than 5% (representing 4452 mg/L COD), the HTL-WW showed signs of toxicity. The percent inhibition (Fig. 1b) ranged from 13.7% to 99.1% at inclusion ratios from 5% to 24%. In particular, at inclusion ratios of 6% and 13%, the percent inhibition was approximately 50% and 80%, respectively. A nearly complete inhibition occurred at an inclusion ratio higher than 24%.

3.3 Effect of adsorption and biofilm on methane production enhancement

3.3.1 Total performance of methane production

Although a methane production enhancement could be obtained at a low inclusion ratio of HTL-WW, considering the treatment efficiency of an anaerobic reactor in a practical process, detoxification at high inclusions of HTL-WW is necessary before or during the anaerobic treatment process. Zeolite, GAC and cubic PM, with a high degree of porosity and an extended surface area, were considered beneficial for the adsorption of toxic compounds and for the promotion of biofilm formation. The detoxification

capability and the anaerobic biodegradability of the HTL-WW with the addition of these three materials were compared with those without any addition (i.e., the control). The average initial SCOD in the test bottles was 6940 ± 399 mg/L.

Fig. 2 Cumulative methane production of the two-round anaerobic batch test. (zeolite, with 2 g/L zeolite; GAC, with 2 g/L granular activated carbon; PM, in the presence of 2 g polyurethane matrices).

The values of total methane production of the two-round feeding anaerobic batch test are shown in Fig. 2 and Table 5. The first feeding test, with 6.5 mL of HTL-WW, was incubated for nearly 90 days, and the second feeding, with 13 mL of HTL-WW, was incubated for more than 130 days. The incubation time was much longer than the retention time of high-strength food wastes or manure wastes (approximately 20~30days), indicating the recalcitrant and toxic effect of HTL-WW. The p values of the methane yield (mL/g COD) of the first feeding, second feeding and the total are 0.005, 0.023, and 0.038 (<0.05). So the methane yields are significantly different at the

p level of 0.05. The total methane yields in the presence of zeolite, GAC and PM were 136 mL/g COD, 169 mL/g COD, and 168 mL/g COD, which were 11%, 37% and 36% higher than the control (suspended biomass without any addition), respectively. The relative methane productions compared with the maximum theoretical methane production, according to Speece et al. (1996) [33], were 39%, 48% and 48% for HTL-WW digested with zeolites, GAC and PM, respectively. The corresponding COD removal efficiencies were 31%, 38%, and 43%.

Table 5 Performance of the two-round feeding anaerobic batch test of detoxified HTL-WW generated from the hydrothermal liquefaction of *Spirulina* (300°C with a retention time of 30 minutes) using different adsorbents.

		Methane yield (mL/g COD)	Relative CH ₄ production (%)*	Methane increment relative to control (%)	COD removal efficiency (%)
The 1 st feeding	Control	216± 18	62	/	36
	Zeolite	227 ± 15	65	5	37
	GAC**	245 ± 20	70	13	52
	PM	278 ± 3	79	29	43
The 2 nd feeding	Control	67±23	19	/	23
	Zeolite	81±7	23	21	20
	GAC	124±16	35	85	23
	PM	103±9	30	54	32
1 st +2 nd feeding	Control	123±19	35	/	33
	Zeolite	136±4	39	11	31
	GAC	169±9	48	37	38
	PM	168±6	48	36	43

* Considering the maximum theoretical methane production according to Speece (1996)[33] relative to the initial COD.

** Zeolite, with 2 g/L zeolite; GAC, with 2 g/L granular activated carbon; PM, in the presence of 2 g polyurethane matrices.

Considering the methane yield, better detoxification abilities were observed in the presence of PM and GAC during the entire anaerobic digestion process. Compounds identified in HTL-WW (e.g. alcohols, ketones, and nitrogen-containing compounds), can be adsorbed by PM and activated carbon [8, 13] and therefore favorable conditions

were provided to overcome inhibition. The non-specific adsorption of organic matter with adsorbents (GAC and PM) inside the anaerobic reactors was most likely followed by desorption, which was mediated by the bioconversion that occurred in the reactors. In this way, the adsorbent acted more like a buffer that could temporally store nutrients and slowly release them to the microbes, and therefore contributed to a higher methane yield and higher conversion rates.

