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Comparing Nutrient Recovery via Rapid (Flash Hydrolysis) and Conventional Hydrothermal Liquefaction Processes for Microalgae Cultivation

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COMPARING NUTRIENT RECOVERY VIA RAPID (FLASH HYDROLYSIS) AND CONVENTIONAL HYDROTHERMAL LIQUEFACTION PROCESSES FOR MICROALGAE CULTIVATION

by

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ABSTRACT

COMPARING NUTRIENT RECOVERY VIA RAPID (FLASH HYDROLYSIS) AND CONVENTIONAL HYDROTHERMAL LIQUEFACTION PROCESSES FOR MICROALGAE CULTIVATION

Caleb Talbot
Old Dominion University, 2015
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Algal biofuels have the potential to provide a scalable source of renewable fuels in the near future. The high nutrient use in algae cultivation and its recovery and recycling is one of the challenges that may limit the scalability and sustainability of algal biofuels. The present study evaluates the use of Hydrolysate obtained after Flash Hydrolysis (FH) of *Scenedesmus* at 280^{o}C as a nutrient source for microalgae cultivation. FH Hydrolysate nutrient recycling was compared with low temperature batch Hydrothermal Liquefaction (HTL) nutrient recycling. *Oocystis* and *Scenedesmus* were cultivated using Hydrolysate as a partial phosphorous (P) and nitrogen (N) source. The study shows that 50% of the phosphorus required in the culture media could be replaced with Hydrolysate from FH; also, 50% nitrogen was provided from the same source. Ammonia toxicity is one limitation for the higher percentages of N replacement in this study. The HTL aqueous phase had a near continuous high soluble ammonia concentration in the media. The effect was significant for the 50% P HTL replacement treatment where almost no algae growth was observed in the first 11 days of the experiment.

This thesis is dedicated to my family, without whom this would never have been possible, and the future, may it be brighter than the past.

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NOMENCLATURE

3N-BB	A type of microalgae culture media
AM-14	Culture media prepared at ODU-CEE
BG-11	A type of microalgae culture media
COMBO	A type of microalgae culture media
DI	Deionized
DOE	Department of Energy
DW	Dry weight
EPA	Environmental Protection Agency
FD	Freeze dried
FH	Flash Hydrolysis
HTL	Hydrothermal liquefaction
R-HTL	Rapid Hydrothermal liquefaction
AP	Aqueous Phase
IC	Ion Chromatography
MPa	Mega Pascal
Psi	Pressure per Square Inch
NREL	National Renewable Energy Laboratory
ODU-CEE	Old Dominion University Civil and Environmental Engineering
PBR	Photobioreactor
SD	Standard deviation
TN	Total Nitrogen
TOC	Total Organic Carbon
TC	Total Carbon
TSS	Total Suspended Solids
VCERC	Virginia Coastal Energy Research Consortium

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CHAPTER 1

INTRODUCTION

Microalgae comprise a mixture of organic and inorganic compounds; at elemental scale the major components are carbon, hydrogen, oxygen, nitrogen, phosphorus, sulfur, potassium, sodium and other elements present in minor quantities. From a biofuels perspective, the carbon and hydrogen content are the most important since they will provide most of the energy yield. From an environmental and scalability point of view other elements, particularly nitrogen and phosphorus, are very important. They are even more so if they could potentially be recovered and recycled in biologically available forms [1]. Nitrogen is biologically available in the form of ammonium (NH_4^+) and nitrate (NO_3^-). Phosphorus is biologically available in the form of phosphate (PO_4^{3-}) [2-4]. Most nitrogen containing fertilizers are produced by reacting atmospheric nitrogen in a process that demands significant amounts of fossil fuels (mainly natural gas as CH_4). The Haber–Bosch process first produces anhydrous ammonia (NH_3) that is then further upgraded [3, 5]. Phosphorus is a mined resource and cannot be substituted. Phosphorous is also a fundamental component in all living organisms and is thus a crucial element in the production of biofuels as well as the food chain [6, 7].

One of the first studies of nutrient recycling after microalgae biomass processing used *Chlorella vulgaris* grown in the recovered solution after low temperature catalytic gasification of itself, a process by which methane rich fuel gas was obtained. The study concluded that *C. vulgaris* could grow in the recovered solution, but mixing with a standard media to provide nutrients such as phosphorus and micronutrients was necessary [8]. Biller et al. reported in a recent study the evaluation of the potential use of recycled aqueous phase (AP) from the Hydrothermal Liquefaction (HTL) process at two different temperatures, 300 and 350 °C, to

grow four different algae strains (*Chlorella vulgaris*, *Scenedesmus dimorphus*, *Spirulina platensis* and the cyanobacteria *Chlorogloeopsis fritschii*). The AP was diluted 50x, 100x and 400x due to the high concentration of nutrients in it compared to the standard media used to grow the algae strains and to avoid the effects of growth inhibitors such as phenols, fatty acids and nickel (from the reactor wall corrosion). All the studied algae strains were able to grow in the recycled water, but different optimum dilutions were observed for each of them. [9].

The cultivation of *Desmodesmus sp*. using AP recovered after HTL of the same microalgae biomass was studied by Garcia-Alba et al. [10] to evaluate the potential of nutrient recycling. HTL experiments were conducted in a batch reactor at 300°C with 5 minutes reaction time (excluding pre-heating time). The study concluded that when using only AP recovered after HTL diluted with deionized water a substantial reduction in growth was observed. When a mixture of growth media (COMBO), DI water and AP was used, a comparable growth rate as that observed in COMBO medium was reported. The most likely cause for this difference was again the lack of essential micronutrients that must be supplied in order to balance the media and optimize the nutrient recycling after HTL [10]. Further studies by Garcia-Alba et al. indicated that the nutrients could be recycled up to five consecutive times using HTL AP [7]. This allowed for recycling of 65.7% of the phosphorous and 39.5% of the nitrogen present in the COMBO medium. Unfortunately, the cell morphology started to change by the fifth recycle. The authors pointed out that aromatic heterocycles were present in the HTL AP and the cell morphology mirrored a study testing the impact of triazines on *Scenedesmus sp.* [11]. The authors also pointed out that the amounts of potential inhibitory compounds (pyrrolidones, phenols, cyclic dipeptides) were increasing with each successive recycle and that supercritical water gasification

of the HTL AP before recycle may be necessary to reduce these compounds to ammonia and CO_2 [7].

Lopez Barreiro et al. recently reported that supercritical water gasification of HTL AP greatly reduces the amount of organic nitrogen to ammonia/ammonium. They also found that up to 75% of the nutrients (using a nitrogen based calculation) present in COMBO and Guillards f/2 media can successfully be recycled depending on algae species [12]. The authors further varied the pH of the COMBO media by adjusting the pH to 7.8 as recommended and by not doing so leaving the media pH at 9.0. The authors observed that the decline in growth for the tested high pH treatments was most likely due to ammonium shifting toward ammonia in equilibrium at levels toxic to the algae. Interestingly, for *Scenedesmus almeriensis*, pH adjustment and supercritical water gasification of the HTL AP did not encourage the algae to grow as well as the COMBO media. It was also reported that most treatments ran out of total nitrogen (TN) before the 14 day experimental period was over, suggesting that a more nutrient replete medium should be developed and used to allow for longer cultivation periods [12].

Rapid Hydrothermal Liquefaction (R-HTL) of microalgae biomass is a promising subcritical water-based continuous flow conversion and extraction method capable of solubilizing more than 60% of the protein present in the biomass and recovering it in the Hydrolysate as organic nitrogen (mix of ammonia, amino acids and soluble peptides) [13, 14]. In a similar way, more than 90% of the organic phosphorus from the microalgae biomass is recovered as soluble orthophosphates along with most of the micronutrients. The short (9 s) residence time of the R-HTL process allows for minimum formation of toxic compounds such as aromatic heterocycles that could potentially impact algae growth [14, 15]. Most of the organic nitrogen present is in the form of soluble peptides and amino acids with small amounts of

ammonia. If the algae cannot use the organic nitrogen directly, it should degrade to ammonia over time. This might especially happen in high pH conditions that should be present during algae photosynthesis. This would potentially allow for the slow release of ammonia to the algae [16-19]. This slow release process would potentially allow for a continuous low level presence of ammonia that would be beneficial to the algae instead of harmful if controlled [20, 21]. From here on, R-HTL will be referred to as Flash hydrolysis (FH) due to the use of the name in prior publications [13, 14].

The objective of this study was to evaluate the potential use of the Hydrolysate obtained after FH as a source of nutrients for a sustainable microalgae production system. The recycling of nitrogen and phosphorus back into the algae culture in theory reduces the requirements of those nutrients in the balanced cultivation media, therefore resulting in a reduction in the total production cost of algal biomass. In order to compare FH Hydrolysate's potential use in nutrient recycling with the established use of HTL AP; batch HTL experiments were conducted using a relatively standard reported residence time (30 minutes) and the same temperature used for FH experiments (280°C). The robustness of the nutrient recycle process was further compared across the different genera: *Scenedesmus* and *Oocystis*.

CHAPTER 2

MATERIALS AND METHODS

2.1 Feedstock Obtainment

Scenedesmus was cultivated in a one acre (4046 m^2) raceway open pond system established near Spring Grove, Virginia (37.1657 N, 76.9733 W). The produced biomass was collected, stored at -4 ^oC, freeze dried, and stored at -4 ^oC until use in preparation of FH Hydrolysate and HTL AP.

2.2 Preparation of FH Hydrolysate and HTL AP

Flash hydrolysis experiments on the algae *Scenedesmus* were conducted in a continuous flow subcritical water reactor as described before in previous publications [13, 14]. The reactor is briefly described again. Using two separate high pressure, high precision pumping systems, algae slurry (7-8% solids) is fed by a pre-loaded stainless steel piston (80 mL) into a mixing chamber where it is mixed with preheated DI water before entering the reactor (3 mL). The final solids loading rate was approximately 1.0% for each run [13]. The reactor is a stainless steel tube (47.5 cm in length, 2.8 mm inner diameter, and 6.4 mm outer diameter) placed in a horizontal furnace with precise temperature control. The temperature is monitored using two thermocouples to monitor the preheated water temperature and the reaction temperature. The reacted slurry is then cooled in a water bath before passing through a back pressure regulator to be collected for analysis. The conditions of reactor temperature, pressure, and residence time were 280°C, 2100 psi (14.5 MPa), and 9 s respectively as these had previously been described as the optimum conditions for nitrogen extraction from the algae biomass [13]. The FH Hydrolysate

was collected and stored at 4°C until analyzed and used. Appendix A.1 details this process by individual study.

HTL experiments on the algae *Scenedesmus* were conducted using a 500 mL batch reactor equipped with a Parr 4848 controller. The reactor was set at 280°C for 30 minutes (plus preheating time of approximately 40 minutes); the pressure was measured to be 1500 psi (10.3 MPa). The experimental temperature was chosen to be similar to that of FH experiments. The reactor was loaded with 400g of slurry with 8.7% solids (prepared by mixing 40g of freeze dried algae and 400mL of water). The methodology for the separation and characterization of the HTL AP is included in Appendix A.1. The experiment was conducted in duplicates and the final AP product from both batches was mixed and used as a nutrient source for the experiments. Appendix A.1 details this process by individual study.

2.3 Preparation of AM-14 Control Media and Inoculating Cultures

A new culture media was prepared to grow algae in the authors' lab having balanced macro and micronutrients to sustain algae growth. The new media (Algae Media 2014 or AM-14) was prepared so it will have slightly more nitrogen and phosphorous than BG-11 growth medium in a 10:1 mass ratio. The final concentration in the media is 300 mg/L N (from $NaNO_3$) and 30 mg/L P (from K_3PO_4) [21, 22]. Vitamins were not included since Croft et al. found that they are supplied in the natural environment by a symbiotic relationship with bacteria [23]. The new media has a final pH of 10.5, is not sterilized to reflect open pond conditions and its composition is detailed in Table 1. The formulation of the media along with the isolation and adjustment of the *Oocystis sp.* and *Scenedesmus* cultures to the new media is detailed in Appendix A.2.

Table 1. Algae culture media composition (AM-14).

Chemical compound	mg/L
$NaNO_3$	1820
K_3PO_4	206
$CaCl_2$	19
$MgSO_4$*7 H_2O	75
NaCl	25
Na_2EDTA	0.75
$FeCl_3$	0.059
$MnCl_2$*4 H_2O	0.041
$ZnCl_2$	0.005
$CoCl_2$*6 H_2O	0.0037
Na_2MoO_4*2 H_2O	0.0053
$CuCl_2$*2 H_2O	0.0025
H_3BO_3	0.168

2.4 Control Experiment Using FH Hydrolysate: No Algae

To verify the nutrient uptake by the microalgae and the evolution of the nitrogen species from the Hydrolysate nutrient supplementation two control experiments were conducted under similar conditions as the algae growing experiment. First, eight 3L bottles were set up on an insulated tabletop for AM-14 Control, FH 10% P, FH 20%, and FH 50% P treatments in duplicate. The temperature was maintained between 24-30°C and a 12 hour light/dark cycle was set for the duration of the experiment by means of artificial light (SUN-904302 ft. Fluorescent Grow Light Fixture full spectrum 6500K). Permanent aeration was also provided using air stones inside each bottle to keep the algae in suspension. For the second experiment, six 2L bottles were placed in the same incubator to replicate the 3 different nutrient replacement levels of 10% P, 20% P, and 50% P in duplicate. For both experiments, no algae were inoculated. Every day a 10mL sample was taken from each bottle, filtered (in glass fiber discs 1.4 micron pore size Whatman 934-AH 47 mm), and preserved at 4°C until analyzed. Please note that this was to

obtain a trend of the species evolution, and more proper storage techniques are required to obtain proper values for other purposes (e.g. kinetics). The parameters measured in the liquid samples were soluble peptides (Lowry's method) and ammonia (HACH DR 2800 spectrophotometer). Tables 2 – 4 detail the preparation of each bottle for the first control experiment, the concentration of major components derived from the FH Hydrolysate, as well as the initial concentration of nitrogen and phosphorous in the media along with the %P and %N replaced in the media respectively. Tables 17 – 19, listed in Appendix B, detail the preparation of each bottle for the second control experiment, the concentration of major components derived from the FH Hydrolysate, as well as the initial concentration of nitrogen and phosphorous in the media along with the %P and %N replaced in the media respectively.

2.5 Cultivation Using FH Hydrolysate and HTL AP

For the *Scenedesmus* cultivation using FH Hydrolysate and HTL AP study, the evaluated nutrient replacement levels using FH Hydrolysate were established at 4%, 8% and 25%; using HTL AP, they were established at 8% and 25% of the total phosphorus provided by the AM-14 media.The amount of nitrogen supplemented by the Hydrolysate and AP was calculated so it could be balanced with the amount provided by the AM-14 media in each experiment. Table 5 shows the formulation used for the five different nutrient replacement levels.

The algae were cultivated for 22 days in 3L plastic bottles placed on an insulated table top where the temperature was maintained between 24-30°C, a 12 hour light/dark cycle was set for duration of the experiment by means of artificial light (SUN-904302 ft. Fluorescent Grow Light Fixture full spectrum 6500K), and permanent aeration was provided using air stones inside each bottle to keep the algae in suspension.

Table 2. Formulation for the first control experiment of *No Algae* using FH Hydrolysate at three different levels of nutrient replacement.

Formulations for Nutrient Replacement with Flash Hydrolyzate (FH) First Control Experiment (*No Algae*)

	Control	10% P Replacement (mL)	20% P Replacement (mL)	50% P Replacement (mL)
Part A	20	18.05	16.10	10.50
Part B	2.00	1.80	1.60	1.00
Part C	10.00	10.00	10.00	10.00
Part D	1.00	1.00	1.00	1.00
Hydrolyzate	0	45	90	220
Water	970	925	880	760
Total Volume	**1000**	**1000**	**1000**	**1000**

Table 3. Concentration of major components derived from the FH Hydrolysate in the first control experiment of *No Algae.*

Flash Hydrolyzate (FH) First Control Experiment
Concentration Derived From Effluent Replacement
No Algae

Solution	NH_3 (mg/L)	TPO_4 (mg/L PO_4)	TP (mg/L P)	Soluble Peptides (mg/L)	TN (mg/L N)	pH	TOC (mg/L)	Phenols (mg/L)	mL FH	Dilution Factor (DF)
FH	43.4	205	67	4982	650	5.3	3350	-	-	-
Control	-	-	-	-	-	-	-	-	0	-
FH 10% P	2.0	9.2	3.0	224.2	29	-	151	-	45	22
FH 20% P	3.9	18.5	6.0	448.4	59	-	302	-	90	11
FH 50% P	9.5	45.2	14.7	1096	143	-	737	-	220	5

Eighteen 3 L plastic bottles were used in order to have six different treatments: control (only AM-14 media), FH 4% P replacement, FH 8% P replacement, FH 25% P replacement, HTL 8% P replacement, and HTL 25% P replacement. Each treatment was set in triplicates, and the bottles were placed randomly on the tabletop between the lights. Table 6 details the concentration of major components derived from the HTL AP and FH Hydrolysate, and Table 7

details the initial concentration of nitrogen and phosphorous in the media along with the %P and %N replaced in the media.

