CHARACTERIZING THE EFFECTS OF HYDROTHERMAL PROCESSES ON BIOACTIVE COMPOUNDS IN WASTEWATER BIOENERGY SYSTEMS

BY

MAI THI VIET PHAM

DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Agricultural and Biological Engineering
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2013

Urbana, Illinois

Doctoral Committee:

Assistant Professor Lance Schideman, Chair
Professor Michael J. Plewa
Professor Yuanhui Zhang
Doctor B.K. Sharma

ABSTRACT

The primary objective of this research was to improve our understanding of the water quality effects of thermochemical bioenergy production processes that can be applied to wet organic-laden wastes, such as animal manures, municipal wastewater, and food processing wastes. In particular, we analyzed the impacts of a novel integrated process combining algal wastewater treatment with hydrothermal liquefaction (HTL) on the fate of emerging bioactive contaminants (i.e., pharmaceuticals, estrogenic compounds, antibiotic resistant genetic material, etc.) and the potential for wastewater reuse. We hypothesized and then confirmed that the elevated temperature and pressure of an HTL process can effectively convert the bioactive organic compounds into bioenergy products or otherwise break them down to inactive forms.

High performance liquid chromatography (HPLC) analysis of samples before and after HTL treatment showed removal of specific emerging bioactive compounds (florfenicol, ceftiofur and estrone) to below detection limits when HTL was operated at temperatures 250°C for 60 min or at 300°C for ≥30 min. Complete destruction and/or inactivation of antibiotic resistant genes in wastewaters by the HTL process was also obtained at all tested HTL conditions (250–300°C, 15–60 min reaction time). The presence of HTL feedstock such as swine manure or *Spirulina* algae reduced the removal of bioactive compounds (11–15%) and plasmid DNA (2–3%) when HTL was operated at lower temperatures (250°C) and short retention time (15 min). However, this effect was negligible when HTL was operated at 250°C for 60 min or at 300°C for ≥ 30 min.

Analysis of the organic compounds in the HTL wastewater using liquid-liquid extraction in conjunction with nitrogen-phosphorus derivatization and gas chromatography-mass spectrometry (GC-MS) showed the occurrence of hundreds of nitrogenous organic compounds

(NOCs). Purified chemical reference standards for nine of the most significant NOC chromatography peaks were obtained and then used to positively identify and quantify the concentrations of these predominant NOCs. The chronic cytotoxicity effects of these NOCs were also evaluated using a Chinese hamster ovary (CHO) cell assay as an indicator of mammalian cell cytotoxicity. This analysis found that the rank order for chronic cytotoxicity of these nine NOCs was 3-dimethylamino phenol > 2,2,6,6-tetramethyl-4-piperidinone >2,6-dimethyl-3-pyridinol > 2-picoline>pyridine > 1-methyl-2-pyrrolidinone > σ-valerolactam > 2-pyrrolidinone > ε-caprolactam. However, none of the individual NOC compounds exhibited cytotoxicity at the concentrations found in HTL wastewater (HTL-WW). In contrast, the complete mixture of organics extracted from HTL-WW showed significant cytotoxicity, with our results indicating that only 7.5% of HTL-WW would induce 50% reduction in CHO cell density.

The effect of identified NOCs in HTL-WW on algal growth was also investigated to provide insight on combining algal wastewater treatment with HTL biofuel production. Experimental results showed three out of eight tested NOCs from HTL-WW could cause at least 50% inhibition of algal growth at their detected concentration in HTL-WW. In addition, we found that treatment of HTL-WW with a batch fed algal bioreactor could effectively remove more than 99% of these eight specific NOCs with 7 days or less of treatment.

HTL-WW was also fractionated into hydrophobic and hydrophilic fractions using XAD 8 resin. Algal bioassays with fractionated HTL-WW demonstrated that dissolved organic nitrogen (DON) in the hydrophilic fraction was effectively utilized for algal growth, whereas hydrophobic DON remained nearly constant during the 3 week incubation period. Removal of total nitrogen and dissolved organic nitrogen in the hydrophilic HTL-WW fraction by algal bioreactor treatment was 99% and 82%, respectively. Meanwhile, only 32% removal of total nitrogen was

obtained for hydrophobic HTL-WW fraction during algal bioreactor treatment, and no removal of dissolved organic nitrogen in the hydrophobic HTL-WW fraction was observed.

The effects of three key HTL operating parameters (reaction temperature, reaction time and feedstock solids concentration) on the chemical characteristics and cytotoxicity of HTL-WW was also investigated for fifteen different combinations of operating conditions in the range considered to be practical for bio-oil production. We found that HTL-WW contained a substantial quantity of suspended solids, nutrients and organics. Comparing the three tested HTL operating parameters, the feedstock solids concentration was the most dominant factor in determining the concentration of nutrients and organics in the HTL-WW. The higher the feedstock solids concentration, the more nutrients and organics were found in HTL-WW. Looking at the effect of different operating conditions on cytotoxicity, we found that prolonged reaction times (≥60 min) generally decreased the HTL-WW toxicity. Meanwhile, cytotoxicity generally increased with increases in either reaction temperature or feedstock solids content.

Unfortunately, increases in reaction temperature or solids content also generally enhanced bio-oil yield, such that there will be trade-offs between oil yield and toxicity. There was also a moderate positive correlation ($r=0.58$ and $p=0.024$) between increasing cytotoxicity of HTL-WW and increasing concentrations of chemical oxygen demand (COD) in HTL-WW. We also noticed that operating conditions providing a high bio-oil yield generally produced a more cytotoxic HTL-WW. The most cytotoxic HTL-WW was generated when 35% solids content *Chlorella pyrenoidosa (C. pyrenoidosa)* was liquefied at 300°C for 30 min reaction time. Experimental data also showed that HTL of 35% solids content *C. pyrenoidosa* feedstock could be carried out at 280°C and 60 min reaction time for an advantageous balance of fairly high oil yield and lower cytotoxicity in the HTL-WW.

Treatment of 10% HTL-WW diluted in municipal wastewater with a semi-batch algal bioreactor provided 50% removal of COD and 30% removal of the HTL-WW cytotoxicity. Subsequent post-treatment of algal treated HTL-WW with granular activated carbon provided an additional of 40% removal of COD and 62% removal of cytotoxicity. Thus, a combination of algal and GAC treatment provided of 90% removal of COD and 92% removal of HTL-WW cytotoxicity. These post-treatments of HTL-WW synergistically integrate with HTL bioenergy production because biomass from algal bioreactor processes and the GAC used to treat HTL-WW can both be fed back into HTL to generate additional bio-crude oil. Full strength HTL-WW was also treated via catalytic hydrothermal gasification (CHG),which provided removal of 96.7% of COD and 37.5% of cytotoxicity. All in all, integration of adsorption and algal bioreactor with HTL bioenergy production offers significant potential for an advanced wastewater treatment system that can simultaneously provide significant biofuels, decrease the cytotoxicity and nutrient levels of effluent wastewater and support water reuse applications.

To Father and Mother

For your love, support and understanding

and

To Long Vu and Linh Vu

Who love me, and whom I dearly love.

ACKNOWLEDGEMENTS

It would not have been possible to write this doctoral thesis without the help and support of the kind people around me, to only some of whom it is possible to give particular mention here.

Foremost, I would like to express my deepest appreciation to my advisor, Professor Lance Schideman, for his excellent guidance, caring, patience, and providing me with an excellent atmosphere for doing research. This dissertation would not have been possible without his supervision and support. I would like to thank my co-advisor, Professor Michael Plewa, for guiding my research for the past four years and helping me to develop my background in toxicology. His wisdom, knowledge and commitment to the highest standards inspired and motivated me. I would like to thank my thesis committees: Professor Yuanhui Zhang and Dr. B.K. Sharma for their encouragement, insightful comments, and support. My sincere thanks also go to Professor Helen Nguyen and Dr. Julie Zilles at the department of Civil and Environmental Engineering, UIUC for teaching me basic microbiology techniques and allowing me to use some facilities in their labs.

I thank my friends and colleagues in the Bioeviromental Engineering group: Dr. Zhichao Wang, Jixiang Zhang; Peng Zhang, Wan-Ting Chen, Yan Zhou, Matthew Ong, Chih-ting Kuo , Chao Gai, Young Hwan Shin, and Peng Li, for the stimulating discussions, valuable support, and for all the fun we have had together. I also thank Nanxi Lu and Heather E. Goetsch from the Civil and Environmental Engineering Department, UIUC for their assistant with development of certain methods and some sample analyses. Finally, I thank John Scott, Dr. Wei Zhang, Dr.

Xiaolin Li, and Dr. Yonghong Zhang at the ISTC for their technical assistance with several sample analyses.

I would also like to thank my parents and my older sister, who have always supported, encouraged and believed in me. I love them so much, and I would not have made it this far without them.

Finally, I would like to thank my husband, Long Vu, and my lovely daughter, Linh Vu. Your love, support and constant patience have taught me so much about sacrifice, discipline and compromise. I love you both.

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LIST OF ABBREVIATIONS

AOF	Animal Operating Facility
B	Bulk's medium
BAC	Bioactive Contaminants
BBM	Bold's Basal Medium
BC	Bulk's medium plus Carbenecillin
BOD	Biological Oxygen Demand
BPA	Bisphenol A
CARB	Carbenecillin
CAFO	Concentrated Animal Feeding Operations
CECs	Chemicals of Emerging Concern
CF	Ceftiofur
CHG	Catalytic Hydrothermal Gasification
CHO	Chinese Hamster Ovary
CLSC	Continuous Liquid Scintillation Counting
COD	Chemical Oxygen Demand

DMSO	Demethylsulfoxide
DON	Dissolved Organic Nitrogen
DNA	Deoxyribonucleic Acid
FDA	Food and Drug Administration
E1	Estrone
E2	Estradiol
EBCT	Empty Bed Contact Time
E2-Energy	Environmental Enhancing Energy
FF	Florfenicol
GAC	Granular Activated Carbon
GC/MS	Gas Chromatography /Mass Spectometry
HPLC/MS	High Performance Liquid Chromatography/ Mass Spectrometry
HTL	Hydrothermal Liquefaction
NOCs	Nitrogenous Organic Compounds
OD680	Optical Density at 680 nm
OD750	Optical Density at 750 nm

TN	Total Nitrogen
TIN	Total Inorganic Nitrogen
Tris-EDTA	Tris-Ethylenediaminetetraacetic acid
TSS	Total suspended solid
UCSD	Urbana-Champaign Sanitary District
WHO	World Health Organization

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

1.1.1 Bioenergy Production from Biomass

Bioenergy production is of growing importance for addressing the environmental concerns associated with fossil fuel usage. Biomass is among the most promising feedstocks for renewable energy, especially liquid biofuels, with the potential to contribute up to 35% of the primary energy needs of modern society for industrialized and developing countries, respectively (Voivontas et al., 2001). Biomass resources can be grouped into wood residues, agricultural residues, dedicated energy crops, and municipal/industrial wastes (Easterly & Burnham, 1996). One of the main proposed strategies for bioenergy production from biomass is widespread planting and harvesting of dedicated energy crops, such as switchgrass (*Panicum virgatum L.*) or miscanthus (*Miscanthus X giganteus*). However, large scale production of bioenergy crops can displace food crops on arable land, which could threaten food security. Converting forest lands into plots for bioenergy-related agriculture could actually accelerate climate change by emitting carbon stored in forests, which can cause a carbon debt projected to take more than a century to repay (Fargione et al., 2008). Raising bioenergy crops such as miscanthus and switchgrass also gives rise to water sustainability concerns because they use approximately 45% more water than corn and soybeans (McIssac et al., 2010). Further, there is insufficient arable land to fully replace petroleum with current or next generation bioenergy crops (DOE, 2011). In contrast, the amount of manure generated in the U.S. was estimated to exceed 135 million tons of dry matter per year (USDA, 2011). If this amount of biowastes can be effectively harvested and converted into biofuels, it has the potential to provide more renewable energy than all current U.S. production of renewable energy from wind, solar and biomass. Bioenergy from wastes also

minimizes the competition for arable land between food and fuel, which currently limits first generation biofuels and second generation "energy" crops from any chance of meeting the full national energy need. Therefore, waste-to-energy has been receiving greater attention as a sustainable, secure and cost-effective domestic energy source.

Given the diverse sources and applications of biomass, a variety of conversion technologies are commercially available or under development. Direct combustion of relatively dry biomass feedstocks, such as wood or crop residues, for heating in domestic and industrial application is the most common use of biomass. However, conversion of biomass into gaseous or liquid fuel via thermal, biological and physical processes is also currently gaining considerable interest among energy policy-makers. Some conventional thermochemical methods for fuel production from biomass (e.g., gasification, pyrolysis) require a dry feedstock or they will suffer a large energy penalty from vaporizing the moisture content. However, many renewable biomass sources, such as food processing wastes, municipal wastes, algae, animal manure and human wastewater biosolids all have high moisture contents. Hydrothermal liquefaction (HTL) is one attractive option for conversion of wet biomass into biofuels because they use pressure to avoid vaporizing the water content of wet feedstocks. HTL has been shown to successfully transform wet bio-solids (up to 85% water content) into valuable, self-separating co-products (bio-oil and bio-char). For example, HTL can convert up to 70% of swine manure volatile solids (dry mass basis) into bio-crude oil with heating values between 32–38 MJ/kg, which is 75–90% of petroleum crude heating value (He et al., 2000a; He et al., 2001a; He et al., 2001b; He et al., 2000b; He et al., 2001c) . This conversion of swine waste into bio-crude oil was accomplished with temperatures between 250–350°C, pressures of 8–12 MPa, relatively short retention times (<40 min), and high feedstock water contents (80%). The energy recovery ratio defined as the

energy output of the HTL bio-crude compared to the process heating energy input (E_{out}:E_{in}) was 3:1 (Zhang, 2008) without accounting for any heat recovery.

Successful HTL conversion of algae and cyanobacteria to bio-crude oil has also been achieved without catalysts and at lower temperatures (240°C), lower pressures (7 MPa), and shorter retention times (10 minutes) than previous studies using manure feedstocks (He et al., 2000a; Yu et al., 2011c). Therefore, the net energy balance for HTL of algae would be even more favorable than for swine manure. Additionally, both high-lipid algae (genera *Chlamydomonas*), and lower-lipid algae (genera *Chlorella*, *Spirulina*) can all be successfully converted into bio-crude oil (Yu, 2012). This is extremely important because previous algae biofuel research was mostly focused on growing and extracting oil from high lipid content algae. However, high oil content algae generally have slower growth rates and are more prone to contamination, which are critical limitations for economic viability and practical operations, especially with wastewater derived algae. Therefore, application of HTL technology can resolve several practical limitations associated with current large-scale algal biofuel production. First, it resolves problems associated with contamination of target high-oil biomass because HTL can convert low-oil algae, bacteria and other grazing microorganisms into oil. Secondly, the parasitic energy demand for dewatering/drying is minimized because HTL can use wet biomass directly, and the resulting bio-crude oil is self-separating. Finally, nutrients and CO_2 released to the HTL aqueous and gaseous fractions can be recycled back into algal cultivation, which lowers the input costs for algal cultivation.

1.1.2 Occurrence of Bioactive Contaminants in Animal Wastes

Occurrence of bioactive contaminants such as pharmaceuticals, hormones, and antibiotic resistant genetic material in animal manure and municipal wastewater discharges has been

reported in numerous studies (Andaluri et al., 2012; Brooks & McLaughlin, 2009; Chee-Sanford et al., 2001a; Chee-Sanford et al., 2009; Raman et al., 2001; Raman et al., 2004). Antibiotics are routinely used in the livestock industry to promote animal growth and treat or prevent disease.. According to recent statistics from the U.S. Food and Drug Administration (FDA), up to 80% of antibiotics produced in the USA are used for livestock animals (FDA, 2009), with most used for growth promotion rather than treating illnesses. The Union of Concerned Scientist also reported that about 87% of antibiotics (11.2 million kg) used in United Stated are administered to livestock as growth promoters while only 13% (1.4 million kg) is for human therapeutic and nontherapeutic use (Mellon M, 2001). It is also important to note that a significant fraction (30–90%) of administered antibiotics will be excreted in an unaltered state (Sarmah et al., 2006). Once these residual antibiotics are released into the environment, they can exert selection pressures on microbial communities that foster the development of antibiotic resistance as a defense mechanism (Khachatourians, 1998). Widespread use of antibiotics for animal livestock is a significant public health concern because of the potential to increase antibiotic resistance among pathogenic microorganisms (Wise et al., 1998). According to the U.S. Centers for Disease and Control (CDC), in 2006, just one organism alone, methicillin resistant Staphylococcus aureus (MRSA), killed more Americans (~19,000) than emphysema, HIV/AIDS, Parkinson disease, and homicide combined (Hidron et al., 2008). The Infectious Diseases Society of America (IDSA) also reported that about 2 million Americans develop hospital acquired infections (HAIs) annually, resulting in 99,000 deaths, the vast majority of which are due to antibiotic resistant pathogens (IDSA, 2012a). The estimated economic impact associated with treatment of antibiotic resistant infections has increased from $5 billion in 1998 to $50 billion by 2009 (IDSA, 2012b; Lederberg, 1998).

In addition to antibiotics, the occurrence of natural and synthetic estrogenic hormones at trace levels in the environment has been reported in numerous studies and is of growing concern due to potential adverse effects on the reproductive biology of vertebrates at very low concentrations (10–100 ng/L) (Routledge et al., 1998; Schuh et al., 2011). Excretion of steroidal estrogens from humans and farm animals is the major source of estrogenic compounds in the environment and can potentially contaminate surface and ground water (Finlay-Moore et al., 2000; Hanselman et al., 2004; Raman et al., 2004; Shore et al., 1993). The annual excretion of estrogens from livestock animals such as cattle, pigs, sheep, and chickens, has been estimated to be 39 tons in the European Union and 41 tons in the United States (Lange et al., 2002). High concentrations of estrogenic hormones and their partial breakdown products are often reported in manure containing wastewaters (Bradford et al., 2008; Hanselman et al., 2003b; Hutchins et al., 2007; Kolodziej et al., 2004). For example, concentration of 17α-estradiol and estrone in surface water and well water near a cattle farm had been reported in the range of 0.05–7.4 ng/L and 4.5 ng/L, respectively (Fine et al., 2003; Irwin et al., 2001). Moreover, concentrations of estrogens in wastewater originating from agricultural activities was found to be three to four times higher than municipal wastewaters (Shore et al., 1993). Excretion of antibiotics and estrogenic hormones from agricultural activities is a significant concern as they can contaminate water resources and cause adverse ecological effects at very low concentrations (Irwin et al., 2001; Jobling et al., 1998; Thorpe et al., 2003). Therefore, it is particularly important to better understand the fate, transport, and transformation of these bioactive compounds in livestock systems and to develop management practices that cost-effectively mitigate the associated risks.

1.1.3 Management of Animal Manure and Municipal Solid Waste

Animal manure and wastewater biosolids are often applied to the land as a nutrient source for crop growth and for reducing sewage disposal pressures on landfills, incinerators, and composting facilities. However, there are several potential drawbacks to land application of manure and wastewater biosolids that could discourage use on cropland. First, land application of manure and wastewater biosolids poses a potential risk of spreading bioactive contaminants, pathogens, nutrients and metals to food products and the environment in general. Second, the transportation and handling costs associated with land application of these wastes for nutrient reuse are relative high compared to commercial fertilizer (Risse et al., 2001) because of the high water content in typical manures. Third, there is often not sufficient cropland within the vicinity of major livestock operations to safely dispose all of the manure produced. Therefore, alternative strategies for animal manure and municipal biosolids treatment and management are needed.

On the other hand, animal manure has been recognized as one of the largest potential sources of biomass for bioenergy production. The amount of manure generated in the U.S. at concentrated animal feeding operations (CAFOs) and animal feeding operations (AFOs) is estimated to exceed 135 million tons of dry matter per year (USDA, 2011). HTL conversion animal manure to oil had been demonstrated with swine manure with a mean efficiency of $70 \pm 7\%$ based on dry matter (He, 2001; He, 2000). At this conversion rate, a market hog's lifetime manure could be converted into 50 L (about 0.3 barrel) of crude oil using HTL. If all swine manure in the U.S. were collected and converted using HTL, it would produce 30 million barrels of crude oil annually (Zhang, 2008). The HTL of livestock manure and municipal biosolid to bio-oil is an alternative strategy to composting, incineration, land application, and landfill of the animal manure and municipal wastes. HTL could also reduce environmental

impacts resulting from traditional manure management, such as pathogen release, nutrient pollution, eutrophication, and the discharge of hormones pharmaceuticals and other bioactive compounds to the environment.

1.1.4 Integrated System for Wastewater Treatment and Bioenergy Production

Some recent studies proposed a highly advantageous integrated wastewater treatment scheme referred to as "Environment-Enhancing Energy" (E2-Energy), which integrates wastewater treatment and bio-crude oil production via HTL (Yu et al., 2011a; Zhou et al., 2011a). Figure 1.1 presents a process schematic for this integrated system that simultaneously provides bioenergy production and treatment for animal manure or human waste streams. As shown in Figure 1.1, this novel approach combines algae bioreactor treatment of various wet biowastes with thermochemical treatment of biosolids with heat and pressure to produce a valuable bio-crude oil product and co-product sidestreams of gas, char, and post-HTL wastewater (HTL-WW), which is rich in nutrients and organic compounds. The process diagram begins with a wet biowastes, such as municipal animal manure or domestic wastewater, which is separated into a dilute liquid fraction and a concentrated biosolids fraction. The concentrated biosolids fraction, which still has a moisture content of 70–85%, is converted using hydrothermal liquefaction (HTL) into a self-separating bio-crude oil and/or bio-char, which are valuable co-products. The HTL process also generates a gas rich in CO_2 (> 95%) and an aqueous fraction that is rich in nutrients and organics. The gas and liquid fractions are combined with the original dilute wastewater in the algal bioreactor, which captures the nutrients and organics into mixed culture algal biomass while cleaning the water. Finally, the algal biomass is separated from the purified water and routed back to the HTL process, where it is converted into more bio-crude oil. Our previous research has shown oil conversion efficiencies of 30–75% for algae and biowaste

feedstocks and a positive energy yield of 3–10 times input heat energy (Zhang, 2008). Equally important, previous work has shown that when the algal biomass undergoes liquefaction, it releases most of the nutrients (50–90%) to the HTL-WW (Yu et al., 2011a), so multiple cycles of algae growth can occur on the influent wastewater (Jena et al., 2011b; Zhou, 2010). Thus, this process can multiply the biosolids and biofuel harvested from the wastewater treatment process by up to 10 times, which make the oil potential from biowastes and algae enormous. Based on the current total organic animal waste produced in the United State of 135 million tons per year and the potential to grow additional algal biomass using waste nutrients as described above, this process has the potential to produce enough bio-crude oil to meet the entire national demand (91.2 billion tons/yr.). Meanwhile, the nutrient removal that occurs in this process can provide a higher quality effluent than conventional wastewater treatment, which enhances environmental quality and water reuse potential.

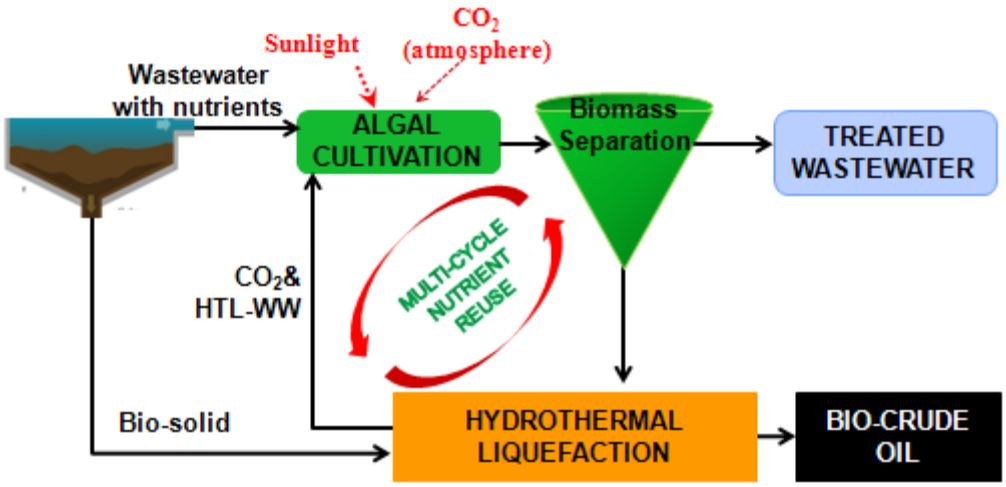

Figure 1.1 Novel integrated process for algal wastewater treatment-bioenergy production, which is referred to as Environment-Enhancing Energy (E2-Energy).

1.1.5 Characterization of Hydrothermal Liquefaction Wastewater

Recycling HTL-WW for algal cultivation is an important component of the E2-Energy system, and a few studies have investigated the feasibility of growing algae in HTL-WW. Zhou et al. found that recycling a higher amount of the aqueous product from HTL swine manure and *Spirulina* feedstocks could inhibit algal growth (Zhou et al., 2010a). Specifically, a supplement of more than 5% (v:v) of HTL-WW from swine manure or more than 1% (v:v) HTL-WW from *Spirulina* into F/2 growth media could inhibit algal growth. Jena et al. found that the aqueous product from HTL conversion of *Spirulina* feedstock has to be diluted to 0.2% in DI water for optimal algal growth (Jena et al., 2011b). These data suggest that the potential to recycle HTL-WW for algal cultivation strongly depends on the specific chemical characteristics of HTL-WW. Therefore, it is important to study the chemical and biological characteristics of HTL-WW.

Past studies showed that HTL-WW contained a substantial amount of nutrient (nitrogen, and phosphate), organics, minerals, and micro-nutrients that are essential for algal growth (Appleford, 2004; Jena et al., 2011b; Zhou et al., 2010b). However, the concentration and compositions of these compounds in HTL-WW are dependent on the feedstock and HTL operating conditions. Since the specific characteristics of HTL-WW will determine the way it was recycled in E2-Energy process, it is of great interest to have a better understanding of the characterization and quantification of organic contaminants in HTL-WW. This study focused on characterizing and quantifying the chemical compositions of HTL-WW with an emphasis on nitrogenous organic compounds (NOCs). NOCs were selected because they can be recycled as nutrients to help support multiple cycles of algal cultivation (Antia et al., 1991; Seitzinger & Sanders, 1997) and are often detected in HTL-WW (Anastasakis & Ross, 2011).

Petroleum and the water found in contact with natural petroleum deposits typically exhibits significant toxicity to aquatic organisms (Girling, 1989; Griffin & Calder, 1977; Henderson et al., 1999; Johnsen et al., 1994; Neff et al., 2006). Thus, it is important to evaluate the toxicity associated with the organic constituents in HTL-WW. Understanding the effects of HTL operating conditions on chemical composition and the toxicity of HTL-WW is also desirable for better recycling of HTL-WW for algal cultivation. Information on the effects of HTL operating conditions on bio-oil yield and toxicity of HTL-WW allows for balancing the desires for maximum bioenergy production and minimum environmental impacts.

1.1.6 Mitigation Measures for Hydrothermal Liquefaction Wastewater

The chemical and biological characteristics of HTL-WW make it not suitable for environmental discharge without extensive treatment. The high concentration of BOD in HTL-WW could directly affect the amount of oxygen in river and streams if HTL-WW is released into these environments. A substantial amount of nutrients (nitrogen and phosphate) in HTL-WW could cause eutrophication of surface waters. In addition, little is known about the potential ecological and health impacts of HTL-WW to aquatic organisms and human. Therefore, it is of great interest to develop and evaluate different treatment schemes for improving the chemical and biological quality of HTL-WW.

Algal-based wastewater treatment has been developed and applied for wastewater from piggery and aquaculture farms (An et al., 2003; Mallick, 2002), paper industry (Tarlan et al., 2002), and carpet industry (Chinnasamy et al., 2010). This was mostly due to the ability of bacteria and algae to utilize, degrade and transform nutrients and organic pollutants in wastewater to produce additional biomass (Ghasemi et al., 2011). Microalgae such as *Chlorella vulgaris* and *Coenochloris pyrenoidosa* have been shown to remove many contaminants,

including phenols, nitrophenols, chlorophenols, and bisphenol A (Hirooka et al., 2003; Hirooka et al., 2006). Algal species such as *Oscillatoria salina, Plectomena terebrabs, Aphanocapsa*, and *Synechococcus* can bio-remediate oil contaminants (Cerniglia et al., 1980a; Raghukumar et al., 2001). HTL-WW contains a significant concentration of carbon, nitrogen and phosphorous, which are essential nutrients for autotrophic growth of algae. In addition, most of the mineral nutrients needed for algal growth were also found in HTL-WW in significant quantities compared to the standard growth media culture media (Jena et al., 2011b). Thus, HTL-WW is a good source of nutrients that can be recycled for growing algae.

Recent studies have explored the feasibility of an integrated wastewater bioenergy system that can sustainably cultivate algal biomass for biofuel production via hydrothermal conversion (Jena et al., 2011b; Roberts et al., 2013; Zhou et al., 2011a; Zhou et al., 2011b). These studies have demonstrated that algal biomass produced during wastewater treatment can be successfully converted into bio-crude oil via hydrothermal liquefaction with a positive energy balance. These integrated systems can recycle the nutrient content of the incoming waste stream to support multiple cycles of algae growth. The present work evaluated the effects of algal wastewater treatment on mitigating nutrients, organics and toxicity of HTL-WW. Batch and semi-batch bioreactors were operated to grow algae in diluted HTL-WW. We anticipated that the toxicity of HTL wastewater would be significantly reduced by treatment with algae that will ultimately be harvested and used as a feedstock for bioenergy production. Through this project we achieved a better understanding of the broader water quality impacts of the proposed integrated wastewater treatment-bioenergy production system.

Even though biological treatment of HTL-WW with algal bioreactors could remove nutrient and organic contaminants, there are still recalcitrant organic compounds that are not

readily biodegraded by bacteria and algae. Thus, employing physical removal process such as membrane biological reactors or biological activated carbon may be needed for complete removal of these compounds. Previous researches have demonstrated that GAC can effectively remove various organic contaminants of concern (Aksu, 2005; Tryba et al., 2003). More importantly, GAC does not generally produce any harmful byproducts and can be readily recovered, regenerated, and reused. In this study, we explored the use of granular activated carbon (GAC) to capture organic contaminants in HTL-WW and mitigate its toxicity. GAC can be used as a subsequent treatment of algal treated HTL-WW or in conjunction with microbial biodegradation processes in the algal bioreactors. In the latter case, the biological removal of adsorbed organics can provide continuous in-situ regeneration of GAC adsorption capacity. Finally, both activated carbon and any biomass grown during treatment of HTL-WW can be recycled back to the HTL process to produce more bio-oil.

The current study also investigated the application of catalytic hydrothermal gasification (CHG) for treatment of HTL-WW, which has not been investigated previously. CHG is a promising technology for converting waste biomass to synthetic natural gas (SNG). Under hydrothermal conditions, water with temperature and pressure near or above its critical point ($T > 374°C$ and $P > 22$ MPa) will act as a homogeneous non-polar solvent of high diffusivity and high transport properties that can convert organic compounds into gases (Hodes et al., 2004; Kritzer & Dinjus, 2001; Savage, 1999; Watanabe et al., 2004). Therefore, organic residues in HTL-WW can be gasified and the toxicity associated with these compounds could be significantly reduced or eliminated.

1.2 RESEARCH OBJECTIVES AND APPROACHES

The primary aim of this research is to characterize the effects of HTL treatment on bioactive contaminants in wastewater bioenergy system, which has not been previously reported. Specifically, we investigated the effect of HTL operating conditions on removing bioactive compounds in biowastes and disrupting antibiotic resistant gene transfer. Our working hypothesis is that the elevated temperature and pressure of HTL processes (250–350°C and 10–20 MPa) can degrade and deactivate a wide range of organic compounds as well as antibiotic resistant genetic materials. Thus, health and ecosystem risks associated with pharmaceuticals and antibiotic resistance in human and livestock wastewaters can be mitigated and water reuse potential can be enhanced. In addition, costs associated with antibiotic resistant treatments and the development of new antibiotics could be reduced over the long-term.

Another important issue to address is whether the HTL treatment of biowastes and algae generates any new toxic compounds not present in the original feedstock. In order to answer this question, we focused on characterizing and quantifying the chemical composition of HTL-WW and evaluating the toxicity associated with the organic constituents in HTL-WW. Different remediation strategies to improve the chemical and biological quality of HTL-WW were also investigated. The current study provides essential evidence to support the feasibility of a novel integrated wastewater treatment and bioenergy production system. Finally, the outcome of this work will support the national goals of increasing bioenergy production, improving water quality, and enhancing the potential for beneficial reuse of wastewaters. Specific objectives and approaches for this research are summarized below:

1. Investigate the effects of hydrothermal liquefaction on fate of bioactive contaminants in biomass feedstocks

- Investigate the impacts of HTL under different practical operating conditions on the fate of bioactive contaminants (antibiotics and estrogens). Specifically, we focused on the effect of HTL temperature (250–350°C), and retention time (15–60 min), on the removal or conversion of pharmaceuticals and estrogenic compounds into valuable bio-crude oil. The emerging contaminants investigated include bisphenol A (BPA), estrone (E1), ceftiofur (CF), and florfenicol (FF) which have been widely detected in human and animal waste. High performance liquid chromatography (HPLC) was the primary method used to separate these emerging contaminants and measure their concentrations before and after HTL treatment.
- Demonstrate the effect of HTL on destructing antibiotic resistant genes and disrupting antibiotic resistant gene transfer. Plasmid DNA with antibiotic resistant coding was extracted from pure *Escherichia coli* (*E.coli*) culture and manure slurry samples and was transferred into common environmental bacteria, such as *E. coli* and *Azotobacter vinelandii*. Both natural transformation and high-efficiency electroporation transformation of plasmid DNA before and after HTL-treated DNA were measured to elucidate the effect of HTL on the transfer of antibiotic resistant genes.

2. Characterize the chemical and biological characteristics of wastewater from hydrothermal liquefaction conversion of biomass to biofuels

- Characterize and quantify the organic nitrogen composition of HTL-WW. Nitrogenous organic compounds (NOCs) in HTL-WW were identified and quantified using a general

scan and high resolution gas chromatography–mass spectrometry (GC/MS) measurements with known analytical standards used for quantification of specific compounds.

- Investigate the mammalian cytotoxicity and algal inhibition effects of detected NOCs and the whole organic mixture in HTL-WW, as well as the hydrophobic and hydrophilic fractions from HTL-WW Bioassays with Chinese hamster ovary (CHO) cells were used to quantify mammalian cytotoxicity of NOCs identified by GC-MS and the complex organic mixture extracted from HTL-WW.
- Algal growth/inhibition tests with specific NOCs and the complex organic mixture were also conducted using *Chlorella protothecoides.*

3. Evaluate the effects of HTL operating conditions on the quality of HTL-WW

- Characterize effect of HTL operating condition on chemical quality of HTL-WW. Specifically, we focused on the effect of HTL temperature (260–300°C), reaction time (30–90 min), and solids concentration (15–35%) on the chemical composition and cytotoxicity of HTL-WW. Typical water quality parameters (total nitrogen, total phosphorous, total suspended solid and chemical oxygen demand) were determined using standard methods.
- Characterize effects of HTL operating conditions on HTL-WW cytotoxicity. Bioassays with CHO cells were used to quantify cytotoxicity of HTL-WW generated from different operating conditions.

4. Develop and evaluate treatment processes for improving HTL-WW quality

- Study the algal uptake of hydrophobic and hydrophilic dissolved organic nitrogen in HTL-WW. HTL-WW was fractionated into hydrophobic and hydrophilic fractions and used to cultivate algae. The removal of dissolved organic nitrogen, inorganic nitrogen, and total nitrogen in hydrophilic and hydrophobic fractions by bacteria and algae was monitored and compared.
- Assess the use of algal bioreactor and activated carbon adsorption for treating HTL-WW diluted with municipal wastewater. Algal wastewater treatment bioreactors were operated to measure algal uptake of NOCs overtime. Semi-batch algal reactors followed with granular activated carbon (GAC) adsorptive treatment were operated to remove organic contaminants and reduce the cytotoxicity of HTL-WW.
- Assess the use of catalytic gasification for treatment of HTL-WW. Direct CHG of HTL-WW at 350°C for 60 min reaction time with a Ra-Ni catalyst was conducted to convert organic residues from HTL-WW into a combustible gas and reduce the toxicity of the HTL-WW.