In addition to showing adsorbent capability, PM and activated carbon also provided a good environment for the biofilm formation, which resulted in better conditions for inhibition recovery compared to the control. This is one of the major advantages of using the three additions (PM, GAC and zeolites) as co-promoters during the anaerobic process to overcome inhibition instead of only using them as pretreatments. Similar results were observed by Palatsi et al. (2009) [38], who verified that the addition of adsorbents was one of the best strategies to recover anaerobic reactors exposed to long chain fatty acid inhibition.

Zeolite has been widely used as an ion exchanger for the removal of ammonium in high ammonia content wastes due to the presence of Na^+ , Ca^{2+} and Mg^{2+} in its crystalline structure [14]. However, under this condition, zeolites did not show significant advantages for methane production compared to PM and GAC. This result is different from reports in which the anaerobic process was favored by the addition of zeolite [23, 39]. This is most likely due to the low inhibition conditions related to the ammonia concentration in HTL-WW. Although the ammonia concentration of the original HTL-WW reached 10117 mg/L, the ammonia concentration in the first and second round feedings was 658 mg/L and 1315 mg/L, respectively, which were much lower than the inhibitory concentrations, as discussed in Section 3.1. Therefore, zeolite could not demonstrate its capacity to remove ammonium in this case. This result also

suggested that zeolites had a limited contribution to the reduction of toxicity of organic compounds identified in HTL-WW.

3.3.2 Comparison of the adsorption and biofilm effect through inference of the kinetic parameters

The modified Gompertz model has been widely used due to good simulation results and wide applications [26, 40]. This model is frequently used to predict important parameters, such as lag-phase time and maximum methane production rate. The experimental methane productions from the first feedings are presented in Fig. 3a, and the calculated parameters are shown in Table 6. The model could well predict the cumulative methane yield. The results from the Gompertz model confirmed that the presence of GAC and PM was important in the process because the maximal methane production rates (k) were relatively improved in presence of GAC and PM, and the corresponding lag phase time (λ) was reduced compared to the control.

Table 6. Parameters of methane production from the modified Gompertz equation.

Additions	The first feeding			
	P_{CH_4} (mL)	k (mL/d)	λ (d)	R^2
Control	208	2.27	13.8	0.92
Zeolite	163	2.37	10.1	0.85
GAC	171	3.75	9.6	0.98
PM	203	3.26	6.6	0.99

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274 Fig. 3 Respective cumulative methane productions of the two feedings and the modified
 275 Gompertz simulation results of the first feeding. (Zeolite, with 2 g/L zeolite; GAC, with 2 g/L
 276 granular activated carbon; PM, in the presence of 2 g polyurethane matrices).

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At the first feeding of the anaerobic batch test of HTL-WW, the initial volumetric loading of HTL-WW was 6.5 mL, which represented 6.5% of the working volume of the reactor. According to the ATA results, the percent inhibition at an inclusion ratio of 6.5% is approximately 60%, which was not fatal to the anaerobe activities. Under this condition, the control without any adsorbents resulted in a methane yield of 216 mL/g COD, implying that the anaerobic system could overcome inhibition after a period of acclimatization. The inhibition caused by the inclusion of 6.5% of HTL-WW was confirmed by the balance of volatile fatty acids observed at the end of the first run (Table 7). Acetic acid, propionic acid and butyric acid were detected in the HTL-WW. At the end of the first run, all the concentrations of the VFAs were slightly lower, particularly propionic and butyric acids, while it was possible to detect, on average, 76% reduction of the acetic acid concentration. This result indicates that acetoclastic microorganisms were present and active during the digestion process. In all flasks, valeric acid was produced and appeared to accumulate in average concentrations of 1150 mg/L, indicating that the valeric acid conversion may be a rate-limiting step in the anaerobic process.

Table 7. VFA concentrations of the original HTL-WW and at the end of the first feeding.