Table 4. Initial concentration of nitrogen and phosphorous in the first control experiment of *No Algae.*

Flash Hydrolyzate (FH) First Control Experiment
Initial Concentration In Media
No Algae

Solution	TN (mg/L N)	TN From Hydrolyzate (mg/L N)	TP (mg/L P)	TP From Hydrolyzate (mg/L P)	%P Replacement	%N Replacement
Control	300	-	30.0	-	0	0
FH 10% P	300	29	30.0	3.0	10%	10%
FH 20% P	300	59	30.0	6.0	20%	20%
FH 50% P	300	143	30.0	14.7	49%	48%

Table 5. Formulation for *Scenedesmus* cultivation using HTL AP and FH Hydrolysate at five different levels of nutrient replacement.

Formulation for Nutrient Replacement
Hydro-Thermal Liquefaction (HTL) vs. Flash Hydrolyzate (FH)
Scenedesmus

	Control AM-14 (mL)	4% P Replacement FH (mL)	8% P Replacement FH (mL)	25% P Replacement FH (mL)	8% P Replacement HTL (mL)	25% P Replacement HTL (mL)
Inoculum	300	300	300	300	300	300
Part A	50	48.5	47	42	46.5	41
Part B	50	45	40	25	40	25
Part C	25	25	25	25	25	25
Part D	2.5	2.5	2.5	2.5	2.5	2.5
Hydrolysate	0	66.5	133	333	21	51.5
Water	2073	2013	1953	1774	2066	2055
Total Volume	2500	2500	2500	2500	2500	2500

Table 6. Concentration of major components derived from the HTL AP and FH Hydrolysate in *Scenedesmus* cultures.

Hydro-Thermal Liquefaction (HTL) vs. Flash Hydrolysate (FH)
Concentration Derived From Effluent Replacement
Scenedesmus

Solution	NH_3 (mg/L)	TPO_4 (mg/L PO_4)	TP (mg/L P)	Soluble Peptides (mg/L)	TN (mg/L N)	pH	TOC (mg/L)	Phenols (mg/L)	mL FH	mL HTL	Dilution Factor (DF)
FH	55	113	36.9	2570	355	5.8	1910	-	-	-	-
HTL	668	729	238	9140	2620	7.4	9150	-	-	-	-
Control	-	-	-	-	-	-	-	-	-	-	-
FH 4% P	1.5	3.0	1.0	68	9	-	51	-	66.5	-	37.6
FH 8% P	2.9	6.0	2.0	137	19	-	102	-	133	-	18.8
FH 25% P	7.3	15.1	4.9	342	47	-	254	-	333	-	7.51
HTL 8% P	5.6	6.1	2.0	77	22	-	77	-	-	21.0	119.0
HTL 25% P	13.8	15.0	4.9	188	54	-	188	-	-	51.5	48.5

Every day TSS was measured as an algae growth indicator (filtration was conducted with glass fiber discs 1.4 micron pore size Whatman 934-AH 47 mm). Temperature and pH (Extech PH220-C pH Meter) were also measured every day during hour 9 of the light period. Every two days a 10 mL sample (after filtration) was collected and preserved at 4°C until analyzed. The parameters that were measured in the liquid samples were: Soluble peptides (Lowry's method), Nitrate (Dionex ICS-5000), Phosphate (Dionex ICS-5000), Sulfate (Dionex ICS-5000), Nitrite (Dionex ICS-5000), Ammonia (HACH DR 2800 spectrophotometer), and Total Nitrogen (Shimatzu TOC/TN model TOC V-csn). Figure 1 shows pictures taken during the *Scenedesmus* experiment.

After finishing the experiment all the bottles used were harvested by centrifuging (Beckman Coulter Avanti Centrifuge J-26 XP). The collected biomass was freeze dried and stored at -4°C until analyzed. All the samples were analyzed for elemental composition using a Thermo Finnigan Flash 1112 Elemental Analyzer with a nicotinamide standard for calibration. Approximately 1-2 mg of each solid sample was placed in a 3.3 x 5mm tin capsule for

combustion. The method used for analysis was a furnace at 900°C, oven at 75°C, and carrier gas helium at 91 mL/min. The cultivation of *Oocystis sp.* using FH Hydrolysate and HTL AP was similar to the conditions stated above and is included in full in Appendices A.3 and A.4.

Table 7. Initial concentration of nitrogen and phosphorous in the *Scenedesmus* cultures.

Hydro-Thermal Liquefaction (HTL) vs. Flash Hydrolysate (FH)
Initial Concentration In Media
Scenedesmus

Solution	TN (mg/L N)	TN From Hydrolysate (mg/L N)	TP (mg/L P)	TP From Hydrolysate (mg/L P)	%P Replacement	%N Replacement
Control	300	-	30.0	-	0	0
FH 4% P	300	9	28.0	1.0	4%	3%
FH 8% P	300	19	26.0	2.0	8%	6%
FH 25% P	300	47	19.9	4.9	25%	16%
HTL 8% P	300	22	26.0	2.0	8%	7%
HTL 25% P	300	54	19.9	4.9	25%	18%

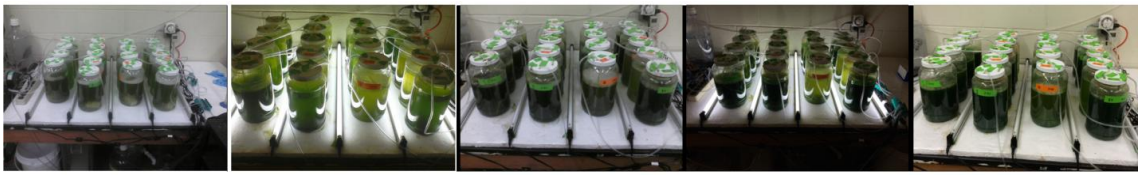

Figure 1. Pictures of different growth stages (Days 0, 5, 10, 15, and 19 from left) during *Scenedesmus* experiment.

CHAPTER 3

RESULTS

3.1 Blank Experiment Using FH Hydrolysate: No Algae

To observe the change in soluble peptides and other nitrogen species in the media, a blank experiment was set up and monitored. Figures 2 – 3 show the change in concentration of soluble peptides and ammonia over time. In all of the following graphs the positive and negative error bars each represent one standard deviation of a sample.

A reduction in soluble peptides concentration was observed along with an increase in ammonia concentration, which then started to decline even though no algae were present. This observation potentially provides evidence that the conversion of soluble peptides (an organic form of nitrogen) to ammonia is occurring under the studied culture conditions of a non-sterile high pH medium.

The soluble peptides concentration shows a similar trend (2) in all the treatments. It started at high values and started decreasing from Day 0 (first sample) until it reached an almost constant concentration, presumably because the conversion to ammonia reached a maximum level. It is a known phenomenon that organic matters are converted into inorganic compounds such as NH_3 or NH_4^+ and CO_2 through the mineralization process. The resultant ammonium can then be nitrified by bacteria if it is not taken up by the algae [18, 24]. The change in concentration of soluble peptides shown in Figure 2 coincides with the observed change in ammoniacal nitrogen concentration in Figure 3. This is a potential cause for concern since an excessive amount of free ammonia in the media may inhibit the normal development of the algae culture [20, 25, 26].

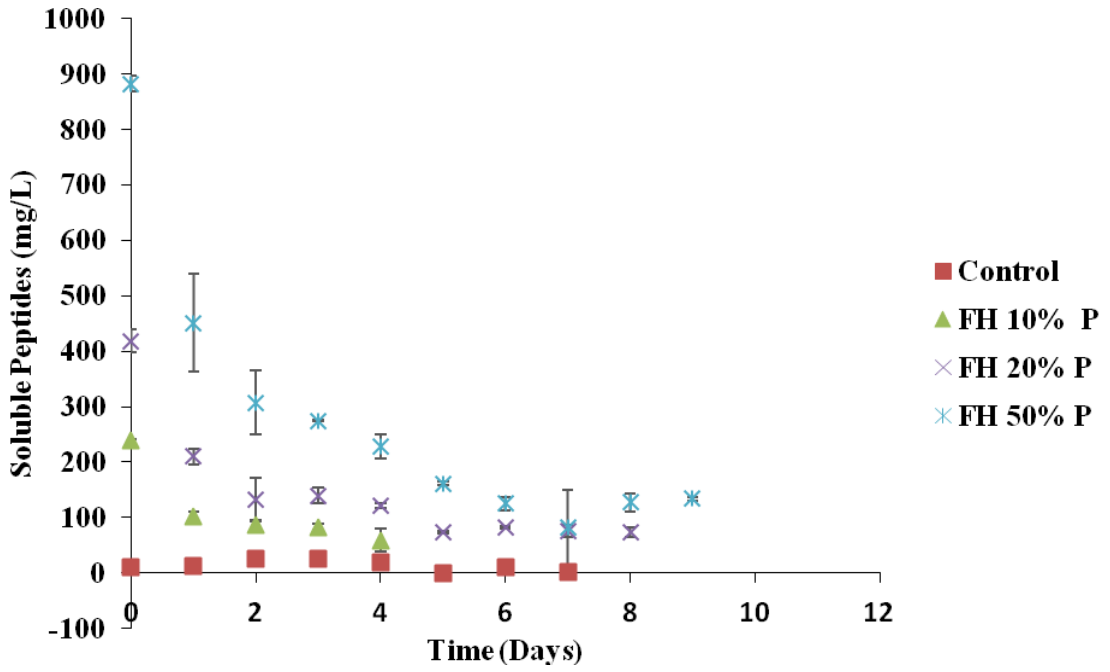

Figure 2. Change in concentration of soluble peptides over time for the Control Experiment using FH Hydrolysate: *No Algae*.

Below the inhibitory level most of the ammonia could be used as the primary nitrogen source to support algae growth [8, 10]. The inhibitory level is reported by Azoz et al. to be relatively constant for the NH_3 concentration but in practical terms is a function of pH and temperature since NH_3 concentration is a function of these parameters. The inhibitory level reported by Azoz et al. is the following: 1.2 mM NH_3 led to 50% reduction in photoassimilation of ^{14}C for the three studied species including *Scenedesmus obliquus* [20].

Transformations between different forms of nitrogen in solution are influenced by environmental conditions like light, pH, and dissolved oxygen among others. In any case, ammonia is the principal nitrogenous byproduct of organic decomposition [17, 27-29]. This process may explain what is observed in Figure 3 where the three different treatments showed an

initial increase in soluble ammonia concentration in proportions that coincide with the amount of Hydrolysate used to replace the available nutrients in the media.

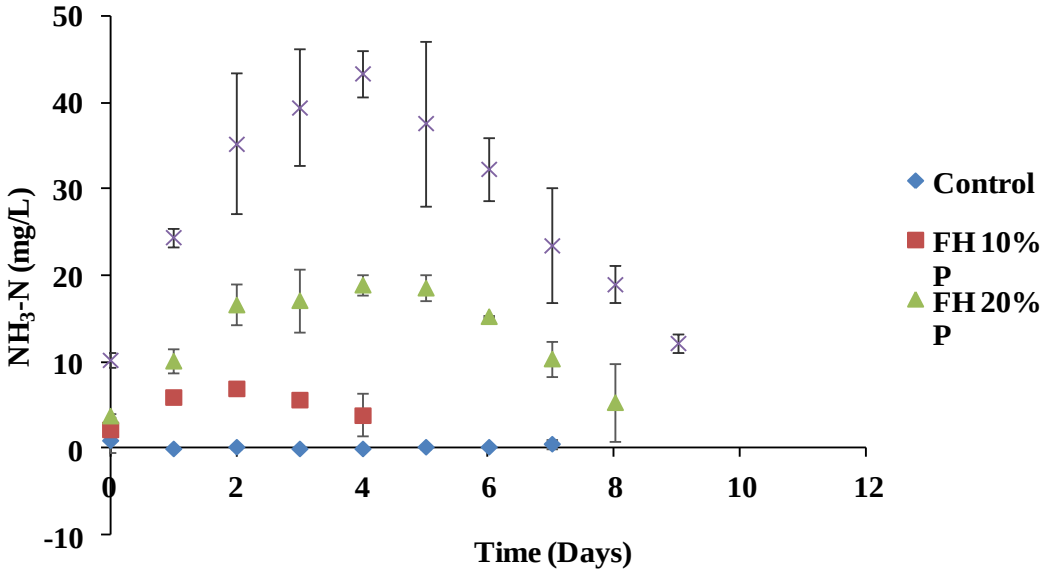

Figure 3. Change in concentration of ammonia over time for the Control Experiment using FH Hydrolysate: *No Algae*.

3.2: Scenedesmus Cultivation Using FH Hydrolysate and HTL AP

The most important parameters that affect algal growth are nutrient quantity and quality, light, pH, turbulence, salinity and temperature. The influence of these factors could be interdependent, and a parameter that is optimal for one specific set of conditions may not automatically be optimal for another [30]. Using HTL AP as a nutrient source to partially replace the phosphate originally provided by the culture media (AM-14) showed significant differences in the growth rates compared to those observed for the FH Hydrolysate and control treatments. Figure 4 shows the algae concentration over time. As can be seen in Figure 4, there is almost no lag phase for any of the FH Hydrolysate treatments or the control since the algae used as inoculum for this study were already acclimated to the new AM-14 media. With that

said, the FH 8%P and FH 25%P treatment did experience a slight lag phase. Interestingly, there was no significant difference in growth between the control and FH Hydrolysate treatments. All bottles started with a 150mg/L TSS concentration. The HTL AP treatments had very little growth throughout the course of the experiment.

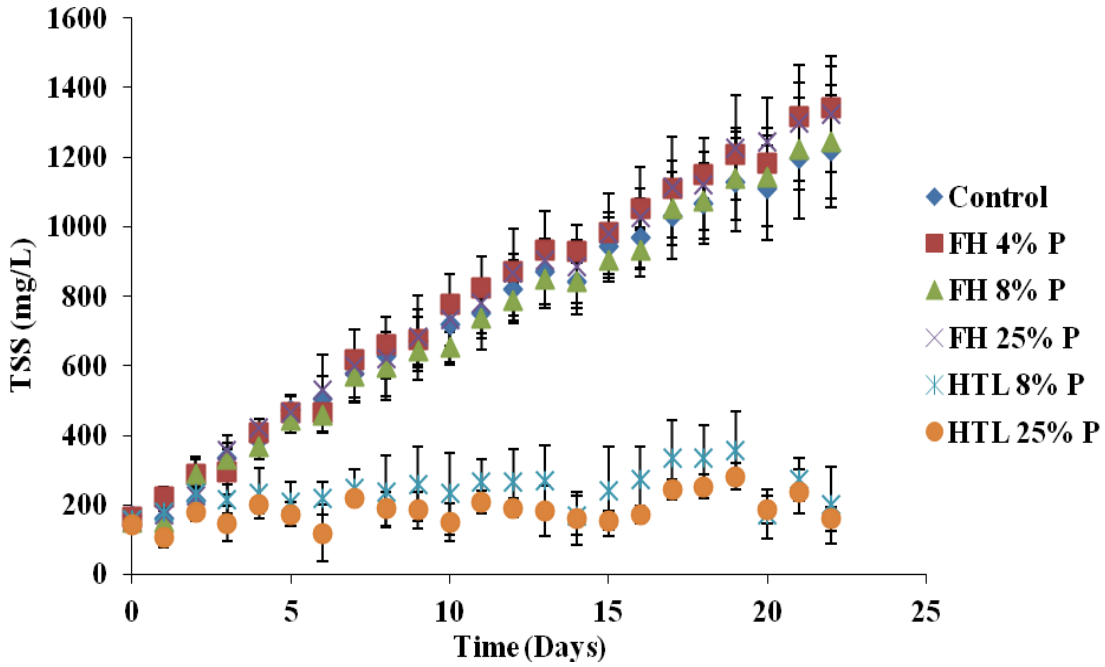

Figure 4. TSS values (average for each treatment) for the *Scenedesmus* cultivation using FH Hydrolysate and HTL AP study.