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CHAPTER 2: LITERATURE REVIEW

2.1 FATE OF BIOACTIVE COMPOUNDS IN ANIMAL WASTES

2.1.1 Agricultural Use of Antibiotics and Estrogenic Compounds

Since the early 1950s, antimicrobial agents and hormones have been used widely in agriculture to treat and prevent disease, promote growth, and enhance feed efficiency in food animals. Addition of antibiotics into animal feed enhances the animal's ability to absorb feed and thus grow faster. In addition, supplementing antibiotics in animal feed helps decrease animal's susceptibility to the effect of crowded living conditions and poor hygiene in intensive animal agriculture (EMS, 2000). According to a recent reported of the U.S. Food and Drug Administration in 2009, a total of 36 million pounds of antibiotic is produced in the U.S and a major portion of this amount (80%) are used in food animal production (Table 2.1).

A significant portion (90%) of antibiotics used for food animals is given as a supplement to promote growth in food animals rather than to treat infection. Commonly used antibiotics in animal agriculture are listed in Table 2.2. The recommended levels of antibiotics for feeds were just 5–10 ppm in the 1950s but have been increased by 10 to 20-fold since then (Khachatourians, 1998). A recent study found that sub-therapeutic levels of antibiotics in feed (3–220 g/Mg feed) is sufficient to enhance animal's growth and immunization (McEwen & Fedorka-Cray, 2002). For example, supplement of antibiotics at a low level (e.g., 35–100 mg of bacitracin, chlortetracycline or erythromycin per head per day or 7–140 g of tylosin or neomycin per ton of feed) to high energy feed for meat and dairy cattle can enhance the rate of weight gain and feed efficiency 3–5%. Addition of 2–500 g of antibiotics (bacitracin, chlortetracycline, erythromycin, lincomycin, neomycin, oxytetracycline, penicillin, streptomycin, tylosin or virginiamycin) to each ton of swine feed and 1–400 g of the same antibiotics per ton of poultry feed, is sufficient to

improve their growth (JR., 1997). Antibiotics are often given to animals at concentrations higher than recommended despite the fact that the low concentration of antibiotics can still encourage selection of antibiotic resistant bacteria. For example, in an examination of 3328 feeds in the US National Swine Survey, up to 25% of the feeds contained antibiotics at concentrations higher than the recommended levels (Dewey et al., 1997).

The scale of agricultural use of antibiotics is also enormous as compared to human use. It has been estimated that the annual quantity of antibiotics used in animals is about 100–1000 times that in human population (Feinman, 1998; Levy, 1998; Witte, 1998). Extensive use of antibiotics in agriculture has raised a concern of antibiotic resistance genes in animal production and the potential risk to human health. Evidence of resistant infections in people associated with agricultural use of antibiotics has emerged over recent decades. According to the Center for Science in the Public Interest (CSPI) there were 38 foodborne outbreaks between 1973 and 2011 and antibiotic resistant patterns were established in 34 of those 38 outbreaks (CSPI, 2012). The bacteria that caused the outbreaks were resistant to 15 different antibiotics, nine of which are classified by the World Health Organization (WHO) as "critically important" to human medicine. More than 20,000 people were sickened in these outbreaks; 3108 were hospitalized and 27 died. According to a report by the WHO, approximately two million Americans are infected annually with antibiotic resistant pathogens, and approximately 14,000 of them die as a consequence of these infections (WHO, 2000). As a result, the annual cost of treating all drug resistant infections in the U.S. has been estimated at $100–200 million (Cassell, 1997). These facts lead to the conclusion that increased use of antimicrobial agents in agriculture increases the risk of resistant infections in people. Therefore, it is of great interest to have a better control of

agricultural uses of antibiotics and develop control measures to mitigate the spread of antibiotics and antibiotic resistant genes into the environment.

Table 2.1 Antibiotic uses in united state for human and animals (FDA, 2009)

Drug class	Kilograms	Pounds	% of total
FOOD-ANIMAL USE			
Aminoglycosides	339,678	748,862	2%
Cephalosporins	41,328	91,113	0%
Ionophores	3,740,627	8,246,671	23%
Lincosamides	115,837	255,377	1%
Macrolides	861,985	1,900,352	5%
Penicillins	610,514	1,345,953	4%
Sulfas	517,873	1,141,715	3%
Tetracycline	4,611,892	10,167,481	28%
NIR	2,227,366	4,910,501	14%
Sub-total	13,067,100	28,808.024	79.8%
HUMAN MED USE	3,300,000	7,275,255	20.2%
TOTAL	16,367,100	36,083,279	100%

Table 2.2 Commonly used antibiotics for therapeutic or sub-therapeutic purposes in food animals

Purpose	Cattle	Swine	Turkey/Chicken	Fish	Sheep
Treatment of Infection	Amoxicillin* Cephapirin Erytromycin* Flouroquinolone Gentamicin* Novobioc Penicillin Sulfonamid Tilmico Tylosin	Amoxicillin* Ampicillin* Chlortetracycline* Gentamicin* Lincomycin lfamethazine Tiamulin Tylosin	Erthythromycin* Flouroquinolone Gentamycin* Neomycin Penicillin* Spectnomycin Tetracyclines Tylosin Virginiamycin	Ormetoprim Sulfonamide Oxytetracycline	Chlortetracycline* Erythromycin* Neomycin Oxytetracycline* Penicillin*
Growth and feed efficiency	Bacitrac Chlortetracycline* Lasalocid Monensin Oxyteracycline* Amoxicillin* Ampicillin* Bacitrac Ceftiofur Dihydrostreptomycin Erythromycin* Furamazone Gentamycin* Neomycin Penicillin* Streptomycin* Tilmicosin	Asasanilic acid Bacitracin Bambermycin Chlortetracycline* Erthythromycin* Penicillin* Tiamulin Tylosin Virginiamycin	Bambermycin Bacitracin Chlortetracycline* Penicillin* Tylosin Virginiamycin		

*Also used in human

Steroid hormone drugs, including natural estrogen, progesterone, testosterone, and their synthetic versions, have been used in animal food production since the 1950s to increase the animals' growth rate. After administered, a significant portion of these compounds was excreted together with animal's manure or urine. Elevated levels of hormones such as 17β-estradiol and estrone had been detected in manure excreted by poultry or livestock (Nichols et al., 1997; Peterson et al., 2000; Short & Colborn, 1999; Ternes et al., 1999a; Ternes et al., 1999b). The concentration of excreted hormone is generally in the range of milligrams per animal per day (Hanselman et al., 2003a; Lange et al., 2002). The annual excretion of estrogens from livestock animals such as cattle, pigs, sheep, and chickens, had been estimated to be 39 tons in the European Union and 41 tons in the United States (Lange et al., 2002). The potential endocrine-disrupting effects of estrogenic compounds in animal manure used as agricultural fertilizer have become an environmental concern as they can contaminate water resources and cause adverse ecological effects at very low concentrations (Irwin et al., 2001; Jobling et al., 1998; Thorpe et al., 2003). For example, 17-β estradiol and estrone could induce vitellogenin production in male at concentrations as low as 1 ng L^{-1} (Purdom, 1994). In addition, mixtures of estrogenic compounds in animal waste may act in combination to produce synergistic estrogenicity response (Daughton & Ternes, 1999; Sumpter & Jobling, 1995). Therefore, it is also equally important to investigate the fate of estrogenic compounds in animal waste.

2.1.2 Fate and Transport of Bioactive Compounds in the Environment

Antibiotics are poorly adsorbed by animal gut, resulting in as much as 30–90% of the parent compound being excreted via feces or urine (Alcock et al., 1999; Elmund et al., 1971). Many of these drugs persist after storage and/or treatment of these wastes and are released into

the environment, where they can exert selective pressures on microbial communities and cause them to develop antibiotic resistance as a defense mechanism. According to the United States Department of Agricultural (USDA), about 1.32 billion Mg of manure is produced annually. The presence and persistence of antibiotics in this large quantity of manure present a significant environmental concern in term of toxicity of these antibiotics to soil microbial communities as well as to an increase in antimicrobial resistance in the environment. Figure 2.1 show the possible entry pathways of antibiotics and estrogenic compounds into the terrestrial and aquatic environment (Kemper, 2008). As demonstrated in Figure 2.1, land application of manure is the dominating pathway for the release of antibiotics and estrogenic compounds in the terrestrial environment. Once applied into the land, antibiotics in manure can accumulate in the soil, be rinsed off into surface water, or leached to ground water where they can impact both human and environmental health (Jongbloed & Lenis, 1998). Similarly, residual of estrogenic compounds in animal manure can reach to surface and ground water via runoff and leaching. (Lange et al., 2002). High concentrations of estrogenic hormones and their partial breakdown products are often reported in manure containing wastewaters (Bradford et al., 2008; Hanselman et al., 2003b; Hutchins et al., 2007; Kolodziej et al., 2004).

Detection of antibiotics and estrogenic compounds in animal manure and soil has been well documented. For example, tetracyclines, sulfonamides, β-lactams, macrolides, and ionophores are antimicrobial classes that commonly detected in manure wastes (K. Kumar, 2005; M.T. Meyer, 1999). Residues of tetracyclines, sulfonamides and lincomycin residues were also found in swine storage lagoon samples at a concentration approached 1 mg/L (Campagnolo et al., 2002). Table 2.3 shows the detection of antibiotics in pig manure samples from different regions. As shown in Table 2.3, antibiotics were detected in pig manure at a high frequency of

40–60% and a maximum concentration of 59 mg/kg. Table 2.4 represents the detection of antibiotics in soil fertilized with pig manure. As demonstrated in Table 2.4, the concentration of detected antibiotics in soil environment varied significantly and was considerably lower than in manure. The data also suggest that the persistence of antibiotics in soil environment depends on their chemical characteristics and the soil environments.

The occurrence of antibiotics in the aquatic environment have also been reported in numerous studies (Ashton et al., 2004; Golet et al., 2001; Hirsch et al., 1999; Kolpin et al., 2002; Olsen et al., 2001; Rice, 2001; Sacher et al., 2001; Teuber, 1999). For example, a wide range of antibiotics had been detected in all surface water samples collected in Northwest Germany, including sulphonamides, macrolides and lincosamides (Christian et al., 2003). Detection of tetracycline resistant bacteria in lagoons and groundwater underlying two swine production facilities was reported (Chee-Sanford et al., 2001). Tetracycline residues and tetracycline-resistance genes were also found in groundwater impacted by swine production facilities (Mackie et al., 2006). Table 2.5 summarizes different classes of antibiotics that have been detected and quantified in various water environments. As demonstrated in Table 2.5, the concentration of antibiotics in aquatic environment was found to be 5–50 ng/L, which is considerably lower than that in manure or soil environment. Despite the information about the concentration and classes of antibiotic detected in animal manure, little is known about the effects of antibiotics in manure on resistance levels of environmental bacteria. However, quantitatively the massive input of selective agents, antibiotic resistant bacteria, and antibiotic resistant genes with manure could well contribute to the antibiotic resistance problem in human and veterinary medicine. The fate of antibiotic resistant bacteria, in particular their resistance genes and mobile genetic elements introduced via manure into soil and water environment, is

also not well studied. Thus, it is particularly important to better understand the fate, transport, and transformation of antibiotics in livestock systems and to develop management practices that cost-effectively mitigate the risks associated with them.

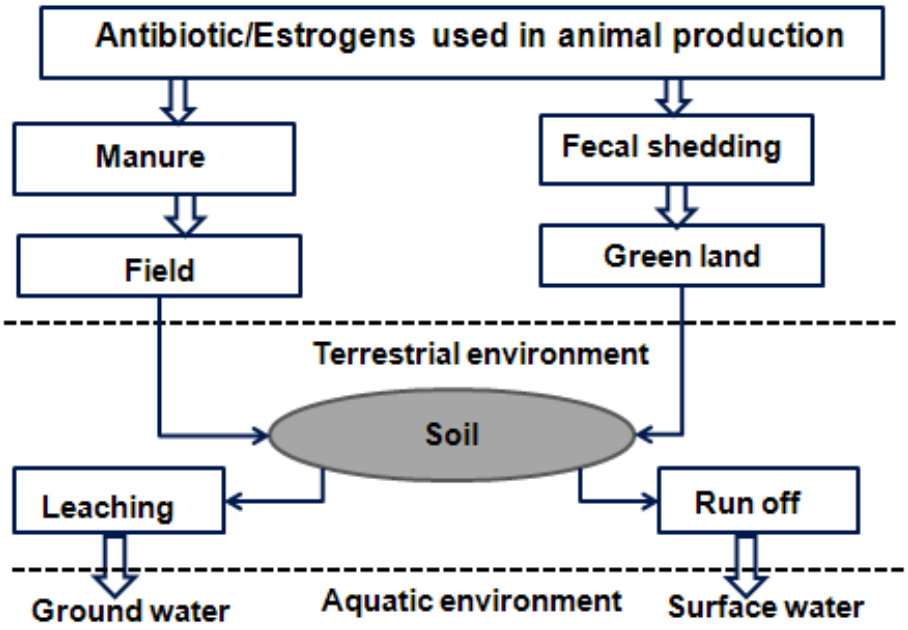

Figure 2.1 Environmental pathways of agricultural antibiotics and estrogens (Kemper, 2008)

Table 2.3 Detection of antibiotic compounds in pig manure samples (Heuer et al., 2011)

Country	Antibiotic compound	Sample size	Detection frequency [%]	Max. Conc. [mg kg^{-1}]	Reference
China	Enrofloxacin	61	49	33	(Zhao et al., 2010)
	Sulfamonomethoxine	61	48	4	
	Oxytetracycline	61	41	59	
Germany	Tetracyclines	305	54	53	(Hölzel et al., 2010)
	Sulfonamides	305	51	38	
Austria	Chlortetracycline	30	57	46	(Martínez-Carballo et al., 2007)
	Sulfadimidine	30	60	20	

Table 2.4 Detection of antibiotics in soils fertilized with pig manure (Heuer et al., 2011)

Country	Antibiotic compound	Sample size	Max. Conc. [µg kg^{-1}]	Reference
USA	Sulfamethazine	43 samples from 2 farms	0.7	(Shelver et al., 2010)
Denmark	Chlortetracycline	several samples from 2 fields	20–30	(Halling-Sørensen et al., 2005)
Denmark	Tylosin A	several samples from 2 fields	25–50	(Halling-Sørensen et al., 2005)
Germany	Tetracycline	1 field sampled in several years	295	(Hamscher et al., 2005)
Germany	Chlortetracycline	1 field sampled in several years	39	(Hamscher et al., 2005)
Germany	Sulfamethazine	1 field sampled in several years	2	(Hamscher et al., 2005)

Table 2.5 Antibiotics detected in water environments (Kemper, 2008)

Class	Substance	Conc. (ng/l)	Source	Literature
Macrolides	Lincomycin	21,100	Surface water	(Boxall, 2005)
		730	Surface water	(Kolpin et al., 2002)
	Clarithromycin	260	Surface water	(Hirsch et al., 1999)
	Erythromycin	1,700	Surface water	(Hirsch et al., 1999)
	Roxithromycin	560	Surface water	(Hirsch et al., 1999)
	Tylosin	50	Surface water	(Ashton et al., 2004; Daughton & Ternes, 2000)
Sulphonamides	Sulphadiazine	4,130	Surface water	(Boxall, 2005)
	Sulphamethazine	240	Ground water	(Hamscher et al., 2005)
	Sulphamethoxazole	410	Groundwater	(Sacher et al., 2001)
		480	Surface water	(Hirsch et al., 1999)
Trimethoprim		20	Surface water	(Boxall, 2005)
		200	Surface water	(Hirsch et al., 1999)
Fluorochinolones	Ciprofloxacin	405	Effluents	(Golet et al., 2001)
	Norfloxacin	120	Effluents	(Golet et al., 2001)
		120	Surface water	(Kolpin et al., 2002)
Tetracyclines	Tetracycline	400	Groundwater	(Krapac, 2005.)
	Oxytetracycline	32,000	Overland flow water	(Kay et al., 2005)
	Chlortetracycline	690	Surface water	(Kolpin et al., 2002)

Table 2.6 Detection of antibiotic resistant genes in water environment

Antibiotic resistant genes	Biological sources	Env. sources[a]	References
Tetracycline resistance genes: tetA; tetB; tet C; ted D; tet E; tet G; tet H; tet H; tet J; tet Y; tet Z; tet 33	Aeromonas; Alcaligenes, Arthrobacter; Comamonas, Escherichia; Afipia; Burkholderia; Pseudomonas	AS, DW, EW, NW, SD, SW, US	Szczepanowski et al. 2004; Srinivasan et al. 2005; Tennstedt et al. 2005; Poppe et al. 2006; Rodríguez et al. 2006; Cernat et al. 2007; Dang et al. 2007
Aminoglycoside resistance genes:aacA4; aacA29b; acC1; aacC2;aacC3; aadA4; aadA5; adA1	Plasmid pTB11; Microbial community; Aeromonas; Citrobacter; Escherichia; Salmonella	NW, SW, US AS, EW	Tennstedt et al. 2005; Mukherjee and Chakraborty 2006;Tennstedt et al. 2003; Henriques et al.2006; Moura et al. 2007; Cernat et al. 2007; Poppe et al. 2006
Macrolide resistance genes: ermA, ermB; ermC; ermE	Enterococcus; Bacillus; Microbial community	EW, SW	Hayes et al. 2005; Chen et al. 2007; Patterson et al. 2007
Chloramphenicol resistance genes: cmlA1; cmlA5; catB2; catB3; catII; catIII; catIV; floR	Plasmid pB2 and pB3; Plasmid pTB11; Aeromonas	AS; SW; NW; DW	Heuer et al. 2004; Tennstedt et al. 2003; Jacobs and Chenia 2007
Sulphonamide and trimethoprim resistance genes: dfrA1; dfrA5; dfrA7; dfrA12; dfrA15; sul I/II/III; sul A	Aeromonas; Escherichia; Salmonella; Vibrio; Listeria; Plasmids pB2, pB3, pB8, and pB10; Acinetobacter	NW, SW; DW, EW	Henriques et al. 2006a; Mukherjee and Chakraborty 2006;Moura et al. 2007; Park et al. 2003; Antunes et al. 2006; Cernat et al. 2007
β-Lactam and penicillin resistance genes: ampC; blaPSE-1; blaTEM-1; blaTEM-1; penA; mecA	Enterobacter; Salmonella; Aeromonas; Vibrio; Plasmid pTB11; Plasmids pB8, pB10; Listeria	DW, NW, SW, US EW, SD, AS	Schwartz et al. 2003; Volkmann et al. 2004; Poppe et al. 2006; Dalsgaard et al. 2000; Taviani et al.2008; Antunes et al. 2006; Moura et al. 2007; Srinivasan et al. 2005

[a]**SW:** Wastewater from hospital, animal production, and aquaculture area**; US:** untreated sewage**; AS:** activated sludge; **EW:** effluent water of sewage treatment plant**; NW:** natural water**; SD:** Sediments; **DW**: drinking water

2.1.3 Antibiotic Resistant Bacteria and Antibiotic Resistant Genes in Animal Manure and Water Environment

Detection of antibiotic resistant bacteria and associated antibiotic resistance genes (ARGs) in agricultural environments such as wastewater lagoons at animal feedlots and in the manure of antibiotic-fed livestock has been documented in numerous studies (Alexander et al., 2009; Aslam et al., 2010; Binh et al., 2008; Cessna et al., 2011; Heuer et al., 2009; Peak et al., 2007). For example, swine manure has been shown to contain high diversity of bacterial communities carrying ARGs encoding resistance to a variety of clinically relevant antibiotic classes (Binh et al., 2008; Heuer et al., 2009). When examining the prevalence and diversity of bacterial fecal pathogens in slurry, slurry solids and liquid fractions from a commercial pig farm in Northern Ireland, Watabe et al. found that the majority of *Salmonella* isolates (57.7%) displayed antibiotic resistance to at least two antibiotic agents, 34.6% of isolates to three agents and the remainder (7.7%) was resistant to four antibiotics (Watabe et al., 2003). Chee-Sanford et al. also reported the detection of tetracycline resistance genes in the swine housing at a concentrated animal feeding operations (CAFO) and also in the manure lagoon serving that CAFO and in groundwater 250 m downstream of the lagoon (Chee-Sanford et al., 2001). Zahn et al. observed a 3 fold higher concentration of tylosin-resistant bacteria in the exhaust air from concentrated animal feeding operations (CAFOs) using medicated feed compared to those using non-medicated feed (Zahn et al., 2001). Antibiotic resistant bacteria and associated ARGs have also been isolated from various water environments such as wastewater from hospital, animal production, aquaculture area, untreated sewage, activated sludge, natural water and drinking water (Table 2.6). The prevalence of antibiotic resistant bacteria and ARGs in animal manures and water environment poses a risk for the proliferation and dissemination of these ARGs to

human and environmental bacteria (Ghosh & LaPara, 2007). When they reach water and soil, many of them have the potential to infect humans and domestic animals and or transfer their antibiotic resistant genes to other microorganism. Antimicrobial-drug resistance has also become a major concern with an estimated economic impact of $4–5 billion (Institute of Medicine, 1998).

A recent study suggested that both antibiotics and antibiotic resistance should be considered as emerging contaminants due to widespread presence in the environment and the potential health and ecosystem risks associated with them (Pruden et al. 2006). As a result, understanding the fate and transport of these contaminants and preventing their spread in the environment are of great interest. It is also desirable to minimize the loading of antibiotics, steroid hormones and their metabolites to the environment and prevent the spread of antibiotic resistance and estrogenicity among organisms. A significant number of studies have been done on the removal of pharmaceuticals and antibiotic resistance in drinking water and wastewater (Gulkowska et al., 2008; Snyder, 2008; Ternes et al., 2002; Ternes et al., 2003; Zorita, Martensson, & Mathiasson, 2009). Although removal of pharmaceuticals has been observed in conventional wastewater treatment processes, most are not effectively designed to remove micropollutants (Janssens et al., 1997; Suidan et al., 2005; Sumpter & Johnson, 2005). Thus, there is a critical need to better understand the fate, transport and transformation of these emerging contaminants in wastewater treatment processes and to develop novel processes that cost-effectively reduce the risks associated with bioactive compounds in wastewaters.

2.2 CHARACTERIZATION OF HYDROTHERMAL LIQUEFACTION WASTEWATER

2.2.1 Hydrothermal Liquefaction Wastewater Quality

There is scattered information available from past studies on the chemical composition and characteristics of the hydrothermal liquefaction wastewaters (HTL-WW). A range of basic water quality parameters for the wastewater from HTL treatment of swine manure and the analytical results are presented in Table 2.7 below (Appleford, 2004). HTL-WW from swine manure showed week acidity around 5.5 by pH testing, while those from algae usually showed a week basicity of 7.5–8. 7 (Jena et al., 2011b; Zhou et al., 2010). HTL-WW from swine manure contains a high concentration of biological oxygen demand (420–59000 mg/L). High concentration of ammonia in the range of 1860–7070 mg/L and 12,700 mg/L has been reported for HTL-WW from manure and *Spirulina*. A large quantity of total nitrogen (16,200 mg/L) was also found in *Spirulina* HTL-WW and 78.4% of the total nitrogen was in the form of ammonia and nitrates (Jena et al., 2011b). Analysis of aqueous products from eleven different HTL feedstocks showed a high concentration of total organic carbon in the range of 9.5–30 g/L (Villadsen et al., 2012). Substantial levels of magnesium, phosphorous, potassium, and in particular, sulfur, were also present in HTL-WW (Table 2.7). HTL-WW also contains significant quantities of metals such as iron, magnesium, manganese, and zinc. Because these elements are necessary for plant and algae growth, the high concentrations suggest that the process water may make a good fertilizer for plant or algae.

Table 2.7 Water quality analysis of HTL-WW (Appleford, 2004)

Water Quality Parameter	Mean	Lowest value	Highest value
Biochemical oxygen demand (mg/L)	35240	420	59000
Total suspended solids(g/L)	33	21	105.52
pH	5.52	4.86	7.98
Ammonia	3413	1860	7070
Chloride	667	84	1378
Nitrate	0.87	0.06	2.21
Phosphate	921	66	1436
Sulfate	427	142	971
Chromium	0.27	0	1
Iron	28	0	78
Magnesium	242	0	579
Manganese	2	0	5
Nitrogen	6360	4752	8651
Phosphorus	434	3	1068
Potassium	1482	56	2411
Rubidium	0.58	0	1
Sulfur	9651	0	35326
Zinc	1.67	0	12

2.2. 2 Organic Compositions of HTL-WW

A wide variety of organic compounds has also been detected in HTL-WW. A wide variety of organic compounds have been found in HTL-WW including sugars, dianhydromannitol, 1-(2-furanyl)-ethanone (acetylfuran), isosorbide`, indole, 3-amino-phenol and 2-cyclopenten-1-one, carboxylic acids, alcohols, ketones, various cyclic hydrocarbons, and many nitrogen-containing compounds (such as amides, azines, and pyrroles (Appleford, 2004).

The chemical composition of HTL-WW is biomass feedstock and operating condition dependent. Table 2.8 presents the organic composition of HTL-WW from different feedstocks. The organic compounds detected in HTL-WW can be categorized into three major groups: oxygen-containing compounds (carboxylic acids, alcohols and ketones), cyclic hydrocarbons and nitrogenous organic compounds (such as amides, azines, and pyrroles). Of these three groups, nitrogenous organic compound (NOCs) is the most frequently detected in HTL-WW and they mostly come from decomposition of proteins (Anastasakis & Ross, 2011; Zhou et al., 2010). High presence of NOCs in HTL-WW is also due to the fact that a major portion of nitrogen in biomass feedstock would partition to aqueous product during HTL treatment (Vardon et al., 2011; Yu et al., 2011a). Acetic acid also commonly detected in HTL-WW at high content as it could form salts with the basic ingredients, which relatively stable and less volatile during the separation and storage (Zhou et al., 2010). The presence of phenol in HTL-WW is also reported in past studies. Phenols and phenolics are toxic compounds and have been shown to have inhibitory effects on algal growth (Nakai et al., 2001; Scragg, 2006).

Table 2.8 Reference composition of aqueous phase produced by similar processes

Feedstocks	HTL-WW Composition
HTL conversion of Macro-alga *L.saccharina* (Anastasakis & Ross, 2011)	Dianhydromannitol; 1-(2-furanyl)-ethanone (acetylfuran); Isosorbide; 2-cyclopenten-1-one; **Pyrrole derivatives; Indole; 3-amino-phenol**
HTL conversion of *E.prolifera* (Zhou et al., 2010)	Acetic acid; Glycerol; Levulinic Acid; Propanoic Acid Benzenepropnoic Acid; **3-Pyridinol;2-Pyrrolidinone; Phenol, 3-amino;Acetamide;2-Piperidinone; Phenol,2-amino; Propanamide;2(1H)-pyridinone; 3,6-dimethyl-Acetamide, N-dimethyl;Phenol-4-amino**
Coal Gasification (Condensate) (Gangwal, 1981)	Phenol; Naphthalene; Fibenzofuran; Fluorene; Pyrene; Benzo[α]fluorence; Bisphenyl; 1-Methylnaphthalene; 2-Methylnaphthalene; **Pyridine; Methyl-,dimethyl-, and ethyl-substituted pyridines; Quinolone; Aacridine; Bbenzoacridine and methyl derivatives**
HTL conversion of Swine manure (Appleford, 2004)	Dianhydromannitol; 1-(2-furanyl)-ethanone (acetylfuran); Isosorbide; 2-cyclopenten-1-one; Carboxylic acids ; Alcohols; Ketones; Cyclic hydrocarbons; **Amides; Azines; Pyrroles ; 3-amino-phenol ; Indole**

*Compounds in bold are nitrogenous organic compounds (NOCs)

2. 3 EFFECTS OF OPERATING CONDITIONS ON HTL CO-PRODUCTS

2.3.1 Effect of Reaction Temperature

HTL reaction temperature has been demonstrated to play an important role on the yield and composition of bio-crude oil. Table 2.9 shows the effect of HTL operating conditions on yield of bio-oil. Initially, increasing in reaction temperature would increase bio-oil yield. Karagoz et al. reported that the total oil yield after liquefaction of sawdust at 180°C, 250°C and 280°C was 3.7%, 7.6% and 8.5% respectively (Karagöz et al., 2006). However, after reaching the maxima for oil yield, further increase in temperature actually inhibits biomass liquefaction. For example, Jena et al. found that the bio-oil yield after liquefaction of *Spirulina* increased with increase in temperature reaching a maximum of 39.9% at 350°C and then dropped down to

36.0% at 380°C (Jena et al., 2011a). That is because high temperature will favor the secondary decompositions and Bourdard gas reactions which lead to formation of gases instead of bio-oil (Akhtar & Amin, 2011). In addition, high temperature would also enhance formation of bio-char due to recombination of free radical reactions. The dominant of these two mechanisms at high temperature reduces production of bio-oil from biomass during HTL conversion.

HTL is also thermodynamically unfavorable at low temperatures. Previous studies have demonstrated that liquefaction at low temperature (<250°C) produced insignificant amount of liquid oils (Karagöz et al., 2004; Sugano et al., 2008; Zhou et al., 2010). That is because bio-oil production is suppressed by incomplete decomposition of individual biomass at low temperature (Akhtar & Amin, 2011). Past studies also suggested that 300–350°C would be an effective temperature range for decomposition of biomass both for sub- and supercritical conditions. For examples, efficient production of liquid oils from *Enteromorpha prolifa,* cattle manure*,* grassland perennials and eucalyptus biomass was obtained at temperature range of 300–315°C (Sugano et al., 2008; Yin et al., 2010; Zhang et al., 2009; Zhou et al., 2010). Qu et al. also reported similar result for liquefaction of *Cunninghamia lanceolata* where they found that final temperature range of 300–330°C was sufficient for production of bio-oil (Qu et al., 2003).

The optimal temperature of liquefaction is also biomass type dependent. For example, the optimal temperature for liquefaction of manure and sewage sludge was 340°C and 275–300°C, respectively (Suzuki et al., 1990; Xiu et al., 2010; Zhang et al., 2011). Meanwhile, optimal temperature for liquefaction of algae such as *Spirulina* and *Laminaria saccharina* was 350°C (Anastasakis & Ross, 2011; Jena et al., 2011b). In general, higher temperature is requires for complete liquefaction of compact biomass. The formation of solid residue is also affected by HTL reaction temperature. For example, the amount of solid residue decreased sharply as

function of temperature for liquefaction of lignocelluloses biomass (eucalyptus) (Sugano et al., 2008). Maximum bio-oil yield and minimum solid residue formation were obtained at 300°C for lignocelluloses biomass. This suggested that varies in solid residue yield can be set as a reference point to determine optimal HTL reaction temperature.

2.3.2 Effect of Biomass Feedstock

The composition of biomass feedstock is also another operating parameter that affects the formation of HTL co-products and overall bio-oil yield. The major components of plant biomass include lignin, hemicelluloses, cellulose, water soluble sugars, amino acids and aliphatic acids, ether and alcohol-soluble constituents (e.g. fats, oils, waxes, resin and many pigments), and proteins. These components react differently to HTL temperature variations. In general, HTL of biomass that contain high cellulose and hemicelluloses content yields more bio-oil. HTL of biomass with high lignin content results in lower bio-oil yield. When studied the effect of temperature on liquefaction of four different woods (*C. lanceolata*, *Fraxinus mandshurica*, *Pinus massoniana* Lamb. and *Populus tomentosa* Carr.). Zhong and Wei found that yield of heavy oil generally decreases with increasing lignin content. The highest heavy oil yield was obtained for *F. mandshurica*, which has lowest lignin contents (Zhong & Wei, 2004). Similarly, Bhaskar et al. reported that hardwood samples (cherry) produced more oils than softwood (cypress) due to the high lignin contents in later biomass (Bhaskar et al., 2008). According to Demirbas, lignin is a macromolecule which consists of alkylphenols and has a very complex structure (Demirbaş, 2000). Therefore, lignin is difficult to degrade and mostly appear in residue fraction. The final reaction temperature to produce maximum oil yield also tends to increase with lignin contents in biomass. For example, a presence of 32.44% lignin in *C. lanceolata* wood sample shifted the final pyrolysis temperature to 613 K (~340°C) compared to 573 K(~300°C) in case of *Fraxinum*

madshuria wood (21% lignin) (Zhong & Wei, 2004). Thus, it is presumable that presence of lignin in biomass leads to low oil production during pyrolysis. Thermal decomposition of lignin at temperature of 525K (~250°C) results in formation of free phenoxyl radicals and these radicals have a random tendency to form solid residue through condensation or repolymerization (Demirbaş, 2000). Therefore, the decreasing of heavy oil yield with increasing lignin content is attributable to the repolymerization of the intermediate products. The yield of heavy oil also decreased as the reaction time was prolonged, owing to the formation of solid residue by repolymerization of the heavy oils once produced. Thus, in the liquefaction process, the amount of solid residue increased in proportion to the lignin content. The correlation between char residue amount and the lignin contents of wood biomass under operating conditions was described in the following equations (Akhtar & Amin, 2011):

$$\text{Maximum heavy oil yield} = 40.525 - 0.583 \times (\text{lignin percentage}) \quad (2.1)$$

On the other hand, liquefaction of biomass with high cellulose and hemicellulose content would result in higher aqueous fraction due to their special structures. Hemicelluloses have amorphous structure, thus they can be easily decomposed. Cellulose contains β (1-4) glucosidic linkages, which make it is hard to decompose (Akhtar & Amin, 2011).

Finally, feedstock type also affects the physical and chemical properties of bio-oil. For example, bio-oil obtained from liquefaction of loosely structured biomass species usually has high oxygen and moisture contents. Thus, the quality of bio-oil produced from these biomass feedstocks is not as high as that of compact biomass feedstocks such as coal. The variations in the bio-oil chemical composition also depend upon the biomass feedstock. This is due to the differences in the building blocks of three major components of lignocellulosic biomass.

Hydrothermal decomposition of cellulose and hemicelluloses lead to the formation of sugars and aqueous decomposition products. Major products of hollocelluloses degradation include cellohexaose, cellopentaose, cellotriose, cellobiose, fructose, glucose, erythrose, glycolaldehyde, glyceraldehydes, pyruvaldehyde, and furfurals. Table 2.10 shows the effect of different biomass feedstocks on the chemical composition of bio-oil obtained from hydrothermal liquefaction.

Table 2.9 Bio-oil yield from hydrothermal liquefaction of various biomass feedstock under different operating conditions

	Biomass	Temp. (°C)	Pressure(MPa)	Reaction time (min)	Bio-oil Yield (wt.%/VS)
Dry Biomass[a]	Pine wood (Karagöz et al., 2005)	280	Ambient/N_2	15	23.8–33.7%
	Corn stover (Derairbas, 1991)	285	Ambient/Air	75	49.2%
	Pineapple stumps (Goudriaan & Peferoen, 1990)	300	0.1/Air	60	75.0%
	Poplar chips (Boocock & Porretta, 1986)	250–350	Ambient/Air	0	~55.0%
	Sawdust (Xu & Lad, 2007)	280–380	2/N_2	10–60	~53.0%
Wet Biomass	Swine manure (He et al., 2000)	275–350	0.34–2.76/CO	5–180	76.2%/VS
	Algae (*Microcystis viridis)* (Yang et al., 2004)	340	3/N_2	30/60	33.0%
	Algae *(C.pyrenoidosa)* (Yu et al., 2011b)	280	NA	120	39.4%
	Algae (*E. prolifera)* (Zhou et al., 2010)	220–300	2/N_2	30	23%
	Algae (*L. Saccharina)*(Anastasakis & Ross, 2011)	250–370	NA	15-120	19.3%
	Domestic garbage (Minowa et al., 1995)	250–340	3/N_2	30	~27.6%/VS
	Sewage waste (Yokoyama et al., 1987)	250–350	3–12/N_2	0–120	25.3–1.8%/VS

Table 2.10 Effect of feedstock types on the chemical composition of bio oil by hydrothermal process

Feedstock	Bio-oil Composition	Temp.	References
Monosaccharide	5-Hydroxymethylfurfural, 2-furaldehyde glycolaldehyde, dihydroxyacetone glyceraldehydes, 1,2,4-benzenetriol pyruvaldehyde, lactic acid, acrylic acid acetaldehyde, formic acid, acetic acid glycolic acid, acetone	340°C	(Srokol et al., 2004)
Cellulose	Acids, cellubiose, erythrose, 1-6 anhydroglucose, 5HMF	350°C	(Sasaki et al., 1998)
Catechol	Cyclopentanone, 5-hydroxymethylfuran, 2-methyl-2-cyclopentenone, phenoxymethane, 3-methyl-2-cyclopentenone, phenol, octamethyltetrasiloxane, *o*-cresol methyl ether, cymene, 2-cyclopenten-1-one, 2,3-dimethyl, *o*-cresol, *p*-guaiacol, *m*-cresol, guaiacol, 2-methylindane, methyl guaiacol, cyclopentasiloxane, *p*-cresol, catechol, *p*-methylguaiacol, 2-formyl-4-methoxyphenoll, *p*-ethylguaiacol 2,5-dimethoxytoluene, dodecamethylcyclohexasiloxane, 1,3-bis(trimethylsiloxy) benzene, 3-isopropylbenzaldehyde, 1-hydroxy-4-aminoanthraquinone, methylisopropylphenanthrene	350°C	(Sasaki & Goto, 2008)
D. tertiolecta	1,1,3-Trimethyl-2-cyclohexanone, 5-Methyl-3-(1-methylethylidene)-4-hexen2-one, -Menth-4-en-3-one,α-Lonene,4a,7,7-Trimethyloctahydro-2(1H)-naphthalenone,β-Lonene,Pentadecene2(4H)-Benzofuranone-5,6,7,7a-tetrahydro-4,4,7a-trimethyl,(8E)-8-Heptadecene,3-Heptadecene,n-Heptadecene,(2E)-3,7,11,15-Tetramethyl-2-hexadecene,2,6,10,14-Teramethyl-2hexadecene,(3E)-3-Lcosene,(2E)-3,7,11,15-Tetramethyl,1-Hexadecen-3-ol,3,7,11,15-tetramethyl,Palmitic acid, methyl ester,Hexadecanoic acid,Cis-9,cis-12-octadecadienoic acid,Paimitamide,Adogen,Hexadecanoic acid, 2-methylpropyl ester,Pyrrolidin,1-stearoyl-,9-Octadecenamide,N	360°C	(Shuping et al., 2010)

Table 2.10 Effect of feedstock types on the chemical composition of bio oil by hydrothermal process (cont.)