Conditions	Concentration (mg/L)				
	Acetic acid	Propionic acid	Iso-butyric acid	Butyric acid	Valeric acid
Initial HTL-WW	369.2 ± 107.2	613.9 ± 54.8	nd**	1346.0 ± 204.6	nd
Control	86.4 ± 23.5	614.0 ± 128.6	nd	109.2 ± 93.2	1171.7 ± 24.3
Zeolite*	55.8 ± 8.6	496.6 ± 98.7	64.37 ± 3.59	748.8 ± 233.5	1188.0 ± 17.2
GAC	68.8 ± 119.2	423.8 ± 13.3	nd	418.4 ± 49.0	1085.0 ± 47.6
PM	149.0 ± 69.8	490.78 ± 64.1	nd	688.01 ± 167.8	1261.8 ± 107.7

* Zeolite, with 2 g/L zeolite; GAC, with 2 g/L granular activated carbon; PM, in the presence of 2 g polyurethane matrices.

** nd = Not detected

After the acclimation during the first feeding, anaerobic microorganisms were expected to adapt to the highly toxic HTL-WW, and biofilms formed to some extent. A second feeding of each reactor with 13 mL of HTL-WW was performed to investigate the effect of detoxification and the response of the anaerobic microorganisms under stress conditions. Equation 1 did not adhere to the accumulated methane production obtained at the second feeding, so its parameters could not be inferred (Fig. 3b). The higher toxic effect of HTL-WW made the process slower, and the biogas was still being produced. Additionally, a diauxic tendency was observed in the presence of PM and zeolites and in the control at the second feeding, implying that two classes of substrates may have been present: easily degradable substrates, such as short chain fatty acids from the first feeding, and recalcitrant compounds from the HTL-WW, provided in the second feeding. In the presence of GAC, however, it was possible to observe a nearly constant rate of methane production. According to the ATA tests, at an HTL-WW concentration of 13%, the percent inhibition potential achieved 80%, which could be fatal to the anaerobes (Fig. 1). As indicated by Fig. 3 and Table 5, all the methane productions of the second feeding were lower than those found in the first feeding. The methane yields from the control and in the presence of zeolite, GAC and PM were 67, 81, 124, and 103 mL/g COD at the second feeding, respectively, which were much lower than the corresponding values observed in the first feeding. Nevertheless, because of the detoxification effect, in the second feeding, the methane yields in the presence of zeolite, GAC and PM were 21%, 85% and 54% higher than the corresponding control, respectively. These increments are all significantly higher than the 5%, 13% and 29% values observed in the first feeding, with zeolite, GAC and PM, respectively. This result also indicates that the adsorbents could behave more effectively under more stressful conditions.

PM appeared to be an excellent colonization matrix for anaerobe growth and retention to enhance the methane production under low or moderate toxic conditions (e.g., the first feeding of 6.5 mL of HTL-WW). However, this was not the case under higher toxic conditions. The methane yield of the second feeding in the presence of PM was 103 mL/g COD, which was lower than that observed in the presence of GAC (124 mL/g COD). GAC presented a better ability than PM to overcome inhibition through adsorption of the toxic compounds. GAC may have served as a buffer through which the initial adsorption process contributed to a decrease in the toxic level, and then the adsorbed organics may have desorbed due to the concentration gradient between the GAC surface and bulk liquid caused by microbial activities. Consequently, the organic compounds were digested. This process is called the bioregeneration of active carbon [41], which provides an attachment surface for biofilm colonization and protects the microorganisms from shock loadings of toxic and inhibitory materials, whereas microorganisms bioregenerate the activated carbon. This function of GAC resulted in its better performance for methane production compared with the treatment with PM at the second feeding., especially during the digestion time from day 60 to day 110 (Fig. 3) . In addition, the bioregeneration process would be economically beneficial for decreasing the requirement for periodic replacement of virgin GAC. Therefore, GAC is more favorable in conjunction with the anaerobic process for the treatment of HTL-WW.