The nitrate data in Figure 5 agrees with growth data where nitrate was taken up by the control and FH treatments but not the HTL treatments. Figure 6 shows the inoculum media were replete with phosphate, indicating the algae were not phosphorous starved. The control and FH algae showed a rapid initial phosphate uptake followed by relatively steady levels. This has been previously observed for *Scenedesmus* [22, 31]. The phosphate levels for the HTL treatments fluctuated slightly, but no overall uptake was observed.

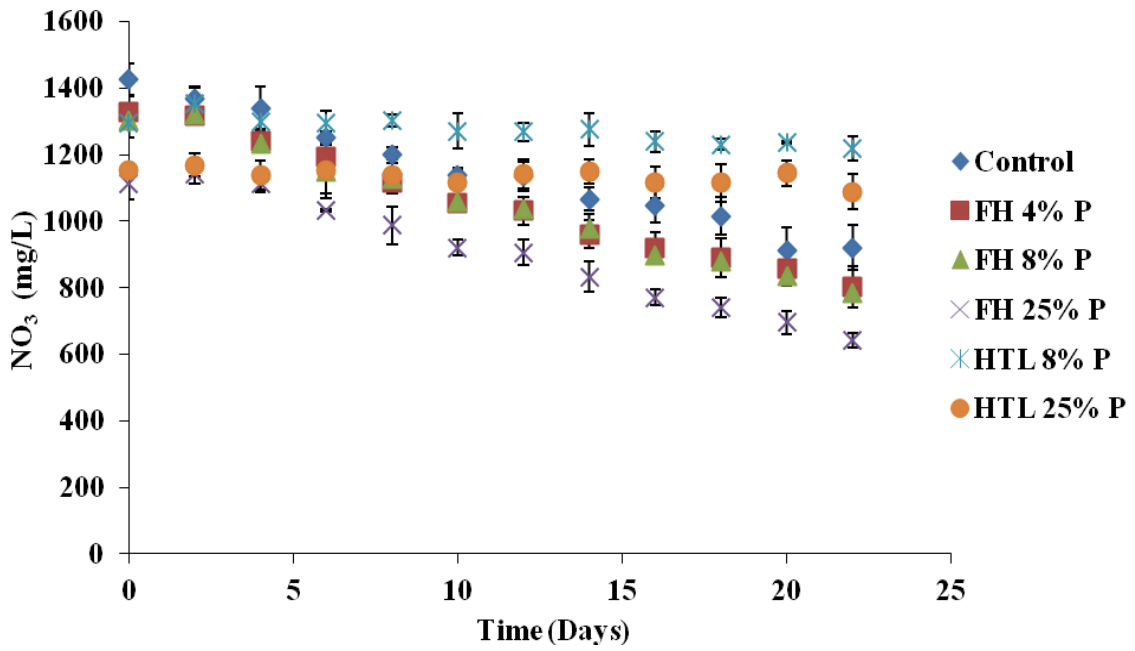

Figure 5. NO_3^- values (average for each treatment) for the *Scenedesmus* cultivation using FH Hydrolysate and HTL AP study.

The soluble peptides appeared to decline again in the same way as observed in the No Algae study, but the ammoniacal nitrogen level did not increase. See Figures 7 – 8. This could potentially be because the *Scenedesmus* algae immediately took it up for use in its cell components. Figure 8 shows that ammoniacal nitrogen is being used until depletion. This observation matches the order of nitrogen assimilation described in the literature review [27, 28]. Finally, there was a significant difference in the elemental carbon and nitrogen content of the algae collected from the FH treatments compared to HTL and the control respectively, suggesting a richer uptake of these nutrients when grown using FH (Table 8).

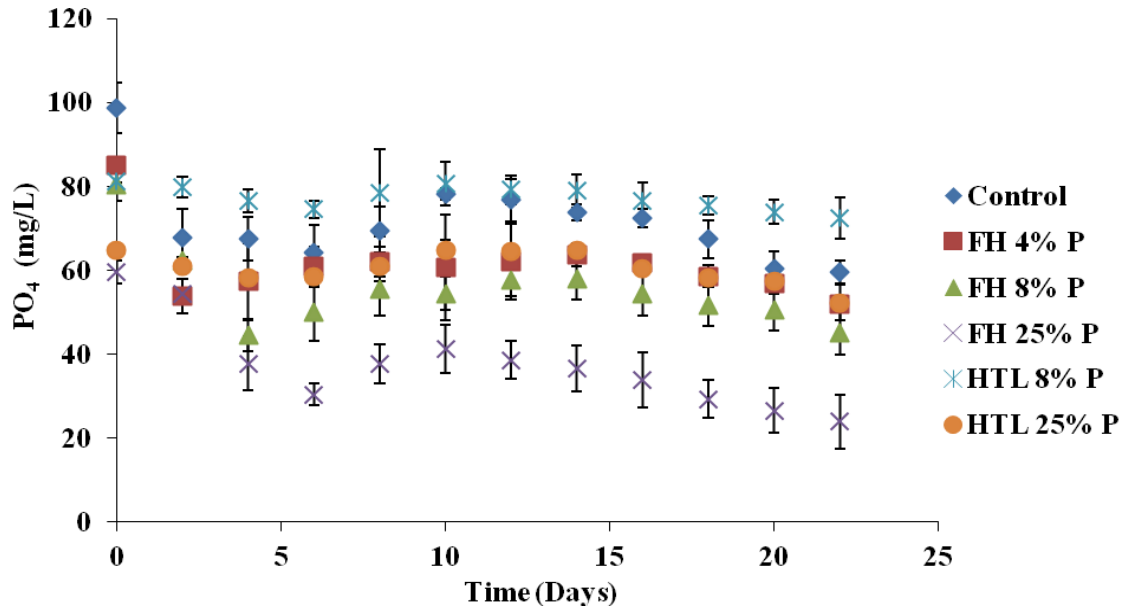

Figure 6. PO_4^{3-} values (average for each treatment) for the *Scenedesmus* cultivation using FH Hydrolysate and HTL AP study.

Table 8. Elemental analysis composition for the algae collected after the *Scenedesmus* cultivation using FH Hydrolysate and HTL AP study.

***Scenedesmus* Elemental Analysis Data Summary**

	% Nitrogen		% Carbon		% Hydrogen	
	Average	**St. Dev.**	**Average**	**St. Dev.**	**Average**	**St. Dev.**
Control	5.70	0.46	47.97	0.61	8.13	1.07
10 % P FH	6.92	0.78	49.30	0.72	7.49	0.83
20 % P FH	7.90	0.98	50.36	0.99	7.01	0.39
50% P FH	7.67	0.38	49.05	0.28	8.02	0.44
20% P HTL	6.81	0.23	45.74	0.19	7.55	1.51
50% P HTL	7.67	0.31	46.75	0.77	7.17	0.73

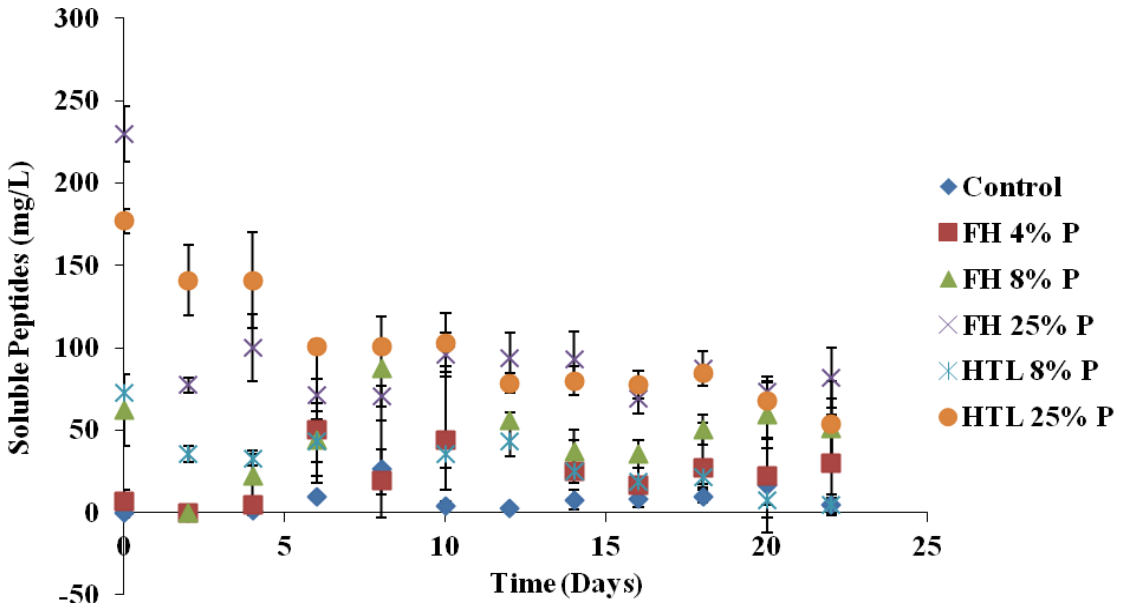

Figure 7. Soluble Peptides values (average for each treatment) for the *Scenedesmus* cultivation using FH Hydrolysate and HTL AP study.

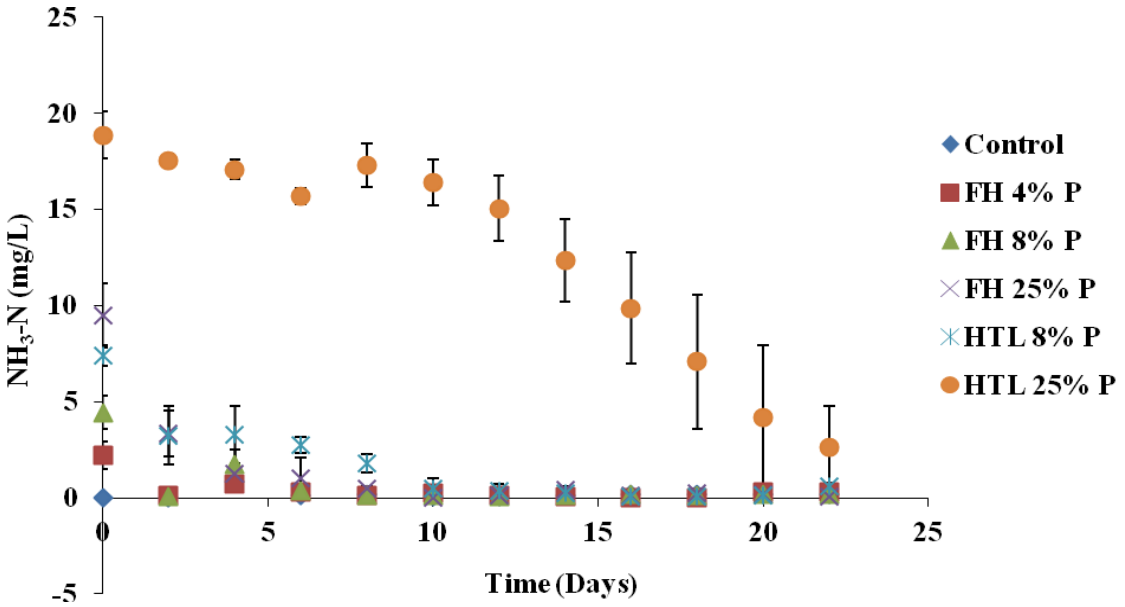

Figure 8. NH_3-N values (average for each treatment) for the *Scenedesmus* cultivation using FH Hydrolysate and HTL AP study.

3.3 Oocystis sp. cultivation using FH Hydrolysate and HTL AP

Oocystis sp. cultivation using FH Hydrolysate was studied because it became the dominant culture in the PBRs used to inoculate the experiments. This also would occur occasionally for unknown reasons in the open pond facility at ODU where the *Scenedesmus* was cultivated, as well as many other open pond facilities [32, 33]. As can be seen in Figure 9, there is almost no lag phase for the FH treatments or the control.

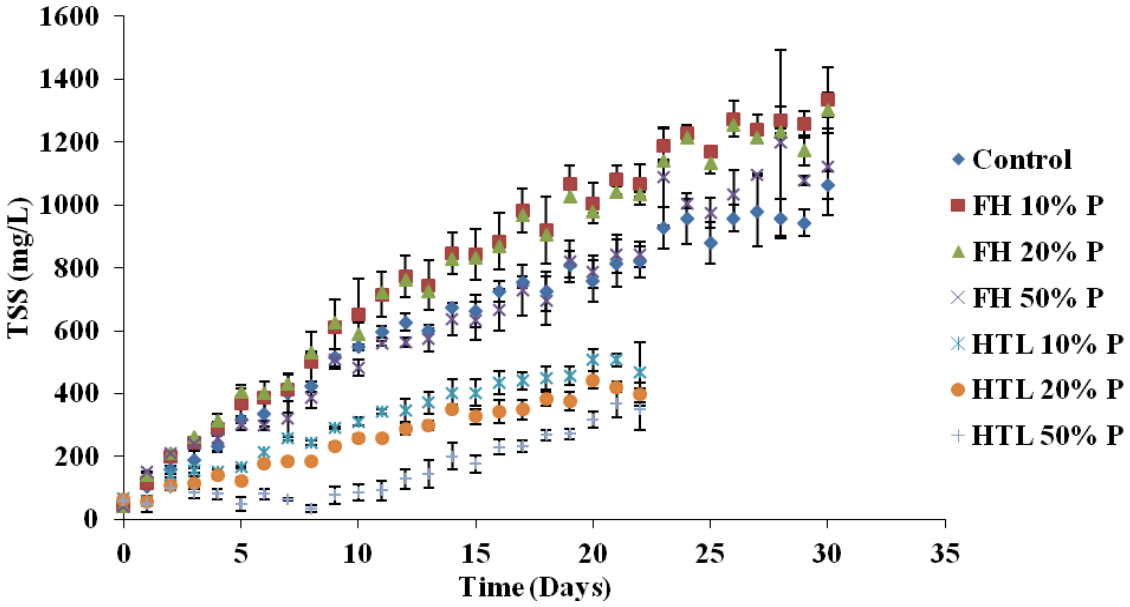

Figure 9. TSS values (average for each treatment) for the *Oocystis* cultivation using FH Hydrolysate and HTL AP study.

Using HTL AP as a nutrient source showed significant differences in the growth rates observed for the different treatments. The algae used as inoculum for this study were already acclimated to the new AM-14 media; this may explain the rapid growth rate observed in the first few days for the control and FH treatments. During the first four days all FH treatments showed higher TSS average values than the control. After the first four days the FH 10% P and FH 20% P replacement treatments grew faster than the control and FH 50% P treatment, a tendency that

was observed for the duration of the study (30 days) until phosphorous became a limiting factor. The TSS values for those two treatments are almost identical and follow the same trend. The recovered HTL AP product had a different composition than that obtained after FH. The 10% P and 20% P replacement treatments using the HTL AP product comparatively showed a significantly slower algae growth (TSS < 500 mg/L), possibly due to an excessive amount of ammonia in the media which may inhibit the normal development of the cultures [20, 25, 26]. The HTL 50% P replacement showed almost no growth for the first 11 days, but at the end of the experiment the algae concentration measured was almost comparable to the HTL 20% P replacement.