Feedstock	Bio-oil Composition	Temp.	References
Spirulina	Phenylglyoxal; Phenol, Phenol, 4-methyl, Indole, Naphthalene, 1,2,3,4-tetrahydro-1,1,6-trimethyl-, 2,5-Pyrrolidinedione, 1-propyl, 1,5-Dioxaspiro[5.5]undecan-9-one, 3,3-dimethyl, Benzonitrile, 2,4,6-trimethyl, Hydroxylamine, O-decyl, 1-Dodecanol, 3,7,11-trimethyl, 1-Dodecanol, 3,7,11-trimethyl, 5-Tridecene	300°C	(Vardon et al., 2011)
Nannochloropsis	$C_8H_{12}O$ cycloalkene ketone,4-methylphenol,1-ethyl-2-pyrrolidinone,trimethyltetralin, dimethylnaphthalene, dimethylindole,dimethylindole,pentadecane3-hydroxy-trimethyltetralin,cycloalkane,1-heptadecene,3-eicosene,nonadecane,palmitic acid amide, alkane, echolestanol,fucosterol,1,30-triacontanediol	350°C	(Brown et al., 2010)
Enteromorpha prolifera	pyrazine, methyl-,2-cyclopenten-1-one, 2-methyl, 2-cyclopenten-1-one, 3-methyl-, phenol, 2-cyclopenten-1-one, 2,3-dimethyl-,phenol, 4-methoxy-,indole, 1-pentadecene, 8-heptadecene, 2-hexadecene, 3,7,11,15-tetramethyl-,hexadecanoic acid, octadecanamide, 2,5-piperazinedione, 3-benzyl-6-isopropyl	300°C	(Zhou et al., 2010)
Swine manure	Pentanoic acid, Hexanoic acid, Pyrazine, Phenol-4 methyl, Mequinol, Phenol, 4-ethyl, Phenol, 4-ethyl-2-methoxy, N-2-Hydroxyethyl-succinimide, ^{1}H-Indole, 4-methyl, 1-Hexadecanol, 2-Propenoic acid, tridecyl ester, Cholest-4-ene, 5.alpha.-Ergost-8(14)-ene	300°C	(Vardon et al., 2011)
Anaerobic sludge	Benzoylformic acid, Phenol, Phenol, 4-methyl, 4-Methylindole, Hydroxylamine, O-decyl-7-Tetradecene, Dodecyl acrylate, Cholest-4-ene, Cholest-2-ene, Cholest-7-ene, (5.alpha.)-	300°C	(Vardon et al., 2011)

2.3.3 Effect of Pressure

Pressure is another important operating parameter during conversion of biomass to bio-oil by HTL. Most researchers agree that the unique properties of water at high temperatures and high pressures play a significant role in subcritical hydrothermal liquefaction of biomass to bio-oil. The dependence of the water dissociation and ionization on temperature (Figure 2.2) and pressure (Figure 2.3) has been fully investigated and a standard formulation exists (IAPWS 2004). The range of pressures for HTL is much smaller than the range shown in Figure 2.3. Thus, temperature has a greater effect on water ionization in HTL systems. Generally, increase in temperature and pressure would increase water dissociation constant and ionization. High pressure also helps to maintain the single-phase media for both sub and supercritical liquefaction during HTL process. This single-phase liquefaction does not require large enthalpy inputs for phase change of solvents as compared to the two-phase system (Goudnaan et al., 2008). In addition, the hydrolysis rate and biomass dissociation during liquefaction can be controlled by maintaining process pressure above the critical pressure of the medium. Thus, the final pressure would enhance thermodynamically favorable reaction pathways for production of liquid fuels or for gas yield. Pressure also increases solvent density which allows the medium to penetrate more efficiently into molecules of biomass components. Thus, the decomposition and extraction of biomass will be enhanced by controlling the pressure. However, once supercritical conditions for liquefaction are achieved, pressure play little or negligible impact on yield of liquid oil or gas yield (Kabyemela et al., 1998; Kabyemela et al., 1997; Kersten et al., 2006; Sangon et al., 2006). For example, Sangon et al. reported that increase in pressure (7–12 MPa) slightly increased liquefied oil yield during coal liquefaction in supercritical conditions (Sangon et al., 2006). In

addition, bio-oil yield decreased with increase in pressure when catalytic was used in the same study. This is because increase in pressure caused high-density solvent, which blocked catalyst active sites and reduced oil formation. Kabyemela et al. also found that rising in pressure resulted in lower glucose degradation rate. (Kabyemela et al., 1997). Similarly, pressure change (30–40 MPa) at 400°C did not affect the hydrolysis rate for cellobiose liquefaction and decreased in rate of pyrolysis of the same feedstock(Kabyemela et al., 1998). Thus, variation of pressure in supercritical liquefaction may not play an important role for overall liquid oil yields.

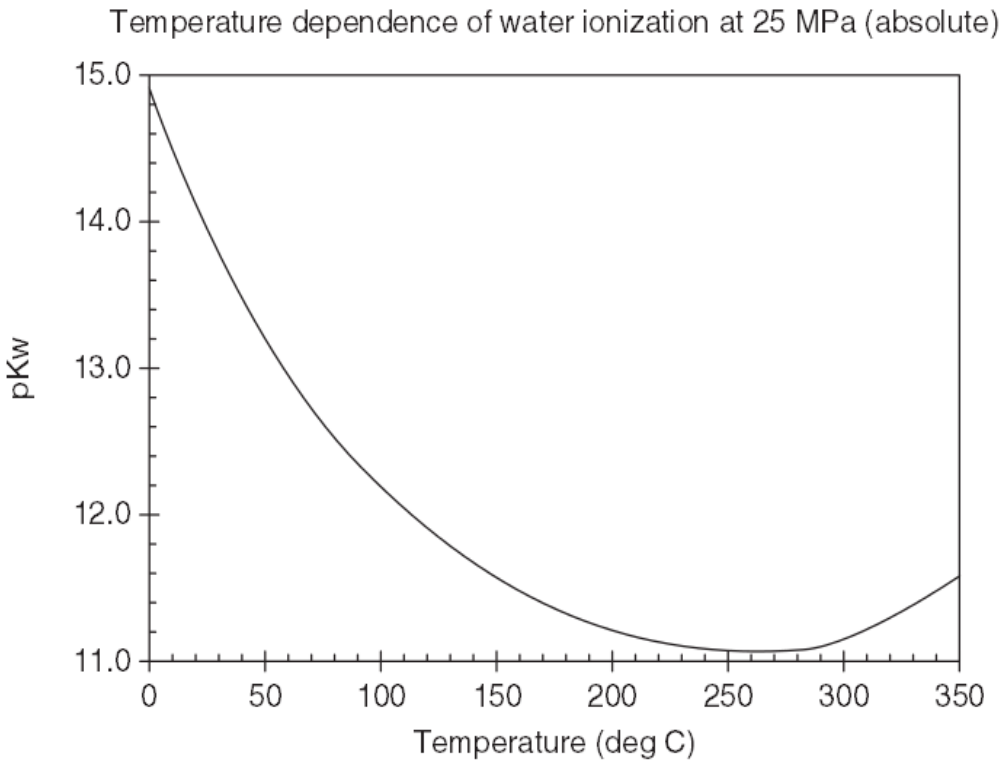

Figure 2.2 Effect of temperature on water dissociation constant at 25 MPa. The dissociation constant KW is expressed as pKW, where pKW= – log10(KW)

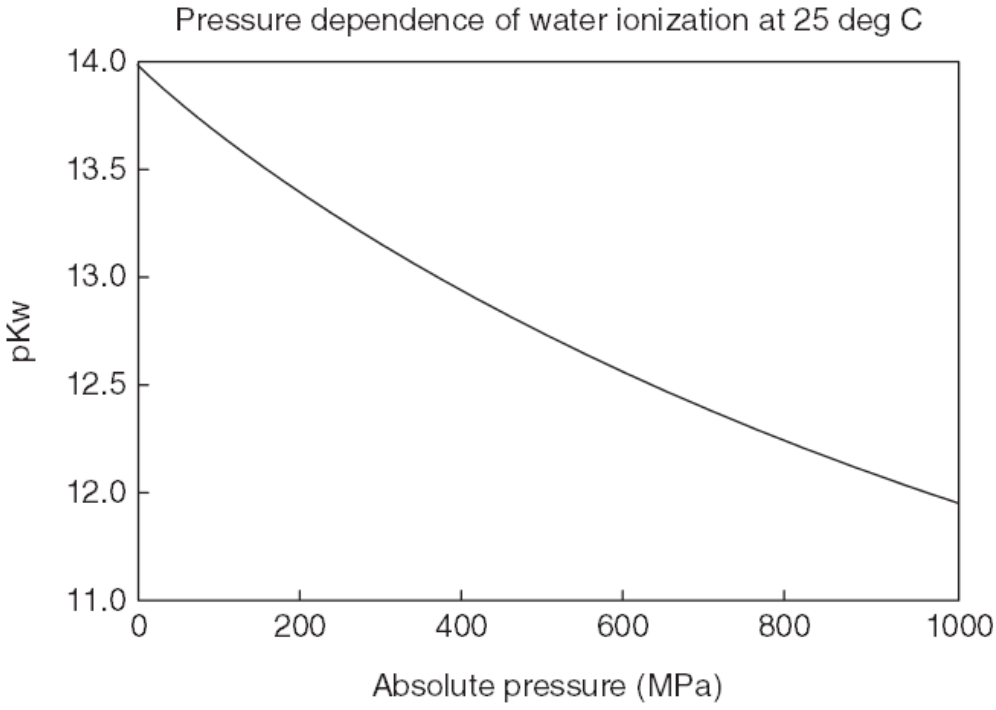

Figure 2.3 Effect of pressure temperature on water dissociation constant K_W at 25°C. The dissociation constant K_W is expressed as pK_W, where $pK_W = -\log 10 (K_W)$

2.3.4 Effect of Reaction Time

The effect of reaction time on bio-oil yield by HTL has been investigated in numerous studies (Qu et al., 2003; Su et al., 2004; Xu & Lancaster, 2008; Yan et al., 1999; Zhang et al., 2007). The reaction time is the actual time period of liquefaction reaction once it reaches to a designated reaction temperature. The duration of reaction time play an important effect on both the distribution of HTL final products and the overall conversion efficiency of biomass. In

general, under supercritical condition, the rate of biomass hydrolysis and decomposition is relatively fast, thus, a short reaction time (<60 min) is sufficient to convert biomass into bio-oil (Sasaki et al., 2003). During hydrothermal biomass liquefaction, small residence times are usually preferred. In addition, optimal reaction time is usually specific for different biomass feedstock. Different studies have reported results for bio-oil yield as a function of reaction time. In most case, it is reported that higher bio-oil yield can be achieved by increasing the reaction time. However, once the maximum oil yield is achieved, further increase in reaction time would decrease oil yield (Boocock & Sherman, 1985; Jena et al., 2011a; Karagöz et al., 2004; Yan et al., 1999). Boocock and Sherman found that longer residence times suppressed the bio-oil yield except for very high biomass to water ratios (Boocock & Sherman, 1985). Similarly, Jena et al. reported that increasing reaction time during liquefaction of *Spirulina* resulted in decreased bio-crude oil yield due to the conversion of the lighter hydrocarbon compounds in the bio-crude into gaseous products (Jena et al., 2011a) . Yan et al. observed negligible increase in liquid yields for longer residence times on liquid yields (Yan et al., 1999). Qu et al. observed that heavy oil yield from direct liquefaction of *Cunninghamia lanceolata* decreased for longer reaction times (Qu et al., 2003; Sugano et al., 2008). In fact, the effect of reaction temperature on oil yield is also influenced by the reaction temperature. Karagoz et al. reported that at low reaction temperature (<150°C) increase in reaction times favored the liquid oil yield and conversion of sawdust (Karagöz et al., 2004). Reverse was true for high temperatures (250–280°C) although overall conversion of biomass and gas yield increased with rise in temperature. There may be many reasons to the effect of residence times in liquefaction. Initial increase in bio-crude yield with prolonged reaction time could be explained by the fact that longer reaction time enhances dehydration of carbohydrates and formic acid/acetic acid intermediates resulted by hydrolysis

reactions that might account for formation of additional bio-crude. Once conversion of biomass reached its saturation point, further prolonged reaction time would enhance the secondary and tertiary reactions in hydrothermal medium. These reactions can convert heavy intermediates either into liquids, gases, or residues species results in lower oil yield. Reaction times may also affect the HTL product distribution and chemical composition of bio oil. According to Jena et al., increasing in reaction time (30–120 min) resulted in higher gases yield, lower in water soluble fraction yield, and no significant changes of solids residues (Jena et al., 2011a). Variations in reaction time can also affect the composition of HTL final products. According to Karagoz et al., the composition oil product was not similar for longer and shorter reaction time the both for low (180°C) and high (250°C) temperatures. The authors found that at the temperatures of 180°C and 60 min reaction time, bio-oil contained 5-(hydroxymethyl)-2-furan carboxaldehyde, 4-hydroxy-3-methoxybenzoic acid, 4-hydroxy-3-methoxybenzeneacetic acid, and bis(2-ethylyhexyl) phthalate were observed. However, these compounds were not observed at the same temperature for 15 min. Similarly, at 280°C and 15 min reaction time, vanillin, phenol, 2,4-dihydroxybenzaldehyde, etc., were detected in bio-oil but were not observed at 60 min reaction time (Karagöz et al., 2004). In general, oil yield reaches a maximum before decreasing for very long residence times while gas yield and biomass conversion increase continuously until the saturation point (Zhang et al., 2007).

2.4 ALGAL-BASED AND ADSORPTIVE TREATMENT OF WASTEWATER

2.4.1 Cultivation of Algae Using HTL-WW

Successful cultivation of algae in poor quality wastewater effluent from various sources such agricultural farm (Wang et al., 2010; Woertz et al., 2009), piggery and aqua cultural farms (An et al., 2003; Mallick, 2002), paper industry (Tarlan et al., 2002), and carpet industry

(Chinnasamy et al., 2010) has been reported in the literature. For example, algal biomass production potential (measured in terms of chlorophyll *a*) for various freshwater and marine strains of algae was 16–24 % higher in treated and untreated carpet wastewater than in standard BG 11 medium (Chinnasamy et al., 2010). Minowa and Sawayama also reported the stimulation of *Chlorella minutissima* growth in wastewater from the gasification of *Chlorella*. Jena et al. found that addition of 0.1–0.2% nutrient rich HTL-WW from liquefaction of *Spirulina* to a nutrient depleted wastewater could enhance algal growth significantly (Jena et al., 2011b). Enhancement of algal growth in wastewater effluent is mostly due to algae's ability to directly utilize, degrade and transform nutrients and organics in wastewater to support their growth (Ghasemi et al., 2011). Table 2.11 summarizes different classes of organic compounds (phenolic compounds, aromatic compounds, oil contaminants, agrochemicals, and steroid compounds) that can be biodegraded and/or bio-transformed by algae. As demonstrated in Table 2.11, microalgae such as *Chlorella vulgaris* and *Coenochloris pyrenoidosa* has been shown to remove many contaminants, including phenols, nitrophenols, chlorophenols, and bisphenol A (Hirooka et al., 2003; Hirooka et al., 2006). Algal species such as *Oscillatoria salina, Plectomena terebrabs, Aphanocapsa*, and *Synechococcus* can bio-remediate oil contaminants (Cerniglia et al., 1980a; Raghukumar et al., 2001). HTL-WW contained a high concentration of carbon, nitrogen and phosphorous, which are the three most important nutrients for autotrophic growth of algae. In addition, most of the mineral nutrients needed for algal growth were also found in HTL-WW in significant quantities when compared to the standard growth medium such as BG 11, modified Allen's medium and Bold's Basal medium (Jena et al., 2011b). Thus, HTL-WW is a good source of nutrients that can be recycled for growing algae.

Recent studies have explored the feasibility of an integrated wastewater algae-to-bio-crude process that can sustainably cultivate algal biomass for biofuel production via hydrothermal conversion (Jena et al., 2011b; Roberts et al., 2013; Zhou et al., 2011a; Zhou et al., 2011b). These studies demonstrated that: (i) algal biomass can be produced during wastewater treatment; (ii) algal biomass can be successfully converted into bio-crude oil via hydrothermal liquefaction with a positive energy balance; and (iii) the system can recycle the nutrient content of the incoming waste stream to support multiple cycles of algae growth. These studies also proposed an integrated biorefinery system which includes algal biomass production during wastewater treatment, conversion of algal biomass to bio-oil through HTL, and recycling of the HTL-WW as low-cost nutrient source for algae cultivation. This integrated wastewater treatment-bio-oil production system can resolve several key bottlenecks for large scale algal biofuel production including the contamination of target high-oil algal cultures, high nutrient cost inputs, as well as the significant energy inputs for dewatering and extracting oil from algae. These studies also established the proof of concept for combining algae cultivation with HTL for recycling the nutrients. In the present work, we explored the ability of using algae to remove nutrients, organics and mitigate cytotoxicity of HTL-WW. Specifically, we operated batch and semi-batch algal bioreactors to grow algae in diluted HTL-WW. Removal of nitrogen and organics was monitored daily. The algal biomass produced can be fed back to HTL process to generate additional bio-oil. All in all, this novel treatment system offers potential advantages for reducing the potential toxicity risks associated with byproducts of HTL bioenergy production and improving wastewater effluent quality.

Table 2.11 Biodegradation and transformation of organic compounds by microalgae

Organic Compounds	Biological Mechanism	Algal Species	Reference
Phenolic Compounds	Aerobic Degradation: Oxidation of organic compounds	*Ochromonas danica; Synechococcus; Phormidium valderianum; Chlamydomonas reinhardtii ; Anabaena cylindrical; Chlorella vulgaris; Coenochloris pyreno ; Pseudokirchneriella*	(Semple et al., 1999); (Lai et al., 2002); (Lika & Papadakis, 2009); (Wurster et al., 2003); (Shashirekha et al., 1997)
Aromatic compounds: PAHs, BaP	Degradation: Enzymic attack on the aromatic rings by the oxygen	*Chlamydomonas sp., Chlorella miniata, C. vulgaris, Scenedesmus platydiscus, S. quadricauda, S. capricornutum*	(Brooks & McLaughlin, 2009; Lei et al., 2006; Lei et al., 2007; Lei et al., 2002) ; (Juhasz & Naidu, 2000) ; (Lima et al., 2004)
Oil contaminants	Biodegradation	*Oscillatoria Salina; Plectomena terebrans, Aphanocapsa ; A. quadruplicatum; Phormidium corium; Microcoleus chthonoplastes*	(Cerniglia et al., 1980a)(Cerniglia et al., 1980a)(Cerniglia et al., 1980a)(Cerniglia et al., 1980a; Cerniglia et al., 1980b); (Raghukumar et al., 2001);(Alhasan et al., 1994)
Agrochemicals	Biotransformation and enzymatic responses	*Skeletonema costatum; Tetraselmis marina; Eichhornia crassipes*	(Yang et al., 2002); (Tikoo et al., 1997); (Petroutsos et al., 2007)
Steroid compounds	Biotransformation; Metabolism via hydroxylation; Reduction; Side-chain degradation	*C. Pyrenoidosa; Chlorella, Scenedesmus, Ankistrodesmus, Selenastrum, Anabaena, and Nostoc Chlorella, Selenastrum, Muriella, Cyanidium, Galdieria Cyanidioschyzon, Scenedesmus*	(Guehler et al., 1962); (Fernandes et al., 2003); (Pollio et al., 1994; Pollio et al., 1996); (Faramarzi et al., 2008)
Terpenes	Biotrasformation: Hydroxylatation the C=C double bond	*Synechococcus sp.; Euglena gracilis and Dunaliella tertiolecta; Chlorella minutissima, Nannochloris atomus;*	(Hamada et al., 2003); (Noma et al., 1992; Noma et al., 1991); (Hook et al., 1999)
Other compounds	Degradation Metabolization	*C. minutissima; N. atomus; D. parva; P. purpureum; I. galbana ; Chlorella pyrenoidosa*	(Hook et al., 2003); (Brown et al., 2010; Yoshizako et al., 1994; Yoshizako et al., 1992; Yoshizako et al., 1991)

2.4. 2 Removal of Organic Contaminants by Granular Activated Carbon

The use of granular activated carbon (GAC) to remove organic contaminants of concern by adsorption is also well documented (Aksu, 2005; Tryba et al., 2003). Previous studies had evaluated the use of activated carbon adsorption and ozonation on a bench scale for treatment of petrochemical wastewater (Farooq & Misbahuddin, 1991; Kunz & Giannelli, 1976). Batch adsorption studies indicated that activated carbon can remove 51–73% of total organic carbon (TOC) in the wastewater. Ozonation can provide 32.5% and 40% reduction in TOC and COD, respectively. The combined ozone and adsorption treatment was found to remove up to 81% of TOC (Farooq & Misbahuddin, 1991). Since HTL-WW was in contact with bio-oil during the HTL conversion, it is expected to have similar chemical properties to petrochemical wastewater. Thus, activated carbon can potentially be used to remove organic contaminants in HTL-WW. As mentioned previously, HTL-WW contains numerous organic compounds. These compounds fall into three major categories: oxygen containing compound, cyclic hydrocarbon, and nitrogen containing compounds. Previous studies had demonstrated that activated carbon can effectively remove oxygen containing compounds, including carboxylic acids (Ward & Getzen, 1970), alcohols and ketones (Aizpuru et al., 2003; Brasquet et al., 1999; Daifullah & Girgis, 1998; How & Morr, 1982; Vazquez et al., 2007; Ward & Getzen, 1970). Activated carbon can also remove alkanes and cyclic hydrocarbons (Diaz et al., 2004; Malek & Farooq, 1996). More importantly, activated carbon does not generally produce any harmful by-products and can be readily recovered, regenerated, and reused. In addition, activated carbon has been widely used in conjunction with microbial biodegradation processes, and the biological removal of adsorbed organics can provide continuous in-situ regeneration of activated carbon adsorption capacity (Dussert & Van Stone, 1994; Kim et al., 1997; Takeuchi et al., 1997; Toor & Mohseni, 2007;

Voice et al., 1992; Walker & Weatherley, 1999). Generally, data from these studies showed that the biological activated carbon (BAC) system can outperform the combination conventional activated carbon and biological water treatment processes. That is because this system provides simultaneous adsorption of non-biodegradable matter and oxidation of biodegradable contaminants in a single reactor (Weber et al., 1978). Once the activated carbon becomes saturated, it can be potentially fed back to HTL process as a feedstock to produce additional bio-oil. Extensive prior studies had demonstrated the potential of coal liquefaction to produce clean liquid fuels and chemical feedstocks (Mochida & Sakanishi, 1994; Ramage & Katzer, 2009; Ross & Hirschon, 1990; Song et al., 2000; Williams & Larson, 2003). GAC is made from coal, thus, it is feasible to use the saturated GAC as HTL feedstock to produce additional bio-oil.

2.4.3 Catalytic Hydrothermal Gasification of Biomass

Bioenergy from biomass may significantly contribute to the growing future demand for energy. The advantages of bioenergy production from biomass are that they do not stand in direct completion to food production and generally have a better energy balance. Conventional thermochemical methods for fuel production from biomass (e.g., gasification, fast pyrolysis) require a dry feedstock or they will suffer a large energy penalty from vaporizing the moisture content. Many potential biomass feedstocks, such as agricultural crop waste and residues, wood waste and residues, municipal wastes, and animal wastes have very high moisture contents. These wet biomasses cause high drying costs if classical gas-phase gasification or liquefaction processes are used. Therefore, direct thermochemical processing of such wet biomass feedstocks is attractive from an energy perspective. Catalyst and non-catalyst hydrothermal gasification are being explored for conversion of wet biomass into biofuels. Hydrothermal gasification refers to the gasification of wet biomass and organic wastes under hydrothermal conditions to produce a

hydrogen rich fuel gas. Under hydrothermal condition, water with temperature and pressure near and above its critical point (T > 374°C and P > 22 MPa) will act as a homogeneous non-polar solvent of high diffusivity and high transport properties and be able to dissolve any organic compounds and gases (Hodes et al., 2004; Kritzer & Dinjus, 2001; Savage, 1999; Watanabe et al., 2004). Therefore, hydrothermal gasification can completely and energetically efficiently convert wet biomass to gases. As a consequent, the yield of hydrothermal gasification conversion is relatively high (>99%) and the formed gases contained mostly hydrogen (up to at least 50%). Past studies had demonstrated that hydrothermal gasification can effectively gasify wet biomass and organic wastes into hydrogen or methane rich fuel gas, depending on the operating conditions (Antal et al., 2000; Brown et al., 2010; Chakinala et al., 2009; Elliott, 2008; Osada et al., 2006; Schmieder et al., 2000; Waldner & Vogel, 2005). Early studies of hydrothermal gasification were conducted on model compounds such as glucose for cellulose, catechol and vanillin for lignin and glycine for proteins. For example, it was found that the main gases produced by hydrothermal gasification of glucose were carbon dioxide, carbon monodioxide, methane, and hydrogen, and there were also significant production of oil and char (Williams & Onwudili, 2005). Influence of hydrothermal gasification operating parameters such as temperature, residence time, and initial concentration of glucose on the yield and composition of co-products was also investigated. The authors found that the product yield and composition do not significantly change with the temperature (and pressure) and residence time. However, high concentration of glucose in the reactor system causes a decrease in the gasification of glucose and results in significant formation of char and oil. Lee et al. investigated gasification of glucose using tubular flow reactor at a temperature range from 480–750°C and 28 MPa with a residence time from 10–50 min (Lee et al., 2002). The authors reported that the yield of hydrogen was

significantly enhanced as the temperature was increased to 660°C and above. On the other hand, the yield of carbon monoxide decreased with temperature, most probably due to the role of a water-gas shift reaction. Maximum (100%) carbon gasification efficiency was obtained at 700°C and maintained as the residence time varied from 10–50 min. Later studies of hydrothermal gasification were generally conducted on real biomass (wood as sawdust, straw) and wastes (sewage, sludge and lignin) with addition of catalysts. In recent years, significant progresses have been achieved in the development of various catalytic hydrothermal biomass gasification (CHG) processes. Table 2.12 summarizes the state of catalytic hydrothermal gasification. Hydrothermal gasification has been confirmed as an environmental friendly process by previous life cycle assessment studies. In a study by Gasafi et al., the environmental impacts of different sewage sludge disposals were accessed and compared with hydrothermal gasification process. The authors found that hydrothermal gasification is a very environmental friendly process in term of energy consumption, particle emission, and eutrophication (Gasafi et al., 2003). Similarly, the global warming potential of methane production from wood chips via hydrothermal gasification is found to be slightly negative (Luterbacher et al., 2009). Thus, hydrothermal gasification is a promising technology for converting waste biomass to synthetic natural gas (SNG).

Past work has demonstrated the ability of CHG to convert various organic compounds into a biogas with methane, hydrogen and carbon dioxide. For instance, Elliott and Sealock achieved 99.9% COD reduction with CHG of olive processing wash water using a Raney nickel catalyst (350°C, 21 MPa) (Elliott & Sealock Jr, 1996). Under similar conditions, the COD concentrations from a nylon manufacturing wastewater were reduced from 1200 mg/L to 50–55 mg/L (Elliott et al., 1993). Hydrothermal gasification processes have been shown to degrade 90–

100% of specific organic compounds such as phenol, analine, tetrahydrofuran, toluene, and cyclohexanol using a Raney nickel catalyst or a Sn-modified Raney nickel catalyst (Li et al., 2008). The present study interested in applying hydrothermal gasification process for treatment of HTL-WW, which has not been investigated previously. Our working hypothesis is that the elevated temperature and pressure of this process can effectively gasify most of organic residues in HTL-WW into gases and thus reduce the cytotoxicity of HTL-WW.

Table 2.12 The state of catalytic hydrothermal gasification of biomass

Feedstock	Catalysts	Reaction condition	Gas products	Reactor	Reference
Wood (saw dust, straw) Wastes (sewage sludge and lignin)	KOH or K_2CO_3	500–700°C 500–1000Bar	H_2 and CO_2	Batch reactor	(Schmieder et al., 2000)
Wood (saw dust)	Raney nickel	300–410 °C 12–34 MPa	CH_4	Batch reactor	(Waldner & Vogel, 2005)
Organic wastewater	Ni/carbon	360°C, 20 MPa	H_2; CH_4	–	(Osada et al., 2006)
Cellulose, softwood, hardwood, and grass lignin	Ni	400°C, 25 MP		Microreactor	(Yoshida & Oshima, 2004)
Corn- and potato-starch gels, sawdust, cornstarch gel, potato wastes	Carbon	650°C, 22 MP	H_2	Tubular flow reactors	(Antal et al., 2000)
Glycerol, glucose, cellobiose, bagasse, sewage sludge	Charcoal activated carbon	600°C, 34.5 M	H_2 and CH_4	Supercritical flow reactor	Xu et al., 1996
Vanillin, glycine K_2CO_3, straw, sewage, sawdust, sludge, lignin pyrocatecho	K_2CO_3, KOH	600–700°C	H_2	Batch and tubular reactor	(Kruse et al., 2000)
Microalgae (*Chlorella vulgaris*)	Ru/TiO_2	400–700 °C	H_2, CO_2, CO, CH_4	Batch (Quartz Capillaries)	(Chakinala et al., 2009)

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CHAPTER 3: EFFECTS OF HYDROTHERMAL LIQUEFACTION ON FATE OF BIOACTIVE CONTAMINANTS IN BIOMASS FEEDSTOCKS

3.1 INTRODUCTION

Biowastes such as manure and municipal wastewater biosolids contain a variety of bioactive chemicals (BACs) of emerging concern (e.g. pharmaceuticals, hormones, and antibiotic resistant genes) that pose a range of risks to human health, economic productivity, ecosystem services, and the long-term sustainability of agricultural activities (Costanzo et al., 2005; Graham et al., 2007; Kümmerer, 2004). For instance, antibiotic resistant infections have been steadily increasing, and the estimated economic impact associated with treatment of antibiotic resistant infections has increased from $5 billion in 1998 to $50 billion by 2009 (Lederberg, 1998; IDSA, 2012). Recent study indicates that up to 90% of the drugs administered to animals are excreted in an unaltered state together with their manure residuals (Sarmah et al., 2006). These drugs can persist through storage and treatment, and then be discharged to the environment by a variety of mechanisms including surface water runoff, groundwater seepage, and land application.

Antibiotics in animal manure and wastewater biosolids that are disposed of in the environment present a significant public health risk associated with the generation of antibiotic resistant microbial pathogens (Alexander et al., 2008; Chander et al., 2008). In the environment, antibiotics exert selective pressures on microbial communities that lead to the development of antibiotic resistance as a defense mechanism. Subsequently, the genetic material coding for antibiotic resistance can be passed from one microorganism to another, including pathogens. Excretion of estrogenic hormones from agricultural activities is also of growing concern as they can cause adverse effects on the reproductive biology of aquatic vertebrates at very low

concentrations (10–100 ng/L) (Hanselman et al., 2006). Therefore, it is important to remove BACs in concentrated biosolids to prevent spreading them into the environment. Past studies indicate many BACs preferentially partition with the biosolids due to adsorptive phenomena (Carballa et al., 2004; Clara et al., 2004; Li & Zhang, 2010). Thus, treatment of the biosolids portion of manure and other wastewater biosolids provides an excellent opportunity to interrupt the flow of BACs into the environment.

Recent studies have explored the feasibility of integrated algal systems for wastewater treatment coupled with bioenergy production (Hu et al., 2013; Nielsen et al., 2012; Zhang et al., 2013). Successful cultivation of algae in manure and other wastewaters has been reported, and the resulting algal biomass will also be laden with bioactive chemicals (An et al., 2003; Kebede-Westhead et al., 2004; Kebede-Westhead et al., 2006; Mallick, 2002; Mulbry et al., 2008; Wang et al., 2010; Woertz et al., 2009). In addition, large-scale algal cultivations for bioenergy are likely to utilize bioactive chemicals for maintaining selectivity of desired species (Hong et al., 2010; Larkum et al., 2012). Thus, it is also important to understand the fate of BACs during conversion of algal biomass to bioenergy products, and to develop management practices that cost-effectively mitigate the risks associated with BACs.

Bioenergy production from algal and manure feedstocks minimizes the competition for arable land between food and fuel, which currently limits both first generation biofuels from food crops and the second generation of "energy" crops from meeting the full national need for biofuels. Therefore, waste-to-energy has been receiving greater attention as a sustainable, secure and cost-effective domestic energy source. Hydrothermal liquefaction (HTL) is a thermochemical conversion process that is particularly attractive for wastewater biosolids and algae because it can convert wet biosolids into bio-crude oil, with a net positive energy balance,

which is difficult to achieve with conversion technologies that require dry biomass (Anastasakis & Ross, 2011; Jena et al., 2011b; Yu et al., 2011b; Zhou et al., 2010). HTL uses elevated temperature (typically 200–350°C) and pressure (10–15 MPa) and has demonstrated bio-oil conversion efficiencies of 30–75% with a net positive energy yield of 3–10 times input heat energy (Zhang, 2008; Zhang & Schideman, 2009). HTL bio-oil is a heavy crude with heating values between 32–38 MJ/kg, which is 75–90% of petroleum crude heating value (He et al., 2001a; He et al., 2001b; He et al., 2000b; He et al., 2001c).

The current study focuses on understanding the effects of HTL operating conditions (reaction temperature and reaction time) on destruction of bioactive contaminants such as antibiotics, antibiotic resistant genes, and estrogenic compounds commonly detected in animal manure and wastewater. The BACs used in this study included florfenicol (FF), ceftiofur (CF), estrone (E1), bisphenol A (BPA). The first two (FF and CF) are broad spectrum antimicrobial agents commonly used for the treatment of respiratory diseases in livestock. FF is a synthetic fluorinated chloramphenicol derivate, and CF is a semisynthetic β-lactam. Occurrence of CF, FF and other antimicrobials in manure, wastewater, river water and groundwater has been reported in previous studies (Berge et al., 2006; Hölzel et al., 2010; Martinez, 2009). E1 and BPA are estrogenic compounds that have been found in wastewater and in soil receiving biosolids from municipal wastewater treatment, animal manure as documented in numerous studies (Furuichi et al., 2006; Hanselman et al., 2004; Jacobsen et al., 2005; Kinney et al., 2008; Liu et al., 2012; Raman et al., 2001; Raman et al., 2004; Zheng et al., 2008). Therefore, it is of great interest to better understand the fate, transport, and transformation of these antibiotics and estrogenic hormones in biowastes and develop proper waste management processes that cost-effectively reduce the spread of these compounds in the environment. The specific objectives of this study

were to: (i) investigate the impacts of HTL on the fate of specific BACs (antibiotics and estrogens) under different practical operating conditions, (ii) study the effect of biowaste feedstocks on the removal of BACs (iii) demonstrate the effect of HTL for disrupting antibiotic resistant genes and gene transfer.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals and Reagents

Bisphenol A and Estradiol labeled with ^{14}C were purchased from American Radiolabeled Chemicals (Saint Louis, MO). Estrone, florfenicol, carbenicillin and ceftiofur were purchased from Sigma Aldrich (Milwaukee, WI). Solvents (phenol, chloroform, and methanol) were purchased from Sigma-Aldrich (Milwaukee, WI). All chemicals and solvents were purchased at the highest level of purity available.