3.3.3 Physicochemical characterization of HTL-WW digestate

The anaerobic digestion of HTL-WW not only recovers energy but also produces a nutrient-rich digestate containing nitrogen and phosphorus. The physicochemical characteristics of the digestate collected during the anaerobic digestion of the two-round

feedings are shown in Fig. 4. The pH of all the digestates was between 7.6 and 7.9.
 Because of the low COD removal efficiency of the second feeding, the COD of all
 digestates was high. However, all the concentrations of NH_4^+ -N and TN (total nitrogen)
 were below 4000 mg/L, which demonstrated that ammonia is not inhibitory during the
 anaerobic digestion process of HTL-WW. The concentration of TP ranged from 438 to
 513 mg/L. Nutrients are a large and expensive input for the mass production of
 microalgal biomass. Ward et al. (2014) [12] reported that the nitrogen to phosphorous
 ratio in the range of 10 to 17:1 is ideal for the cultivation of algal species. The ratio of
 nitrogen to phosphorus is 5 to 8:1 in this case. Therefore, the addition of relatively small
 amounts of nitrogen can easily make the digestate suitable to provide nutrients for the
 algal cultivation. This finding highlights the role of anaerobic digestion in the recycling
 of nutrients that are vital to the sustainability and economic feasibility of commercial-
 scale algal biofuel industries.

Fig. 4 Physicochemical characteristics of HTL-WW digestates detoxified by different adsorbents during anaerobic digestion. (Zeolite, with 2 g/L zeolite; GAC, with 2 g/L granular activated carbon; PM, in the presence of 2 g polyurethane matrices)

As discussed in Section 3.1, more than 100 compounds were identified in HTL-WW using the GC-MS method. However, after anaerobic digestion, the identified compounds sharply decreased, as shown in Fig. 5. The digestates with or without adsorbents shared nearly identical GC-MS spectra profiles. Complex compounds were degraded into smaller ones during the anaerobic process. Therefore, a new compound was formed during the biodegradation (peak 1, Fig. 5), and it was identified as 1, 1-dicyanoethane. Peak 2 was identified as valeric acid, which may be indicative that its conversion is a rate-limiting step in the anaerobic process of the treatment of HTL-WW. Peak 3 was identified as benzeneacetic acid, which also existed in the original HTL-WW.

Fig. 5 GC-MS spectra of original HTL-WW and digestate detoxified by different adsorbents. (Zeolite-DIGE, digestate with 2 g/L zeolite; PM, digestate in the presence of 2 g polyurethane matrices; GAC, digestate with 2 g/L granular activated carbon).

However, it is important to highlight that most of the peaks observed in the HTL-WW disappeared after anaerobic digestion, such as peaks 4 and 5 in Fig. 5, which were the two most significant peaks and were identified as methyl-pyrazine and glycerin, respectively. Glycerol is a carbon-rich by-product of the trans-esterification conversion of lipids to bio-oil, and it is an excellent carbon source to maximize gas production in anaerobic digestion. These results indicated that most compounds were adsorbed or degraded by anaerobes.

Bacterial consortia can degrade organic pollutants that algae cannot utilize because only some algal species can conduct mixotrophic or heterotrophic metabolism. Furthermore, the range of organics that algae can digest is much narrower than that of heterotrophic bacteria [7]. Organic compounds, such as phenol and benzene, identified in the HTL-WW are known to be toxic to algal growth, but these compounds were mitigated during the anaerobic digestion process (i.e., those compounds were not detected using the reported GC-MS method). Anaerobic treatment was demonstrated as an effective step to remove the recalcitrant compounds identified in HTL-WW for the down-stream algal cultivation. Further investigation and optimization of reactor configuration design and operation are still required to provide higher carbon recovery and higher process rates, as well as to optimize system costs.

4. Conclusion

Anaerobic digestion is a promising method to recover residual energy remaining in the HTL-WW. The ATA results showed that inclusions of HTL-WW higher than 5% presented high toxicity to anaerobes. In the presence of PM, GAC and zeolites, the total methane yields were 10%, 38% and 37% higher than the control, respectively. GAC and PM greatly improved the methane yields and the maximal methane production rates, while zeolites did not show significant advantages. Considering the consistently superior performance at the two feedings, GAC is recommended as the preferred adsorbent for the pretreatment of HTL-WW as the substrate in methane production.

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Graphical Abstract

Environment-Enhancing Energy (E2-Energy) system