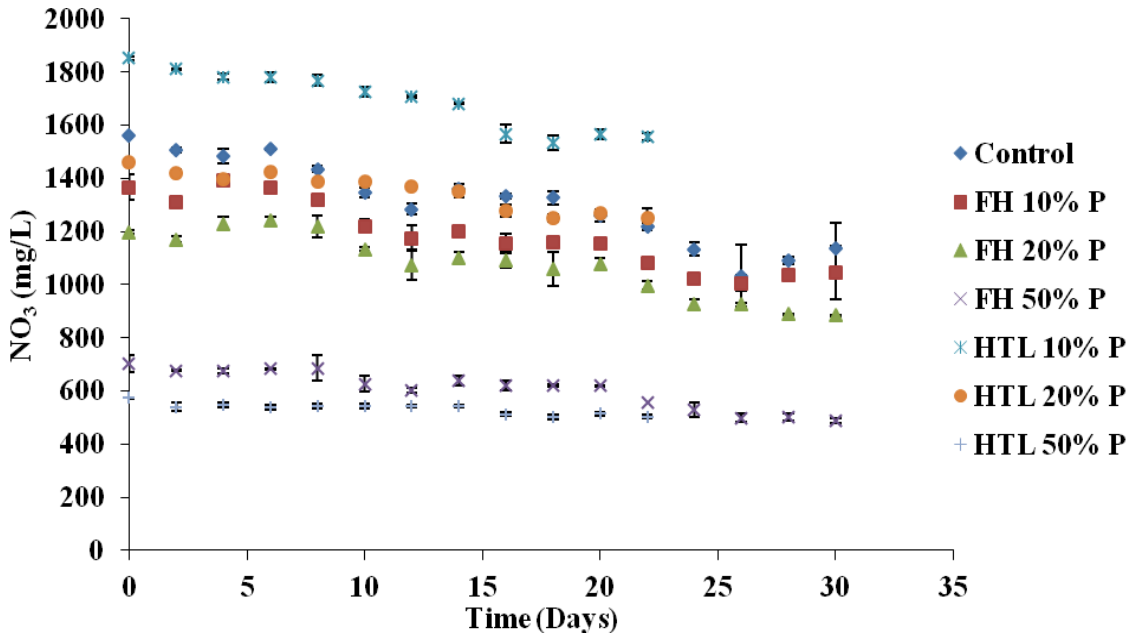

Figure 10. NO_3^- values (average for each treatment) for the *Oocystis* cultivation using FH Hydrolysate and HTL AP study.

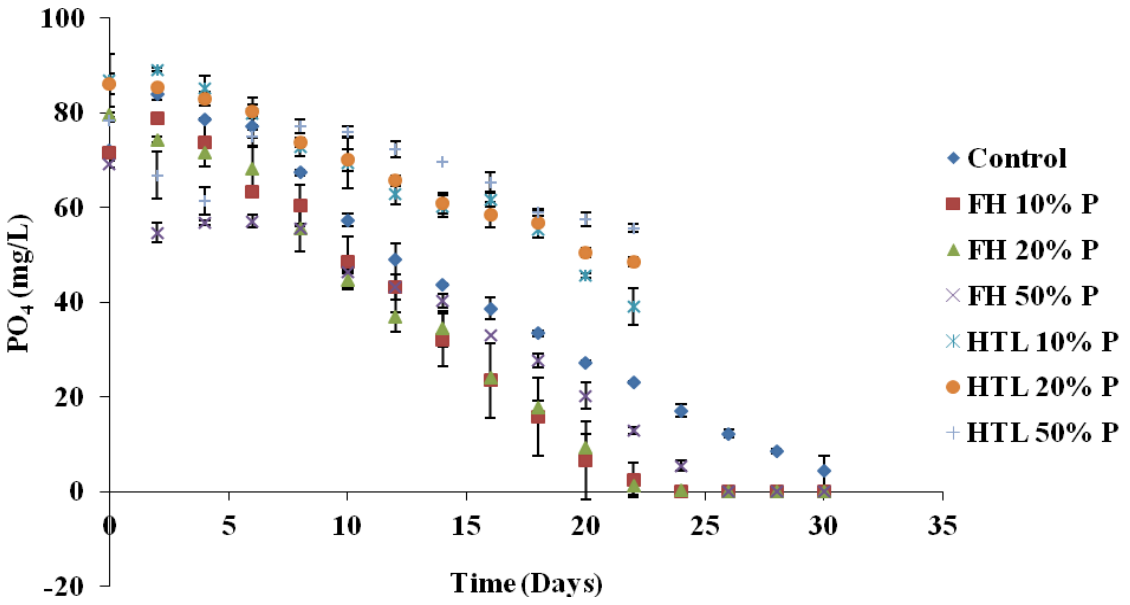

Figure 11. PO_4^{3-} values (average for each treatment) for the *Oocystis* cultivation using FH Hydrolysate and HTL AP study.

The control along with the FH 10% P, FH 20% P, HTL 10% P, and HTL 20% P treatments showed a similar trend in the nitrate reduction measured over time (Figure 10). In those cases, nitrate was the most abundant nitrogen species in the media, which can explain the observed uptake by the algae. The 50% P replacement treatment for FH and HTL showed almost no variation in nitrate concentration coinciding with the respective 53% N and 70% N replacements (Tables 12 and 15). In the case of phosphate concentration, all the bottles (treatments and control) started with a similar concentration. It is possible that the initial difference observed was due to the presence of organic molecules containing phosphorus since the analytical method employed to make the percent replacement calculation required a digestion time and the results are expressed as total phosphate. The reduction in its concentration follows a similar trend (Figure 11) in the FH treatments and the control whereby in the last days of the experiment it was close to the detection level, and values of 0 mg/L were reported. It is suspected

that if kept for a longer time the phosphorus concentration would have become the limitation factor restricting algae growth, and the decaying phase would have started. For the HTL treatments the phosphate concentration declined but not at the rate of the control and FH treatments. This data reflects the TSS values.

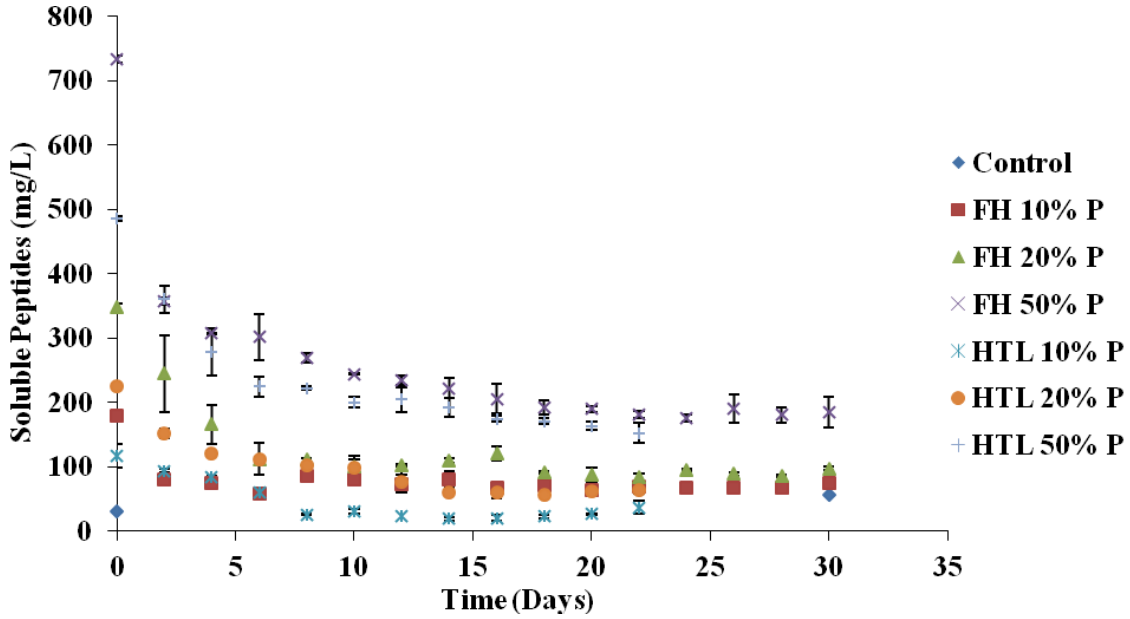

Figure 12. Soluble Peptides values (average for each treatment) for the *Oocystis* cultivation using FH Hydrolysate and HTL AP study.

The soluble peptides concentration shows a similar trend (Figure 12) in all the treatments; it started at high values and started decreasing from Day 0 until it reached an almost constant concentration. The change in concentration of soluble peptides shown in Figure 12 coincides with the observed change in algae growth and also the presence of ammoniacal nitrogen. Since FH 50% P showed inhibited growth after the first four days but recovered after the eighth, a time coinciding with elevated ammoniacal nitrogen levels, these observations give rise to the potential of toxic ammonia levels as a factor directly limiting higher levels of nutrient recycling. For HTL

50% P the algae started growing only after 12 days and at a very slow rate when compared to the other treatments. This time coincides with the presence and removal of high levels of ammoniacal nitrogen. As observed in the *Scenedesmus* study, the ammoniacal nitrogen concentration decreased significantly as this should be the preferred nitrogen source to support algae growth.

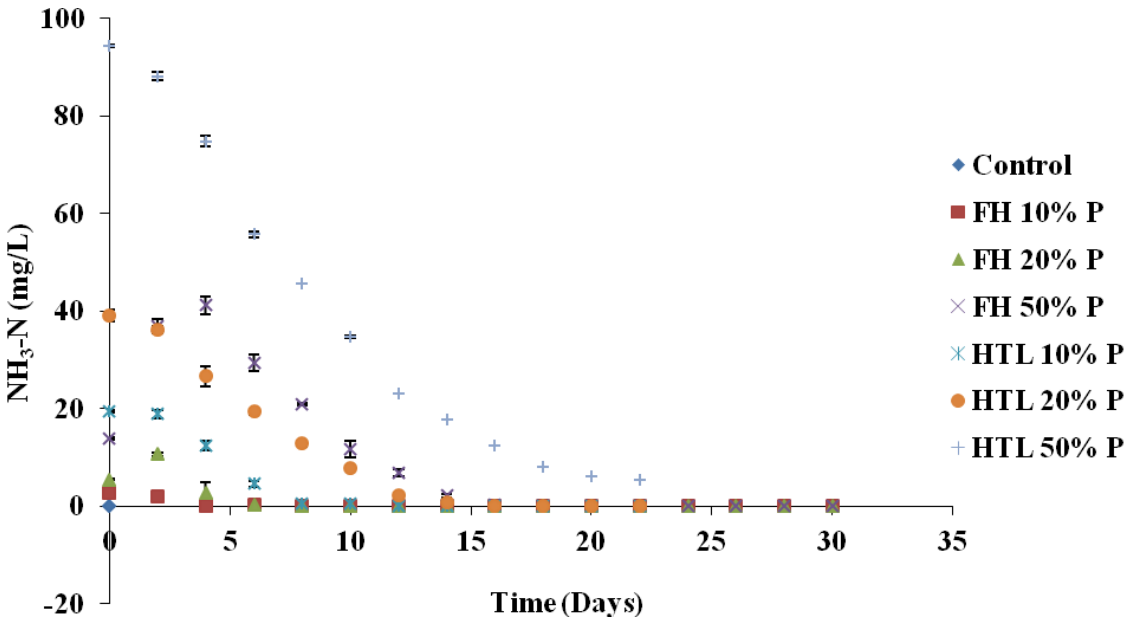

Figure 13. NH_3-N values (average for each treatment) for the *Oocystis* cultivation using FH Hydrolysate and HTL AP study.

Table 9 summarizes the elemental analysis composition for the collected algae after the nutrient recycle experiment. There was a significant difference in elemental nitrogen and carbon contents of the collected algae between the control and FH treatments. The FH again had higher nitrogen and carbon content than the control. Unfortunately the HTL algae were not collected for elemental analysis. Interestingly, the *Scenedesmus* treatments had higher carbon and nitrogen contents than the respective *Oocystis* treatments, showing the importance of species in algae biomass production.

Table 9. Elemental analysis composition for the algae collected after the *Oocystis* cultivation using FH Hydrolysate study.

***Oocystis* Elemental Analysis Data Summary**

	% Nitrogen		% Carbon		% Hydrogen	
	Average	**St. Dev.**	**Average**	**St. Dev.**	**Average**	**St. Dev.**
Control	4.58	0.18	42.05	0.32	8.10	0.84
10% P FH	4.48	0.16	43.62	0.48	8.35	0.44
20% P FH	4.74	0.23	44.06	0.66	8.39	0.79
50% P FH	6.02	0.44	46.06	1.19	8.13	0.83

CHAPTER 4

DISCUSSION

Both *Scenedesmus* and *Oocystsis* grew as well as, if not better than, the control AM-14 media using FH nutrient recycling. Unfortunately, HTL AP did not grow as well as the control. The AM-14 media only provides nitrate as a nitrogen source while the FH and HTL AP added provides a mixture of ammonia and organic nitrogen (soluble peptides). The difference in nitrogen sources in the various treatments appeared to impact both the growth rate and the final elemental composition of the algae. The phosphate uptake reflected the growth rate for all treatments and seems to be a potential limiting nutrient for AM-14.

Anderson et al. and Belay reported that pulses of 1 to 2 mM ammonia, followed by a 30% dilution of the culture, was an effective treatment against culture contaminants due to differential sensitivity of various algae species to ammonia and that while this treatment worked to purify cultures of *Spirulina*, *Oocystis sp.* was still a problem contaminant [21, 32]. The results of this study agree with this observation, and the need for a dilution step following the ammonia pulses indicates that long exposure periods to ammonia are toxic to most algae strains. Toxicity is further indicated by the flocculation observed at 100x magnification for the HTL 25% P replacement treatment in Figures 14 – 15 since algae are known to flocculate when in the presence of high amounts of ammonia [25].

This is a potential problem since the sustained dose of ammoniacal nitrogen coming from the decay of the soluble peptides possibly limits higher levels of direct nutrient recycling. Fortunately, pH adjustment has been found to fix ammonia toxicity by shifting the equilibrium to ammonium and is recommended for future studies [12]. The extra colonial cell wall present in the cell structure of *Oocystis sp*. probably gives this algal species the ability to endure a high

sustained dose of ammonia, recover, and begin exponential growth again, as was observed in the *Oocystis sp.* cultivation using HTL AP study (Figure 9, 13). Since *Scenedesmus* does not have this colonial cell wall, their tolerance to free ammonia is likely lower. De Mott and Donk even isolated the control *Oocystis* strain used in their study from the guts of field collected zooplankton [34].

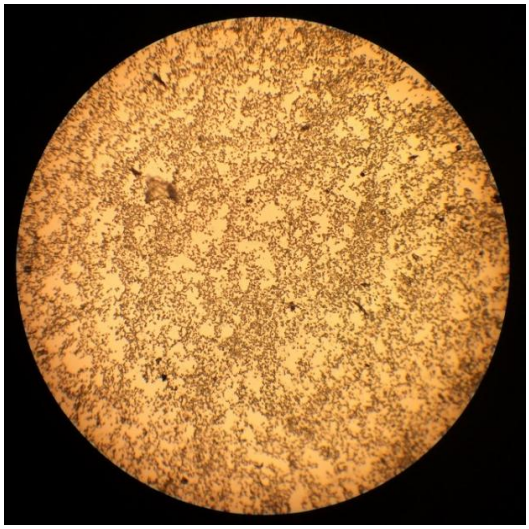

Figure 14. Picture of *Scenedesmsus* Bottle 13 (FH 8% P) sample slide at 100x magnification on Day 13.

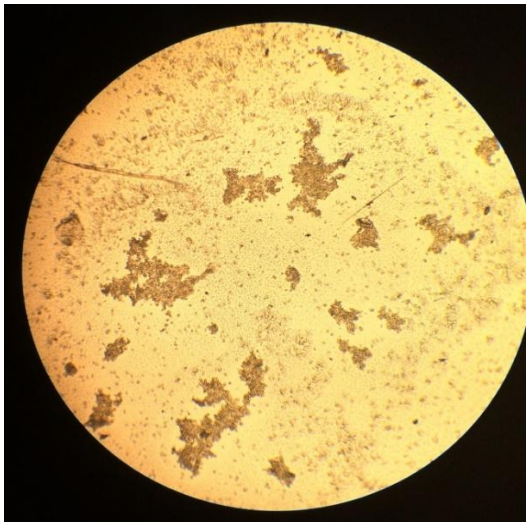

Figure 15. Picture of *Scenedesmsus* Bottle 16 (HTL 25% P) sample slides at 100x magnification on Day 13.

CHAPTER 5

CONCLUSION

The FH Hydrolysate contains significant amounts of soluble nutrient compounds that can be recycled to grow microalgae. Algae successfully grew at a rate similar to the control media when using FH Hydrolysate. A slower growth rate, if any growth occurred at all, was observed when using HTL AP. The effect was significant for the HTL 50% P replacement treatment where almost no algae growth was observed in the first 11 days of the experiment. Starting on day 12 of the experiment algae started growing, coinciding with a significant soluble ammonia concentration decrease. *Scenedesmus* had a high initial phosphorous uptake rate compared to *Oocystis sp.*, but the latter completely removed it over the course of 30 days for the FH treatments. The recycling of nutrients after processing the algae biomass will reduce production costs of algal products since the amount of money required for fertilizers will reduce proportionally to the level of replacement. It can be expected that 50% of the phosphorus required in the newly formulated AM-14 culture media could be replaced with that recovered after FH; also, 53% or more nitrogen could be provided from the same source.