3.2.2 Hydrothermal Liquefaction Feedstocks

Spirulina biomass (solids content of 95%) were obtained in a dry powder form from Cyanotech (Kailua-Kona, Hawaii) and stored at 4°C prior to processing. Fresh swine manure was collected from the grower-finisher pen floors of the Swine Research Center at the University of Illinois. The manure sample was blended with tap water using a commercial Warning® blender and then homogenized by a high shear mixer to achieve a total solids content of 20%. The homogenized manure samples were stored in a cold room at 4°C before being used in the HTL tests. The total solids content of swine manure was determined by heating the sample at 105°C for 24 hours in a convection oven (DKN 400, Yamato Co.). The volatile solids content was measured by burning the swine manure in a furnace (Barnstead Thermolyme Co.) at 600°C for 3 hours or until the weight stabilized. The volatile fraction of the total solids was 80–85%.

3.2.3 Bacterial Strains, Culture Conditions, and Preparation of Inocula

Plasmid encoded carbenicillin resistance was maintained in *Escherichia coli (E. coli)* strain S17-1 λpir. This strain was referred to as Carb-R- *E. coli* S17-1 λpir throughout this chapter to distinguish it from the unmodified strain of *E. coli* S17-1 λpir, which is not resistant to carbenicillin. Stock strains were kept at −80°C in 10% glycerol until used. To start a fresh culture, a loop of frozen culture was inoculated into a sterile Luria-Bertani (LB) broth (Difco, Sparks, MD, USA) containing 5µg/mL of carbenicillin and incubated overnight at 37°C and 300 rpm shaking. After the incubation time had passed, the pure culture was streaked onto an LB plate containing carbenicillin and incubated overnight at 37°C to create single colonies. This plate was then kept at 4°C and used for inoculation in all experiments. Subculturing onto new plates was performed every two weeks to keep the culture pure and active. *E. coli* strain S17-1 λpir were used as recipient cells in high-efficiency electroporation transformation experiments. These strains were also stored at −80°C and then subsequently revived and maintained using the same techniques described above. The competent cells were prepared following a previously published method (Dower et al., 1988).

Wild-type cells of *A. vinelandii* strain DJ were used as the recipient in natural transformation assays. The competent cells were prepared by streaking *A.vinelandii* from −80°C stock onto a plate containing Burk's (B) medium and incubated for two days to form separate individual colonies. Then, a single colony from this plate was inoculated into B medium and incubated at 30°C and 170-rpm shaking for 18–20 h to grow a culture of competent cells (Lu et al., 2010).

3.2.4 Hydrothermal Liquefaction Experiments

The HTL experiments were conducted at two different temperatures (250 and 300°C) and three different reaction times (15, 30, and 60 min). These selected reaction conditions were shown in previous studies to provide a reasonably good bio-oil yield for HTL of *Spirulina* (25−30%) and swine manure (32.5–60%) feedstocks (He et al., 2000; Dong et al., 2009; Jena et al., 2011). The reaction time reported here was the actual time period of HTL reaction once it reaches to a designated reaction temperature. Destruction of bioactive compounds by HTL treatment was studied using small HTL batch reactors made from pipe and fittings from Swagelok. Each reactor included a cap (Part # 1 SS-600-C), a 0.38 inches O.D. ×3.24 inches L SS tubing (Part # SS-T6-S-049-20), and an N-series needle valve (Part # SS-6NBS6-G). A set of four reactors were run in parallel to spontaneously test the effect of different operating conditions on the destruction of bioactive compounds. The starting test solution was prepared in DI water with the following concentrations of bioactive compounds: ceftiofur (50 ppm), florfenicol (50 ppm), and estrone (2 ppm). Before each HTL experiment, 2.5 mL of the starting solution was added into each reactor. The last reactor was loaded with 2.5 mL of DI water only and used as the blank control. The reactors were then loaded into a preheated furnace (Barnstead Thermolyme Co.) at desired reaction temperature and maintained for a desired reaction time. After the reaction time had passed, the reactors were rapidly cooled down by soaking into a water bath. Samples from each reactor were then collected into separate HPLC vials and used for HPLC analysis.

For testing the effects of HTL operating conditions on destructing genetic materials (plasmid DNA), we used 100 mL stainless steel batch HTL reactors with a coupled magnetic stirrer (Parr Instrument Co., Moline, IL). A set of three reactors was run in parallel to

simultaneously test the effects of different HTL operating conditions (i.e. temperature and reaction time). Each reactor was loaded with 70 g of feedstock (either Carb-R-*E. coli* S17-1 λpir or swine manure spiked with Carb-R-*E. coli* S17-1 λpir), sealed, and then purged 3 times with pure nitrogen. The initial pressure was set at 600-635 kPa (88–92 PSI) to prevent water from boiling during the experiment. Finally, the reactor was heated to the desired reaction temperature (250–300°C) and the reaction temperature was maintained for a reaction time of 15–60 min. Subsequently, the reactor was rapidly cooled and the gaseous product was vented. HTL aqueous product was collected and used for DNA extraction.

3.2.5 HPLC Analysis of Florfenicol, Ceftiofur and Estrone

Concentrations of florfenicol (FF), ceftiofur (CF) and estrone (E1) in post-HTL wastewater were analyzed using a Waters 2695 Separations Module HPLC equipped with a Waters 996 PDA detector. Separations were performed using a Phenomenex Luna 5-µm C18 (2) 100 Å 250 mm × 4.6 mm column. An isocratic separation method was used with two mobile phases: 48% solvent A (acetonitrile) and 52% solvent B (0.1% formic acid in DI water at pH=5). The flow rate was maintained at 0.8 mL/min and the injection volume was 30 µL. Wavelengths for quantification for CF, FF and E1 were 290 nm, 224 nm, and 205 nm respectively, and the corresponding retention time for CF, FF and E1 were 4.6 and 5.5 and 16.4 minutes. The detection limit for CF and FF was 0.05 mg/L and 0.08 mg/L for E1.

3.2.6 HPLC Analysis for C-labeled BPA

Detection of ^{14}C-labeled BPA was performed with an HPLC (LC-20 Shimadzu) with a PDA detector (SPD-20A, Shimadzu) and a continuous liquid scintillation counter (CLSC) (β-RAM Model 2, IN/US) connected directly after the PDA detector. IN-Flow 2:1 scintillation cocktail (IN/US) was used as the mobile phase for the CLSC detector to make radioactive decay

events measurable as fluorescent emissions. Two connected columns, a Waters 4 μm Nova-Pak® C18 analytical column (3.9 × 150 mm) and a Waters 4 μm Nova-Pak® C18 guard column (3.9 × 20 mm), were used for reverse-phase separation with a 100 μL sample loop. A binary gradient elution consisting of phosphoric acid (10 mM) solution (A) and pure acetonitrile (B) was used. Details of gradient elution method are shown in Table 3.1, which resulted in a reaction time of 33 min and detection limit 203.8 ng/L for BPA.

Table 3.1 Gradient elution method for newly developed HPLC-CLSC technique

Time (min)	Acetonitrile (%)	Phosphoric acid (%)
0.01	10	90
12	20	80
60	50	50
76	80	20
84	80	20
100	10	90
140	10	90

3.2.7 Liquid Scintillation Counting of Radiolabeled Carbon in Post-HTL Products

The fate of bioactive compounds in biowastes subjected to HTL treatment was studied by spiking radio-labeled compounds (^{14}C-BPA or ^{14}C-Estradiol) into the 20% swine manure feedstock before the HTL process. In this case, the HTL treatment was carried at 300°C and 60 min reaction time for both compounds. Following the liquefaction, the process gas was bubbled into 1N NaOH solution to capture $^{14}CO_2$. After HTL treatment, the radio-labeled compounds were distributed into the different components of post-HTL products (gas, wastewater, and bio-crude oil), which were individually analyzed via scintillation counting with a liquid scintillation spectrometer (Packard TRI-CARB 2100TR). Based on the concentration of radio-labeled compounds in each fraction, a mass balance was conducted to determine the distribution of

radio-labeled carbon after HTL treatment and identify the fraction that contains a majority of the radio-labeled carbon.

3.2.8 DNA Extraction/Purification

Plasmid DNA from pre- and post-HTL treatment of Carb-R- *E. coli* S17-1 λpir culture was extracted using a previously reported method (Sambrook, 2001). Extraction of DNA from swine manure spiked with Carb-R- *E. coli* S17-1 λpir was conducted following a previously published method (Trochimchuk et al., 2003). DNA concentration and size distribution were determined by Nanodrop® ND-1000 (Thermo Scientific) and gel electrophoresis, respectively. The DNA samples were divided into aliquots and stored at −20°C until used.

3.2.9 Natural Transformation of *Azotobacter vinelandii*

Natural transformation of plasmid DNA extracted from the pre-and post-HTL treatment of Carb-R- *E. coli* S17-1 λpir and swine manure to *A.vinelandii* strain DJ (wild type) was conducted following a previously published method (Goetsch et al., 2012). Transformation frequencies were calculated by dividing the number of the colony forming units (CFUs) on selective Burk's medium with carbenicillin (transformants) by the total number of CFUs on Burk's medium alone. Negative controls without DNA were performed for each batch of competent cells and were used to determine detection limits. The average detection limit was a transformation frequency of 1.4×10^{-6}. Frequencies below the detection limit were included in the calculations as the detection limit, providing a conservative estimate of transformation frequency.

3.2.10 High-Efficiency Transformation of *E. coli* by High-voltage Electroporation

Electroporation transformation of plasmid DNA extracted from Carb-R- *E. coli* S17-1 λpir culture pre- and post-HTL treatment was conducted according to previously reported

methods (Dower et al., 1988). This method allows *E. coli* to be transformed at extremely high efficiency by subjecting a mixture of cells and DNA to brief but intense electrical fields of exponential decay waveform (electroporation). The transformation frequency was determined by dividing the number of CFUs on selective LB medium with carbenicillin (transformant) by total number CFUs on LB medium alone. Negative controls without DNA were performed for each batch of competent cells and were used to determine detection limits. No colonies were found on selective plates when no DNA was added, or when DNA and cells were mixed but not subjected to an electric pulse.

3.3 RESULTS AND DISCUSSIONS

3.3.1 Removal of Bioactive Compounds via HTL Treatment without Feedstock Present

Figure 3.1 presents the percent removal of the three bioactive compounds: Estrone (A), Florfenicol (B) and Ceftiofur (C), at six different HTL operating conditions. When the concentration of tested compounds was below the detection limit, we used half of the detection limit to calculate the percent removal. Thus, the maximum percent removal reported using our methods was 98% for Estrone (E1) and was 99.95% for both Florfenicol (FF) and Ceftiofur (CF). Experimental data showed that the descending rank order for bioactive compound removal was CF>FF>E1. Better removal of each compound was generally observed with increasing reaction time or reaction temperature. For example, with a reaction time of 15 min, the percent removal of E1 increased from 76.7% to 99.2% as temperature increased from 250°C and 300°C (Figure 3.1 A, white bar vs. black bar). Removal of FF increased from 94.2% to 99.3% as the temperature increased from 250°C and 300°C, respectively (Figure 3.1 B). Looking at the effect of reaction time, we observed that the removal of E1 at 250°C increased from 76.7% to 98% as the reaction time increased from 15 to 60 min (Figure 3.1 A, white bars). Similarly, removal of

FF at 250°C increased from 94.2% to 99.9% as the reaction time increased from 15 min to 60 min (Figure 3.1 C, white bars). More than 99% removal of CF was obtained at all tested operating conditions (Figure 3.1 C). It is noteworthy that when the reaction temperature was ≥ 300°C, virtually all of the bioactive compounds were removed, and thus increasing reaction time had a negligible effect. Removal to below detection limits was observed at 300°C and 30 min for all tested compounds. However, when the HTL treatment was operated at temperature of 250°C, it required 60 min of reaction time to achieve removal below detection limits for all bioactive compounds.

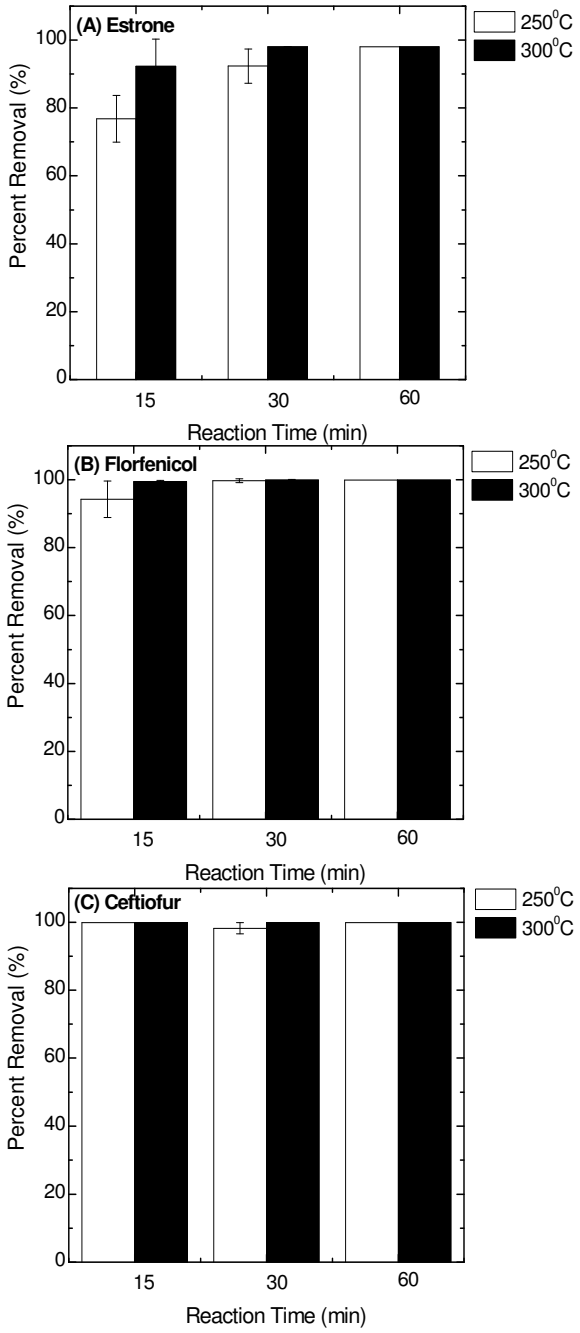

Figure 3.1 Percent removal of estrone (A), florfenicol (B), and ceftiofur (C) by HTL treatment without feedstock present. The average and standard deviation values of three measurements for each tested compound were plotted

3.3.2 Removal of Bioactive Compounds by HTL in the Presence of *Spirulina* Feedstock

In order to study the effect of HTL feedstock on removal of bioactive compounds, we gradually added different amounts of *Spirulina* (from 0.1 to 20% solids concentration) into the starting solution, which contain 50 ppm CF, 50 ppm FF and 2 ppm E1. As the solids content of the *Spirulina* feedstock increased to 10% we encountered analytical problems due to sample matrix interference, and were unable to accurately quantify the concentration of tested compounds post-HTL treatment. Thus, we only reported the percent removal of tested compounds in the presence of 0.1–5% solids content. Figure 3.2 presents the percent removal of E1 in the presence of various concentrations of *Spirulina* solids. Under the HTL conditions of 250°C and 15 min reaction time, the removal of E1 generally decreased as the solids content of *Spirulina* in the HTL reactor increased. Specifically, the removal of E1 decreased from 76% to 61% as the solids content increased from 0.1% to 5%. This suggests that at some HTL operating conditions the presence of biomass feedstocks would interfere with the removal of bioactive compounds. Previous studies have reported that E1 is a hydrophobic compound of low volatility, low water solubility of 0.8 to 12.4 mg/L, and a log K_{OW} value of 3.43 (Hurwitz & Liu, 1977; Shareef et al., 2006; Ying et al., 2002). The low aqueous solubility of E1 can influence its partitioning between the aqueous and solids phase (*Spirulina*), and make it more resistant to breakdown during HTL treatment. Previous studies have shown that sorption is the major mechanism for removing E1 in an algal-based wastewater treatment system, and it can be well sorbed by various strains of algae, including *Spirulina* (Shi et al., 2010). One possible explanation for this phenomenon is that the hydrophobic nature of E1 enhances its sorption to *Spirulina* and thus, makes E1 less accessible during HTL treatment, especially when a low reaction

temperature and short reaction time was used. However, as the reaction time increased to 30 min and above, the effect of the sample matrix on the removal of E1 became negligible (Figure 3.2 A). Additionally, variations in the solids content of HTL feedstock did not affect removal of E1 (Figure 3.2 B) when the HTL was operated at 300°C.

Similar results were also obtained for FF removal in the presence of *Spirulina* feedstock. As demonstrated in Figure 3.3 A, the removal of FF also decreased as the feedstock solids content increased under HTL conditions of 250°C and 15 min reaction time. For example, the removal of FF decreased from 87% to 76% as the solids content increased from 0.1% to 5%, confirming that the presence of feedstock could also provide some protective effect for this bioactive compound. However, when the reaction time was extended to 30 min and longer, the presence of feedstock did not show any clear effect on FF removal (Figure 3.3 A). The protective effect provided by the *Spirulina* feedstock was smaller with FF than E1. This is likely due to the higher water solubility (1.32 mg/mL) and lower log K_{ow} value of 0.37 (FDA, 2004) for FF, which indicates that FF will preferentially be in water rather than sorbed to *Spirulina.* When the HTL process was operated at 300°C, the percent removal of FF was greater than 95%, suggesting that any protective effect provided by the presence of feedstock could be overcome by increasing the reaction temperature (Figure 3.3 B). For HTL at 300°C and reaction times of 30 minutes or more, removal of FF was observed to near or below detection limits.

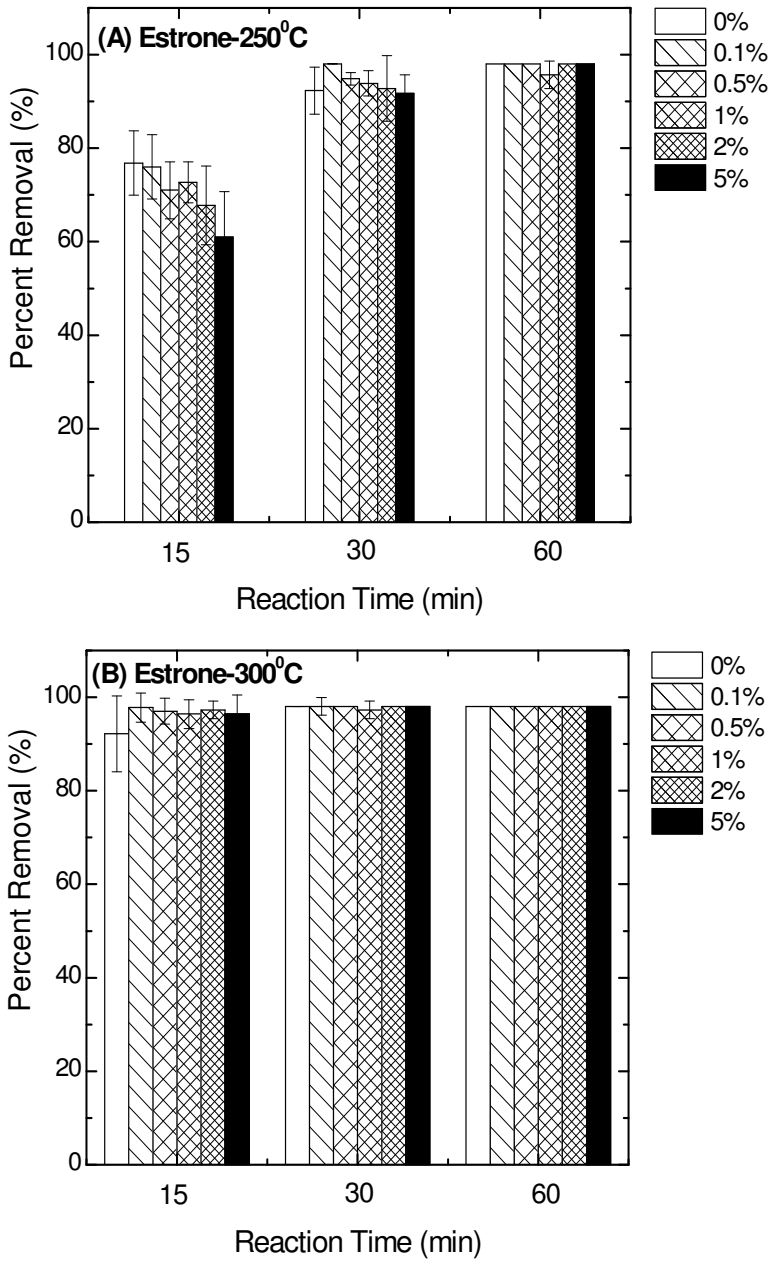

Figure 3.2 Percent removal of estrone in the presence of 0.1–5% *Spirulina* feedstock at (A) 250°C and (B) 300°C. The average and standard deviation values of three measurements were plotted.

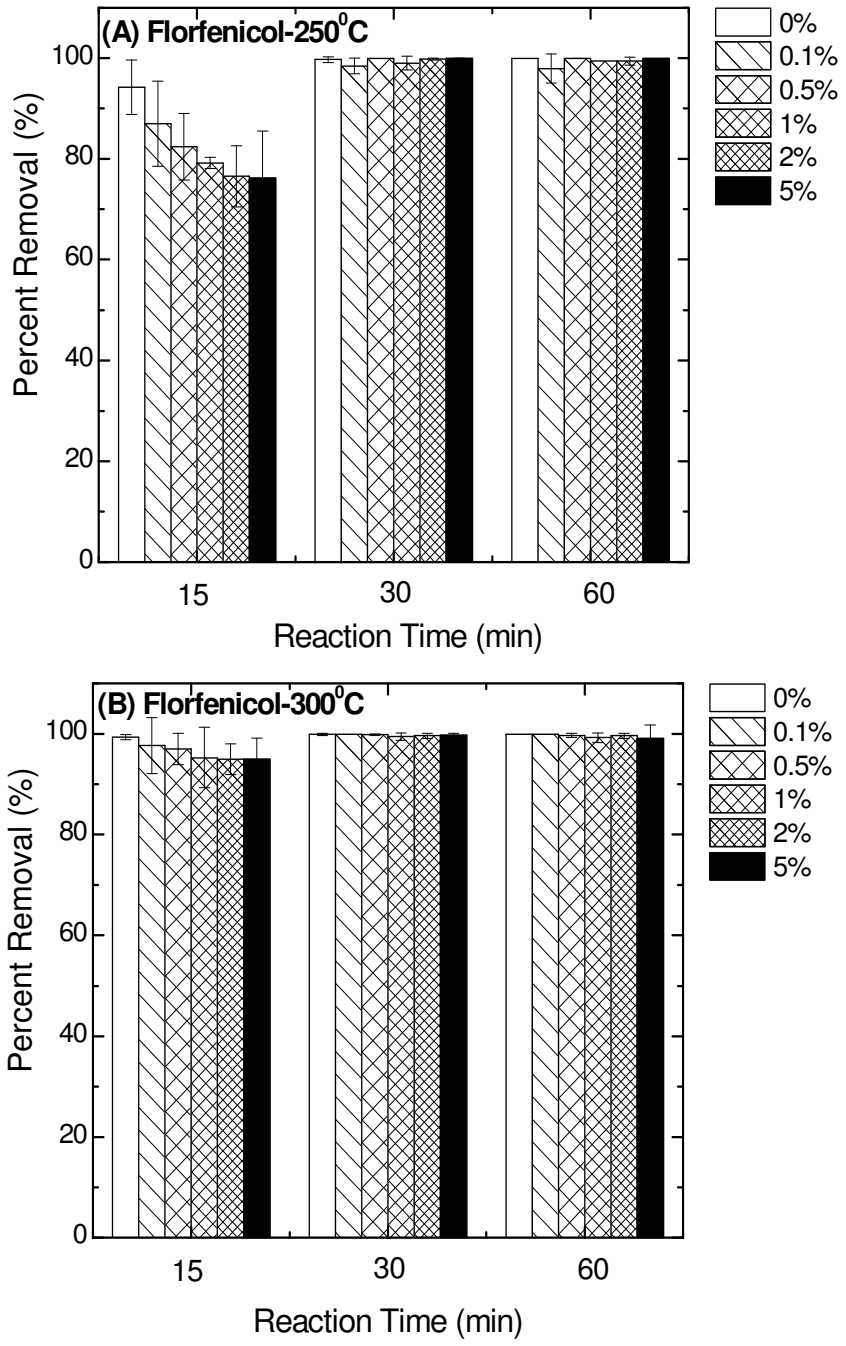

Figure 3.3 Percent removal of florfenicol in the presence of 0.1–5% *Spirulina* feedstock at (A) 250°C and (B) 300°C. The average and standard deviation values of three measurements were plotted.

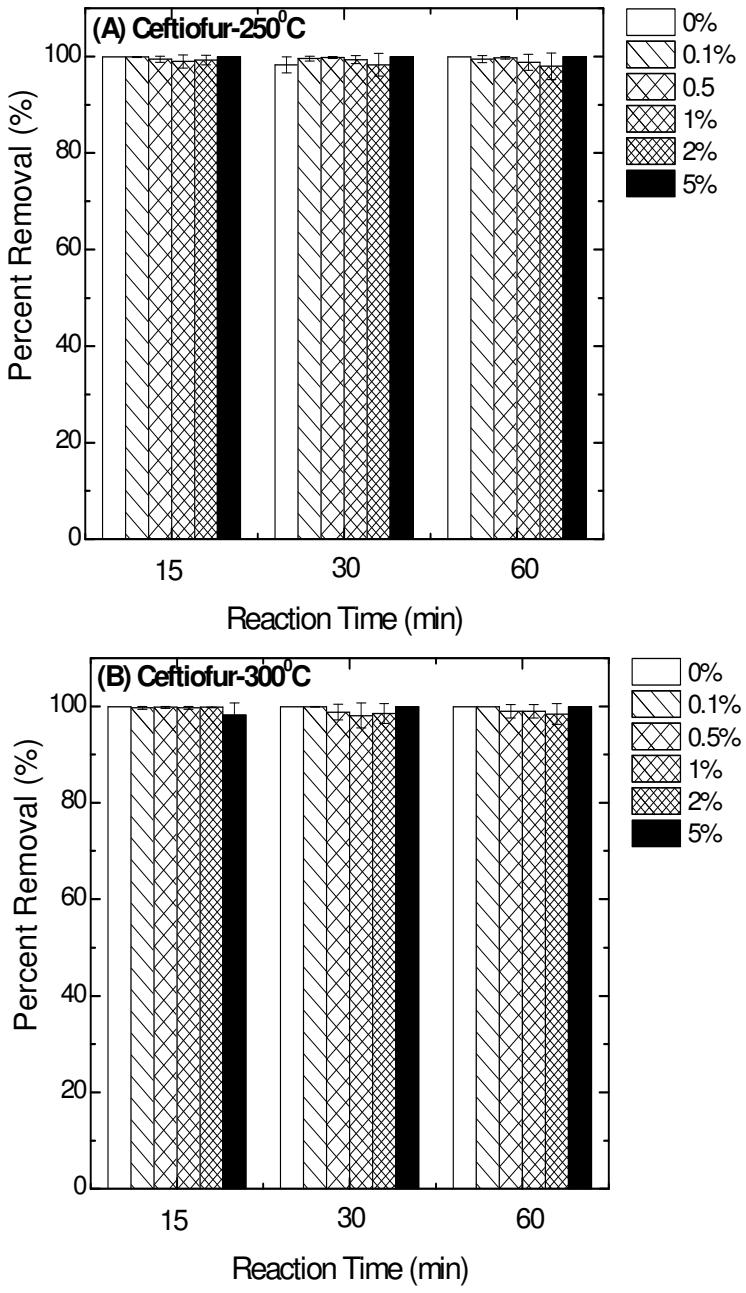

Figure 3.4 Percent removal of ceftiofur in the presence of 0.1–5% *Spirulina* feedstock at (A) 250°C and (B) 300°C. The average and standard deviation values of three measurements were plotted.

Figure 3.4 presents the percent removal of CF in the presence of various amount of *Spirulina* feedstock. Unlike FF and E1, removal of CF was not affected by the presence of *Spirulina* feedstock. Essentially complete removal of CF was obtained for all tested HTL operating conditions. This could result from the fact that CF is highly soluble in water and thus, it is more accessible during the HTL treatment. This result was also consistent with the data we obtained for HTL treatment of pure CF solution in DI water, where essentially complete removal of CF was achieved for all tested conditions. Previous studies have shown that CF could be rapidly degraded in aqueous solution and destruction rate increases with increasing temperature and pH (Li et al., 2011; Sunkara et al., 1999). This past work noted that CF degradation was most accelerated at pH 7.4, which happened to be the pH of our HTL feedstock (Sunkara et al., 1999).

Comparing the behavior of all the tested compounds highlights that the effectiveness of HTL for destructing bioactive compounds is specific to the chemical characteristics of each compound, but the differences primarily occur at lower HTL temperatures and shorter reaction times. Specifically, we found that hydrophobic compounds such as E1 are likely to be more persistent after HTL treatment than hydrophilic compounds such as CF and FF. Interactions between test compounds and the feedstock would make bioactive compounds less accessible and/or more resistant to degradation during HTL treatment. These interaction effects became less important when HTL reaction time was extended to 60 min with a temperature of 250°C, and were also minimal or insignificant when the reaction temperature was above 300°C. Optimal HTL operating conditions are specific to the feedstock, the reactor configuration and desired product characteristics. Nonetheless, the HTL conditions that provide a high level of removal for bioactive chemicals like estrogenic compounds and pharmaceuticals (250°C for ≥ 60 min or at

300°C for ≥15 min) also provide desirable yields of bio-crude oil (Jena et al., 2011a; Jena et al., 2011b; Yu, 2012; Yu et al., 2011a). Thus, there is good compatibility between the goals of bioenergy production via HTL and removal of bioactive compounds from wastes.

3.3.3 Destruction of Bisphenol A by HTL in the Presence of Swine Manure Feedstock

As mentioned above, we encountered difficulty of detecting CF, FF, and E1 post-HTL treatment due to sample matrix interference when more than 5% solids content feedstock was used. Meanwhile, the typical solids content of HTL feedstock would be around 5–20%. Thus, we developed a new HPLC-CLSC technique that allows detection of bioactive compounds at trace levels in the presence of higher amounts of biosolids feedstock. 20% swine manure feedstock was spiked with radio-labeled ^{14}C-BPA and used for HTL at 300°C and three different reaction times 15, 45 and 60 min. As demonstrated in Figure 3.5, no peak for ^{14}C-BPA was detected after HTL treatment of swine manure feedstock. Instead, the chromatograms show that there is a breakdown product of ^{14}C-BPA with a much shorter retention time (11 min vs. 33 min) in all of three tested samples after HTL treatment. Variations in HTL reaction time did not significantly affect the retention time of BPA breakdown products suggesting that the destruction of ^{14}C-BPA occurred fairly quickly after the reaction time started and the breakdown product is relatively stable. The amount of the BPA breakdown product at 15 min of reaction time was noticeably less than at 45 min and 60 min. A previous study showed that the amount of raw oil product from HTL processing of swine manure was slightly decreasing as reaction time increased from 15 to 60 minutes, and the organic content of HTL wastewater was increasing as HTL reaction time increased (He et al., 2000a). This would explain why less BPA breakdown product was found in post HTL-WW at short reaction times, because more of it is associated with the raw oil product at shorter reaction times. These results suggest that operation of HTL at

temperature ≥300°C and for 30 min reaction time would be sufficient to eliminate the protective effect of HTL feedstock and provide essentially complete removal of bioactive compounds even in the presence of high levels of feedstock biosolids.

3.3.4 Fate of Bioactive Compounds in Biowastes after HTL Treatment

Figure 3.6 presents experimental data tracking the fate of radiolabeled carbon in HTL products and showed that after HTL treatment, the majority of the radiolabeled carbon in two estrogenic compounds were distributed to the crude oil fraction (BPA-79% and Estradiol-60.23%), and most of the rest was found in the post-HTL wastewater (Figure 3.6). Because the oil fraction will eventually be combusted, this product will ultimately lead to complete destruction of the bioactive compounds. And the previous section showed that the bioactive compounds in the post-HTL wastewater have also been destructed from their original bioactive form. Thus, the HTL process can indeed provide significant removal of a range of trace level bioactive contaminants in biowastes while producing bio-crude oil.

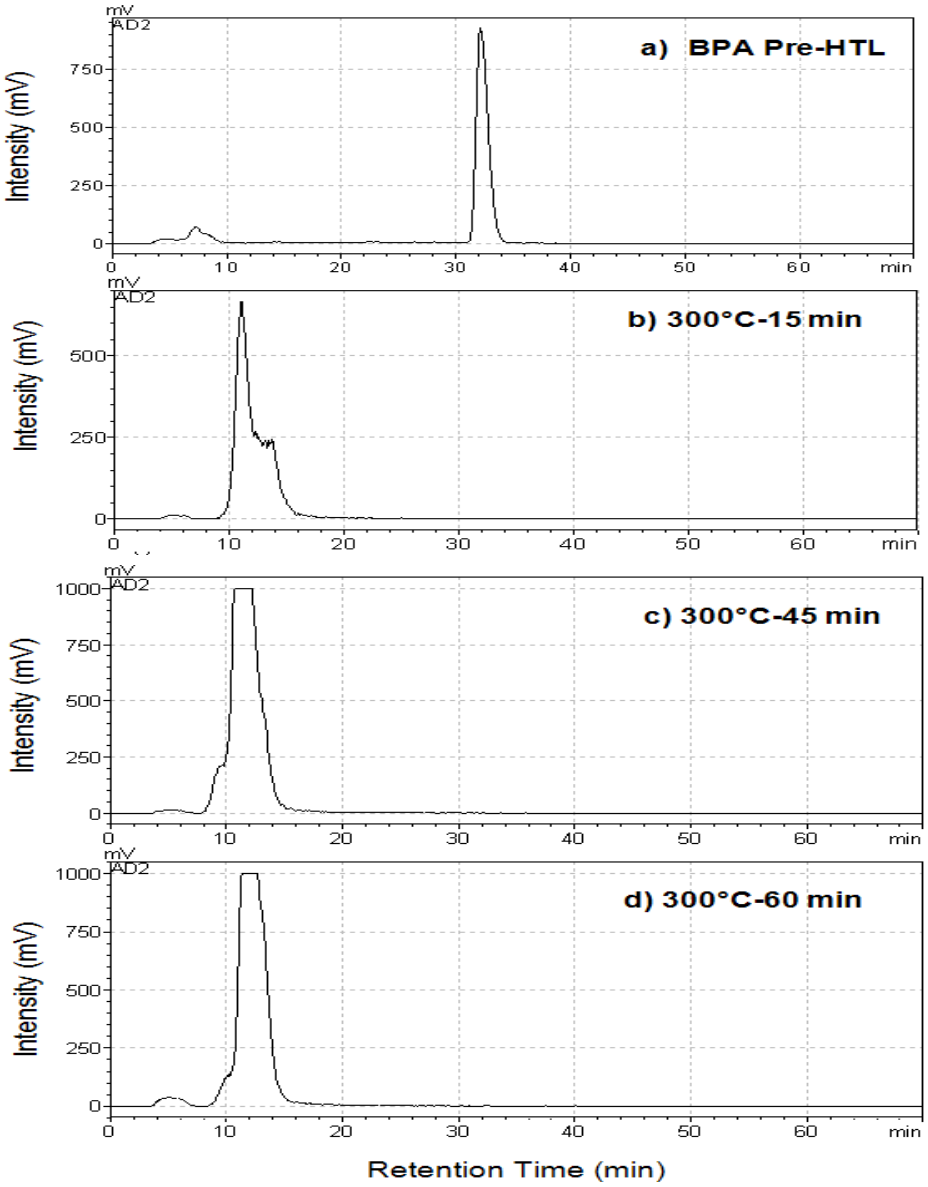

Figure 3.5 Chromatograms of 14C-BPA (A) before HTL treatment and its breakdown product(s) after HTL treatment with 20% swine manure feedstock processed at 300°C and three different reaction times: (B) 15 min, (C) 45 min, and (D) 60 min.