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APPENDICES

APPENDIX A

A.1 Preparation of FH Hydrolyzate and HTL AP

Flash hydrolysis experiments on the algae *Scenedesmus sp*. were conducted in a continuous flow sub-critical water reactor as described before in previous publications [13, 14]. The conditions of reactor temperature, pressure, and residence time were 280°C, 2100 psi (14.5 MPa), and 9 s respectively as these had previously been described as the optimum conditions for nitrogen extraction from the algae biomass [13]. The FH Hydrolysate was collected and stored at 4°C until analyzed and used. For the *Oocystis* and No Algae control experiments, the FH experiments produced a very dilute Hydrolysate due to experimental conditions (less than 1% solids load); to concentrate it, the samples were freeze dried and reconstituted with DI water to a more concentrated solution. For the *Oocystis* FH experiment the FH Hydrolysate was prepared by completely freeze drying 1.6L of aqueous phase product and reconstituting it with 300mL of DI water. For the first control experiment the FH Hydrolysate was prepared by freeze drying 4.0L of aqueous phase product and reconstituting it with 1.0L of DI water. For the second control experiment the FH Hydrolysate was prepared by freeze drying 1.5L of aqueous phase product and reconstituting it with 500mL of DI water. For the *Oocystis* HTL experiment the FH Hydrolysate was also prepared by freeze drying 1.5L of aqueous phase product and reconstituting it with 500mL of DI. For the *Scenedesmus* experiment, the FH Hydrolysate was not freeze dried and reconstituted; rather, it was stored at 4°C until used in the experiment treatments. This allowed for the testing of direct nutrient recycling without the freeze drying and reconstituting step.

HTL experiments on the algae *Scenedesmus sp*. were conducted using a 500 mL batch reactor equipped with a Parr 4848 controller. The reactor was set at 280°C for 30 minutes (plus a preheating time of approximately 40 minutes), the pressure was measured to be 1500 psi. The experimental temperature was chosen to be similar to that of FH experiments. The reactor was loaded with 400g of slurry with 8.7% solids (prepared by mixing 40g of freeze dried algae and 400mL of water). After HTL was finished most of the product (a mixture of liquid and solids) was recovered; extraction with a solvent was required to collect the maximum amount of product, a mixture of Dichloromethane (DCM, Sigma-Aldrich, 99% purity) and water was used for that purpose (250mL DCM and 200mL DI water). The HTL products (both liquid and solids) were mixed with the DCM and DI mixture and placed in a separation funnel to separate the different phases (water soluble products, DCM soluble and solids). To recover most of the water-soluble products, 500mL of DI water was used to rinse the decanted DCM phase. After decanting the DCM phase it was vacuum filtered to recover the solids in it; the solvent was removed using a rotavapor (Cole Palmer N-1100). The amount of DCM soluble compounds (lipids) was quantified gravimetrically by weighing the glassware used before and after the solvent evaporation to complete dryness. After all these steps, three different products were collected: lipids from the DCM soluble fraction, water-soluble compounds (550mL at the first separation step and 500mL of rinsing DI) and solid residue. The solid residue was dried at 55°C for 72 hours, weighted once completely dry and analyzed for its elemental composition. The experiment was conducted in duplicates, and the final aqueous phase product from both batches was mixed and used as a nutrient source for the experiments.

The liquid products from Batch 1 and Batch 2 were mixed and used as a recycled nutrient source for the *Oocystis* HTL AP experiment. For the *Scenedesmus* experiment, the HTL was

prepared in the same way using two separate batches mixed together, and the tables in the following sections contain the characterization of each AP used in each respective experiment along with estimates about the amounts of characterized compounds present in each treatment resulting from the AP. See Tables 6, 11, and 14.

A.2: Preparation of AM-14 Control Media and Inoculating Cultures

A new culture media was prepared to grow algae in the authors' lab. The new recipe is easier to prepare and provides balanced macro and micronutrients to sustain algae growth. The new media (Algae Media 2014 or AM-14) was prepared to have slightly more nitrogen and phosphorous than BG-11 growth medium in a 10:1 mass ratio; the final concentration in the media is 300mg/L N (from $NaNO_3$) and 30mg/L P (from K_3PO_4). Interestingly, it provides at least 20 times the Nitrogen and Phosphorus present in other standard culturing medias such as COMBO and Guillard's f/2 [21, 22]. The rest of the nutrients were added by taking elements of different commonly used media (3N-BB, BG-11 and Guillard's f/2 media) [21, 35]. Vitamins were not included since Croft et al. found that they are supplied in the natural environment by a symbiotic relationship with bacteria [23]. Furthermore, since vitamin additions will more than likely be too expensive for any commercial application of algal biofuels and the bacteria will supply them naturally, the vitamin free media was also not autoclaved and the algae were grown under non-sterile conditions to reflect conditions at any potential commercial scale open pond application. The new media has a final pH of 10.5, and its composition is detailed in Table 1.

To formulate the AM-14 media, several concentrated stock solutions were prepared and added to the amount of DI water needed to balance the desired final concentration. The stock solutions are prepared and mixed as follows: **Part A**: 50x $NaNO_3$, **Part B**: 50x K_3PO_4, **Part C**: 100x $MgSO_4$*7 H_2O + NaCl + $CaCl_2$, **Part D**: 1000x micronutrients. This is also done to prevent the precipitation of some of the components when they are mixed in high concentrations.

The algae *Oocystis* was cultivated in 90L Photo-bioreactors (PBRs) using a modified BG-11 media which differs significantly from the new AM-14 formulation [13]. To acclimate the algae to the new media, 1L of algae suspension was removed from one PBR with an algae biomass concentration TSS ≈ 600 mg/L; the algae suspension was centrifuged at 1500 RPM for 5 minutes, then rinsed and re-suspended with DI water. This last step was repeated two times to remove as much culture media as possible before inoculation in the new media. The recovered concentrated algae were used as inoculum in 2L glass bottles containing the new modified media (AM-14). The *Scenedesmus* inoculating culture was started by inoculating several 3L plastic bottles of AM-14 media with pure culture obtained from petri dishes cultivated in almost sterile conditions.

The first algae genera cultivated for this study was identified as *Oocystis*, and the population in the sample given was described as unicellular, pairs, or groups of 4. The cells are roughly oval shaped, approximately 10 microns in length, and 6 microns in width. The cells/colonies are surrounded by a thin membrane sheath. The second genera cultivated for this study was *Scenedesmus*, and the population in the sample given was described as 2-4 celled coenobia with spines on the terminal cells (communications with Dr. Todd Egerton, Department of Biological Sciences, ODU). Figure 16 and Figure 17 are pictures of *Oocystis* and *Scenedesmus*.

A.3: Oocystis cultivation using FH hydrolyzate

For the *Oocystis* cultivation using FH Hydrolysate study, the evaluated nutrient replacement levels using FH Hydrolysate were established at 10%, 20% and 50% of the total phosphorus provided by the AM-14 media. The amount of nitrogen supplemented by the Hydrolysate was calculated so it could be balanced with the amount provided by the AM-14

media in each experiment. Table 10 shows the formulation used for the control and the three different nutrient replacement levels.

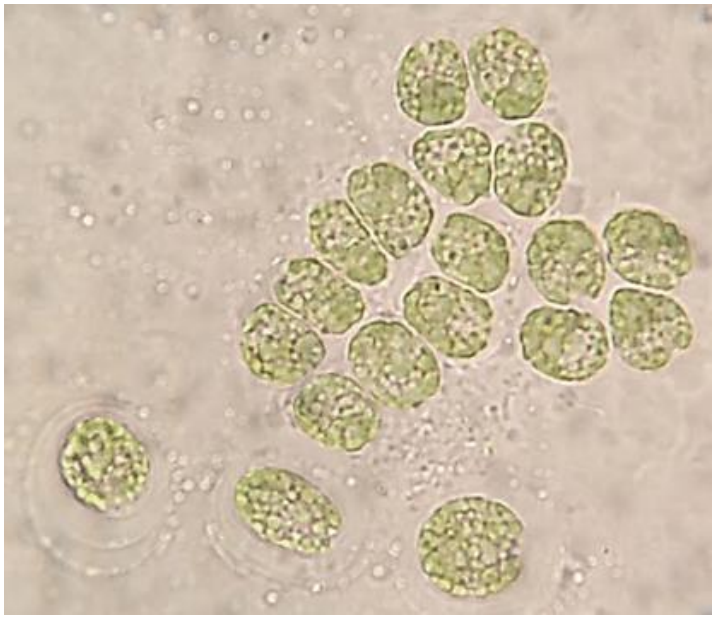


Figure 16**.** Pictures of microalgae *Oocystis.* Notice the colonial cell wall around the cells.

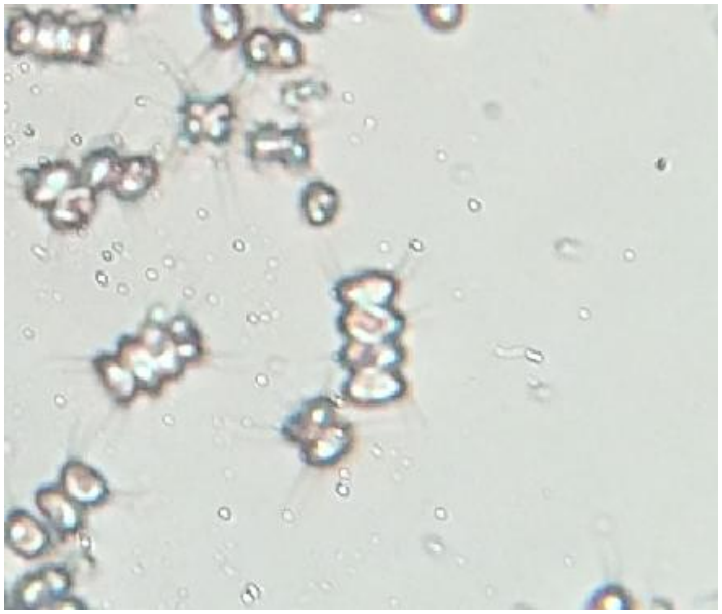


Figure 17. Pictures of microalgae *Scenedesmus*. Notice the spikes on the ends of the colonies.

The algae were cultivated for 30 days in 2L glass bottles placed inside an incubator where the temperature was kept stable between 32-36°C; a 12 hour light/dark cycle was set for the duration of the experiment by means of artificial light (SUN-904302 ft. Fluorescent Grow Light Fixture full spectrum 6500K), and permanent aeration was provided using air stones inside each bottle to keep the algae in suspension.

Twelve 2L glass bottles were used in order to have 4 different treatments: control (only AM-14 media), FH 10% P replacement, FH 20% P replacement, FH 50% P replacement. Each treatment was set in triplicates, and the bottles were placed randomly inside the incubator. The data were reported in duplicate due to a border effect reported by Garcia-Alba et al. [10]. Table 11 details the concentration of major components derived from the FH Hydrolysate, and Table 12 details the initial concentration of nitrogen and phosphorous in the media along with the %P and %N replaced in the media.

Table 10. Formulation for *Oocystis* cultivation using FH Hydrolysate at three different levels of nutrient replacement.

Formulations for Nutrient Replacement with Flash Hydrolysate (FH)
Oocystis

	Control AM-14 (mL)	**10% P Replacement (mL)**	**20% P Replacement (mL)**	**50% P Replacement (mL)**
Inoculum	100	100	100	100
Part A	30	26.7	23.5	14
Part B	30	27	24	15
Part C	15	15	15	15
Part D	1.5	1.5	1.5	1.5
Hydrolysate	0	11	22	54.5
Water	1323.5	1318.8	1314	1300
Total Volume	**1500**	**1500**	**1500**	**1500**

Algae were grown under the detailed conditions for 30 days. Every day TSS was measured as an algae growth indicator (filtration with glass fiber discs 1.4 micron pore size

Whatman 934-AH 47 mm). Every two days a 10 mL sample (after filtration) was collected and preserved at 4°C until analyzed. The parameters that were measured in the liquid samples were: Soluble peptides (Lowry's method), Nitrate (Dionex ICS-5000), Phosphate (Dionex ICS-5000), Sulfate (Dionex ICS-5000), Nitrite (Dionex ICS-5000), and Ammonia (HACH DR 2800 spectrophotometer). Figure 18 shows pictures taken during the FH Hydrolysate *Oocystis* experiment. Note the visual difference in growth rates between the bottles placed inside the incubator as well as the discoloration of the media by the Hydrolysate.

Table 11. Concentration of major components derived from the FH Hydrolysate in *Oocystis* cultures.

Flash Hydrolysate (FH)
Concentration Derived From Effluent Replacement
Oocystis

Solution	NH_3 (mg/L)	TPO_4 (mg/L PO_4)	TP (mg/L P)	Soluble Peptides (mg/L)	TN (mg/L N)	pH	TOC (mg/L)	Phenols (mg/L)	mL FH	Dilution Factor (DF)
FH	430	1270	414	25400	4400	7.2	-	0.18	-	-
Control	-	-	-	-	-	-	-	-	-	-
FH 10% P	3.2	9.3	3.0	186	32	-	-	0.00	11.0	136
FH 20% P	6.3	18.6	6.1	373	65	-	-	0.00	22.0	68.2
FH 50% P	15.6	46.1	15.0	923	160	-	-	0.01	54.5	27.5

Table 12. Initial concentration of nitrogen and phosphorous in the *Oocystis* cultures.

Flash Hydrolysate (FH)
Initial Concentration In Media
Oocystis

Solution	TN (mg/L N)	TN From Hydrolysate (mg/L N)	TP (mg/L P)	TP From Hydrolysate (mg/L P)	%P Replacement	%N Replacement
Control	300	-	30	-	0	0
FH 10% P	300	32	30	3.0	10%	11%
FH 20% P	300	65	30	6.1	20%	22%
FH 50% P	300	160	30	15.0	50%	53%

After finishing the experiment, all the bottles used were harvested by centrifuging (Fisher Scientific accuSpin™ 400) the entire culture at 10000 xg for 3 to 6 minutes. The centrifuged cultures were decanted and centrifuged again with more culture until each respective culture was concentrated in one centrifuge tube. The collected biomass was freeze dried and stored at -4°C until analyzed. All the samples were analyzed for elemental composition using a Thermo Finnigan Flash 1112 Elemental Analyzer with a nicotinamide standard for calibration. Approximately 1-2mg of each solid sample was placed in a 3.3 x 5mm tin capsule for combustion. The method used for analysis was a furnace at 900°C, oven at 75°C, and carrier gas helium at 91 mL/min.

Figure 18. Pictures of different growth stages (Days 0, 5, 10, 19, and 26 from left) during FH Hydrolysate *Oocystis* experiment. Note the visual difference in growth rates between the bottles placed inside the incubator as well as the discoloration of the media by the Hydrolysate.

A.4: Oocystis Cultivation Using HTL AP

For the *Oocystis* cultivation using HTL AP study the evaluated nutrient replacement levels using HTL AP were established at 10%, 20%, and 50% of the total phosphorus provided by the AM-14 media. A 20% total phosphorous nutrient replacement using FH Hydrolysate was added as a control to compare the differences in the growth of *Oocystis* using HTL AP versus FH Hydrolysate. The amount of nitrogen supplemented by the AP and Hydrolysate was calculated

so it could be balanced with the amount provided by the AM-14 media in each experiment. Table 13 shows the formulation used for the four different nutrient replacement levels.

The algae were cultivated for 22 days in 2L glass bottles placed inside an incubator where the temperature was kept stable between 32-36°C, a 12 hour light/dark cycle was set for the duration of the experiment by means of artificial light (SUN-904302 ft. Fluorescent Grow Light Fixture full spectrum 6500K), and permanent aeration was provided using air stones inside each bottle to keep the algae in suspension.

Eight 2L glass bottles were used in order to have 4 different treatments: HTL 10% P replacement, HTL 20% P replacement, HTL 50% P replacement, and FH 20% P replacement. Each treatment was set in duplicates, and the bottles were placed randomly inside the incubator. Table 14 details the concentration of major components derived from the HTL AP and FH Hydrolysate, and Table 15 details the initial concentration of nitrogen and phosphorous in the media along with the %P and %N replaced in the media.

Table 13. Formulation for *Oocystis* cultivation using HTL AP and FH Hydrolysate at four different levels of nutrient replacement.

Formulation for Nutrient Replacement
Hydro-Thermal Liquefaction (HTL) vs. Flash Hydrolysate (FH)
Oocystis

	10% P Replacement HTL (mL)	**20% P Replacement HTL (mL)**	**50% P Replacement HTL (mL)**	**20% P Replacement FH (mL)**
Inoculum	100	100	100	100
Part A	26	21	8	26.5
Part B	27	24	15	24
Part C	15	15	15	15
Part D	1.5	1.5	1.5	1.5
AP/Hydrolysate	15	30	75	24
Water	1315.5	1308.5	1285.5	1309
Total Volume	**1500**	**1500**	**1500**	**1500**

Algae were grown under the detailed conditions for 22 days. Every day TSS was measured as an algae growth indicator (by filtration with glass fiber discs 1.4 micron pore size Whatman 934-AH 47 mm). Every two days a 10mL sample (after filtration) was collected and preserved at 4°C until analyzed. The parameters that were measured in the liquid samples were: Soluble peptides (Lowry's method), Nitrate (Dionex ICS-5000), Phosphate (Dionex ICS-5000), Sulfate (Dionex ICS-5000), Nitrite (Dionex ICS-5000), and Ammonia (HACH DR 2800 spectrophotometer).

Table 14. Concentration of major components derived from the HTL AP and FH Hydrolysate in *Oocystis* cultures.

Hydro-Thermal Liquefaction (HTL) vs. Flash Hydrolysate (FH)
Concentration Derived From Effluent Replacement
Oocystis

Solution	NH_3 (mg/L)	TPO_4 (mg/L PO_4)	TP (mg/L P)	Soluble Peptides (mg/L)	TN (mg/L N)	pH	TOC (mg/L)	Phenols (mg/L)	mL FH	mL HTL	Dilution Factor (DF)
FH	-	1165	380	11900	2200	-	-	-	-	-	-
HTL	1550	920	300	12500	4200	8.4	10550	4.5	-	-	-
FH 20% P	-	18.6	6.1	190	35	-	-	-	24	-	62.5
HTL 10% P	15.5	9.2	3.0	125	42	-	106	0.05	-	15	100
HTL 20% P	31.0	18.4	6.0	250	84	-	211	0.09	-	30	50.0
HTL 50% P	77.5	46.0	15.0	625	210	-	528	0.23	-	75	20.0

Table 15. Initial concentration of nitrogen and phosphorous in the *Oocystis* cultures.

Hydro-Thermal Liquefaction (HTL) vs. Flash Hydrolysate (FH)
Initial Concentration In Media
Oocystis

Solution	TN (mg/L N)	TN From Hydrolysate (mg/L N)	TP (mg/L P)	TP From Hydrolysate (mg/L P)	%P Replacement	%N Replacement
FH 20% P	300	35	30.0	6.1	20%	12%
HTL 10% P	300	42	30.0	3.0	10%	14%
HTL 20% P	300	84	30.0	6.0	20%	28%
HTL 50% P	300	210	30.0	15.0	50%	70%

A.5 Scenedesmus pH and TN:

Due to the potential inhibitory effect of free ammonia on the algae and the need to find a potential mechanism for the decomposition of the soluble peptides into free ammonia, the pH and Total Nitrogen (TN) values were measured for this study. See Figures 19 – 20.

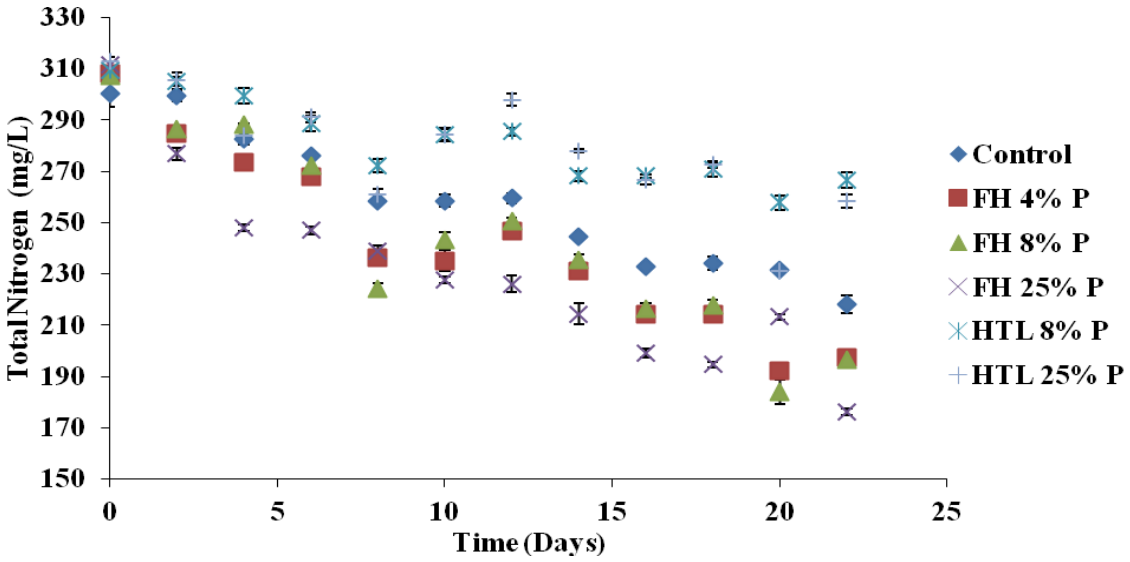


Figure 19. Total Nitrogen (TN) values (average for each treatment) for the *Scenedesmus* cultivation using FH Hydrolysate and HTL AP study.

The pH appeared to be related to the growth of the algae population, with the pH increasing from 8 to as high as 11 and then slightly leveling off as the cultures matured. The TN also appeared to be related to growth where the highest change in TN occurred for the 25% P FH treatment. This treatment also had the highest average %N in the elemental analysis of the final solid algae samples (Table 8). While this indicates that a larger amount of nitrogen went into the cells, the high loss of TN could also be partially explained by possible ammonia stripping since the pH increased to levels where ammonia stripping can occur [19].

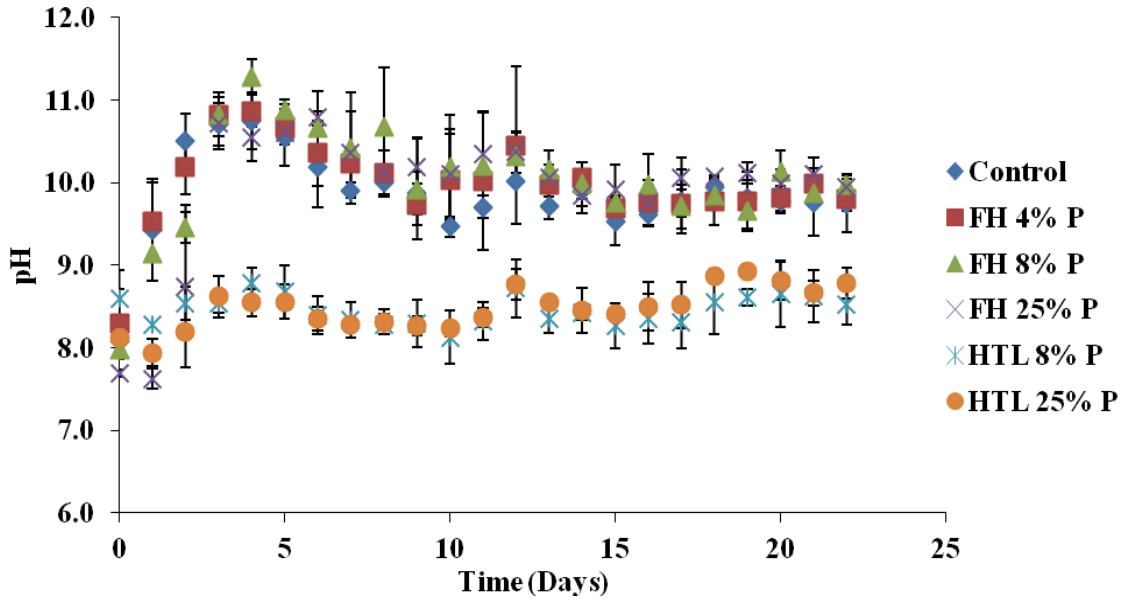


Figure 20. pH values (average for each treatment) for the *Scenedesmus* cultivation using FH Hydrolysate and HTL AP study.

A.6 Phenols:

Both the HTL AP and FH Hydrolysate used in this study contained a small amount of phenolic compounds (4.5 and 0.18mg/L respectively). Some phenols, quinones, and phenylpropanoids have been confirmed to have an inhibitory effect on algae growth in concentrations as low as 1.5 to 4.9mg/L [36]. In all of the studies, the AP and Hydrolysate treatments were diluted at least 7 times, greatly reducing the possibility of direct growth inhibition due to the presence of phenolic compounds.

APPENDIX B

Table 16. Nutrient Recycle Treatment Summary.

Nutrient Recycle Experimental Treatment Summary

	%P	%N	Average Final TSS (mg/L)	Standard Deviation (mg/L)	RSD	Growth Period (Days)
No Algae						
FH 1**						
Control						7
10 %P	10	10	-	-	-	4
20% P	20	20	-	-	-	8
50% P	50	48	-	-	-	9
FH 2**						
10 %P	10	6	-	-	-	4
20% P	20	12	-	-	-	4
50% P	50	30	-	-	-	4
Scenedesmus						
FH						
Control	0	0	1218	161	13%	22
4 %P	4	3	1342	117	9%	22
8% P	8	6	1244	163	13%	22
25% P	25	16	1324	167	13%	22
HTL***						
8% P	8	7	197	156	79%	22
25% P	25	18	160	35	22%	22
Oocystis						
FH						
Control	0	0	1063	42	4%	30
10 %P	10	11	1334	104	8%	30
20% P Trial 1*	20	22	1340	57	4%	30
20% P Trial 2*	20	12	717	5	1%	22
50% P	50	53	1123	156	14%	30
HTL						
10% P	10	14	518	26	5%	22
20% P	20	28	412	16	4%	22
50% P	50	70	337	27	8%	22

*Trial 1 was grown with Control and FH treatments and Trial 2 was grown with HTL treatments for different amounts of time.

**FH1 'No Algae' Treatments lasted a varying number of days since algae started to grow. This defeated the purpose of the experiment and the bottles were cut at the respective days shown on individual bottle graphs. The days reported here are for how long the treatments were in duplicate.

**FH 2 'No Algae' Treatments lasted only four days since soluble peptide conversion was almost completed by Day Four and algae started growing on Day 5.

***_Scenedesmus_ HTL treatments flocculated and settled by Day 13 of the experiment resulting in a high TSS RSD.

Table 17. Formulation for the second control experiment of *No Algae* using FH Hydrolysate at three different levels of nutrient replacement.

Formulations for Nutrient Replacement with Flash Hydrolysate (FH) Control Experiment (*No Algae*)

	10% P Replacement (mL)	20% P Replacement (mL)	50% P Replacement (mL)
Part A	28.3	26.6	21.4
Part B	27	24	15
Part C	15	15	15
Part D	1.5	1.5	1.5
Hydrolysate	11.7	23.5	58.7
Water	1416.5	1409.4	1388.4
Total Volume	**1500**	**1500**	**1500**

Table 18. Concentration of major components derived from the FH Hydrolysate in the second control experiment of *No Algae.*

Flash Hydrolysate (FH) Control Experiment
Concentration Derived From Effluent Replacement
No Algae

Solution	NH_3 (mg/L)	TPO_4 (mg/L PO_4)	TP (mg/L P)	Soluble Peptides (mg/L)	TN (mg/L N)	pH	TOC (mg/L)	Phenols (mg/L)	mL FH	Dilution Factor (DF)
FH	290	1180	385	12100	2300	6.8	-	-	-	-
FH 10% P	2.3	9.2	3.0	94	18	-	-	-	11.7	128
FH 20% P	4.5	18.5	6.0	190	36	-	-	-	23.5	63.8
FH 50% P	11.3	46.2	15.1	474	90	-	-	-	58.7	25.6

Table 19. Initial concentration of nitrogen and phosphorous in the second control experiment of *No Algae.*

Flash Hydrolysate (FH) Control Experiment
Initial Concentration In Media
No Algae

Solution	TN (mg/L N)	TN From Hydrolysate (mg/L N)	TP (mg/L P)	TP From Hydrolysate (mg/L P)	%P Replacement	%N Replacement
FH 10% P	300	18	30.0	3.0	10%	6%
FH 20% P	300	36	30.0	6.0	20%	12%
FH 50% P	300	90	30.0	15.1	50%	30%

Table 20. X-Ray Fluorescence Analysis (XRF) of *Scenedesmus* Biomass and Hydrolysate. The lack of Fe, Zn, and Mn highlights the need for the addition of nutrients through a synthetic media to the hydrolzyzate. This addition will ensure effective algae growth.

XRF Of *Scenedesmus*

Elements	Untreated Algae (wt %)	Freeze-dried Hydrolyzate 280˚C (wt %)
S	2.12	2.87
Ca	1.32	0.41
K	1.06	1.65
P	1.00	1.37
Fe	0.53	0.03
Zn	0.10	0.00
Mg	0.07	0.06
Cl	0.07	0.12
Mn	0.05	0.00
Si	0.04	0.03
Al	0.02	0.01

APPENDIX C

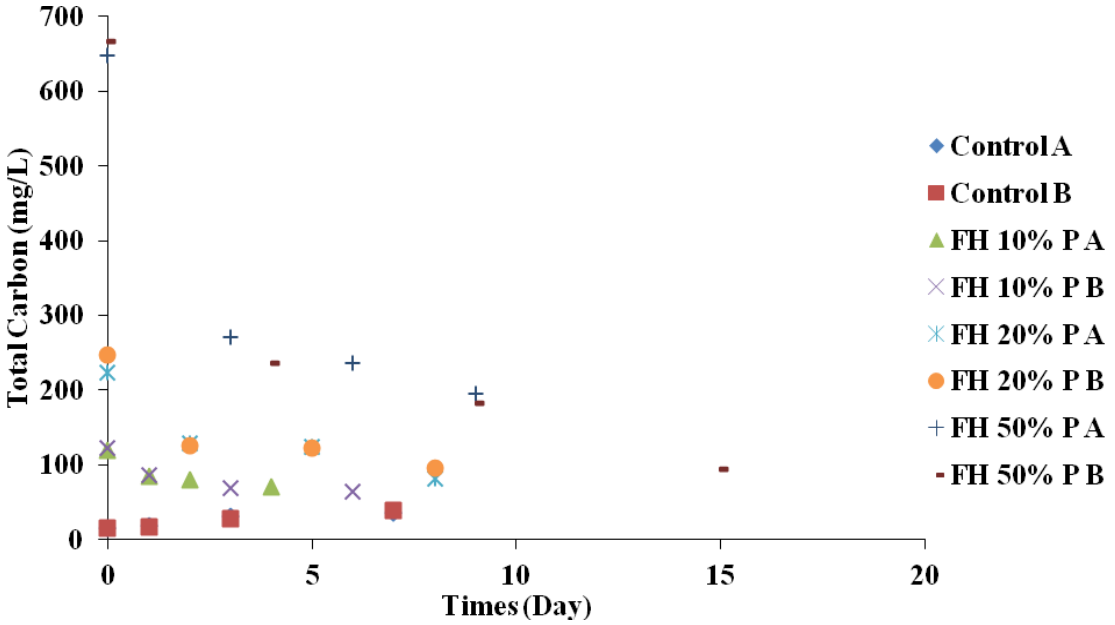


Figure 21. Total Carbon data for the Control Experiment using FH Hydrolysate: *No Algae*.