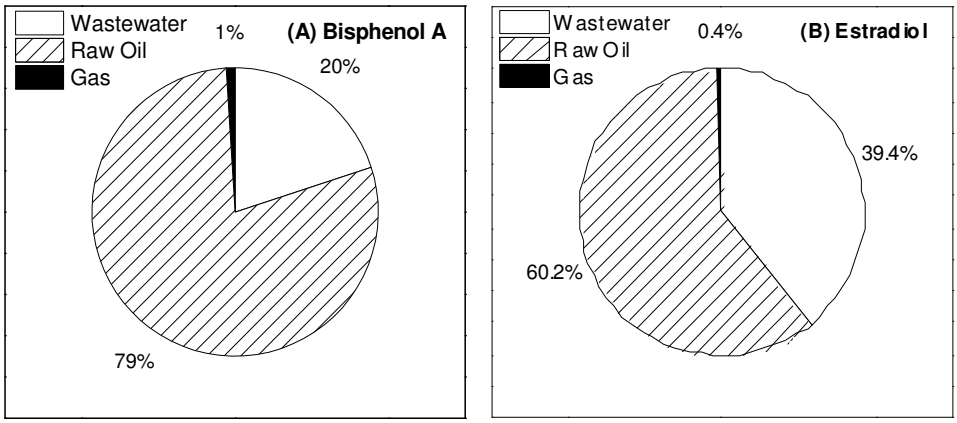

Figure 3.6 Distribution of 14C from bioactive compounds into the various products of HTL treatment for 20% swine manure processed at 300°C and 60 min reaction time: (A) Bisphenol A; (B) Estradiol

3.3.5 Destruction of Plasmid DNA via HTL Treatment

Figure 3.7 presents the percent removal of plasmid DNA during HTL treatment of a Carb-R- *E. coli* S17-1 λpir culture by itself and 20% solid swine manure spiked with Carb-R- *E. coli* S17-1 λpir culture. Experimental data showed more than 99% removal of plasmid DNA from Carb-R- *E. coli* S17-1 λpir culture under all tested operating conditions without any manure present in the reaction mixture. Variation in reaction temperature and reaction time did not considerably affect the removal of plasmid DNA.

HTL treatment of 20% solid swine manure spiked with Carb-R-*E. coli* S17-1 λpir at 250°C for 15–60 min provided 95–97% removal of plasmid DNA. In comparison to results without manure present but with the same reaction temperature and time, percent removal of plasmid DNA by HTL treatment was on average a few percent lower in the presence of 20% solid swine manure (Figure 3.7, white bars vs. striped bars). This observation suggests that

swine manure matrix can provide some small amount of protection for genetic materials and make them less accessible during HTL treatment.

Figure 3.8 shows the size and yield of plasmid DNA pre- and post-HTL treatment of Carb-R-*E. coli* S17-1λ culture when extracted DNA is run on an electrophoretic agarose gel. As indicated in Figure 3.8, plasmid DNA was successfully extracted from fresh liquid culture of Carb-R-*E. coli* S17-1λ and appeared as a clear band in well 2 and 3. However, the DNA extracted after HTL treatment of Carb-R-*E. coli* S17-1λ culture did not appear in the main body of the gel under all HTL treatment conditions (Wells 4 to 9), suggesting that plasmid DNA was broken down into small fragments that were not detectable in this assay.

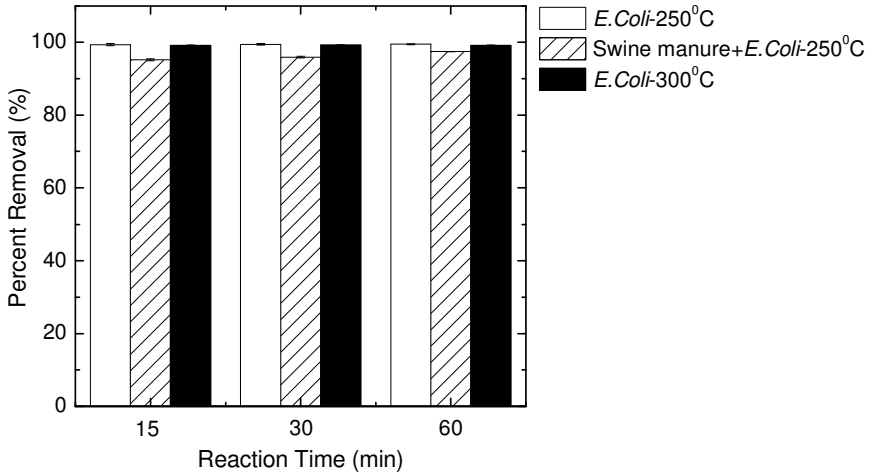

Figure 3.7 Percent removal of plasmid DNA extracted before and after HTL treatment of Carb-R-*E. coli* S17-1 λpir culture under different operating conditions (temperature, reaction time, and presences/absence of swine manure feedstock). The average and standard deviation values of three DNA extractions were plotted.

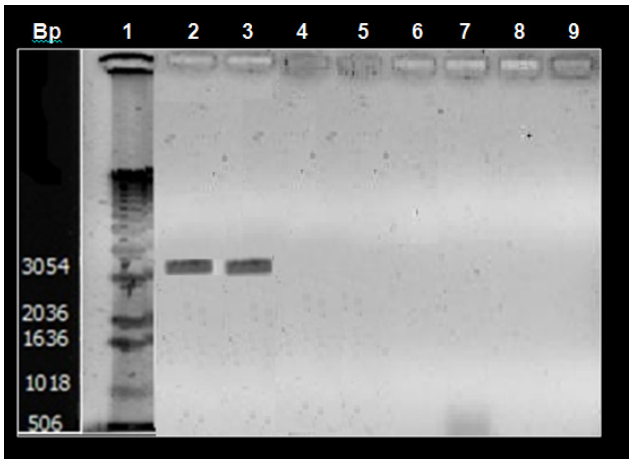

Figure 3.8 Agarose gel of plasmid DNA extracted from pure Carb-R-*E. coli* S17-1 λpir culture pre-HTL (Wells 2 and 3) and post-HTL treatments (Wells 4–9) versus DNA fragment size standards and the associated number of base pairs (Bp, Well 1)

3.3.6 High-Efficiency Electro-Transformation of *E. coli*

To further confirm that post-HTL plasmid DNA had been completely deactivated by HTL treatment, we also conducted a series of high-efficiency electro-transformation experiments to represent idealized conditions for gene transfer. The electro-transformation technique can improve on the natural transformation efficiencies of *E. coli* by subjecting a mixture of cells and DNA to brief but intense electrical fields of exponential decay waveform (electroporation). Thus, if the post-HTL plasmid DNA fragments are still active and carry the antibiotic resistant gene, we would have a better chance of being able to transfer it to other bacteria by using this electro-transformation technique. The DNA extracted from pre- and post-HTL treatments of Carb-R-*E. coli* S17-1λ culture was transferred into *E. coli* S17-1 λpir. Table presents the transformation frequencies of *E. coli* S17-1 λpir with plasmid DNA extracted before and after HTL treatment of Carb-R-*E. coli* S17-1 λpir culture and for treatment of 20% swine manure spiked with Carb-R-*E. coli* S17-1 λpir. As shown in Table 3.2, the pre-HTL treatment DNA extracted from Carb-R-*E.*

coli S17-1 λpir culture was successfully transferred to *E. coli* S17-1 λpir at a frequency of $1.65\times10^{-4}\pm10^{-5}$. Plasmid DNA extracted from 20% swine manure spiked with Carb-R-*E. coli* S17-1 λpir pre-HTL treatment was also transferred to *E. coli* S17-1 λpir at a lower frequency of $1.57\times10^{-5}\pm1.6\times10^{-6}$. Transformation frequencies of different *E. coli* strains using electroporation have been reported in the range of 1.6×10^{-6} to 7.8×10^{-1}, depending on the concentration of DNA and the strain of *E. coli* used (Dower et al., 1988). Thus, our transformation frequencies are in the middle of the range found in previously published work. Once again, after all HTL treatments, no successful transformation of DNA was observed with *E. coli* S17- 1 λpir even with the added efficiency of electro-transformation, indicating that HTL had indeed completely deactivated the DNA. The effects of HTL operating conditions on destruction of DNA and transformation frequency might be more visible at lower operating temperature and shorter reaction times. However, we did not investigate such conditions as we focused on the range of practical HTL operating conditions that provide good bio-crude oil yields. As demonstrated in both natural transformation and electro-transformation experiments, plasmid DNA can be successfully transferred to other environmental bacteria, and it would be good to remove and/or deactivate this DNA in animal waste prior to releasing it to the environment. The present study used multiple experimental approaches to confirm that HTL treatment can effectively destroy genetic materials (plasmid DNA) by breaking them up into small, inactive fragments. Thus, HTL treatment can effectively deactivate genetic materials in biowastes and prevent the potential of transferring antibiotic resistant materials from biowastes into the environment.

Table 3.2 High efficiency electro-transformation of *E. coli* with plasmid DNA extracted before and after HTL treatment of Carb-R-*E. coli* S17-1 λpir alone and 20% swine manure spiked with Carb-R-*E. coli* S17-1 λpir

DNA Donor	HTL Operating Conditions	Recipient	Transformation Frequency (±SD[a])
Carb-R-*E. coli* S17-1 λpir	Pre-HTL treatment	*E. coli* S17-1 λpir	$1.65\times10^{-4}\pm10^{-5}$
	250°C-15 min RT	*E. coli* S17-1 λpir	Not detected (ND)
	250°C-30 min RT	*E. coli* S17-1 λpir	ND
	250°C-60min RT	*E. coli* S17-1 λpir	ND
	300°C-15 min RT	*E. coli* S17-1 λpir	ND
	300°C-30 min RT	*E. coli* S17-1 λpir	ND
	300°C-60 min RT	*E. coli* S17-1 λpir	ND
20% Swine manure spiked with Carb-R-*E. coli* S17-1 λpir	Pre-HTL treatment	*E. coli* S17-1 λpir	$1.57\times10^{-5}\pm1.6\times10^{-5}$
	250°C-15 min RT	*E. coli* S17-1 λpir	ND
	250°C-30 min RT	*E. coli* S17-1 λpir	ND
	250°C-60min RT	*E. coli* S17-1 λpir	ND

[a]SD: Standard deviation

3.4 CONCLUSIONS

In conclusion, we confirmed with a variety of experiments that HTL treatment can effectively destroy genetic materials (plasmid DNA) by breaking them up into small, inactive fragments that are not active if transferred to other bacteria. Removal of DNA from the biowastes by HTL treatment was in the range of 95% to 99.8% for all tested HTL operating conditions. At lower operating temperatures (≤250°C), extending the retention time would slightly enhance the destruction of DNA in biowastes. However, at an operating temperature of 300°C and above, the effect of retention time was insignificant. Since most HTL treatments would be conducted at a temperature above 250°C and a retention time of 60 min or longer for optimal oil yield, all genetic materials in the biowastes are expected to be destroyed. The biosolids matrix could provide some protective effect for genetic materials in biowastes during

HTL treatment. This protective effect, however, can be overcome by extending the retention time and/or increasing operating temperature. Though there was a certain amount of DNA remaining in post-HTL wastewater, this DNA was completely deactivated, as demonstrated in a variety of transformation experiments. Under both high efficiency electro-transformation and natural transformation conditions, we observed 100% reduction in transformation frequencies. Thus, we conclude that HTL treatment can effectively deactivate genetic materials in biowastes and prevent the potential of transferring antibiotic resistant materials from biowastes into the environment.

HTL processing of livestock manure can also effectively destruct a broad range of bioactive compounds under practical operating conditions (>250°C and 60 min retention time). Extending HTL reaction time from 15 to 60 minutes provided some additional removal of bioactive compounds when HTL was operated at temperature ≤250°C. However, when HTL was operated at a temperature of 300°C and above, the effect of HTL reaction time on bioactive compounds removal was minimal. The presence of HTL feedstock lowered the removal of bioactive compounds by 5–10%, when the HTL was operated at a lower temperature (≤250°C) and shorter retention times (≤ 30 min). Experimental results also showed essentially complete removal of all tested compounds in presence of *Spirulina* (up to 5% solids content) or swine manure (20% solids content) when HTL was operated at 300°C and ≥30 min reaction time. These HTL operating conditions are also practical conditions for providing good oil yield. Thus, the HTL process can be successfully utilized to simultaneously produce valuable bio-crude oil and destruct bioactive compounds in animal waste. As a result, health and ecosystem risks associated with bioactive compounds in biowastes can be mitigated via HTL treatment. In

addition, the costs associated with antibiotic resistant treatments and the development of new antibiotics could be reduced over the long run.

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CHAPTER 4: CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF WASTEWATER FROM HYDROTHERMAL LIQUEFACTION CONVERSION OF BIOMASS TO BIOFUELS

4.1 INTRODUCTION

Hydrothermal liquefaction (HTL) is a thermochemical conversion process that can transform wet bio-solids (up to 85% water content) into valuable, self-separating co-products, namely bio-oil and bio-char (Anastasakis & Ross, 2011; Brown et al., 2010; Duan & Savage, 2011; He et al., 2000; Valdez et al., 2012; Yu et al., 2011). This conversion process also produces a gaseous product (mostly CO_2), and an aqueous wastewater product with high concentrations of nutrients and dissolved organics. In essence, HTL removes oxygen, nitrogen, and other micro-constituents from biomass, and liberates them into the gaseous and aqueous fractions. Thus, the bio-crude oil fraction has a much higher energy density than the parent material, which can approach that of petroleum.

HTL conversion of algal biomass is an attractive biofuel alternative that resolves several practical limitations associated with current large-scale algal biofuel production. First, it resolves problems associated with contamination of target high-oil algal species, because HTL can also convert low-oil algae, bacteria, and other grazing micro-organisms into oil. Secondly, dewatering/drying energy is minimized because HTL can use wet feedstocks, and the resulting bio-oil is self-separating from the aqueous fraction. Finally, nutrients and CO_2 released to the aqueous and gaseous fractions can be recycled back into algal cultivation, lowering the cost of these major inputs for algal cultivation. These factors make the production of algal biofuels via HTL conversion a promising alternative for sustainable and cost-effective bioenergy. Thus, it is

also important to characterize the co-products of this process and potential environmental impacts.

Extensive work has been done to optimize HTL conversion of bio-solids to a valuable bio-oil, which has shown oil conversion efficiencies of 30−75% for algae and biowaste feedstocks and a net positive energy yield of 3−10 times input heat energy (Zhang, 2008). The chemical properties of bio-oil from HTL conversion of different feedstock such as *Spirulina*, swine manure, and anaerobically digested sewage sludge have also been well characterized (Anastasakis & Ross, 2011; Brown et al., 2010; Duan & Savage, 2011; Jena et al., 2011a; Jena et al., 2011b; Zhou et al., 2010). However, a much smaller amount of information is available from past studies on HTL-WW. One previous study, showed that the wastewater from HTL conversion of swine manure contained a very high concentration of biological oxygen demand (420−59000 mg/L), a high concentration of ammonia (1860−7070 mg/L) and had other characteristics that make HTL-WW unsuitable for surface water discharge (Appleford, 2004). Few studies have characterized the organic composition of HTL-WW (Anastasakis & Ross, 2011; Villadsen et al., 2012; Zhou et al., 2010). However, the previous studies mostly relied on GC-MS library identification of organics in HTL-WW, and did not use external chemical standards to confirm or quantify the concentrations of these compounds.

This work focuses on chemical and biological characterization of HTL-WW composition with an emphasis on nitrogenous organic compounds (NOCs). NOCs were selected in this study because they can potentially be recycled and utilized as nutrients to support algal cultivation (Antia et al., 1991; Seitzinger & Sanders, 1997). Analysis of HTL products has shown that the majority of the nitrogen in algal feedstock was distributed to aqueous phase product of HTL (Yu et al., 2011). Past research on HTL treatment of brown macro-algae, showed a substantial portion

of aqueous organics were NOCs, including indoles, pyrrole derivatives and 3-amino-phenol (Anastasakis & Ross, 2011). The speciation of nitrogenous compounds in HTL-WW also affects the ability to treat and/or safely discharge HTL-WW to the environment (Berman et al., 1999; Berman & Chava, 1999; Lee et al., 2007). Thus, it is desirable to have a better understanding of the characterization and quantification of NOCs in HTL-WW.

Petroleum and the water found in contact with natural petroleum deposits typically exhibits significant toxicity to aquatic organisms (Girling, 1989; Griffin & Calder, 1977; Henderson et al., 1999; Johnsen et al., 1994; Neff et al., 2006). Thus, it is important to evaluate the toxicity of NOCs and other organic compounds in HTL-WW, both for environmental protection and to evaluate the potential for inhibitory effects when recycling these compounds back into algal cultivation systems. To date, there are no toxicity studies published for HTL-WW. However, a previously published list of 48 hazardous constituents likely to be found in the post-HTL wastewater (Elliot, 1992) did contain several compounds with reported toxic effects, specifically, phenol, toluene, benzene, 2-methylarizidine, and aziridine (Netzeva et al., 2004; Verschaeve & Kirschvolders, 1990; Weisburger et al., 1981; Yardleyjones et al., 1991; Zhao et al., 2009). High concentrations of ammonia in HTL-WW may also be toxic to many aquatic organisms (Camargo & Ward, 1995; Scott & Crunkilton, 2000). Previous studies also indicated that HTL-WW has to be diluted at least 20-fold in order to culture algae (Jena et al., 2011b; Zhou, 2010; Zhou et al., 2011). These results suggest that HTL-WW is likely to have toxic effects in natural ecosystems and require treatment prior to discharge.

The objectives of this chapter were to: i) characterize and quantify organic nitrogen composition of HTL-WWW; (ii) quantify and compare the inhibition effect of individual NOCs

on algal growth; and (iii) quantity and compare the toxicity of individual NOCs, the organic mixture in HTL-WW, the fractionated HTL-WW and the original stock HTL-WW.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals and Reagents

2-Picoline (Cas# 109-06-8), 2-Pyrrolidinone (Cas #616-45-5), phenol (CAS# 108-95-2) were purchased from Alfa Aesar; 2,6-dimethyl-3-pyridinol (CAS# 1122-43-6), ε-caprolactam (CAS#106-60-2), and δ-valerolactam (CAS#675-20-7), 3- dimethylamino-phenol (CAS#99-07-0), 2,2,6,6-tetramethyl-4-piperidinol (CAS# 2403-88-5), pyridine (CAS 110-86-1), and 1-methyl-2-pyrrolidinone (CAS# 872-50-4) were purchased from Sigma Aldrich (Milwaukee, WI). Solvents (methanol, ethyl acetate, dichloromethane, and dimethylsulfoxide) were purchased from Sigma-Aldrich (Milwaukee, WI). All chemicals and solvents were purchased at the highest level of purity available.

4.2.2 HTL Feedstock and Experiment

Spirulina biomass (solids content of 95%) were obtained in dry powder form Cyanotech (Kailua-Kona, Hawaii) and stored at 4°C prior to processing. HTL experiments were conducted following the same method described in Chapter 2. However, a bigger reactor with the capacity of 1000mL was used to produce larger volume of HTL-WW for extraction purpose.

4.2.3 Liquid-Liquid Extraction of Heteroaromatic Compounds from Post-HTLW

Heteroaromatic compounds in HTL-WW were extracted using a published method(Johansen et al., 1996). Fifty mL of dichloromethane (DCM), 2.5 mL of HTL-WW and 5 mL of potassium hydroxide (5M) were added to a 250 mL seperatory funnel and slowly inverted several times. The emulsion was released (2-5 min) and the DCM layer was collected as

extraction part 1. The remaining layer was recovered and adjusted to pH 5 with 6M HCl. The pH adjusted sample was poured back into the separatory funnel and further extracted with DCM. Finally, the DCM layer was collected as extraction part 2. Both extraction part 1 and 2 were concentrated to 1 mL with the aid of a TurboVap concentrator, transferred to a GC vial, and stored in −20°C prior to GC-MS analysis.

4.2.4 Gas Chromatography Analysis

Specific NOCs were profiled with an Agilent Technologies 7820A gas chromatogram with thermionic detection (GC-NPD). Separation was achieved with a Varian CP-sil 8 column (30m×0.25mm×0.25 μm) with helium at a flow rate of 1 ml/min. A 1-μL injection of the extract was performed at 275°C at a spilt ratio of 1:100. The column was initially held at 35°C for 5 min, then increased at a rate of 25°C /min to 130°C, then held for 4 min and then increased at a rate of 25°C /min to 240°C, held for an additional 4 min, and finally increased at a rate of 25°C /min .to a final temperature of 280°C and held for 7 min.

General scan of organic compounds in HTL-WW was performed under the same conditions as GC-NPD analysis. The magnetic sector mass spectrometer (Waters Ultima) collected and measured all masses ranging from 35 Daltons to 350 Daltons. The data was processed with AMDIS (National Institute of Standards and Technology-NIST) with a NIST 2002 mass spectra library. Peaks matching retention time data from the GC-NPD analysis were searched with a nitrogen constraint algorithm.

Identification and quantization of the NOC's was performed by calibration with reference materials procured from commercial sources. Identical instrumental conditions were utilized from general scan measurements; however the instrument was operated in high-resolution (HR)

mode (10,000). Single ion recording programs (SIR) were generated based on injection of reference compounds. Analysis with SIR programs can greatly enhance instrument sensitivity and reduce matrix interference since only ions of interested are collected and measured.

Analysis of phenols was also performed by GC-HRMS. Separation was achieved with a Restek Rtx-5MS column (30m×0.25mm×0.25 μm) with helium at a flow rate of 1 ml/min. A 1μL spiltless injection of each fraction was performed at 300°C. The column was initially held at 40°C for 2 min, then increased at a rate of 25°C /min to 245°C, then increased at a rate of 6°C /min to a final temperature of 330°C. Single ion recording programs (SIR) for phenol analysis were also generated based on injection of reference compounds.

4.2.5 Extraction of Organic Compounds from HTL-WW

Organic compounds in HTL-WW were extracted following an EPA's standard method for extracting disinfection byproducts (Richardson, 2011). Due to the high concentration of organic compound in HTL-WW, only 1L of 10% HTL-WW was used for extraction. The XAD-2 (CAS# 10357) and XAD-8 (CAS# 20278) resins were purchased from Sigma-Aldrich. The resins were prepared by consecutively washed with 0.1 N NaOH, distil water, and methanol for 30 min. After that, XAD resins were further washed for 24 h using Soxhlet extraction with methanol, ethyl acetate, and methanol. Washed XAD resins were stored in methanol at 4°C prior to uses. A chromatography column (i.d. × length: 28 mm × 400 mm with a 1000 mL reservoir) was packed with 100 mL of XAD-2 resin followed by 100 mL of XAD-8 resin. The column was consecutively rinsed with three resin volumes of ultrapure water, two resin volumes of 0.1 N HCl, a single resin volume of 0.1 N NaOH, and a single rinse of ultrapure water. Due to the high concentration of organic compound in HTL-WW, only 1 L of 10% HTL-WW was used for extraction. Prior to the extraction, HTL-WW samples were adjusted to a pH <1 using 12 N HCl

and slowly passed through the packed resin beds and allowed to drain completely. Organic compounds that adsorbed to the resin were then eluted with 400 mL of ethyl acetate. The ethyl acetate was collected in a separatory funnel, and the bottom layer was discarded. The remaining ethyl acetate was passed through organic free sodium sulfate to remove water and concentrated to 1mL with the aid of a RotoVap concentrator. The extracts were transferred to 1 mL conical vials and stored at –20°C before processing.

4.2.6 Fractionation of HTL-WW

HTL-WW was fractionated into hydrophobic and hydrophilic fractions using Amberlite XAD-8 resin (now called Superlite DAX-8 resin, Sigma Aldrich, Inc.). The resin was selected because it has long been used as standard materials for extraction of humic substances. HTL-WW was fractionated into hydrophilic, hydrophobic base, hydrophobic neutral and hydrophobic acid fractions following a previously published study (Leenheer, 1981). Prior to the fractionation, XAD resin was subsequently cleaned for 24 hour with acetone and hexane using Soxhlet extractions. Cleaned resin was store in methanol prior to be used for column packing. The XAD column then rinsed with 0.1N NaOH, 0.1N HCl, and distilled water just before application of the HTL-WW sample. HTL-WW was pumped through the column at the flow rate of 1mL/min and the effluent was collected as the hydrophilic fraction. Hydrophobic base fraction was obtained by back flush eluted with 0.25 bed volume of 0.1N HCl followed by 1.5 bed volume of distilled water. Hydrophobic acids were desorbed by back flush eluted with 0.25 bed volume of 0.1 NaOH followed by 1.5 bed volume of distilled water. After the elution of hydrophobic acid fractions, the XAD resin was pump dried . The resin was then unpacked and spread upon sheets of aluminum foil and air dried at room temperature for 15 hours. The dried

resin was then packed into a Soxhlet extractor and extracted with anhydrous methanol to obtain the hydrophobic neutral fractions. Fractionated HTL-WW was stored at 4°C prior to processing.

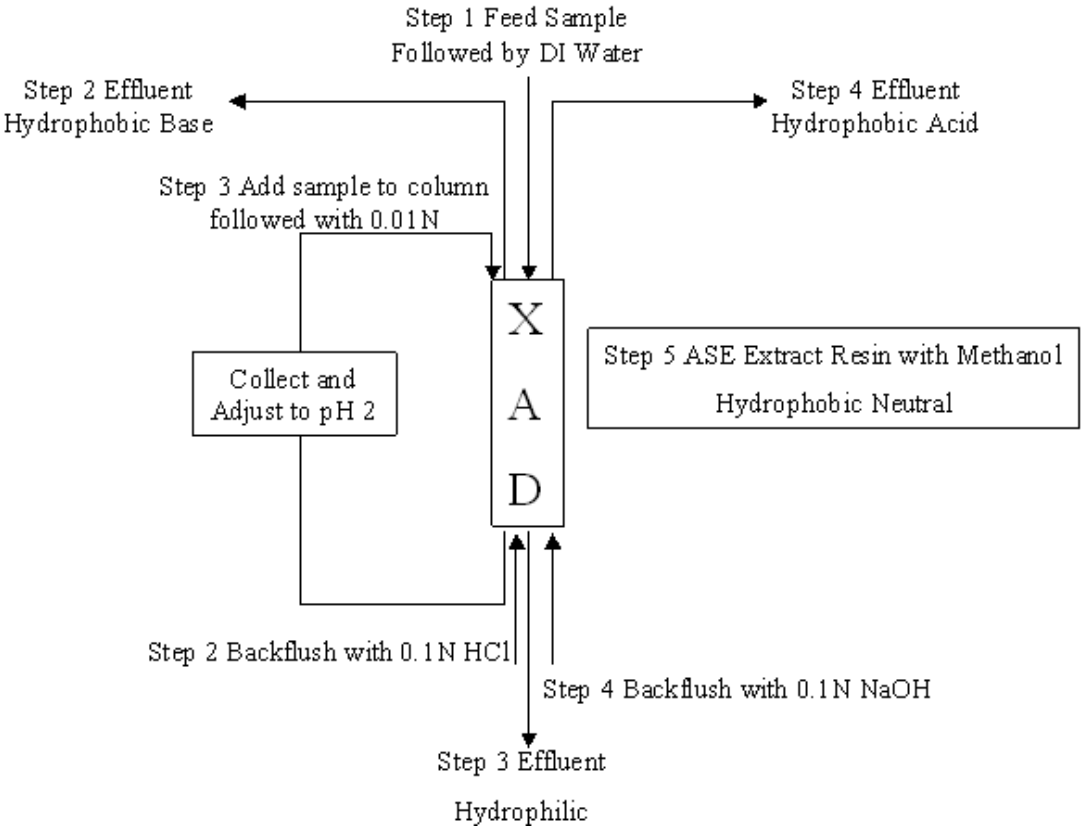


Figure 4.1 Analytical procedure for fractionating HTL-WW into hydrophobic and hydrophilic fractions

4.2.7 Chinese Hamster Ovary Cells

Chinese hamster ovary (CHO) cell line AS52, clone 11-4-8 was used for the cytotoxicity assay (Hsie AW, 1975;Wagner et al., 1998). CHO cells were maintained on glass culture plates in Ham's F12 medium containing 5% fetal bovine serum (FBS), 1% antibiotics (100 U/mL sodium penicillin G, 100 µg/mL streptomycin sulfate, 0.25 µg/mL amphotericin B in 0.85% saline), and 1% glutamine at 37°C in a humidified atmosphere of 5% CO_2.

4.2.8 CHO Cell Chronic Cytotoxicity Assay

This assay measures the reduction in cell density on flat-bottom 96-well microplates as a function of the concentration of the test sample over a period of 72 h (~3 cell cycles) (Plewa et al., 2002; Plewa and Wagner 2009). Flat-bottom, tissue culture 96-well microplates were employed; 8 replicate wells were prepared for each concentration of a specific organic extract for each chemical or HTL-WW sample. Eight wells were reserved for the blank control consisting of 200 µL of F12 medium + 5% fetal bovine serum (FBS). The negative control consisted of 8 wells containing 100 µL of a titered CHO cell suspension (3×10^4 cells/mL) plus 100 µL F12 + FBS. The wells for the remaining columns contained 3,000 CHO cells, F12 + FBS and a known molar concentration of the NOC agents or a known concentration factor of an HTL-WW sample organic extract for a total of 200 µL. To prevent cross-over contamination between wells due to volatilization of the organic extract, a sheet of sterile AlumnaSeal™ (RPI Corporation, Mt. Prospect, IL) was pressed over the wells before the microplate was covered. The microplate was placed on a rocking platform for 10 min to uniformly distribute the cells, and then placed in a tissue culture incubator for 72 h. After incubation, each well was gently aspirated, fixed in 100% methanol for 10 min, and stained for 10 min with a 1% crystal violet solution in 50% methanol. The plate was gently washed in tap water, inverted and tapped dry upon paper towels, and 50 µL of dimethyl sulfoxide/methanol (3:1 v/v) was added to each well for 10 min. The plate was analyzed in a BioRad microplate reader at 595 nm. The data were automatically recorded and transferred to an Excel spreadsheet on a microcomputer connected to the microplate reader. The blank-corrected absorbency value of the negative control (cells with medium only) was set at 100%. The absorbancy for each treatment group well was converted into a percentage of the negative control. For each organic extract concentration, 8-16 replicate

wells were analyzed per experiment, and the experiments were repeated at least 2 times. A concentration-response curve was generated for each sample. A regression analysis was conducted with each curve. The LC_{50} (%C½) values were calculated from the regression analysis and represents the sample concentration factor that induced a 50% reduction in cell density as compared to the concurrent negative controls. The CHO cell chronic cytotoxicity assay has been used to evaluate individual water contaminants as well as complex mixtures (Jeong et al., 2012; Plewa et al., 2012).

4.2.9 Algal Growth Inhibition Assay

4.2.9.1 Algal Culturing

Chlorella protothecoides (C. protothecoides) was provided by the Culture Collection of Alga at the University of Texas (Austin, TX, USA). Stock cultures of *C. protothecoides* were maintained routinely on both liquid and agar slants of Bold's Basal Medium (BBM) by regular sub-culturing at a 7 day interval. The algae were grown autotrophically in batch cultures at 27°C with continuous illumination. The purity of the culture was established by repeated streaking and routine microscopic examination. An inoculum culture in test medium was prepared 4 days prior to the start of the inhibition test to ensure that the algal cultures are in the exponential growth phase when inoculated the test solution. The growth of algae was monitored spectrophotometrically by reading the absorbance at 680 nm and 750 nm.

4.2.9.2 Algal Growth Inhibition Assays

Algal growth inhibition assays were performed in sterile 24-well polystyrence microplates (Multi dishes, Cat. No 144530 Nunc, Thermo Fisher Scientific) following a

published method (Eisentraeger et al., 2003). Each plate contained four growth controls located near the samples with the lowest concentration to avoid cross contamination. NOCs identified in post-HTL wastewater were assayed from low to high concentration with 2 replicate cultures per concentration. Two plates were prepared for each tested compound to provide 4 replicates of each test concentration and 8 replicates of the control. Two milliliter aliquots of each treatment solution were added to each microplate well. Algal stock solution containing 10^6 cells/mL was added (20 µL) to each well to achieve an initial concentration of 10^4 cells/mL. Then, the microplates were covered with the microplate lid and additionally sealed with Parafilm ‘‘M’’ and placed on the shaker table under continuous illumination. Microplates were rotated 90° each day. Inhibition tests were terminated after 96 hours of exposure to the test compounds, which was enough time for the biomass in the controls to increase by a factor of at least 16. Algal growth was monitored every 24 h after the beginning of the exposure. Algal average growth rate for a period is calculated as the logarithmic increase in the biomass using the following equation:

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_i - t_j}$$

where

μ_{i-j} is the average specific growth rate between time i and j;

X_i is the biomass at time i

X_j is the biomass at time j

t_i is the time (day) of i^{th} biomass measurement after beginning the exposure;

t_j is the time (day) of j^{th} biomass measurement after beginning the exposure;

The percent inhibition of growth rate for each treatment replicate was calculated from the equation below:

$$\mathbf{I_\mu = \frac{\mu_c - \mu_\tau}{\mu_c} \times 100}$$

Where:

I_μ is the percent inhibition in average specific growth rate;

μ_c is the mean value for average specific growth rate (μ) in the control group;

μ_τ is the average specific growth rate for the treatment replicate

4.3 RESULTS AND DISCUSSIONS

4.3.1 Detection and Quantification of NOCs

The GC-NPD and GC-MS data indicate that many classes of (NOCs) were detected in the DCM extracts of HTL-WW resulting from liquefaction of *Spirulina* at 300°C with 30 min of retention time (Figure 4.2). Nine of these compounds with relatively large chromatogram peaks have been quantified using reagent grade calibration standards and are listed in Table 4.1. Quantification of these nine NOCs showed a wide range of concentrations from 139 mg/L (δ-valerolactam) to 0.052 mg/L (2-picoline). Of these nine NOCs, the most prevalent peaks included δ-valerolactam followed by ε-caprolactam, 2,6-dimethyl-3-pyridinol, and 2,2,6,6-tetramethyl-4-piperidinone. Previously, indole, pyrrole derivatives and 3-amino-phenol were reported in the aqueous product from liquefaction of the macro-alga *Laminaria saccharina (L. saccharina)* . GC-MS full scan analysis of wastewater from HTL conversion of *Enteromorpha prolifera (E. prolifera)* also showed the occurrence of several NOCs also detected in our work,

including 3-aminophenol, 2-piperidione, 2-pyrrolidinone (Zhou et al., 2010). The presence of pyridine, methyl-, dimethyl-, and ethyl-substituted pyridines was also reported in raw wastewater from coal gasification (Gangwal, 1981). Although the chemical composition of HTL-WW is dependent on the specific feedstock and operating conditions (Anastasakis & Ross, 2011; Eager et al., 1981; Jena et al., 2011a), we noticed that amino-phenol, 2-piperidione, 2-pyrrolidinone, pyridine and its derivatives, piperidinone and its derivatives are commonly detected NOCs in wastewater from HTL conversion of algal biomass. Information on HTL-WW characteristics remains limited mainly because of analytical difficulty related to the complexity of the sample matrix. The GC-NPD scan of the HTL-WW extract provides the retention times of NOC's, however it does not provide the identities of the specific compounds. When the data is integrated with a GC-MS general scan of the same extract under the same chromatographic conditions, then probabilities for the specific NOCs are obtained. The extract is then analyzed by GC-HRMS to verify the formula and retention time of the target NOCs. Once a NOC formula and retention time was identified, then the appropriate reference material was procured. In several instances, the appropriate reference material was not available. For NOCs where reference materials were available, the instrument was calibrated with the appropriate reference materials and the extract was analyzed by GC-HRMS. This systematic approach for characterizing nitrogen-containing compounds in complex matrices presented in this study provides an effective analytical tool to characterize and quantify different types of HTL-WW.