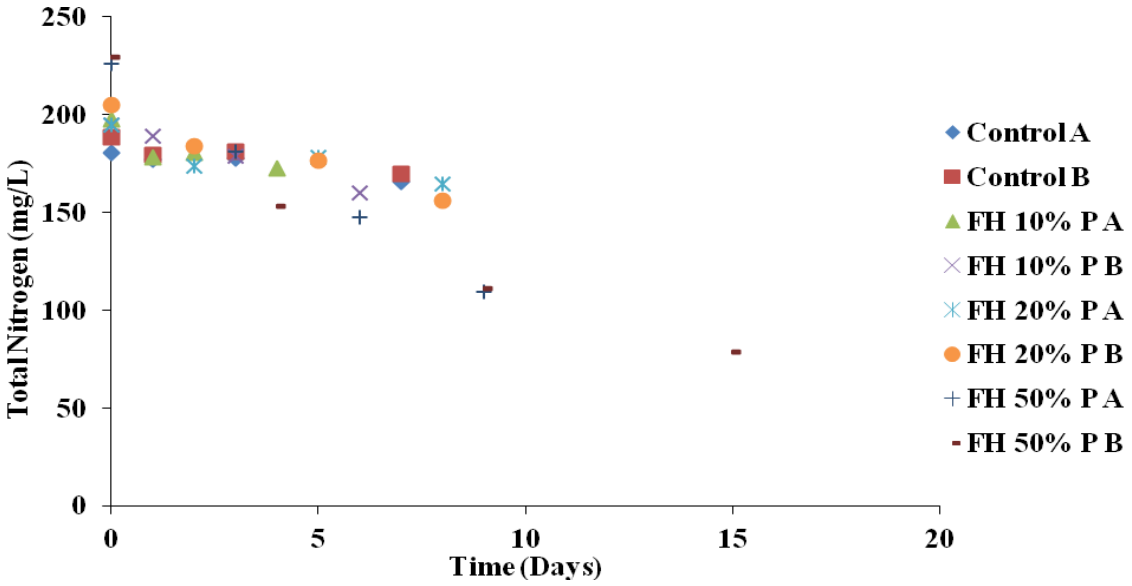


Figure 22. Total Nitrogen data for the Control Experiment using FH Hydrolysate: *No Algae*.

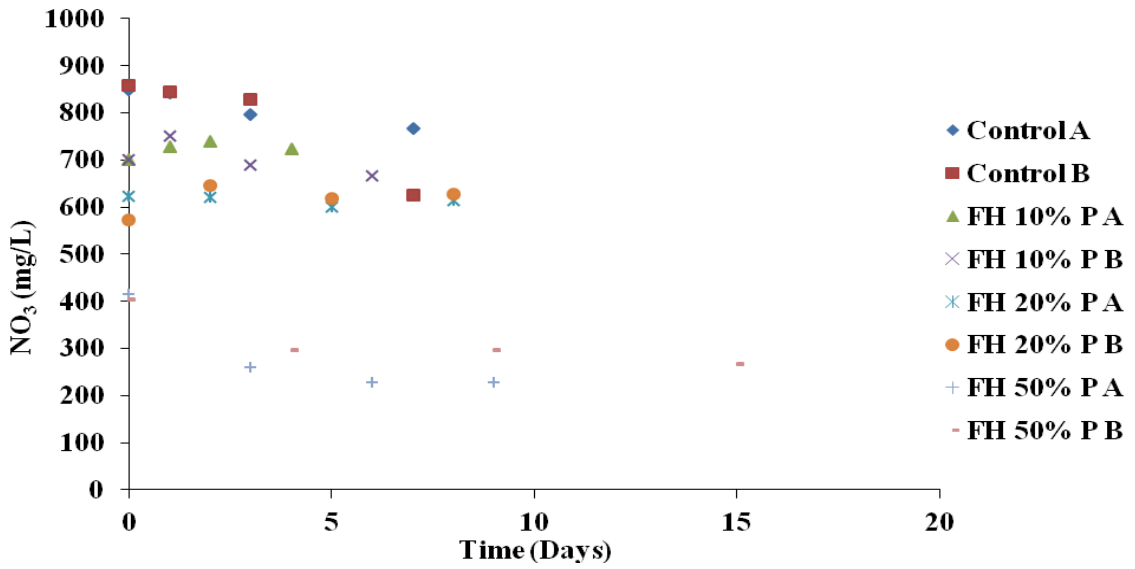


Figure 23. Nitrate data for the Control Experiment using FH Hydrolysate: *No Algae*.

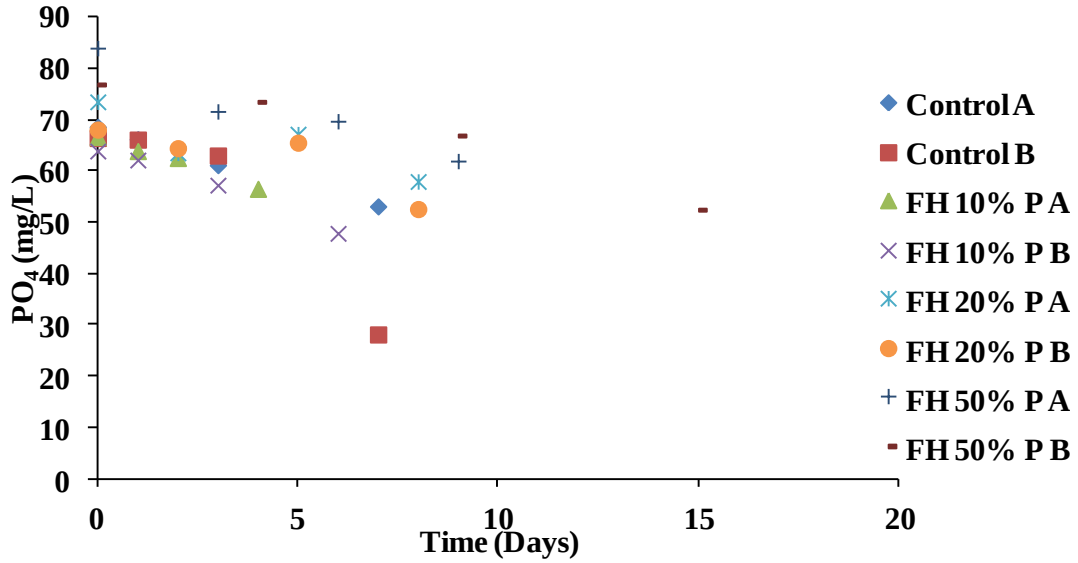


Figure 24. Phosphate data for the Control Experiment using FH Hydrolysate: *No Algae*.

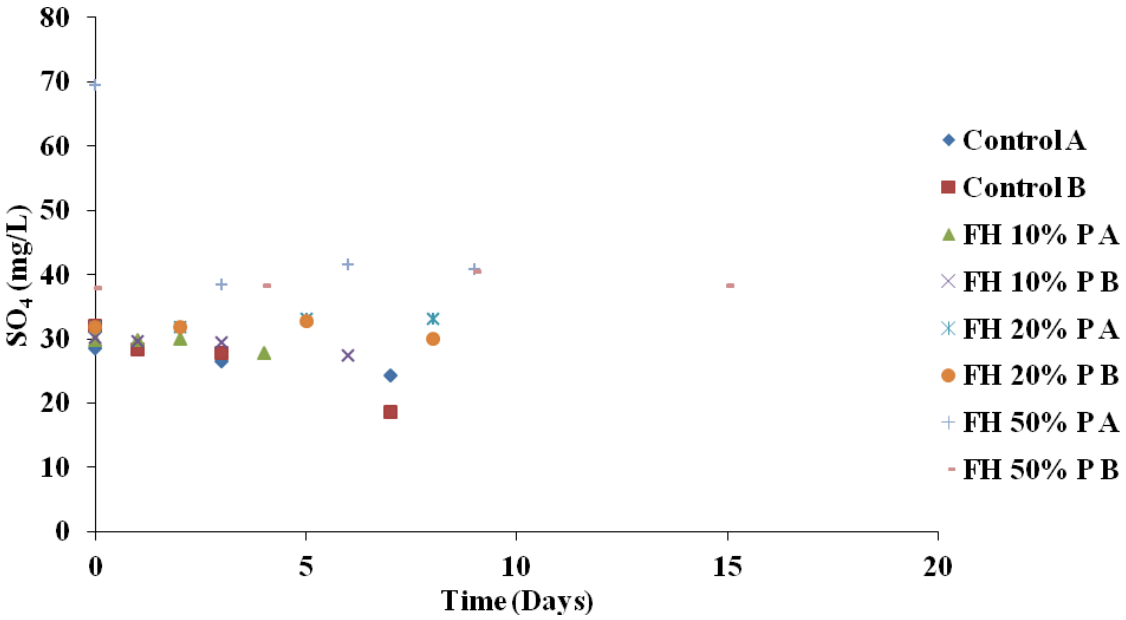


Figure 25. Sulfate data for the Control Experiment using FH Hydrolysate: *No Algae*.

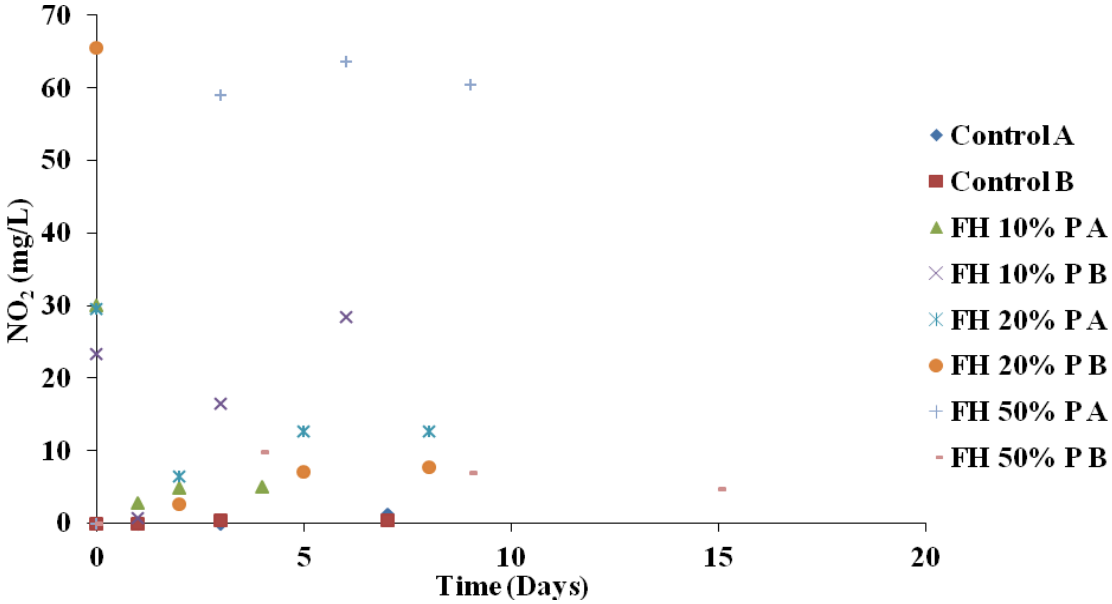
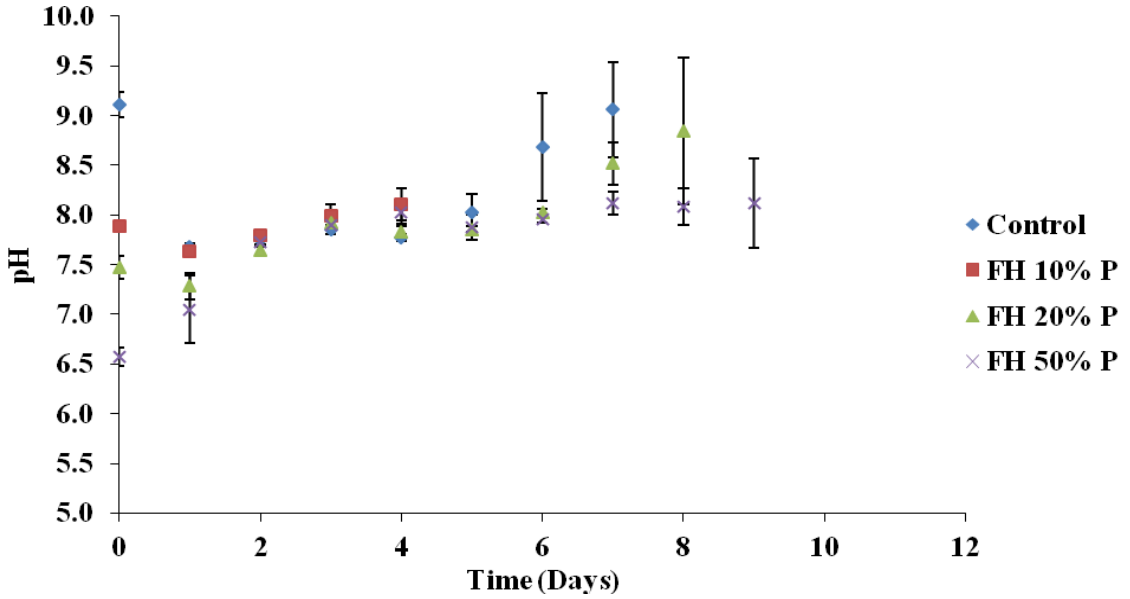


Figure 26. Nitrite data for the Control Experiment using FH Hydrolysate: *No Algae*.



Figure

Figure 27. pH data for the Control Experiment using FH Hydrolysate: No Algae.

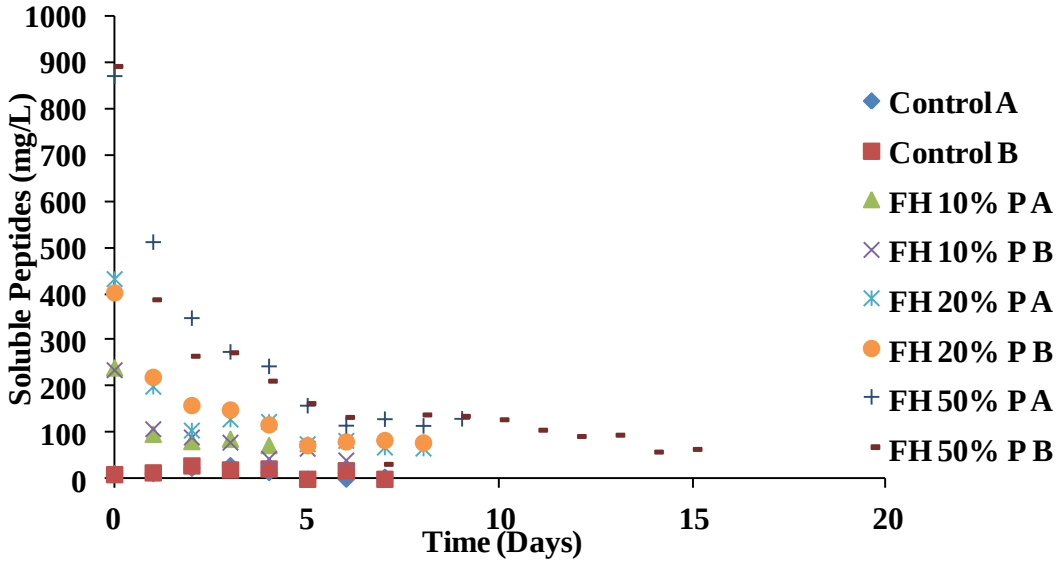


Figure 28. Soluble Peptides data for the Control Experiment using FH Hydrolysate: *No Algae*.

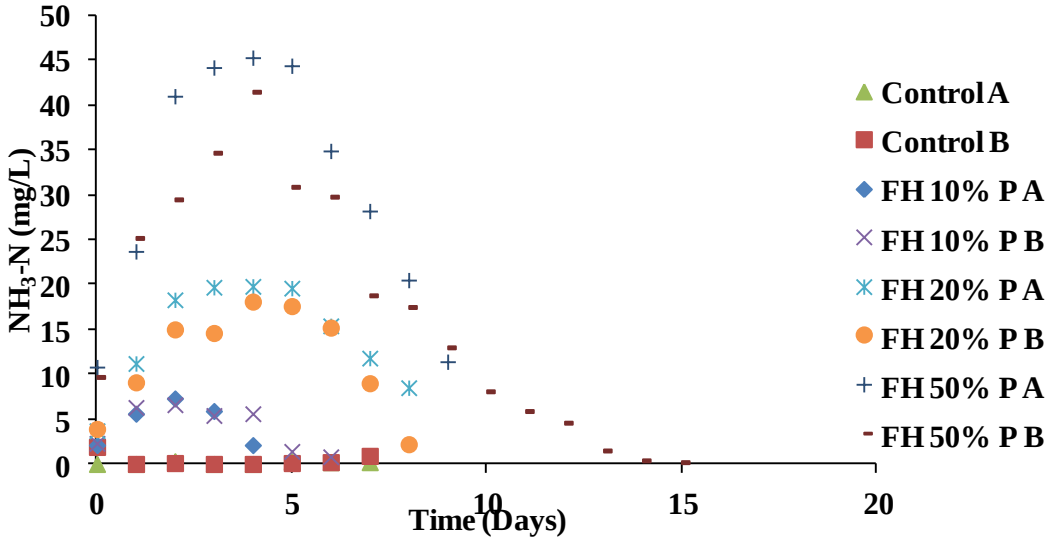


Figure 29. Ammoniacal Nitrogen data for the Control Experiment using FH Hydrolysate: *No Algae*.