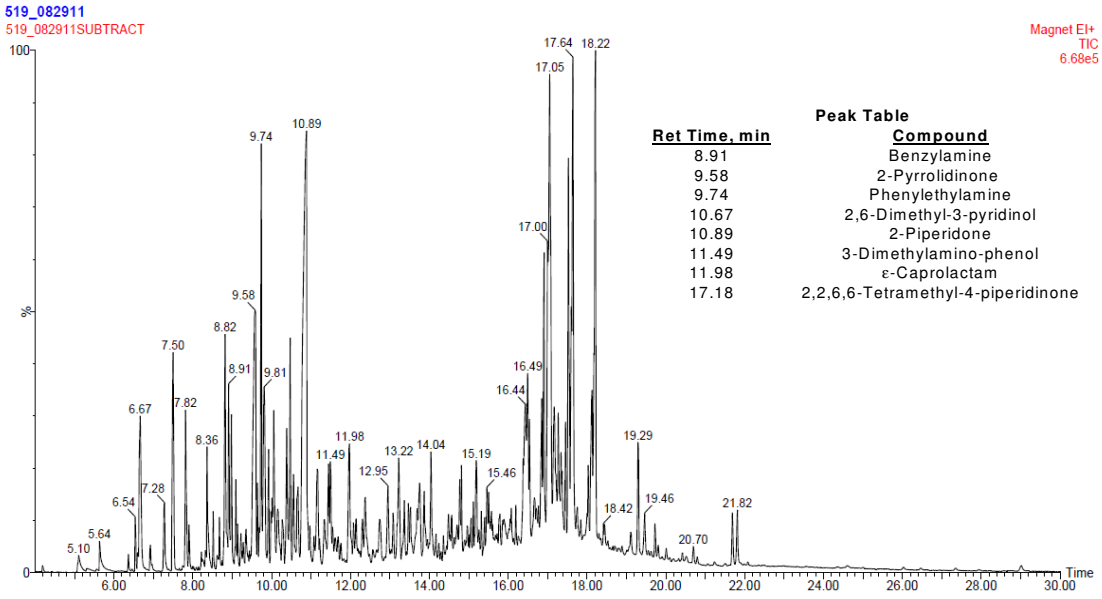
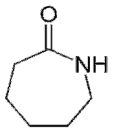
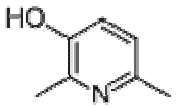
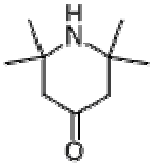
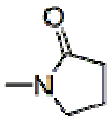
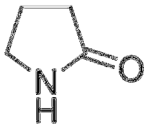
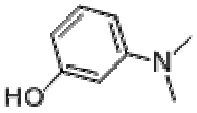
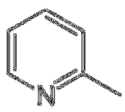


Figure 4.2 Example of GC-MS general scan 35-200 m/z chromatogram.

Table 4.1 Characteristics of nitrogenous organic compounds detected in HTL-WW generated from HTL conversion of *Spirulina* at 300°C and 30 min reaction time

Compound name	Structure and formula	MW (g/mole)	CAS #	Conc. (ppm)
σ-valerolactam or 2-piperidone	 C_5H_9NO	99.13	675-20-7	139
ε-caprolactam	 $C_6H_{11}NO$	113.16	150-60-2	10
2,6-dimethyl-3-pyridinol	 C_7H_9NO	123.1525	1122-43-6	8.2
2,2,6,6-tetramethyl-4-piperidinone	 $C_9H_{19}NO$	157.25	2403-88-5	3.5
1- methyl-2-pyrrolidinone	 C_5H_9NO	99.13	872-50-4	1.5
2-pyrrolidinone or butyrolactam	 C_4H_7NO	85.1	616-45-5	0.82
3-dimethylamino-phenol	 $C_8H_{11}NO$	137.18	99-07-0	0.37
pyridine	 C_5H_5N	97.1	110-86-1	0.16
2-picoline or 2-methylpyridine	 C_6H_7N	93.13	109-06-8	0.052

4.3.2 Biological Characterization of NOCs Detected in HTL-WW

4.3.2.1 Cytotoxicity of NOCs Detected in HTL-WW

Figure 4.3 A provides an example concentration-response curve for CHO cell chronic cytotoxicity for 2-picoline. This plot shows average toxicity data points for each concentration (8–16 independent clones), and the standard error is indicated as whiskers extending from the data points. CHO chronic cytotoxicity analysis for the 9 nitrogen based compounds detected and quantified in HTL-WW is presented Figure 4.3 B. LC_{50} was calculated as the concentration that induced 50% reduction of cell density as compared to the concurrent negative control, and the LC_{50} values are reported in Table 4.2. This table also presents ANOVA statistics and the lowest concentration with a significant difference from the negative control. To directly compare the cytotoxicity of each tested NOCs, we calculated a cytotoxicity index (Table 4.2). The cytotoxicity index value was determined as $(LC_{50})^{-1}(10^3)$, where a larger value represents greater toxic potency (Figure 4.4)

CHO cell cytotoxicity responses varied significantly among the 9 tested NOCs, with LC_{50} values ranging from 500 μM (3-dimethylamino-phenol) to 12500 μM (ε-caprolactam). The rank order for CHO cytotoxicity (highest to lowest) based on their LC_{50} value was 3-dimethylamino phenol> 2,2,6,6 tetramethyl-4 piperidinone >2,6-dimethyl-3pyridinol> 2-picoline> pyridine> methyl-2 pyrrolidinone> δ-valerolactam>2-pyrrolidinone>ε-caprolactam. All ten tested NOCs have a lowest cytotoxic concentration that is higher than the measured concentrations in HTL-WW, suggesting that individual NOCs are not significantly cytotoxic to CHO cell. It is also noteworthy that NOCs with methyl groups (3-dimethylamino phenol, 2,2,6,6, tetramethyl-4 piperidinone, and 2,6-dimethyl-3-pyrrolidinol) are more toxic than those without them. This

finding agreed with previous data, where compounds with two or more methyl groups were more toxic to *Tetrahymena pyriformis* than those with one or no alkyl substitutions (Schultz et al., 1978). An increase in alkyl substitution generally increases the resistance to retardation, decreases the solubility, and increases the toxicity the compound. Heteroatom substitution into or onto the ring also alters both toxicity and solubility. Our data showed that the three most cytotoxic NOCs containing methyl groups and heteroatoms on their rings.

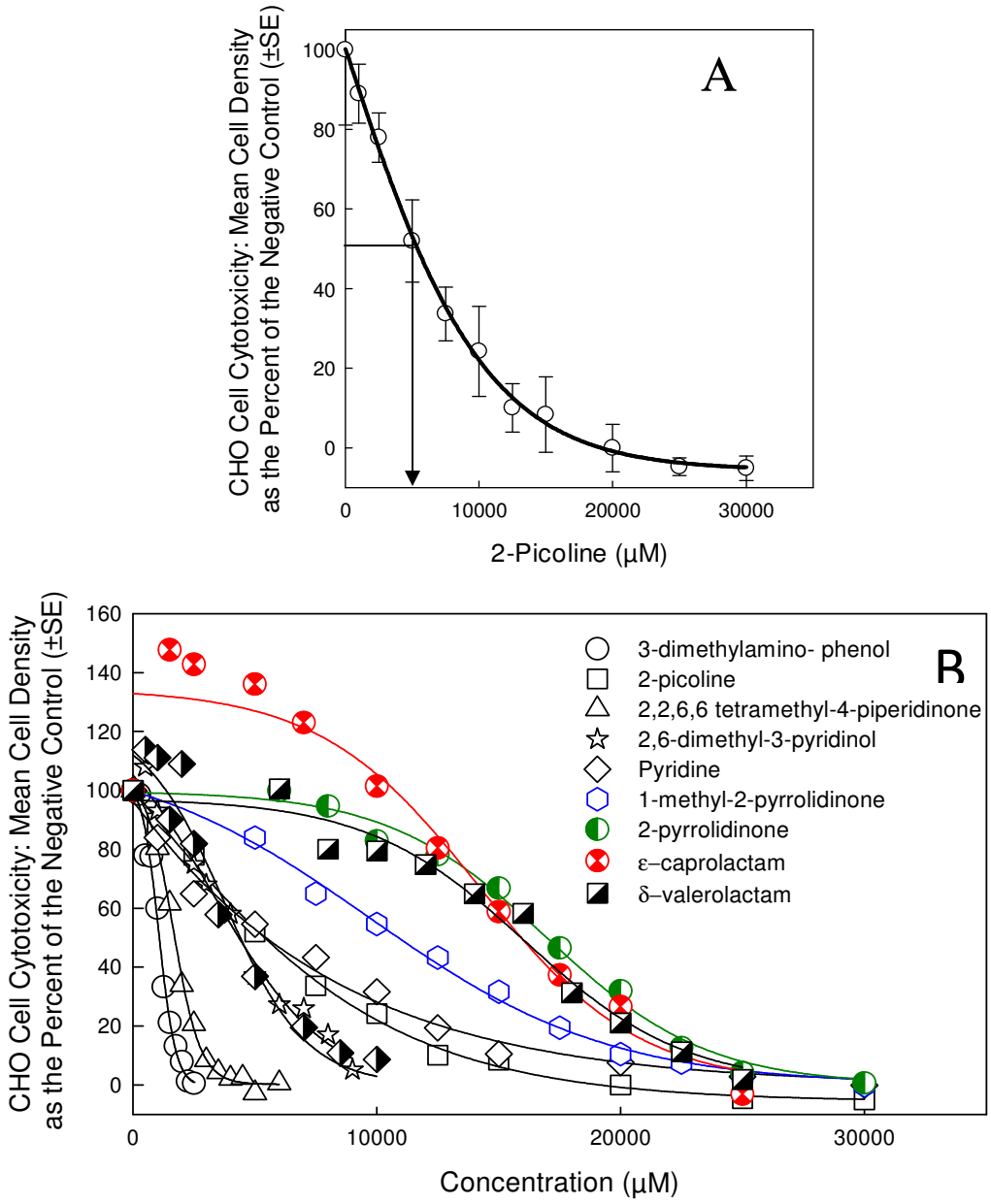


Figure 4.3 (A) Cytotoxicity concentration-response curve for 2-picoline illustrating the regression of the data. The response at each concentration was generated from 8-16 independent clones of CHO cells. The determination of LC50 value is indicated by the arrowed line. (B) A comparison of cytotoxicity concentration response curves from 9 NOCs detected in HTL-WW.

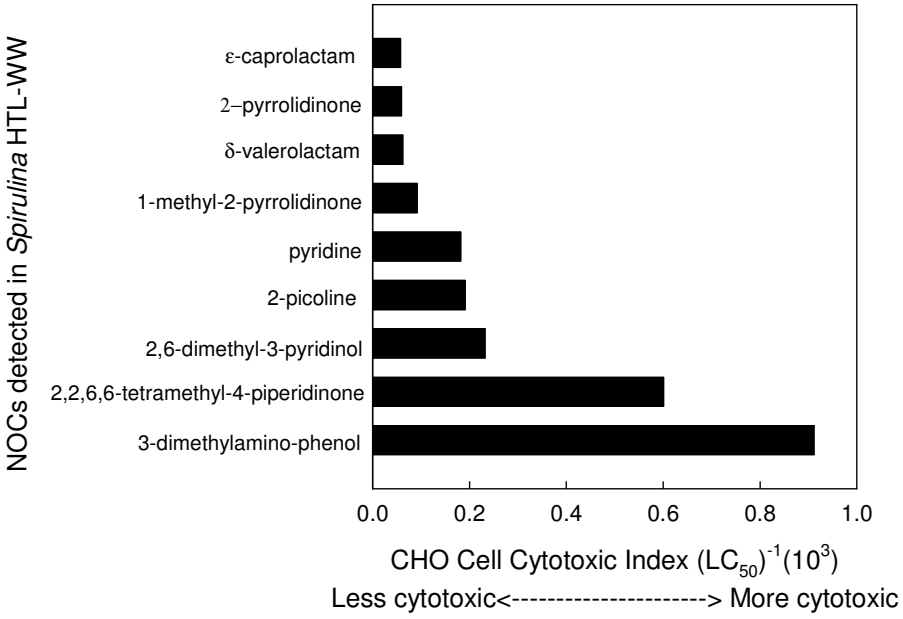


Figure 4.4 Comparison of the CHO cell cytotoxicity index values for NOCs detected in HTL-WW. Index values are expressed in dimensionless units.

Table 4.2 Induction of chronic cytotoxicity in CHO cells by nitrogenous organic compounds

Nitrogenous organic compounds	Lowest cyto. conc. (μM)[a]	R^{2b}	LC_{50}(μM)[c]	Cytotox. Index Values $(LC_{50})^{-1}(10^3)$	ANOVA test statistics
3- dimethylamino phenol	500	0.99	1,100 ± 11.9	0.9	$F_{10,77} = 124.11$; $P \leq 0.001$
2,2,6,6-tetramethyl-4-piperidinone	1000	0.99	1,670 ± 10.1	0.6	$F_{10,77} = 83.33$; $P \leq 0.001$
2,6-dimethyl-3-pyridinol	2500	0.99	4,310 ± 13	0.2	$F_{10,77} = 54.12$; $P \leq 0.001$
2-picoline	1000	0.99	5,230 ± 8.0	0.2	$F_{10,77} = 146.76$; $P \leq 0.001$
pyridine	1000	0.99	5,500 ± 8.0	0.18	$F_{10,77} = 111.66$; $P \leq 0.001$
1-methyl- 2-pyrrolidinone	5000	0.98	10,900 ± 7.8	0.09	$F_{10,77} = 120.51$; $P \leq 0.001$
σ-valerolactam	8000	0.98	16,100 ± 11.9	0.22	$F_{10,77} = 58.53$; $P \leq 0.001$
2- pyrrolidinone	10000	0.99	16,900 ± 11.4	0.06	$F_{10,77} = 72.24$; $P \leq 0.001$
ε-caprolactam	12500	0.91	17,300 ± 12.0	0.058	$F_{10,77} = 130.91$; $P \leq 0.001$

[a]The lowest cytotoxicity concentration was the lowest concentration of the tested compound in the concentration-response curves that induced a significant amount of cytotoxicity as compared to the negative control. [b]R^2 is the coefficient of determination for the regression analysis upon which the LC_{50} value ($\%C^1/_2$ value) was calculated. [c]The LC_{50} is the sample concentration that induced a cell density that was 50% of the negative control. The estimated SE of the LC_{50} was derived as the averaged SE of all the data points in the concentration-response curves. NOCs were listed according to their descending CHO cell cytotoxicity.

4.3.2.2 CHO Cell Cytotoxicity of NOC Mixture

Figure 4.5 illustrates the concentration-response curves of NOC mixture and individual NOCs where the concentration was expressed as concentration factor ×. The synthetic mixture of NOCs was prepared in DMSO by adding the 9 quantified NOCs at 1000of their detected concentration in HTL-WW. The concentrations of individual NOCs were also recalculated and expressed as concentration factor compared to the detected concentration of individual NOCs in HTL-WW (i.e., 1× of δ-valerolactam equal to 139 mg/L) for direct comparison of individual NOC cytotoxicity with that of NOC mixture. Details of these calculations can be found in Table 4.3. As demonstrated in Figure 4.5, the LC_{50} value of NOC mixture was 16×, which is much lower than the LC_{50} value of all tested NOCs except for δ-valerolactam. The LC_{50} values of other NOCs vary from 64.7× to 3336× concentration factor. This result suggests that there was a potential synergistic cytoxicity effect among NOCs in HTL-WW. Thus, HTL-WW toxicity should be evaluated as a mixture of organic compounds in the wastewater rather than as single compounds.

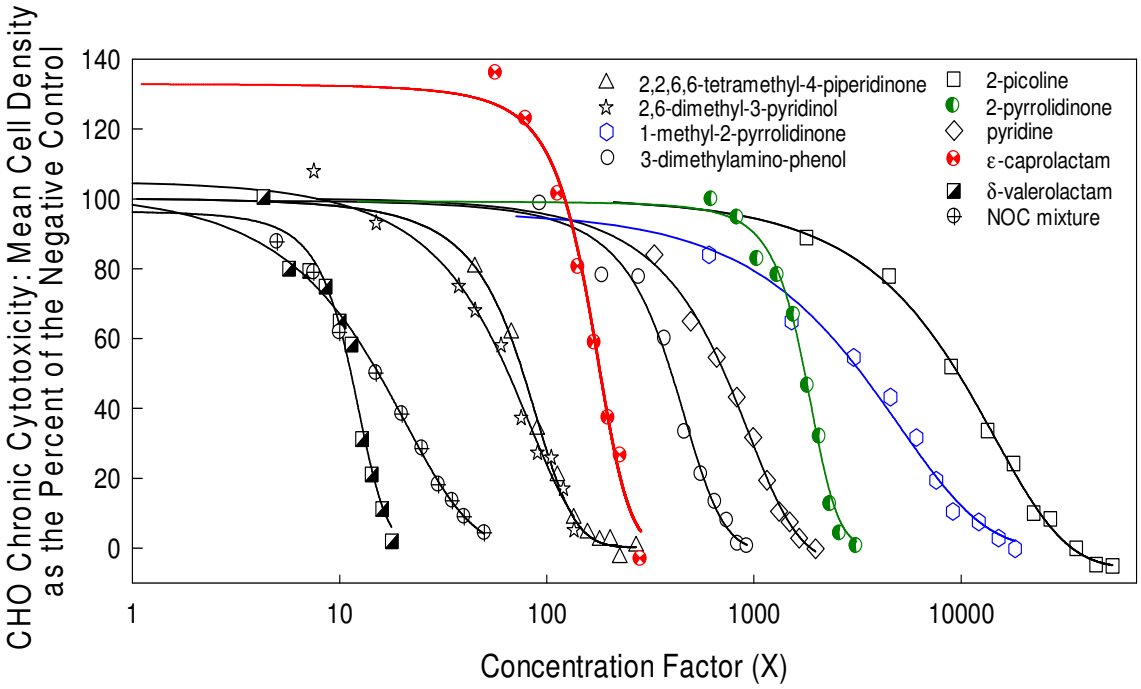


Figure 4.5 Comparison of the CHO cell cytotoxicity concentration response curves for individual NOCs and NOC mixture. The concentration of individual NOCs and NOC mixture are expressed as the concentration factor for direct comparison of the cytotoxicity among individual NOCs and the NOC mixture (1× is equal to the detected concentration of each NOC in HTL-WW).

Table 4.3 Conversion of NOCs concentration from µM to concentration factor (X)[a]

3-dimethyl amino phenol		2-picoline		2,2,6,6-tetramethyl-4-piperidinone		2,6 dimethyl-3 pyridinol		pyridine		1-methyl-2-pyrrolidinone		2-pyrrolidinone		ε-caprolactam		δ-valerolactam	
Conc. (µM)	Conc. (X)	Conc. (µM)	Conc. (X)	Conc. (µM)	Conc. (X)	Conc. (µM)	Conc. (X)	Conc. (µM)	Conc. (X)	Conc. (µM)	Conc. (X)	Conc. (µM)	Conc. (X)	Conc. (µM)	Conc. (X)	Conc. (µM)	Conc. (X)
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
250	92.7	1000	1791	1000	45	500	7.5	5000	330	1000	607	6000	623	1500	17	6000	4.3
500	185	2500	4477	1500	67	1000	15	7500	496	2500	1517	8000	830	2500	29	8000	5.7
750	278	5000	8955	2000	90	2500	38	10000	661	5000	3034	10000	1038	5000	57	10000	7.1
1000	371	7500	13432	2500	112	3000	45	12500	826	7500	4552	12500	1297	7000	79	12000	8.6
1250	463	10000	17910	3000	135	4000	60	15000	991	10000	6069	15000	1557	10000	113	14000	10
1500	556	12500	22387	3500	157	5000	75	17500	1157	12500	7586	17500	1816	12500	141	16000	11.4
1750	649	15000	26864	4000	180	6000	90	20000	1322	15000	9103	20000	2075	15000	170	18000	12.8
2000	742	20000	35819	4500	202	7000	105	22500	1487	20000	12137	22500	2335	17500	1980	20000	14.3
2250	834	25000	44774	5000	225	8000	120	25000	1652	25000	15172	25000	2594	20000	2260	22500	16
2500	927	30000	53729	6000	270	9000	135	30000	1983	30000	18206	30000	3113	25000	283	25000	17

[a] The concentrations of individual NOCs were recalculated and expressed as concentration factor (×) compared to the detected concentration of individual NOCs in HTL-WW (i.e., 1× of δ-valerolactam equal to 139 mg/L) for direct comparison of individual NOC cytotoxicity with that of NOC mixture.

4.3.2.3 Algal Growth Inhibition Effect of NOCs

Algal inhibition tests were conducted with 8 of the NOCs detected in *Spirulina* HTL-WW. Of 8 tested nitrogen-based organic compounds, ε-caprolactam and δ-valerolactam did not show any inhibition effect at their maximum solubility in BBM medium. Table 4.4 presents the inhibition data of six NOCs to *C. protothecoides,* with all concentrations presented in ppm. In this table, the I_{50} value was the concentration of tested NOCs that inhibits 50% of cell growth as compared to the concurrent negative control. The R^2 refers to the goodness of fit of the regression analysis from which the I_{50} was calculated. The I_{50} value ranged from 0.2 ppm (3-dimethylamino phenol) to 960 ppm (2-pyrrolidinone). The rank order for algal inhibition (highest to lowest) based on their I_{50} value was 3-dimethylamino-phenol > 2,6-dimethyl-3-pyridinol > phenol > methyl-2-pyrrolidinone > 2-pyrrolidinone > 2,2,6,6-tetramethyl-4-piperidinone. A comparison of relative inhibition effect of NOCs analyzed in this study is presented in Figure 4.6. Our data were in agreement with the ecological information reported for several of the same compounds in the literature. For example, Scragg found that the initial growth of *Chlorella* vulgaris and *Chlorella* VT-1 was inhibited to varying degrees by 100-400 mg/l phenol. *Chlorella* VT-1 was found to be more tolerant being able to grow, albeit slowly, in the presence of 400 mg/L phenol, whereas this concentration inhibited the growth of *C. vulgaris* (Scragg, 2006). Klekbner and Kosaric also reported that phenol can be readily degraded by different algae (*Chlorella sp., Scenedesmus obliquus* and *Spirulina maxima*) at a concentration of 1000 mg/L (Klekner & Kosaric, 1992). Megharaj et al. reported that p-aminophenol, which has a similar structure with 3-dimethylamino-phenol, would inhibit growth of *C. vulgaris* at a concentration of 5-50 mg/L (Megharaj et al., 1991). Our data showed an I_{50} value of 10.2 mg/L for 3-dimethylamino-phenol. Mann and Florence reported that presence of 1-methyl-2-

pyrrolidinone would enhance rather than inhibit growth of *Nitzschia closterium*, a common Great Barrier Reef diatom (Mann & Florence, 1987). Similarly, the OECD Guideline 201 for algal growth inhibition testing suggested that the EC_{50} value of 1-methyl-2-pyrrolidinone is >500 mg/L. Our data also showed 1-methyl- 2-pyrrolidinone did not inhibit algal growth at a concentration < 300 mg/L, and it inhibited 50% of growth for *C. prototheco*ides at a concentration of 945 mg/L. Of all the tested compounds, 3-dimethylamino-phenol and 2,6-dimethyl-3-pyridinol were the most toxic ones with an I_{50} value close to their detected concentration in HTL-WW (0.2 vs. 0.37 and 10.2 vs. 8.2 ppm, respectively). The other compounds only caused inhibition effects on algal growth at concentrations much higher than their detected concentration in HTL-WW. Though most of individual nitrogen based compounds did not inhibit algal growth, significant algal growth inhibition still occurs when more than 5% of HTL-WW was present in algal culture medium (Zhou, 2010). This is because NOCs are only one class of compounds that are present in the HTL-WW, the inhibition effects might also result from other types of compounds that have not yet been investigated.

Table 4.4 Algal inhibition analysis of nitrogen organic compounds detected in HTL-WW

Nitrogenous Organic Compounds	Significant Conc. Range (ppm)	R^{2a}	I_{50} (ppm)[b]	Detected Conc. in HTL-W (ppm)
3-dimethylamino- phenol	0.05-0.6	0.99	0.2	0.37
2,6-dimethyl-3-pyridinol	0.5-100	0.98	10.2	8.2
phenol	100-800	0.98	395	0.56336
2,2,6,6-tetramethyl-4- piperidinone	400-1600	0.98	1010	3.5
1-methyl-2-pyrrolidinone	100-20000	0.99	945	1.5
2-pyrrolidinone	1000-20000	0.97	960	0.825

[a]R is the coefficient of determination for the regression analysis upon which the I_{50} value was calculated. [b]I_{50} is the sample concentration that inhibited 50% of algal growth as compared to the negative control. Note that two other compounds, ε-caprolactam and δ-valerolactam, were also tested for algal inhibition but did not show any inhibition effects at their maximum solubility.

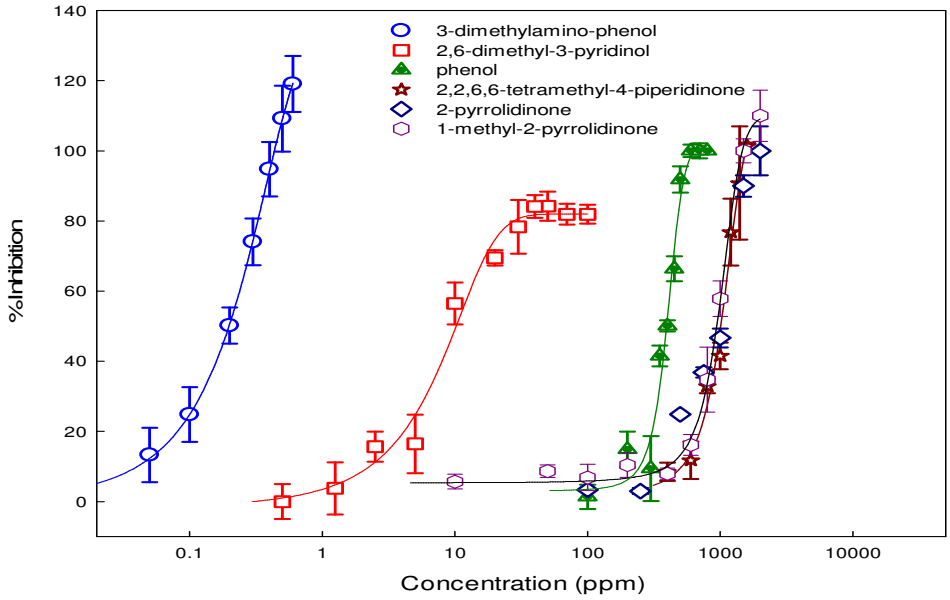


Figure 4.6 Comparison of the concentration response curves for the algal inhibition test with six nitrogen based organic compounds.

4.3.3 Cytotoxicity of Organic Mixture Extracted from HTL-WW

Figure 4.7 compares the concentration-response curves for experiments that measured CHO cell chronic cytotoxicity of the complex mixture of all organics extracted from HTL-WW of swine manure and *Spirulina*. The concentration is expressed as concentration factor compared to the original sample (i.e., 1× = 100% HTL-WW). The average and standard error for each concentration were the average of 8-16 independent clones ± their standard error. As demonstrated in Figure 4.7, the organic mixture in HTL-WW was highly cytotoxic to CHO cells. The LC_{50} value of raw *Spirulina* HTL-WW and swine manure HTL-WW was 0.075× (7.5% HTL-WW) and 0.066× (6.6% HTL-WW), respectively, and full strength HTL-WW would have induced 100% reduction in CHO cell density for both feedstocks. Previous research has identified a list of 48 hazardous constituents likely to be found in HTL-WW and some of these compounds have known toxicity (Elliot, 1992). For example, aziridine has been shown to be toxic and mutagenic in various biological systems including chromosome aberrations and sister chromatid exchanges in human cells (Verschaeve & Kirschvolders, 1990). 2-methylarizidine is also anticipated to be a human carcinogen based on carcinogenicity data from animal studies (Weisburger et al., 1981). Benzene has been shown to cause many types of genetic damage and is considered a Group I carcinogen for man and laboratory animals (Yardleyjones et al., 1991). Although the acute toxicity threshold of several components in Elliot's list had been tabulated, the potential toxic interaction among these components has not been investigated. To our knowledge, this study is the first to investigate the toxicity of the complex matrix of organic compounds in HTL-WW, and the data presented here is sufficient to prove that organic mixture in HTL-WW can be highly toxic to mammalian cells. Further research is needed to understand the degree to which the toxicity results from individual compounds versus interactions between

different organic constituents in HTL-WW. In addition, further study is recommended to understand the effects of HTL operating conditions and feedstock properties on the degree of toxicity in HTL wastewaters.

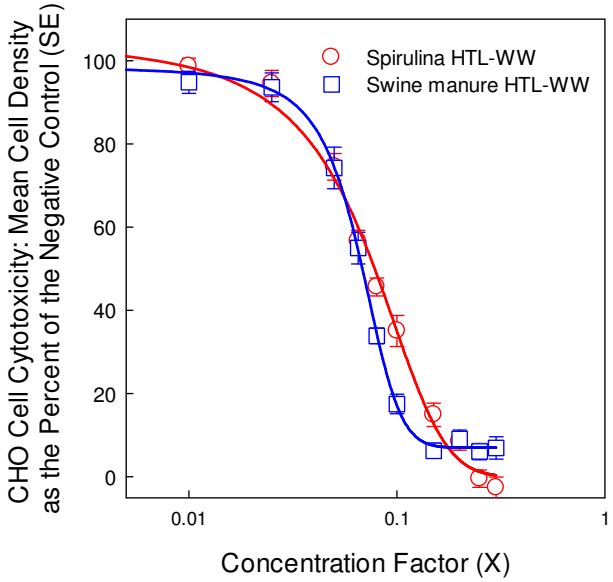


Figure 4.7 Comparison of cytotoxicity concentration response curves for organic mixtures extracted from *Spirulina* and swine manure HTL-WW (300°C and 30 min reaction time)

4.3.4 Cytotoxicity of Fractionated HTL-WW

CHO chronic cytotoxicity analysis for fractionated HTL-WW and the original *Spirulina* HTL-WW is presented in Figure 4.8. The samples were directly tested for cytotoxicity without the organic extraction step. The concentration is expressed as the percent of HTL-WW in growth medium. The average and standard error for each concentration were the average of 8–16 independent clones ± their standard error. LC_{50} was calculated as the concentration that induced 50% reduction of cell density as compared to the concurrent negative control, and the LC_{50} values are reported in Table 4.5. The rank order for CHO cytotoxicity (highest to lowest) based

on their LC_{50} values was HTL-WW> hydrophobic fraction > hydrophobic basic fraction> hydrophilic fraction > hydrophobic neutral fraction> hydrophobic acidic fraction. Figure 4.9 presents the average cytotoxicity indexes ± their standard error obtained for fractionated HTL-WW and the original HTL-WW. As demonstrated in Figure 4.9, the hydrophobic fraction is about two times more cytotoxic than hydrophilic fraction with the corresponding cytotoxicity indexes of 1000 vs. 435. Further fractionation of hydrophobic fraction showed that hydrophobic base fraction is about 2–3 times more cytotoxic than the hydrophobic acid and neutral fractions.

Previous studies showed that the hydrophobicity of organic chemicals is a key factor for their aquatic toxicity because it can drive their partitioning from water into lipid membrane and storage lipids of organisms (Mayer & Reichenberg, 2006; Sabaliunas et al., 2000). The bioaccumulation of a chemical is affected by its solubility in both water and lipid. A chemical that is lipophilic tends to have low solubility in water (hydrophobic); thus the terms hydrophobic and lipophilic are often used synonymously. As hydrophobic compounds are lipophilic, they have a tendency to partition out of the aqueous solution and permeate into cell lipid membranes (Mayer & Reichenberg, 2006). It was also found that the mean effective concentration ($EC_{50)}$ values of organic compounds generally decrease with increasing log K_{OW} values up to 5 to 6 (a high K_{OW} indicates lipophilicity/hydrophobicity of a compound). On the other hand, hydrophilic compounds in HTL-WW tend to stay in aqueous solution. Therefore, the exposure of CHO cells to hydrophilic compounds is generally less than to hydrophobic compounds. As a result, the hydrophilic fraction induced a less toxic effect on CHO cells than the hydrophobic fraction.

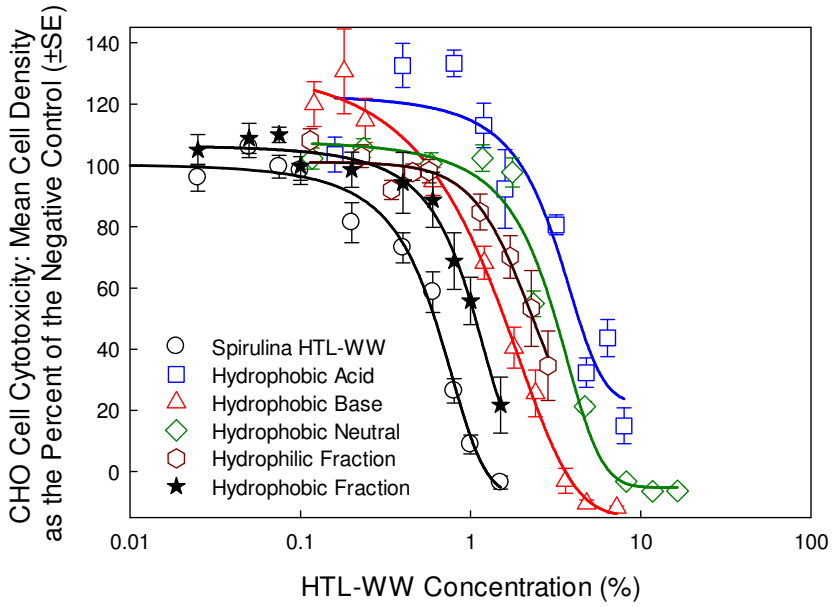


Figure 4.8 Comparison of CHO cell cytotoxicity concentration response curves for various fractions of *Spirulina* HTL-WW.

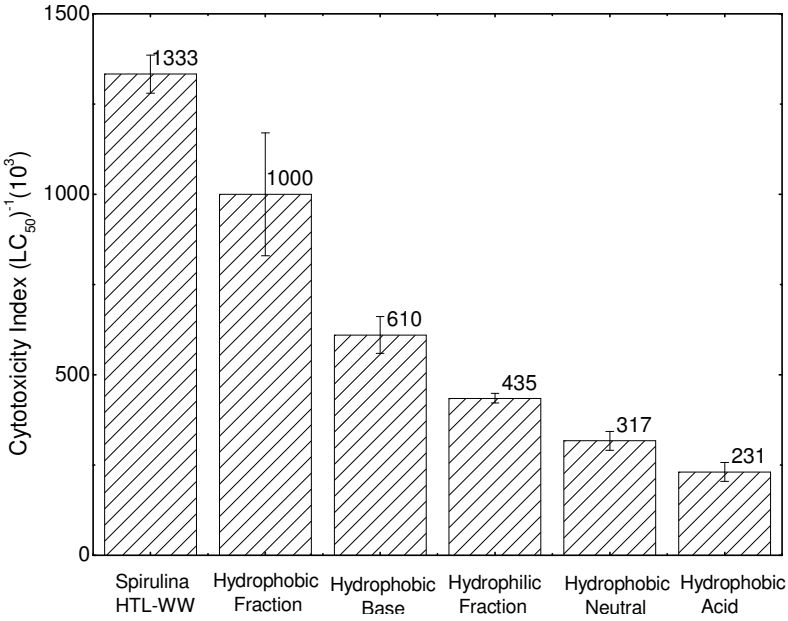


Figure 4.9 Comparison of the CHO cell cytotoxicity index values for various fractions of *Spirulina* HTL-WW. Index values are expressed in dimensionless units.

Table 4.5 CHO cell cytotoxicity analysis for fractionated *Spirulina* HTL-WW

Fractionated HTL-WW	R^{2a}	LC_{50} (%)[b]	Cytotoxicity Index (±SE)
Spirulina HTL-WW	0.99	0.75	1333±52.8
Hydrophobic Fraction	0.98	1.0	1000±169.9
Hydrophobic Base	0.98	1.64	609±50.56
Hydrophobic Neutral	0.98	2.9	345±26.1
Hydrophobic Acid	0.99	3.24	306±26.35
Hydrophilic Fraction	0.97	1.95	513±13.16

[a]R^2 is the coefficient of determination for the regression analysis upon which the I_{50} value was calculated. [b]LC_{50} is the sample concentration that caused 50% reduction in CHO cell density as compared to the concurrent negative control.

4.4 CONCLUSIONS

Numerous NOCs were detected in HTL-WW and nine of the most prevalent ones were quantified using external standards. A CHO chronic cytotoxicity assay showed that all nine detected NOCs in HTL-WW were not cytotoxic to mammalian CHO cells at their detected concentration. Inhibition of algal growth was also measured for eight of these compounds, and of them showed measurable algal inhibition. Of all tested NOCs, 3-dimethylamino phenol and 2,6-dimethyl-3-pyridiniol caused 50% algal growth inhibition at their detected concentration in HTL-WW. It is noteworthy that NOCs with methyl groups (3-dimethylamino phenol, 2,2,6,6, tetramethyl-4 piperidinone, and 2,6-dimethyl-3-pyrrolidinol) were more toxic to mammalian cells and caused more algal inhibition than those without them. Comparison of LC_{50} and I_{50}

values of the same NOCs indicated that LC_{50} is always higher than I_{50} values, suggesting that algae are more sensitive to NOCs than mammalian cells.

Although the detected NOCs showed minimal mammalian toxicity effects, the organic mixture extracted from HTL-WW was found to be highly toxic to mammalian cells. HTL-WW induced 50% reduction in CHO cell density at a concentration factor of only 0.075× (7.5% HTL-WW). The whole HTL-WW was about 10 times more cytotoxic than the organic extract with the LC_{50} of only 0.0075× (0.75% HTL-WW). Hydrophobic fraction was found to be more cytotoxic than the hydrophilic HTL-WW fraction. Within the hydrophobic portion of HTL-WW, hydrophobic basic fraction was the most cytotoxic one. This finding suggests that characterization of HTL-WW should focus on hydrophobic fraction, especially the hydrophobic basic fraction, as toxicants in HTL-WW are more likely to be found in this fraction than in hydrophilic fraction. Additionally, the high cytotoxicity of HTL-WW to mammalian cells and its inhibition effect on algal growth make it not suitable for direct environmental discharge without extensive treatment. Therefore, it is desirable to development treatment measures that can improve the chemical and biological quality of HTL-WW.

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CHAPTER 5: EFFECTS OF OPERATING CONDITIONS ON HYDROTHERMAL LIQUEFACTION WASTEWATER QUALITY

5.1 INTRODUCTION

Hydrothermal liquefaction (HTL) is a thermochemical conversion process that converts wet biosolids into crude oil by applying elevated heat (typically 200–350°C) and pressure (10–15 MPa). Different types of biomass have been converted to bio-oil by HTL, including wood chips (Boocock & Porretta, 1986), corn stover (Derairbas, 1991), sawdust (Xu & Lad, 2007), swine manure (He et al., 2001a; He et al., 2001b; He et al., 2001c) , domestic garbage (Minowa et al., 1995), sewage waste (Yokoyama et al., 1987), and algae (Anastasakis & Ross, 2011; Brown et al., 2010; Jena et al., 2011a; Jena et al., 2011b; Torri et al., 2012; Yu et al., 2011a; Yu et al., 2011b; Zhou et al., 2010). Previous studies demonstrated that bio-oil yield and composition varies with biomass feedstocks and operating parameters such as reaction temperatures, reaction time, initial conversion pressure, residence time, and mass ratios of biomass feedstock to water (Brown et al., 2010; Shuping et al., 2010; Vardon et al., 2011; Zhou et al., 2010).

Scattered information about the effect of HTL processing parameters on the yield of HTL co-products has been discussed in various research articles, technical notes, and review papers (Anastasakis & Ross, 2011; He et al., 2001b; Jena et al., 2011a; Yu et al., 2011a). For example, it has been reported that initially increases of temperature and reaction time could increase bio-oil and gas yields, but once the maximum bio-oil yield is reached, further increases in temperature and reaction time could actually reduce the formation of bio-oil (Brown et al., 2010; Jena et al., 2011b; Yang et al., 2004; Zou et al., 2009). However, the previous work mostly focused on evaluating the effects of operating conditions on bio-oil composition and yield. The

effect of HTL operating conditions on characteristics of other HTL co-products (i.e. solid residue, wastewater, and gases) remains limited.

HTL wastewater (HTL-WW) is the most abundant co-product of the HTL process. Recent studies had investigated the feasibility of recycling HTL-WW for algal cultivation (Jena et al., 2011b; Roberts et al., 2013; Zhou et al., 2011a; Zhou et al., 2011c). These studies found that HTL-WW contained a high concentration of carbon, nitrogen and phosphorous, which are important nutrients for autotrophic growth of algae. In addition, most of the mineral nutrients needed for algal growth were also found in HTL-WW in significant quantities when compared to the standard growth medium such as BG 11, modified Allen's medium and Bold's Basal medium (Jena et al., 2011b). While it is advantageous to cultivate algae in HTL-WW to produce additional algal biomass for HTL biofuel conversion, previous research found that recycling higher amounts of the aqueous product from HTL of swine manure and *Spirulina* feedstocks could inhibit algal growth (Zhou et al., 2011b). Specifically, supplementing F/2 growth media with HTL aqueous product at more than 5% (v:v) for swine manure or more than 1% (v:v) for *Spirulina* could inhibit algal growth. Others have reported that aqueous product from HTL conversion *Spirulina* feedstock needed to be diluted to 0.2% in DI water for optimal algal growth (Jena et al., 2011b). These data suggest that the recycling potential of HTL-WW for algal cultivation strongly depends on specific chemical characteristics of HTL-WW. As with HTL bio-oil, variations in HTL operating conditions are expected to affect the chemical composition of HTL-WW. Therefore, it is important to understand the effects of HTL operating conditions on the chemical characteristics of HTL-WW to better facilitate recycling of HTL-WW for algal cultivation.

Our past study demonstrated that wastewater from HTL of *Spirulina* was extremely toxic to mammalian cells with a LC_{50} value of 7.5% (Pham et al., 2013). Since HTL toxicity is strongly related to the chemical composition, it is expected that variations in operating conditions could also affect HTL-WW toxicity. Therefore, it is also equally important to understand the effects of HTL operating conditions on toxicity of HTL-WW. Information on the effects of HTL operating conditions on HTL-WW toxicity would allow a better balance between optimizing bioenergy production and avoiding negative environmental impacts of HTL processes.

The specific objectives of this study were to: (i) investigate the effect of HTL operating parameters (reaction temperature, reaction time and feedstock solids concentration) on the chemical composition of HTL-WW (ii) investigate the effect of HTL operating parameters (reaction temperature, reaction time and feedstock solids concentration) on cytotoxicity of HTL-WW, (iii) evaluate the correlation between the overall organic concentration and cytotoxicity of HTL-WW, and (iv) identify the key operating parameter affecting the cytotoxicity of HTL-WW.

5.2 Materials and Methods

5.2.1 HTL Feedstocks

Chlorella pyrenoidosa (green microalgae) was purchased from a health food store and stored at 4°C prior to processing. Physicochemical characterization of this alga is summarized in Table 5.1. The crude fat value in the table represents the initial lipid content this microalgae.

5.2.2 Hydrothermal Liquefaction Experiment

For studying the effects of HTL operating conditions on the chemical and biological characteristics of HTL-WW, we used the same HTL batch reactor equipment (Model 4593, Parr Instrument Co., Moline, IL) as described in Chapter 3. The HTL reactors were operated at three different reaction temperatures (260°C, 280°C and 300°C), three reaction times (30, 60 and 90

min) and three solids concentrations (15%, 25% and 35%), and a total of 15 different operating conditions were tested. Prior to each HTL test, the algal feedstock was prepared by mixing algal powder with Nanopure water to create slurry feedstock containing between 15–35% dry matter and 65–85% water by weight. Nanopure water was used to eliminate any effects caused by minerals in the tap water. Then, about 70 g of the slurry feedstock was loaded into the reactor and run at different operating conditions. HTL experiments were conducted following the same method described in Chapter 3. All experiments were run in duplicate or triplicate. After each experiment, HTL products were separated following the procedure described in Figure 5.1. Yields of HTL co-product was quantified as described in Table 5.2.

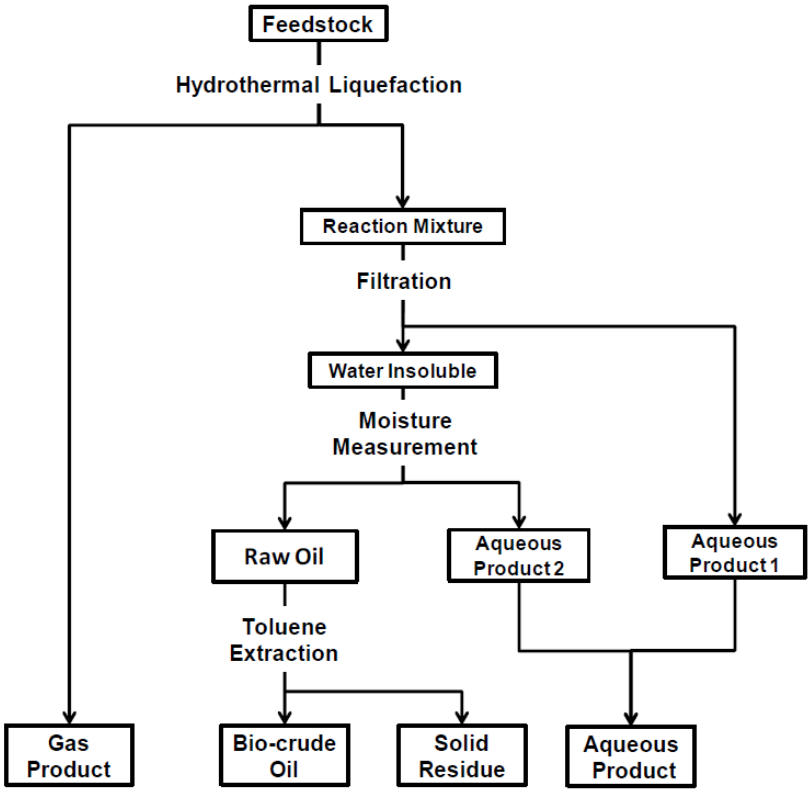

Figure 5.1 Recovery procedures for products from a hydrothermal liquefaction process (Yu et al., 2011b)

Table 5.1 Characteristics of *C. pyrenoidosa* powder (Yu et al., 2011b)

Properties	*C. pyrenoidosa*
Moisture content (wt %)[a]	6.3
Dry solids content (wt %)[a]	93.7
Volatile solids content	94.4
Ash content	5.6
Crude protein	71.3
Crude fat	0.1
Acid detergent fiber	0.5
Neutral detergent fiber	1.0
Lignin	0.2
Non-fibrous carbohydrate[b]	22.0
Elemental composition	
C	51.4
H	6.6
N	11.1
O[b]	30.9

[a]On the total weight basis; [b]calculated by difference

5.2.3 Water Quality Analysis

HTL-WW sample were first filtered by 1μm pore size glass filter (Whatman, 1821-150) and stored at 4°C to use. The chemical oxygen demand (COD) of HTL-WW was determined by visible light absorbance after dichromate digestion according to standard methods (Clesceri, 1999) using a visible light spectrophotometer (HACH Model DR/2010). Ammonia was measured using salicylate method (HACH Method 8155). Total nitrogen was determined by the persulfate digestion method (HACH Method 10072). Total phosphorous was quantified following a HACH method that has been accepted by USEPA for reporting wastewater analyses (HACH Method 10127). Total suspended solids was measured following APHA standard methods (APHA, 1995).

Table 5.2 Measurements and equations for calculating product yields (Yu et al., 2011b)

Measurement (g)	Symbol
Mass of Feedstock	F
Mass of dry matter of microalgae	C
Mass of reaction mixtures	RM
Mass of water insoluble product	WI
Mass of moisture in the water insoluble product	MWI
Mass of toluene insoluble fraction in raw oil	SR
Product	**Equation**
Gas yield (%)	$= \frac{F - RM}{C} \times 100$
Raw oil yield (%)	$= \frac{WI - MWI}{C} \times 100$
Solid residue yield (%)	$= \frac{SR}{C} \times 100$
Bio-crude oil yield (%)	$= \frac{WI - MWI - SR}{C} \times 100$
Aqueous product yield (%)	$= \frac{RM - WI - MWI - (F - C)}{C} \times 100$
Toluene solubility (%)	$= (1 - \frac{SR}{WI - MWI}) \times 100$

5.2.4 CHO Chronic Cytotoxicity Assays

Cytotoxicity of HTL-WW generated from different operating conditions was conducted using the same CHO chronic cytotoxicity assays described in Chapter 3 (Materials and Methods). However, it is important to note that in this case the HTL-WW samples were sterile filtered and tested directly for cytotoxicity effect without the XAD extraction step described in Chapter 3. Thus, the cytotoxicity data presented in this chapter accounted for both organic and inorganic compounds in HTL-WW.

5.3 RESULTS AND DISCUSSIONS

5.3.1 Effect of HTL Operating Parameters on Chemical Characteristics of HTL-WW

The effects of HTL-WW operating conditions on HTL-WW quality was studied by measuring typical water quality parameters such as pH, total suspended solid (TSS), chemical oxygen demand (COD), ammonia (NH_3-N), total nitrogen (TN), and total phosphorous (TP). Table 5.3 summarizes a range of basic water quality parameters for wastewater from HTL conversion of *C. pyrenoidosa* under different operating conditions (250–300°C, 30–90 min reaction time, and 15–35% solids concentration). The pH values of the HTL-WW samples were determined immediately after completion of the reaction and were found to range between 8 and 9, which is consistent with reported values in a previous study (Anastasakis & Ross, 2011). Variation in reaction temperature, reaction time, and solids concentration did not show any clear trends in the effect on the pH of HTL-WW.

The TSS concentration of HTL-WW was in the range of 2–24 mg/L. Generally, liquefaction of feedstock with a higher initial solids concentration resulted in a higher TSS concentration in HTL-WW. When a low solids concentration (15%) was used, increasing the temperature and/or reaction time generally lowered the concentration of TSS in HTL-WW. This effect was not observed when 25% or 35% solids content was used. All HTL-WW samples contained a substantial amount of nutrients (nitrogen and phosphorus) and had high COD concentrations. Carbon and nitrogen are the two most important nutrients for autotrophic growth of algae. Therefore, it is very important to understand how the HTL operating conditions affect the distribution of these nutrients into HTL-WW. The following sections discuss the effects of HTL operating parameters on concentrations of these nutrients

Table 5.3 Water quality analysis of *C. pyrenoidosa* HTL-WW generated from different operating conditions

Temp. (◦C)	Reaction time (min)	Solids Conc. (%)	pH	TSS (mg/L)	COD (g/L)±SD	Total-N (g/L)±SD	NH3-N (g/L)±SD	Total-PO_4^{3-} ((g/L) ±SD
260	30	15	8.2	6	75.4±34.2	12.5±0.7	3.8±0.07	7.9±4.5
260	90	15	9	3.8	86.6±3.8	13.8±0.85	3.4±0.1	5.9±0.64
300	30	15	8.4	3.7	99.9±1.2	12.7±0.14	6.4±0.085	5.5±0.8
300	90	15	8.4	2	79.2±1.4	13.3±0.14	6±0.014	5.7±0.4
280	60	15	8.6	10.3	69.4±2.2	10±0.85	2.98±0.1	5.4±0.2
260	60	25	8.5	9.6	96.9±1.4	22.9±2.7	7.6±0.085	11.6±4.9
280	30	25	8.2	8.7	102.2±0.4	25.6±0.28	9.4±0.03	15.5±0.68
280	60	25	8.1	4.8	91.±4.2	17.9±0.4	8.2±0.06	8.7±1.8
280	90	25	8.2	8.6	95.8±3.4	21.4±1.4	8.8±0.21	15.6±0.16
300	60	25	8.4	10.4	91.5±7.1	19.9±0.14	6.7±0.04	10.3±4.3
260	30	35	8.7	14.8	104.8±1.2	19.9±0.98	6.1±0.085	10.8±5.2
260	90	35	8.6	13.7	94.8±9.7	30.3±1.27	10.8±0.085	17.8±1.7
300	30	35	9.0	15.5	99.8±0.6	29.2±0.56	14.8±0.3	14.7±3.1
300	90	35	8.2	22.4	99.2±5.9	26.6±0.85	14±0.23	14±3.6
280	60	35	8.6	24.2	101.3±0.4	29.4±3.4	12.6±0.1	18.3±1.4

5.3.1.1 Effect of HTL Operating Parameters on COD Concentration

All the HTL-WW samples had high COD concentrations ranging from 69.4–104 g/L (Table 5.3). This was expected as more than one-third of the total carbon in the original material is typically converted into water soluble organic matter at temperature above 220°C (Yu et al., 2011a). Generally, liquefaction of higher solids concentrations feedstock resulted in HTL-WW with higher COD concentration. The highest COD concentration was found in HTL-WW generated from liquefaction of 35% solids concentration at 260°C for 30 min. Experimental results did not show a consistent relationship between COD concentration and tested operating parameters. This may due to the complexity of chemical reactions happening during the HTL process and the fact that dissolved organics are both being formed by hydrolysis of the feedstock and consumed by oil formation mechanisms. The effect of HTL operating conditions on COD levels had distinctly different trends at each of the three initial solids concentrations used in this study. For example, when 15% solid feedstock was used, HTL-WW COD concentration was found to increase with reaction temperature when a short reaction time (30 min) was used and the reverse happened when a long reaction time (90 min) was used. When 25% solids content feedstock was used, COD concentrations slightly decreased as the reaction time increased from 30 to 60 min and then gradually increased back up as the reaction time was extended to 90 min. When 35% solids content was used, COD concentration did not vary considerably with reaction time at the reaction temperature ≥ 280°C.

A past study showed that carbon recoveries of bio-oil generally increased with reaction time and decreased with reaction temperature (Yu et al., 2011a). Thus, it was expected that the COD of HTL-WW would decrease with reaction time and increase with reaction temperature. The experiment data, however, did not always follow this trend. In case of 15% solids content

feedstock, the COD concentration increased with reaction when a short reaction time was used. However, when a long reaction time was used (90 min), increasing in reaction temperature at first increased COD concentration in HTL-WW until it reached to a maximum solubility and then gradually decreased. Our hypothesis is that further increases in reaction temperature can enhance the transformation of organics in HTL-WW into bio-crude or gases, and thus, decreased the COD concentration in HTL-WW. When ≥25% solids content feedstock was used, we observed consistent negative effect of temperature on COD concentration. Since there were more organics in the feedstock, it only required a reaction temperature of 260°C to dissolve all soluble organics into HTL-WW. Once the maximum solubility has reached, further increases in the reaction temperature would facilitate conversion of soluble organics in HTL-WW into bio-oil and/or gases and thus decrease the concentration of COD in HTL-WW. The initial negative effect of reaction time on COD concentration can be explained by the fact that longer reaction time usually favors the recovery of carbon in bio-crude oil, and thus, lower the recovery of carbon in HTL-WW. However, prolonged reaction time can also initiate the transfer of soluble organics into the HTL-WW. Thus, we can see a gradual increase in COD concentration as reaction time was extended to 90 min. Overall our experiment data suggested that extreme HTL operating conditions (high temperature and long reaction time) would potentially lower the concentration of organics in HTL-WW.

5.3.1.2 Effect of HTL Operating Parameters on TN Concentration

HTL-WW samples contained a substantial amount of nitrogen with the concentration ranging from 10–30.3 g/L, which was close to the TN concentration reported for HTL-WW from liquefaction of swine manure and *Spirulina* (Appleford, 2004; Jena et al., 2011a). TN concentrations resulted from the thermal decomposition of proteins, which accounted for 71.3%

of the mass of *C. pyrenoidosa* feedstock. In addition, the HTL process was conducted in a nitrogen atmosphere at high pressure, which could have possibly resulted in the diffusion of nitrogen gas into the aqueous product. Under the same reaction temperature and time, liquefaction of higher solids concentration feedstock resulted in higher TN concentration in HTL-WW (Table 5.3). When 15% solids content feedstock was liquefied at the same reaction temperature, TN concentration slightly increased as the reaction time increased. For example, TN concentration of HTL-WW resulting from liquefaction of 15% solid feedstock at 260°C increased from 12.5 g/L to 13.8 g/L as the reaction time increased from 30 to 90 min. The effect of reaction time on TN concentrations in HTL-WW did not follow a consistent trend when ≥25% solid feedstock was used. A previous study showed that nitrogen generally accumulated in bio-crude oil with longer reaction time (Yu et al., 2011a). This helps to explain the initial decrease in TN concentration as the reaction time increased when low solids content feedstock (15%) was used.

Looking at the effect of reaction temperature on TN concentration when 15% solids content was used, we found that reaction temperature did not strongly affect the concentration of TN. That may due to the fact that the initial nitrogen content in 15% solids feedstock was relatively low and a reaction temperature of 260°C would be sufficient to liquefy all nitrogen in the feedstock. Further increases in reaction temperature did not affect TN concentration as all soluble nitrogen had been recovered in HTL-WW. As the feedstock solids concentration increased to 25%, we observed a slight decrease in TN concentration as the temperature increased from 260 to 280°C. The concentration of TN however, gradually increased back up as the temperature increased from 280 to 300°C. Our hypothesis is that the initial increase in reaction temperature could potentially enhance the conversion of organic nitrogen into gases, and

thus, decrease the TN concentration in HTL-WW. However, further increases in reaction temperature would convert nitrogenous compounds in bio-crude oil into soluble nitrogenous compounds, and thus, increase the concentration of TN in HTL-WW. A past study on nitrogen recovery of raw oil and the aqueous product from HTL treatment of 20% solids content *Spirulina* feedstock also demonstrated that nitrogen recovery in the aqueous fraction increased from 1% to 73.5% as the temperature increased from 100 to 300°C. Our experimental data shows the same effect of reaction temperature on TN concentration when >15% solids content feedstock was used. Overall, increases in temperature and retention time could potentially increase TN concentration in HTL-WW when high solids concentration feedstock (≥25%) was used.

5.3.2 Effect of HTL Operating Parameters on Cytotoxicity of HTL-WW

Figure 5.2 shows the concentration-response curves for CHO cell chronic cytotoxicity for HTL-WW generated from HTL conversion of 15%, 25% and 35% solids concentration at different reaction temperatures and times. These plots show average toxicity data points for each HTL-WW concentration (8–16 independent clones), and the standard error is indicated as whiskers extending from the data points. LC_{50} was calculated as the concentration that induced 50% reduction of cell density as compared to the concurrent negative control, and the LC_{50} values were reported in Table 5.4. This table also presents ANOVA statistics and the lowest concentration with a significant difference from the negative control. To directly compare the cytotoxicity of each HTL-WW sample, we calculated a cytotoxicity index. The cytotoxicity index value was determined as $(LC_{50})^{-1}(10^3)$, where a larger value represents greater toxic potency. Our data showed that the cytotoxicity of HTL-WW varied significantly among the 15 tested operating conditions. All tested HTL-WW samples were extremely cytotoxic to CHO cells, with the LC_{50} varied from 0.36–1.6 % of the HTL-WW. The most cytotoxic HTL-WW was

produced when 35% solids concentration feedstock was liquefied at 300°C for 30 min reaction time. The least cytotoxicity HTL-WW was obtained when 15% solids concentration feedstock was liquefied at 260°C for 90 min of reaction time. Individual effects of tested HTL operating parameters on cytotoxicity of HTL-WW are discussed in the following sections.

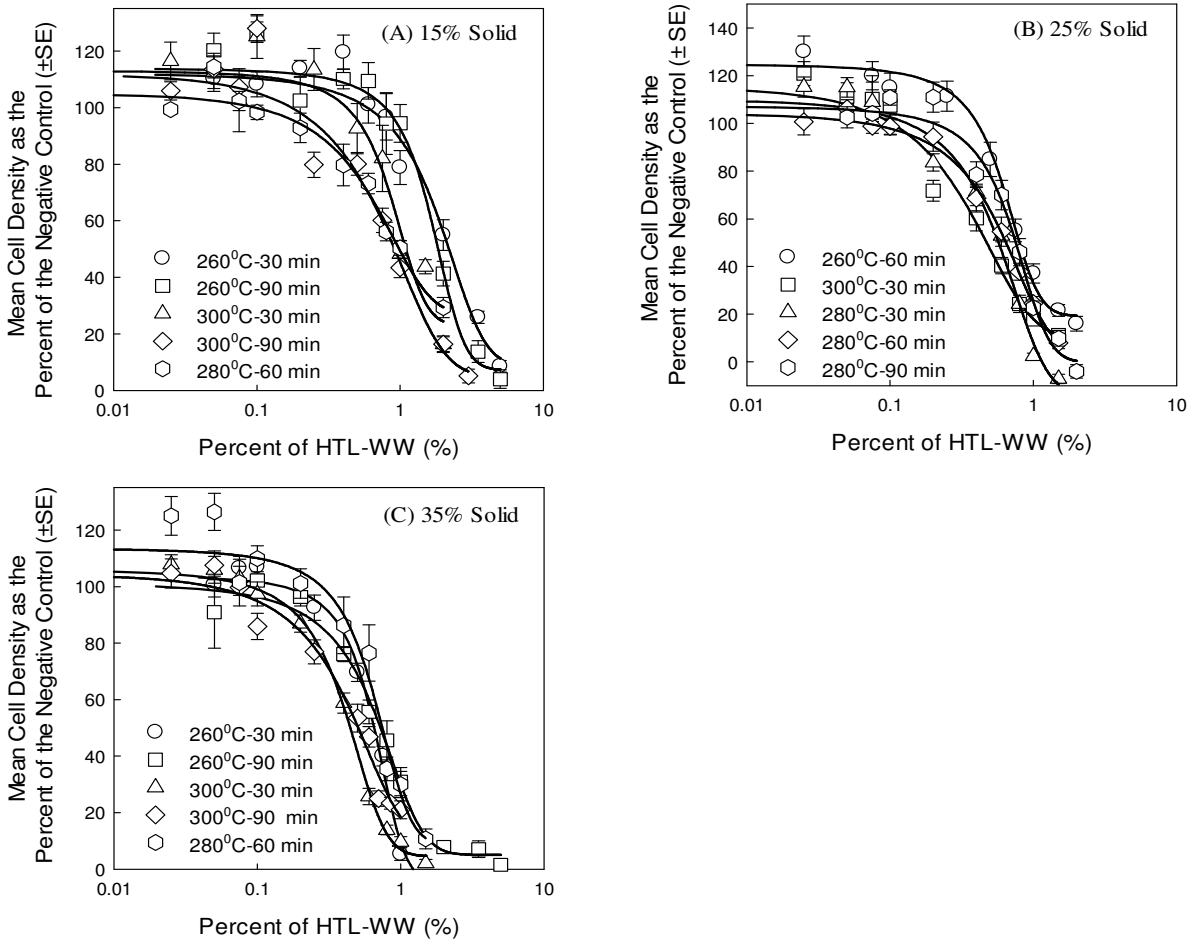

Figure 5.2 Comparison of the CHO cell cytotoxicity concentration response curves for wastewater generated from HTL conversion 15% solid feedstock (A), 25% solid feedstock (B) and 35% solid feedstock (C) at different reaction temperature and times.

Table 5.4 Induction of chronic cytotoxicity in CHO cells by HTL-WW generated from different operating conditions

Reaction Temp. (°C)	Reaction time (min)	Solids Conc. (%)	Lowest cytotox. conc. (% HTL-WW)[a]	R^{2b}	LC_{50} (% HTL-WW)[c]±SE	Cytotoxicity Index	ANOVA test statistic
260	30	15	0.004	0.98	1.33±0.15	818±69.6	$F_{10,77}$=55.6; P≤0.001
260	90	15	0.02	0.98	1.71±0.085	596±33.1	$F_{10,77}$=47.4; P≤0.001
280	60	15	0.0075	0.94	0.9±0.055	1130±57.9	$F_{10,77}$=22.9; P≤0.001
300	30	15	0.005	0.96	0.93±0.97	1109±65.2	$F_{10,77}$=119.8; P≤0.001
300	90	15	0.004	0.97	0.96±0.76	1283±137.7	$F_{10,77}$=42.7; P≤0.001
260	60	25	0.00025	0.93	0.63±0.033	1612±89.3	$F_{10,77}$=87.1; P≤0.001
280	30	25	0.00025	0.97	0.47±0.08	2452±286.3	$F_{10,77}$=60.5; P≤0.001
280	60	25	0.002	0.97	0.6±0.043	1720±125.5	$F_{10,77}$=109.5; P≤0.001
280	90	25	0.004	0.99	0.79±0.048	1295±65	$F_{10,77}$=93.1; P≤0.001
300	60	25	0.004	0.99	0.67±0.11	1714±216	$F_{10,77}$=68.1; P≤0.001
260	30	35	0.005	0.99	0.64±0.11	1574±27.3	$F_{10,77}$=224.7; P≤0.001
260	90	35	0.004	0.99	0.69±0.046	1509±111.4	$F_{10,77}$=56.7; P≤0.001
280	60	35	0.004	0.99	0.43±0.03	2435±200	$F_{10,77}$=135.4; P≤0.001
300	30	35	0.001	0.98	0.63±0.1	1835±225.5	$F_{10,77}$=79.7; P≤0.001
300	90	35	0.0005	0.95	0.77±0.13	1573±279	$F_{10,77}$=36.4; P≤0.001

[a]The lowest cytotoxicity concentration was the lowest concentration of the tested compound in the concentration-response curves that induced a significant amount of cytotoxicity as compared to the negative control. [b]R^2 is the coefficient of determination for the regression analysis upon which the LC_{50} value ($\%C^1/_2$ value) was calculated. [c]The LC_{50} is the sample concentration that induced a cell density that was 50% of the negative control. The estimated SE of the LC_{50} was derived as the averaged SE of multiple LC_{50} values determined from regression analysis for 8-16 concentration-response curves.

5.3.3 Effect of Solids Content on Cytotoxicity of HTL-WW

Solids concentration of the feedstock was the most important operating parameter that determined product yield and cytotoxicity of HTL-WW. Generally, liquefaction of higher solids concentration of algal slurry resulted in better bio-oil yield (Table 5.5) and more cytotoxic HTL-WW (Figure 5.3). For example, the cytotoxicity indexes of wastewater generated from HTL of 15% and 35% solids concentration feedstock at 260°C for 30 min were 818 and 1574, respectively (Figure 5.3 A and C). Cytotoxicity indexes of HTL-WW resulting from HTL conversion of feedstocks with a solids content ≥25 were not considerably different (Figure 5.3B). One previous study reported that increasing solids concentration of *Spirulina* caused increased bio-oil yield and decreased yield of organics in HTL-WW (Jena et al., 2011a). Even if aqueous yield of organics decreases, the aqueous concentration can still increase because more feedstock solids are in contact with each unit of water, resulting in a higher concentration of soluble organic compounds being dissolved into the HTL-WW. Therefore, high concentration of solids will generally cause a negative effect on HTL-WW cytotoxicity. These authors also found that solids concentration greater than 20% had no significant effect on the product yields (Jena et al., 2011a). In our case, we found that solids concentration greater than 25% did not cause a clear effect on bio-oil yield and cytotoxicity of HTL-WW. Thus, the ideal solids concentration of *C. pyrenoidosa* for optimal HTL conversion should be around 25%.

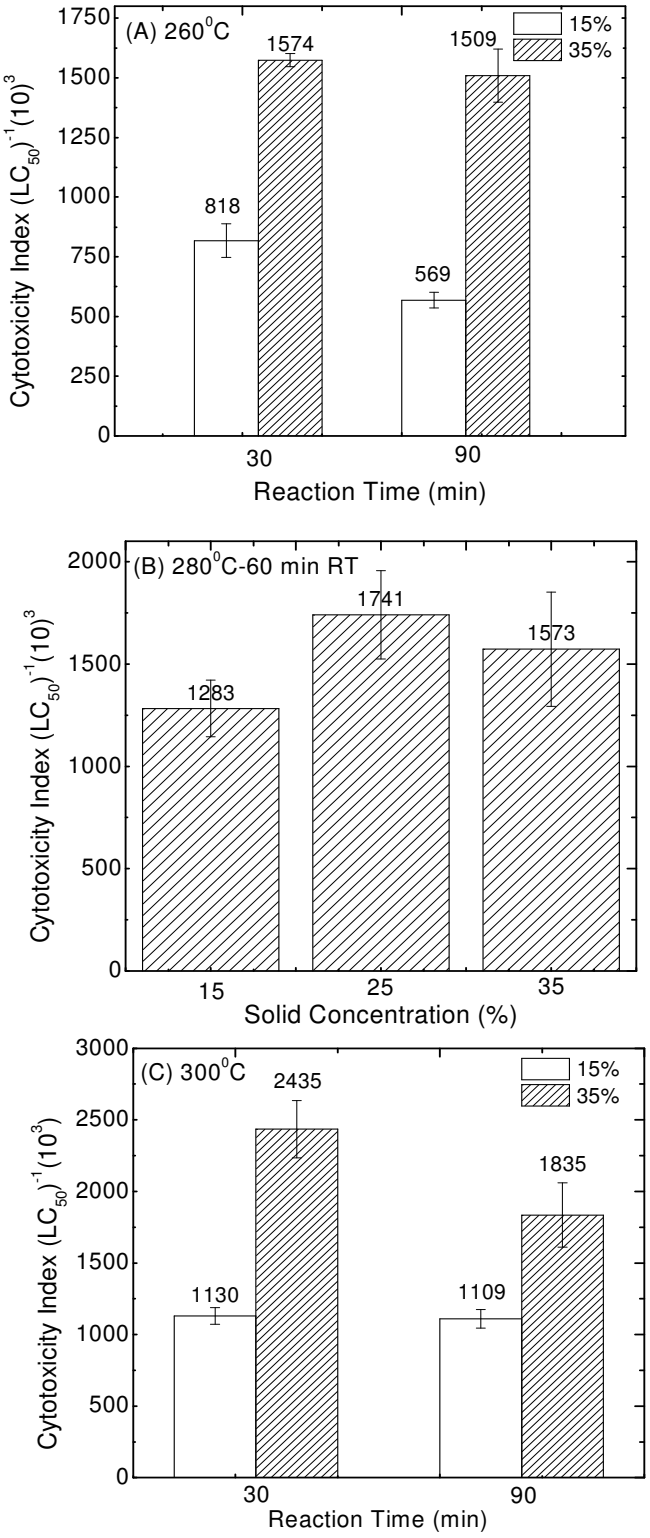

Figure 5.3 Effects of feedstock solids concentration on cytotoxicity of HTL-WW.

5.3.4 Effect of Reaction Time on Cytotoxicity of HTL-WW

Experimental data suggested that extending reaction time can potentially reduce cytotoxicity of HTL-WW. For example, at 260°C the cytotoxicity of HTL-WW generated by liquefaction of 15% solids content feedstock decreased significantly from 818 to 596 as the reaction time increased from 30 to 90 min (Figure 5.3 A, white bars). However, varies in reaction time did not considerably affect cytotoxicity of HTL-WW when the same when feedstock was liquefied at 300°C (Figure 5.3 A, dashed bars). Similar effect was also observed when 35% solids concentration feedstock was used (Figure 5.3 B). For example, cytotoxicity index of HTL-WW decreased considerably from 2435 to 1835 as reaction time increased from 30 to 90 min (Figure 5.3 B, dashed bars).

The positive effect of prolonged reaction time on HTL-WW cytotoxicity because long reaction time can enhance the secondary and tertiary reactions in hydrothermal medium. These reactions can convert heavy intermediates either into liquids, gases, or residues species and lower the bio-oil yield. The toxicity of HTL-WW is partially contributed by the participation of soluble toxic compounds in bio-oil into the wastewater during HTL conversion. Since less bio-oil and more HTL-WW were produced when prolonged reaction time was used, the concentration of soluble toxic compounds participated into HTL-WW became lower. Thus, HTL-WW was less toxic when extended reaction time was used. Our experimental data showed that bio-oil yield decreased from 34.9% to 27.8% as reaction time increased from 30 to 90 min reaction (data related to 260°C and 15% solids content). This 7% reduction in bio-oil yield resulted in 18% reduction in cytotoxicity of HTL-WW. Meanwhile, the bio-oil yield from liquefaction of 35% solids content feedstock at 260°C was not changed as reaction time increased from 30 to 90 min (25.65% vs. 25.67%). Consequently, cytotoxicity indexes of HTL-WW were relatively close

(1575 vs. 1509). Overall, the HTL-WW cytotoxicity could potentially be lessened by extending reaction time. The effect of reaction time on HTL-WW cytotoxicity is more visible when low solids content feedstock (15%) and temperature (260°C) were used. Even though extension of reaction time would potentially lower the toxicity of HTL-WW, it would require additional energy input and result in lower bio-oil yield, which is not desirable from an energy production perspective. Therefore, extending reaction time is not a favorable option for minimizing the toxicity of HTL-WW.

5.3.5 Effect of Operating Temperature on Cytotoxicity of HTL-WW

As reaction temperature increased there was also generally an increase in HTL-WW cytotoxicity. As demonstrated in Figure 5.4, for the same solids concentration and reaction time, HTL at higher reaction temperature produced more cytotoxic HTL-WW. For example, cytotoxicity indexes of wastewater generated from HTL of 25% solids concentration for 60 min at 260, 280 and 300°C were 1612, 1714, and 2452, respectively (Figure 5.4 B). Effect of reaction temperature on HTL-WW cytotoxicity was more potent when higher solids concentration was used (Figure 5.4 C). Looking at the sensitivity of HTL-WW toxicity to reaction temperature, we noticed that initial increase of temperature from 260°C to 280°C did not show a significant effect on toxicity of HTL-WW. However, a further increase in temperature from 280°C to 300°C resulted in a considerably more toxic HTL-WW (Figure 5.4 B). The effect of reaction temperature on HTL-WW cytotoxicity is strongly associated with variations in bio-oil yield. Our experimental data showed that when the same solids content feedstock and reaction time was used; increasing the temperature from 260°C to 300°C would result in about 10–12% increases in bio-oil yield (Table 5.5). Past studies demonstrated that increasing in reaction temperature would increase bio-oil yield and decreased yield of HTL-WW. For example, Karagoz et al.

reported that the total oil yield after liquefaction of sawdust at 180°C, 250°C and 280°C was 3.7%, 7.6% and 8.5% respectively (Karagöz et al., 2006). Similarly, Jena et al found that the yield of bio-crude from HTL of *Spirulina* increased with increase in temperature reaching a maximum of 39.9% at 350°C and then dropped down to 36% at 380 °C (Jena et al., 2011a). Also, a decrease in the yield of water solubles from 56.3 to 30.2% was obtained as the temperature increased from 200°C to 380°C due to conversion of intermediate water soluble products into gases and bio-crude oil. Since less HTL-WW was in contact with more bio-oil, HTL-WW became more concentrated with soluble organics from bio-oil and thus, more cytotoxic. More importantly, experimental data consistently showed a high oil yield obtained at 280°C for 60 min reaction time for all three tested solids concentrations. In addition, further increased of temperature from 280 to 300°C did not cause a considerable difference in bio-oil yield. Therefore, it is recommended that HTL of *C.pyrenoidosa* should be carried at a temperature of 280°C for optimal bio-oil yield and lower energy input.