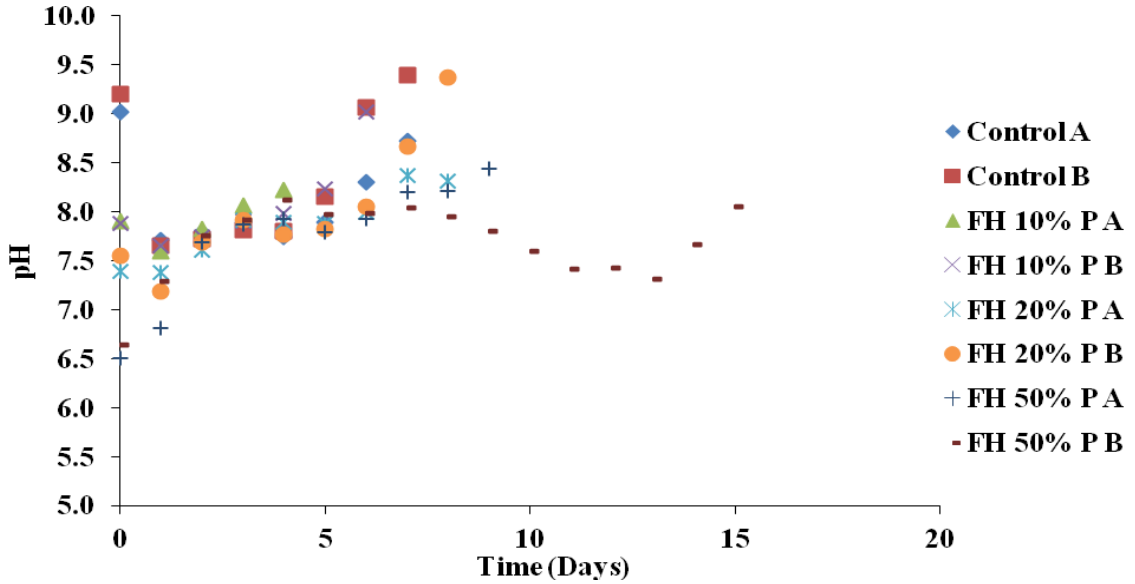


Figure 30. pH data for the Control Experiment using FH Hydrolysate: *No Algae*.

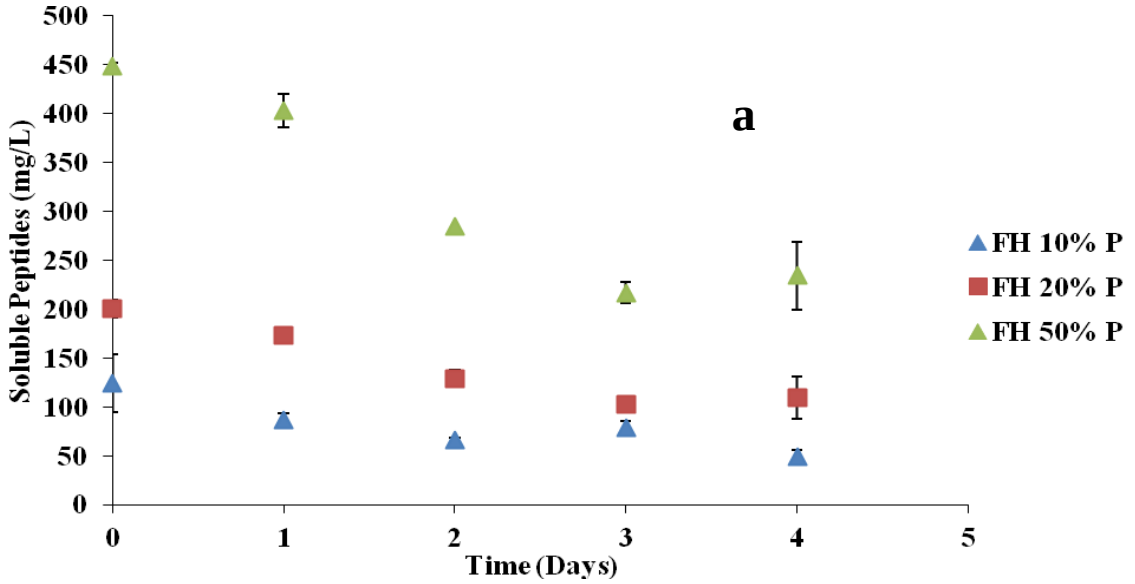


Figure 31. Change in concentration of soluble peptides over time for the second Control Experiment using FH Hydrolysate: *No Algae*.

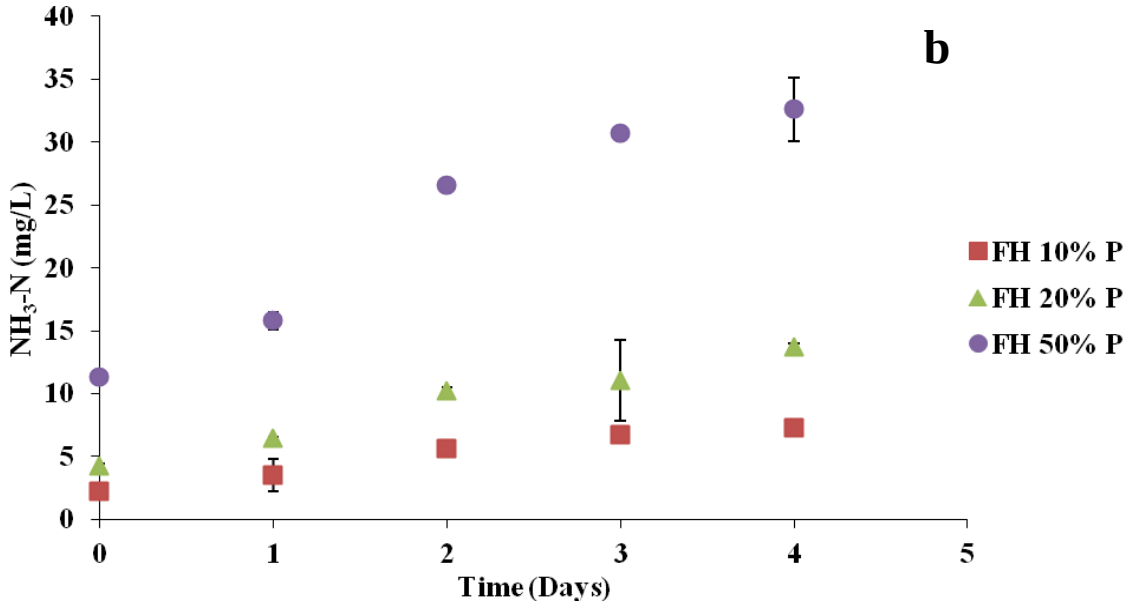


Figure 32. Change in concentration of ammonia over time for the second Control Experiment using FH Hydrolysate: *No Algae*.

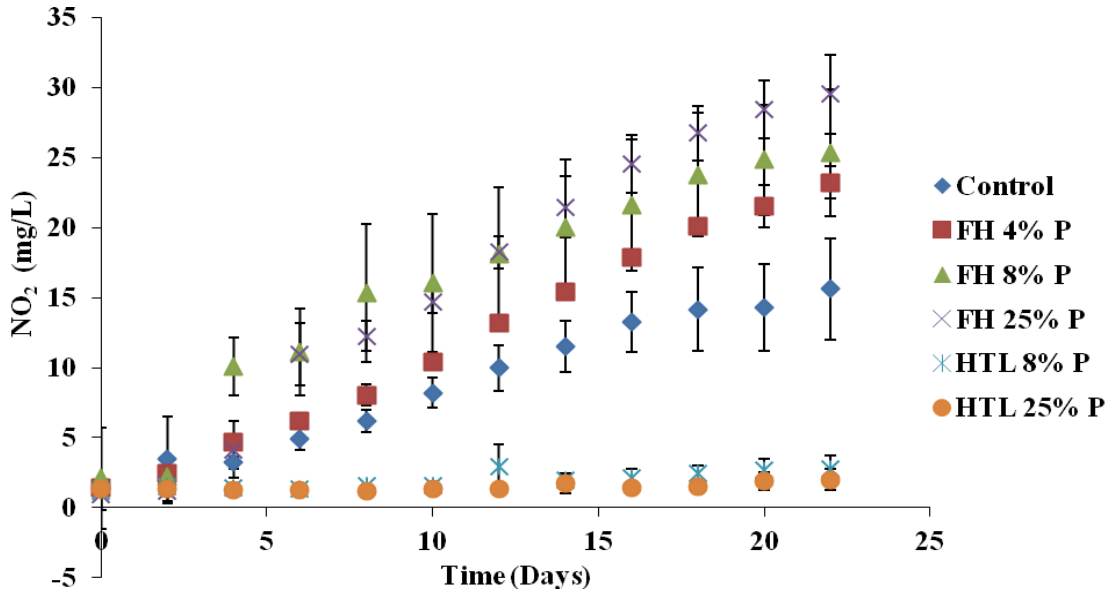


Figure 33. Nitrite for the *Scenedesmus* cultivation using FH Hydrolysate and HTL AP study.

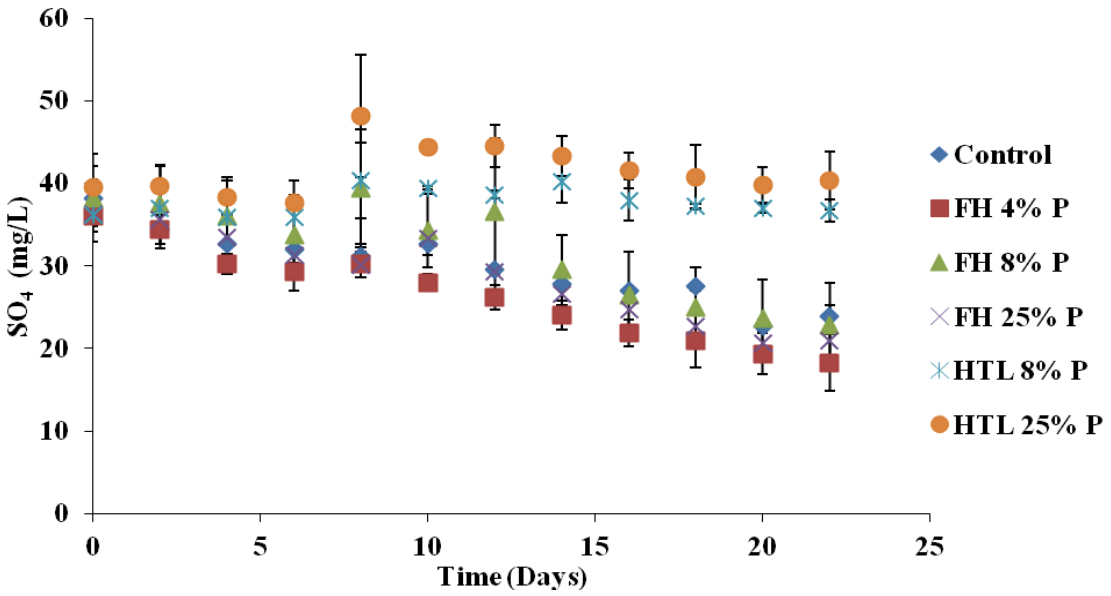


Figure 34. Sulfate for the *Scenedesmus* cultivation using FH Hydrolysate and HTL AP study.

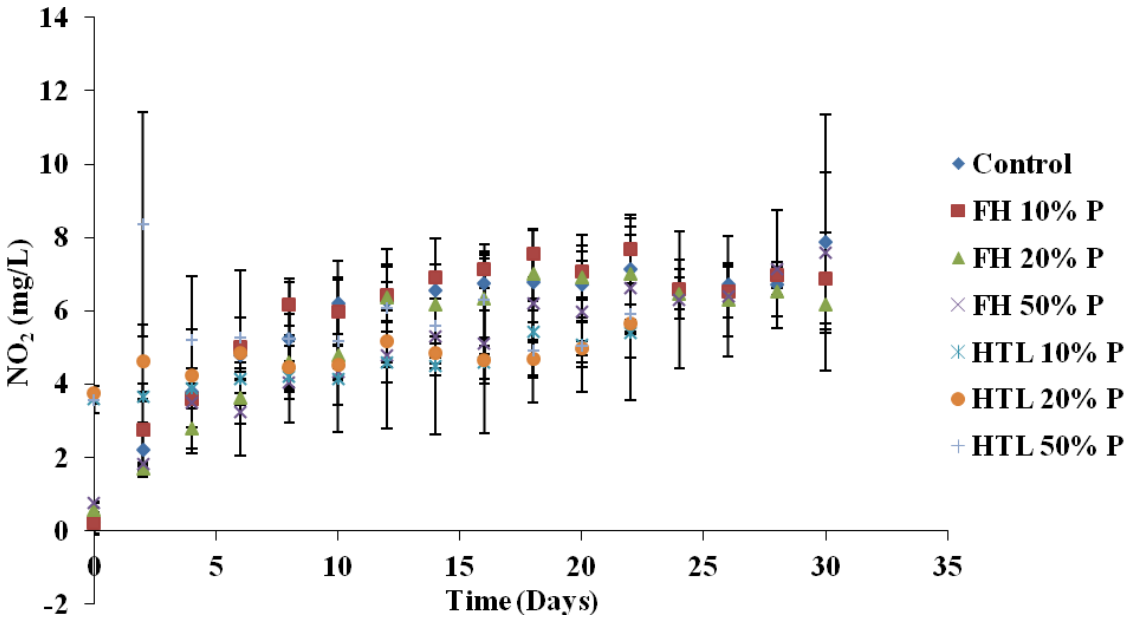


Figure 35. Nitrite for the *Oocystis* cultivation using FH Hydrolysate and HTL AP study.

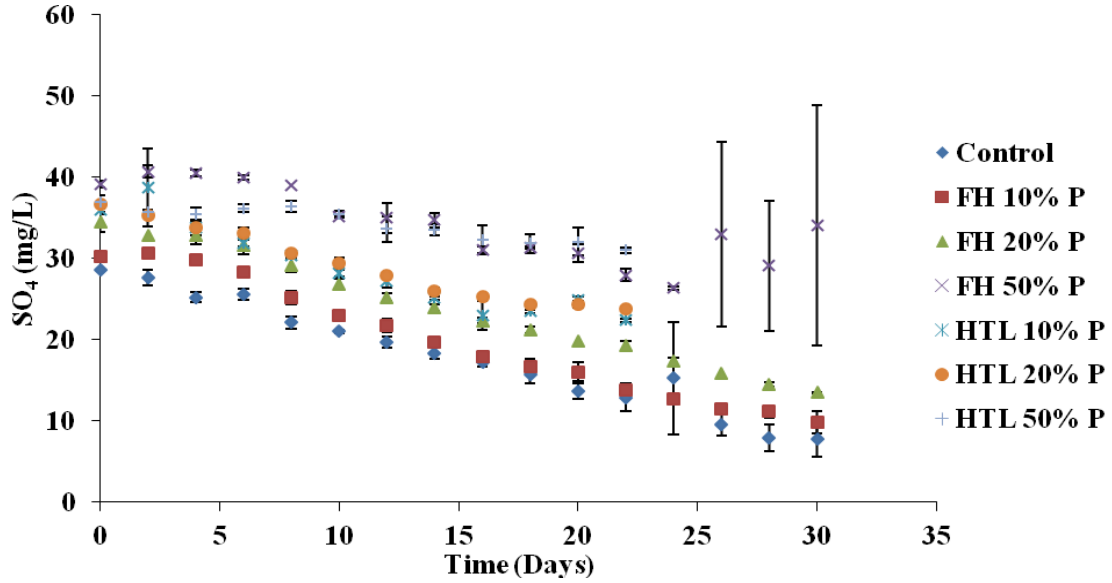


Figure 36. Sulfate for the *Oocystis* cultivation using FH Hydrolysate and HTL AP study.

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Publications

Caleb Talbot, Jose Garcia-Moscoso, Hannah Drake, and Sandeep Kumar, Microalgae Cultivation Using Nutrients Recovered via Flash Hydrolysis, *Algal Research* (manuscript no. ALGAL-S-15-00300, April 2015), under review.

Patents

Bachmann, C.; Talbot, C.; Method and Apparatus for Recovering Non-hydrophilic Components From Algae-Containing Water, US patent application#14/632,633 filed 02/26/15.

Conferences and Presentations

Oral Presentation, American Institute for Chemical Engineers Annual Meeting 2015 at Salt Palace Convention Center, Salt Lake City, Utah. November 2015.