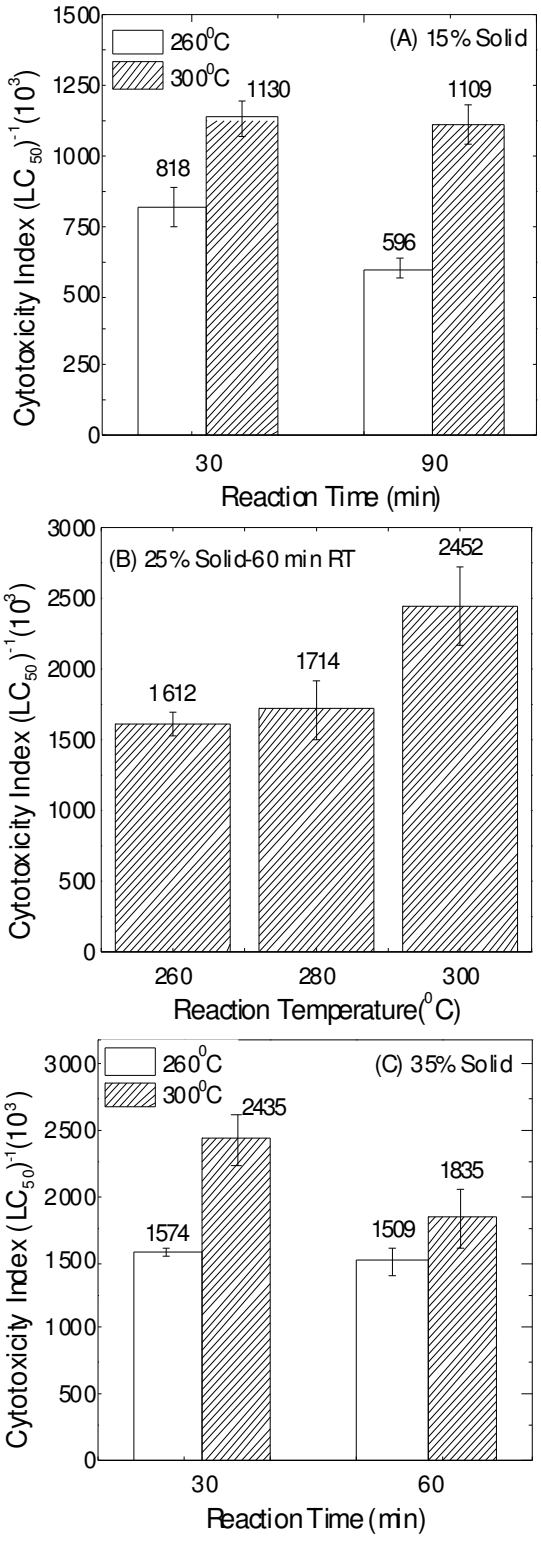

Figure 5.4 Effects of reaction temperature and reaction time on cytotoxicity of HTL-WW.

Table 5.5 Comparison of HTL-WW cytotoxicity and bio-crude oil yield

Run #	Reaction Temp. (°C)	Reaction Time (min)	Solids Conc. (%)	LC_{50} (% HTL-WW)	Cytotoxicity Index	Oil Yield (%)	Oil Yield (g)
1	260	30	15	1.33±0.15	818±69.6	34.93±1.14	5
2	260	90	15	1.71±0.085	596±33.1	27.81±0.39	5
11	280	60	15	0.9±0.055	1283±137.7	41.73±0.41	4.3
5	300	30	15	0.93±0.97	1130±57.9	35.59±0.76	4.4
7	300	90	15	0.96±0.0.76	1109±65.2	29.51±0.83	3.3
9	260	60	25	0.63±0.033	1612±89.3	31.79±0.42	8.8
13	280	30	25	0.47±0.080	1720±125.5	34.52±1.63	8.5
15	280	60	25	0.6±0.433	1714±216	41.74±0.37	9.7
14	280	90	25	0.79±0.048	1295±65	33.27±0.45	8.4
10	300	60	25	0.67±0.11	2452±286.3	39.55±0.79	7.7
2	260	30	35	0.64±0.11	1574±27.3	25.65±0.41	14.8
4	260	90	35	0.69±0.046	1509±111.4	25.67±0.82	12.9
12	280	60	35	0.43±0.03	1573±279	43.26±2.04	14.3
6	300	30	35	0.63±0.1	2435±200	36.74±2.04	13.6
8	300	90	35	0.77±0.13	1835±225.5	38.36±2.45	11.6

5.3.6 Combination Effect of HTL Operating Parameters on Cytotoxicity of HTL-WW

As demonstrated in the previous sections, HTL operating parameters act dependently on the cytotoxicity of HTL-WW. Under a specific operating condition, one parameter can play a more dominant role in forming the cytotoxicity of HTL-WW than others. Figure 5.5–5.7 demonstrated the cytotoxicity index of HTL-WW at different temperatures, reaction times and solids contents. Figure 5.5 shows the response surface and contour plots of the cytotoxicity of bio-HTL-WW generated from liquefaction for 15% solids concentration feedstock at different temperature and reaction time. As indicated in Figure 5.5, under a fixed temperature, the cytotoxicity of HTL-WW would first increased with reaction time until it reached to 60 min and then gradually increased as the reaction was further increased from 60 to 90 min. On the other hand, when a fixed reaction time was used, the cytotoxicity of HTL-WW increased continuously with increasing reaction temperature and reached a maximum at 300°C.

Similar effects of reaction time and temperature on HTL-WW cytotoxicity was also observed when 25% solids content feedstock was used (Figure 5.6). We found that HTL-WW toxicity increased with reaction temperature and decreased with reaction time. It also important to note that when the same temperature and reaction time was used, HTL-WW generated from 25% solids content feedstock was considerably more cytotoxic than that of 15% solids concentration feedstock. Reaction temperature was still the dominant factor determining the toxicity of HTL-WW.

Figure 5.7 shows the response surface and contour plots of the cytotoxicity of bio-HTL-WW generated from liquefaction of 35% solids content feedstock at different temperature and reaction time. Cytotoxicity of HTL-WW was first decreased as the reaction time increased from 30 to 70 min and then gradually increased back up as the reaction time increased. Under a fixed

reaction time, HTL-WW cytotoxicity increased continuously with reaction temperature. This observation suggested that when higher solids content feedstock was used, increasing in reaction time and temperature would potentially increase the toxicity of HTL-WW. Increasing reaction temperature and time, however, can also enhance the yield of bio-crude oil. Therefore, it is important to balance between optimizing the process yield and minimizing the process environmental impact.

Experimental data showed that the highest oil yield was obtained for liquefaction of 35% solids *C. pyrenoidosa* feedstock at 280°C for 60 min reaction time and this operating condition also created the most cytotoxicity HTL-WW. Direct manipulation of HTL-WW operating conditions such as increasing reaction time or decreasing reaction temperature and feedstock solids concentration can potentially reduce the toxicity of HTL-WW. However, this would result in considerable lower bio-crude oil yield, which is not favorable from energy production perspective. Therefore, it is important to investigate alternative options for reducing cytotoxicity of HTL-WW.

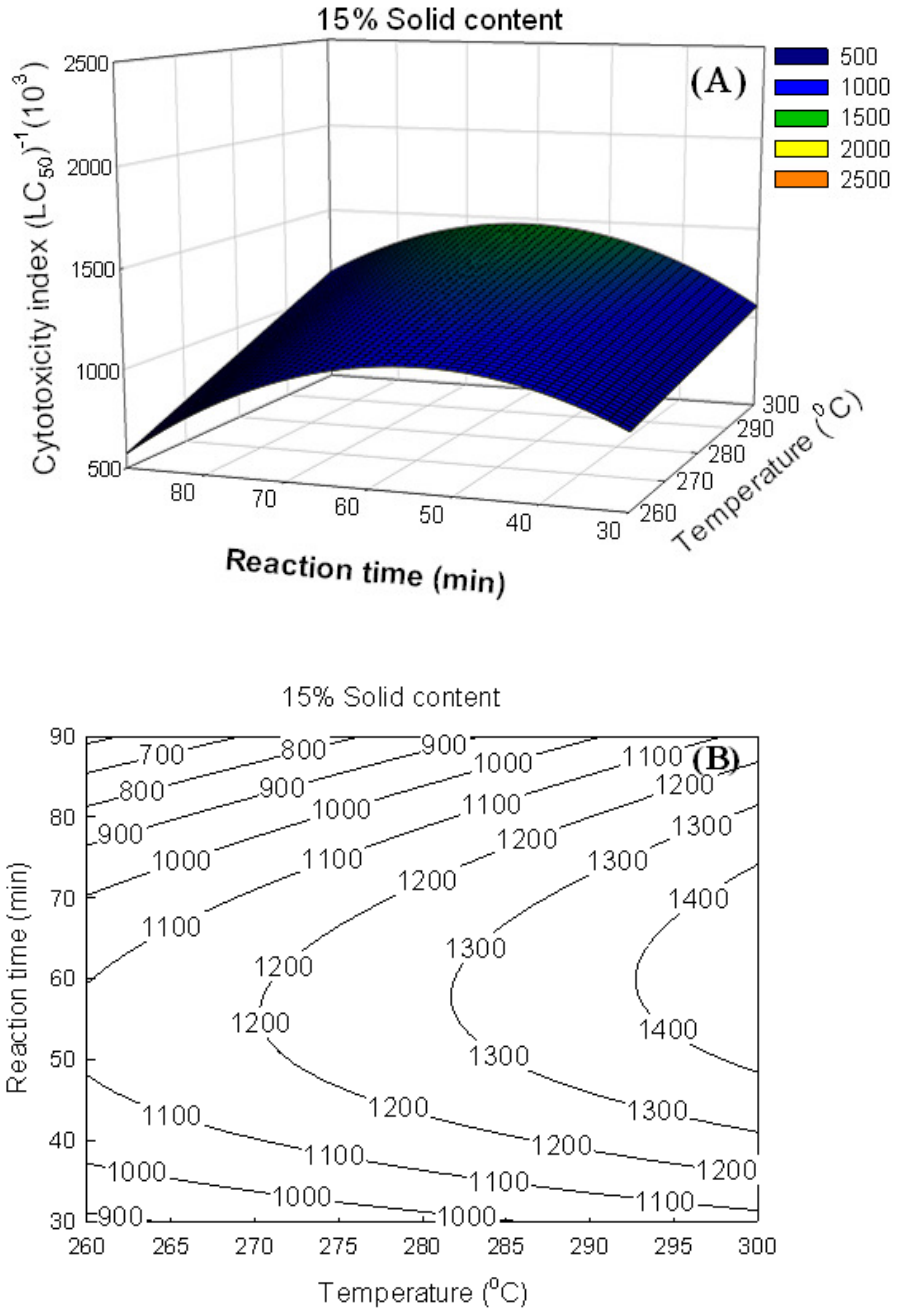

Figure 5.5 Response surface (A) and contour plot (B) of cytotoxicity of HTL-WW generated from liquefaction of 15% solids content feedstock at different temperature and reaction time

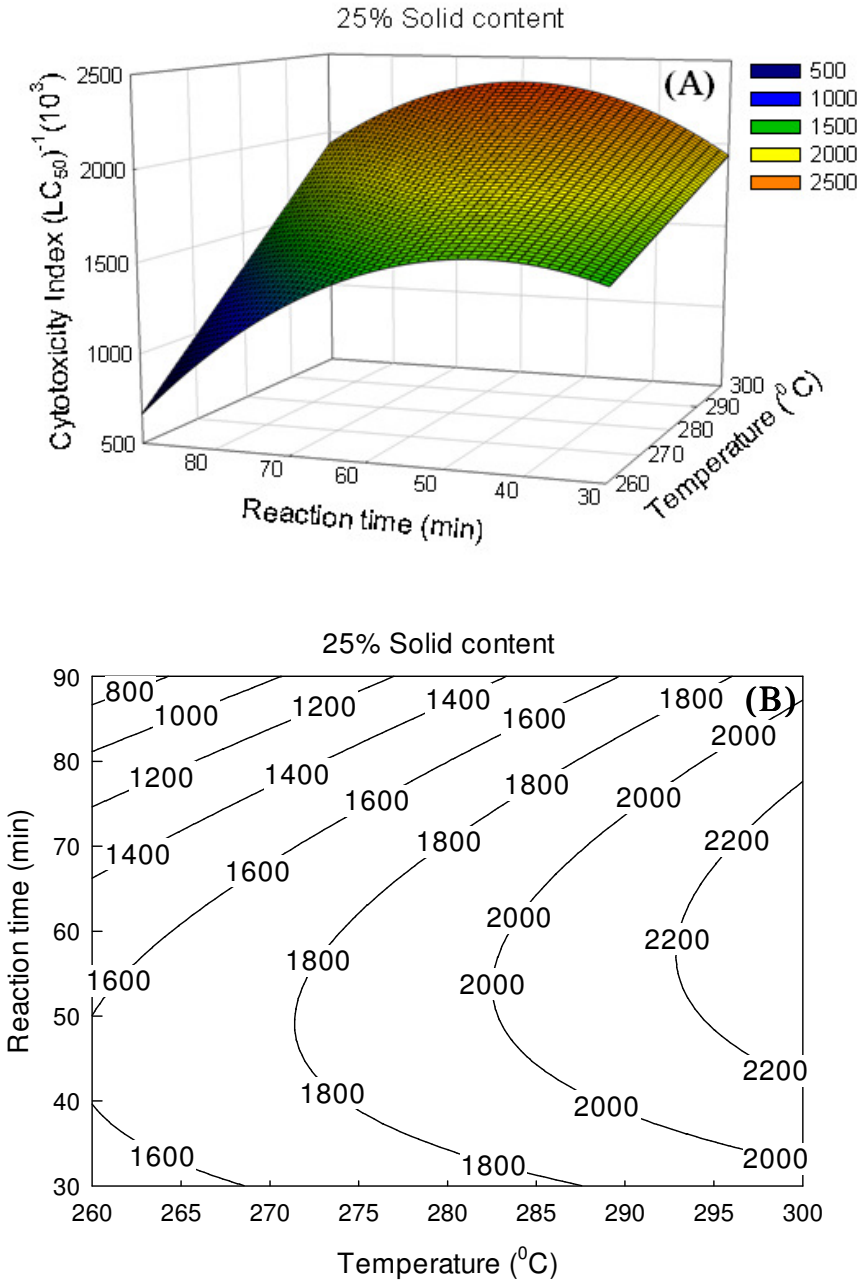

Figure 5.6 Response surface (A) and contour plot (B) of cytotoxicity of HTL-WW generated from liquefaction of 25% solids content feedstock at different temperature and reaction time

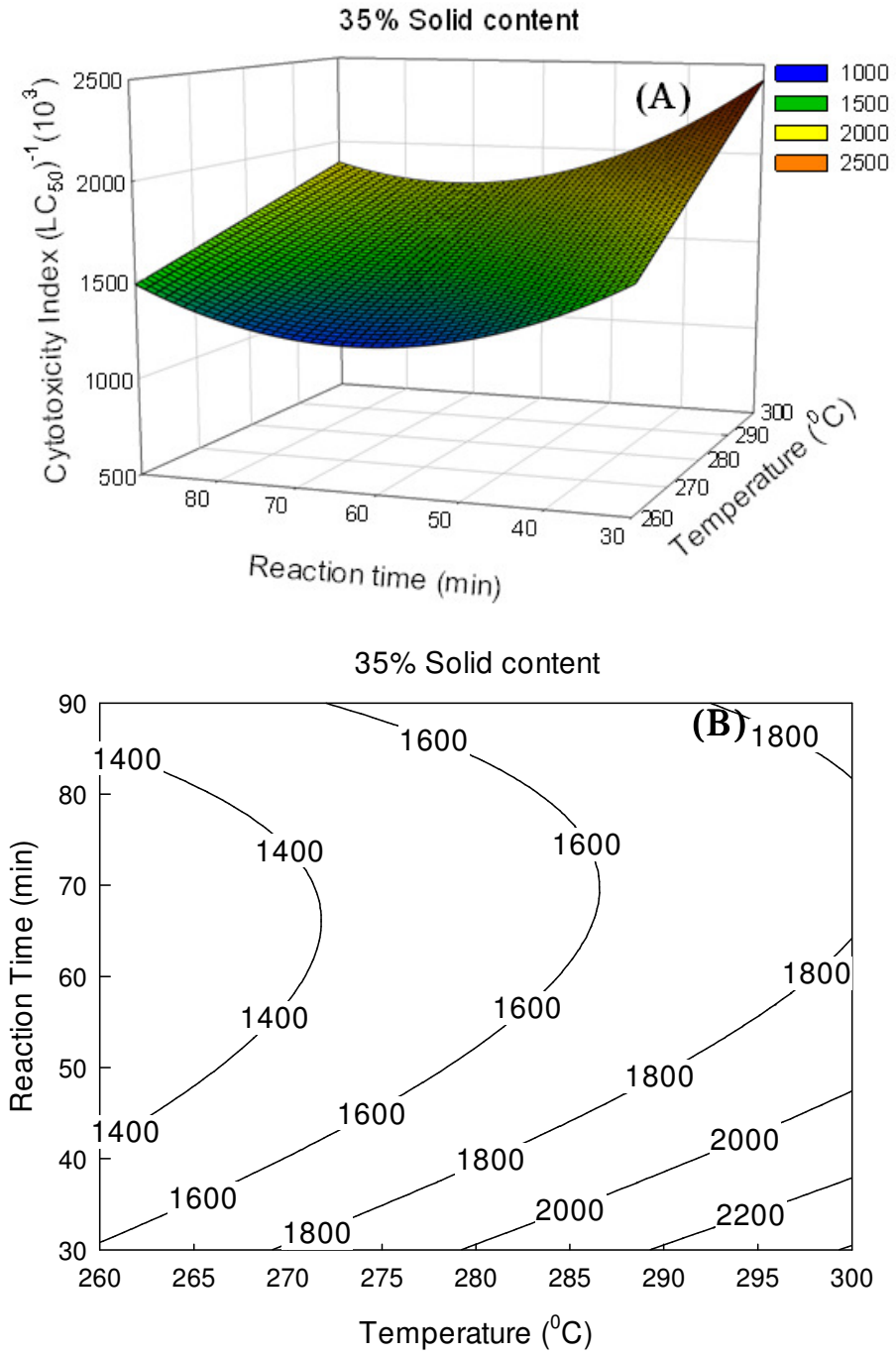

Figure 5.7 Response surface (A) and contour plot (B) of cytotoxicity of HTL-WW generated from liquefaction of 35% solids content feedstock at different temperature and reaction time

5.3.7 Correlation Between HTL Operating Parameters, COD Concentration and HTL-WW Toxicity

To evaluate the effect of HTL operating parameter on HTL-WW toxicity , we conducted a Pearson correlation analysis. The Pearson product-moment correlation coefficient is a measure of the linear correlation (dependence) between two variables *X* and *Y*. The correlation coefficient ranges from −1 to 1. A value of 1 implies that a linear equation describes the relationship between *X* and *Y* perfectly, with all data points lying on a line for which *Y* increases as *X* increases. A value of −1 implies that all data points lie on a line for which *Y* decreases as *X* increases. A value of 0 implies that there is no linear correlation between the variables. Table 5.6 summarizes the correlation coefficient and the P value of different operating parameter and the cytotoxicity of HTL-WW. The pair(s) of variables with positive correlation coefficients and P values below 0.05 tend to increase together. For the pairs with negative correlation coefficients and P values below 0.05, one variable tends to decrease while the other increases. For pairs with P values greater than 0.05, there is no significant relationship between the two variables. As demonstrated in Table 5.6, feedstock solids concentration is the only operating parameter that shows a direct positive relationship with the HTL-WW toxicity. Increases in solids concentration of the feedstock consequently result in more cytotoxicity HTL-WW. The relationship between reaction time or reaction temperature with HTL-WW toxicity was found to be not significant.

Figure 5.17 shows the corresponding cytotoxicity index of HTL-WW with its COD concentration. As indicated in Figure 5.8, there is a positive correlation between HTL-WW toxicity and COD concentration. The statistical analysis also demonstrated that there was a significant relationship between the two variables. between COD concentration and HTL-WW

toxicity with a correlation coefficient of 0.58 and the P value of 0.024. Therefore, it is expected that HTL-WW with higher COD concentration would also have high cytotoxicity index.

Table 5.6 Correlation between HTL operating parameters and HTL-WW cytotoxicity

HTL Operating Parameter	HTL-WW Cytotoxicity	
	Correlation Coefficient (r)	P value
Feedstock solids concentration (%)	0.66	0.008
Temperature (°C)	0.207	0.460
Reaction time (min)	-0.285	0.303
COD concentration	0.58	0.024

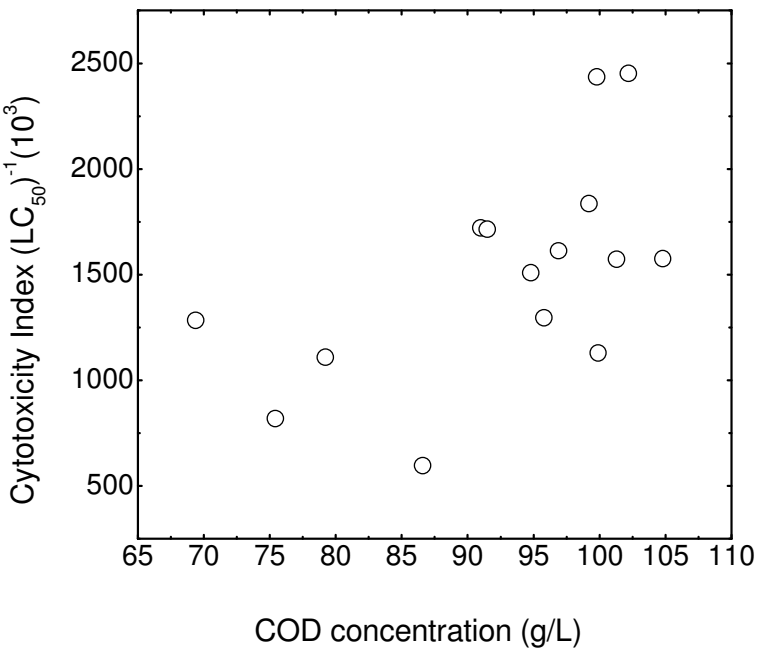

Figure 5.8 Correlation between COD concentration and HTL-WW cytotoxicity

5.4 CONCLUSIONS

In conclusion, the chemical and biological characteristics of HTL-WW were strongly affected by HTL operating condition. All HTL-WW samples have high pH, high concentration of nutrients (TN and TP) and high COD concentration that make it unsuitable for discharge to surface waters without significant additional treatment. The concentration of HTL-WW quality parameters was mostly determined by the initial solids concentration. The higher the solids concentration was use, the more nutrients and organics were found in HTL-WW. There was a positive correlation between feedstock solids concentration and the concentration of organics and nutrients in HTL-WW. However, there was no linear relationship between nutrient concentration and reaction temperature or reaction time. Instead, these parameters act dependently on the concentration of nutrients and organics in HTL-WW. Generally, COD concentration was found to increase with reaction time and temperature when ≥25% solids concentration or a short reaction time (≤30 min) was used, respectively. When high solids concentration was used COD concentration was found to decreased with increasing reaction time. The concentration of TN in HTL-WW increased with prolonged reaction times (≥60 min) and temperature (≥280°C). At temperature ≥280°C, increasing in temperature result in higher TN concentration in concentration of TN in HTL-WW. TP concentration in HTL-WW decreased with increasing reaction time and temperature when ≥15% solids concentration was used. When higher feedstock solids concentration was used (≤25%), TP concentration increased with increasing reaction time and temperature until it reached to 280°C. Further increasing in temperature would lower the concentration of TP in HTL-WW.

The cytotoxicity of HTL-WW is also strongly affected by HTL operating conditions. Experimental data showed that HTL-WW cytotoxicity generally increased with feedstock solids

concentration, which was expected because more organic materials are converted into water soluble organics per amount of water in the reaction. However, when the solids concentration reached to 25%, the cytotoxicity of HTL-WW appeared to be stable. Increase in reaction temperature generally enhanced HTL-WW toxicity and oil yield. On the other hand, prolonged reaction time reduced the toxicity of HTL-WW. Correlation analysis showed that there was a strong positive correlation between feedstock solids concentration and HTL-WW cytotoxicity. The higher the feedstock concentration, the more cytotoxic the HTL-WW. There was also a moderate positive correlation between HTL-WW cytotoxicity and the concentration of COD in HTL-WW. HTL-WW with high COD concentration usually more cytotoxic than those with lower COD concentration. In addition, variations in operating condition also affect the chemical composition of HTL-WW and thus, change its cytotoxicity. The criteria for optimal operating condition are high oil yield and low cytotoxicity index of HTL-WW. Based on these criteria, we recommended that HTL of *C.pyrenoidosa* should be conducted at 280°C for 60 min using 35% solids concentration for optimal oil yield (43%) and low cytotoxic HTL-WW. It is also important to note that under the identified optimal operating condition for *C.pyrenoidosa,* the HTL-WW is still highly toxic and requires additional treatment prior to environmental discharge.

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CHAPTER 6: MITIGATION MEASURES FOR HYDROTHERMAL LIQUEFACTION WASTEWATER

6.1 INTRODUCTION

Hydrothermal liquefaction (HTL) is a thermochemical conversion process that transforms wet bio-solids (up to 85% water content) into valuable, self-separating co-products (bio-oil and bio-char). A large quantity of aqueous product or wastewater that contains high level of organics, nitrogen, phosphorus, and other nutrients is also generated after HTL of biosolids. Previous studies reported that the aqueous product from HTL of swine manure contained high concentration of N, P, K and was not suitable for directly discharge to the environment (Appleford, 2004; He et al., 2000b). Wastewater generated from HTL of swine manure and *C. vulgaris* (at 87% moisture content) was reported to have ammonia concentrations as high as 7070 mg/L and 9000 mg/L, respectively (Appleford, 2004; Minowa & Sawayama, 1999). High concentrations of ammonia may be toxic to many aquatic organisms (Camargo & Ward, 1995; Scott & Crunkilton, 2000). For example, Tsukahara et al. found that the high concentration of ammonia (16.62g/L) in a condensate solution recovered from gasification was toxic to *C.vulgaris* (Tsukahara et al., 2001). In addition, HTL-WW also contains many different classes of soluble organics resulting from intimate contact with the produced bio-oil. Elliot had summarized a list of 48 hazardous constituents likely to be found in HTL-WW that contains several components with reported toxic effects, specifically, phenol, toluene, benzene, 2-methylarizidine, and aziridine (Elliot, 1992). In Chapter 4 and 5, we have demonstrated that HTL-WW from various feedstocks (swine manure, *Spirulina and C. pyrenoidosa*) is highly cytotoxic to CHO cells**.** Specifically we found that only 6.9-7.5% HTL-WW would induce 50% reduction in CHO cell density (Pham et al., 2013). In addition, previous batch studies in our research group showed that

algal growth was inhibited when more than 5% of HTL-WW was added into the growth medium. These results demonstrate that HTL-WW is likely to have toxic effects in natural ecosystems and require treatment prior to environmental discharge. Thus, it is important to develop treatment technologies that can remove organics and nutrients in HTL-WW and mitigate its toxicity. The objectives of this work were to: i) investigate the use of a batch-algal bioreactor to remove dissolved nitrogenous organic compounds (NOCs) in HTL-WW; (ii) evaluate the use of semi-batch algal bioreactor to improve the chemical and biological quality of HTL-WW; (iii) examine the use of granular activated carbon (GAC) adsorption to reduce the toxicity of HTL-WW and (iv) study the use of catalytic hydrothermal gasification (CHG) to remove organics in HTL-WW.

6.2 MATERIALS AND METHODS

6.2.1 Algal Culturing

Algal mixture was obtained from the clarifier outlets at a local wastewater treatment plant (Urbana-Champaign Sanitary District, UCSD) and was used as one of the inoculums (USCD algae). The composition of this mixed culture of algae was characterized roughly under the microscope. It was mainly composed of single cell cyanobacteria (blue green algae), green algae and filamentous green algae. Pure culture of *C. protothecoides* was provided by the Culture Collection of Alga at the University of Texas (Austin, TX, USA). Stock cultures of *C. protothecoides* were maintained routinely on both liquid and agar slants of Bold's Basal Medium (BBM) by regular sub-culturing at 7 days interval. The algae were grown autotrophically in batch cultures under $27 \pm 1^{\circ}C$ with continuous illumination. The purity of the culture was established by repeated streaking and routine microscopic examination. An inoculum culture was prepared 4 days prior to the start of the inhibition test to ensure that the algae are in the

exponential growth phase when used to inoculate the test solution. The growth of algae was monitored spectrophotometrically by reading the culture absorbance at 680 nm and 750 nm.

6.2.2 Wastewater Sample Collection

Wastewater samples were collected from a local wastewater treatment plant (Urbana-Champaign Sanitary District) at different treatment stages including raw wastewater influent, primary effluent, and secondary effluent. After collection, samples were sequentially filtered through 1.0 µm and 0.45 µm glass filters to remove suspended solids. All samples were acidified to pH 2.0 with 12 N HCl and stored at 4°C until use for XAD resin extraction of organic compounds, which occurred within 7 days.

6.2.3 Batch Algal Bioreactor for Removal of Nitrogenous Organic Compounds Detected in HTL-WW

A set of three batch algal bioreactors was operated in parallel to investigate the removal of NOCs by via algal uptake of NOCs into their cells. A pure stock culture of *C. protothecoides* was used to inoculate the reactors. Reactor 1 was spiked with NOCs at concentrations that did not express any inhibition effect based on our previous inhibition experiment data (Chapter 4). Reactor 2 also contained the same compounds at the same concentration as reactor 1 but was not inoculated with algae. This reactor was setup to evaluate reduction of tested compounds due to volatility. Reactor 3 contained only medium and algae and used as a negative control of the experiment. Samples from these reactors were collected on daily basis for 2 weeks and subjected to liquid-liquid extraction prior to GCMS analysis. A set of three batch algal bioreactors was operated in parallel to investigate the removal of NOCs by via algal uptake of NOCs into their cells. Reactor 1 was spiked with NOCs at concentrations that did not express any inhibition effect based on our previous inhibition experiment data (Chapter 4). Reactor 2 also contained the

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6.2.4 Batch Algal Bioreactor for Removal of Hydrophobic and Hydrophilic Dissolved Organic Nitrogen in HTL-WW

Batch algal bioreactors were operated to access the potential of different DON fractions to simulate algal growth. Algal mixture collected from Urbana Champaign Sanitary District (UCSD) was used to inoculate into the reactors. Because bacteria often enhance the uptake of DON, a bacterial inoculum was added into each reactor. Specifically, about 1L aliquot of mixed liquor collected from UCSD was filtered through a 1 µm glass fiber filter to remove large particles and then through a 0.2 µm filter. Particle collected on the 0.2 µm filter were resuspended in 100 mL of 0.2 µm filtered UCSD wastewater effluent as a site specific bacterial inoculum.

To start a batch reactor, HTL-WW hydrophobic and hydrophilic fractions were first diluted in BBM medium to a concentration of 0.5%. Then, 250 mL of each diluted fraction was added to 500 mL sterilized Erlenmeyer flasks, adjusted to pH 7.0 by drop wise addition of 1M HCl or NaOH solution. Then, 3 mL of algal inoculum and 2 mL of bacterial inoculum were added to each reactor. Algal bioreactors were incubated on a shaker at 25±2°C under continuous illumination. Each fraction was tested in duplicate, along with a negative control that contains deionized water. Growth of algae was monitored by measuring the fluorescent intensity with a Tecan Infinite 200 reader. The spectrophotometer was set to measure fluorescent intensity at an

excitation wavelength of 488 nm and an emission wavelength of 680 nm. The absorbency measured at 680 nm and 750 demonstrated the growth of both algae and bacteria in the reactors. 5mL of sample was collected daily for measurement of total nitrogen (TN), inorganic nitrogen (IN), and COD. TN and COD analyses were conducted following standard methods described in Chapter 5 (Materials and Methods). IN was measured using titanium trichloride reduction method (HACH Method 10021).

6.2.5 Semi-batch Algal Bioreactors for Mitigation of HTL-WW Cytotoxicity

A pure culture of *C. protothecoides* was grown in HTL-WW diluted in municipal wastewater from primary effluent (UCSD). We started with a diluted HTL-WW, approximately 1%, and periodically added a certain amount of HTL-WW to gradually build resistance to HTL-WW over time. The growth of algae was monitored spectrophotometrically at 680 nm. Wastewater quality was examined by measuring chemical oxygen demand (COD) with APHA's standard method (APHA, 1995). We stopped adding HTL-WW into the reactor once we observed stagnant algae growth. Algae treated HTL-WW was then be extracted for organic compounds and tested for toxicity using methods described above.

6.2.6 Adsorptive Treatment with Granular Activated Carbon (GAC).

Various amounts of virgin GAC made from bituminous coal (Calgon F-400), were packed into a 2.5 cm OD glass chromatography column to provide performance data for two different empty bed contact times (EBCT) of 5 and 20 min. The GAC layer was packed in the middle of two layers of sand to ensure a uniform flow distribution. Algae treated HTL-WW was fed into the GAC column at a flow rate of 1.0 mL/min using a high performance liquid chromatography pump. The effluents from GAC treatment were collected, and organic compounds were extracted for toxicity analyses.

6.2.7 Catalytic Hydrothermal Gasification of HTL-WW

CHG of HTL-WW was conducted using a 300 mL stainless steel batch HTL reactor (Parr Instrument Co., Moline, IL). The reactor was loaded with 100 mL of HTL-WW and 22.6 g of Raney-Nikel catalyst, sealed, and then purged 3 times with pure nitrogen. The initial pressure was set at 150 PSI to prevent water from boiling during the experiment. Finally, the reactor was heated to the desired reaction temperature of 350°C and the reaction temperature was maintained for a reaction time of 60 min. Subsequently, the reactor was rapidly cooled and the gaseous product was collected into the Tedlar® gas sampling bag for later GC analysis. HTL aqueous product was collected, filtered through 0.2 µm filtered and used for water quality analysis and cytotoxicity assays.

6.2.8 Gas Product Molecules Identification Using GC Analysis

Gas sampled collected after the CHG was analyzed Varian CP-3800 Gas chromatography equipped with an Alltech Hayesep D 100/120 column and a thermal conductivity detector (TCD). The carrier gas was helium with a flow rate at 30 ml/min. The temperature of injection and detection were both 120°C. A standard gas with a known composition of hydrogen, nitrogen, oxygen, carbon monoxide, methane, and carbon dioxide were used to quantify these gasses, which account for most of the gaseous product.

6.2.9 Extraction of Organic Compounds and CHO Chronic Cytotoxicity Assays

Organic compounds in HTL-WW and municipal wastewater samples were extracted on XAD-2 and XAD-8 resins (Richardson, 2011). Details on extraction procedure can be found in Chapter 4 (Materials and Methods). The organic extracts were tested for cytotoxicity effect using the CHO chronic cytotoxicity assays. Details on this assay can be found in Chapter 4 (Materials and Methods).

6.3 RESULTS AND DISCUSSIONS

6.3.1 Removal of NOCs via Batch-algal Bioreactor

Figure 6.1 compares algal growth of *C. protothecoides* in reactor 1 (BBM and NOCs) and reactor 3 (BBM only). As demonstrated in Figure 6.1, algae grew well in the presence of low NOCs concentrations. However, the density of algae in reactor 1 was much lower than in reactor 3 (negative control). This was expected and further supported our hypothesis of a synergistic effect of the organic compounds in HTL-WW on algal growth.

Figure 6.2 illustrates the removal of nitrogen organic compounds (NOCs) detected in *Spirulina* HTL-WW by a batch algal bioreactor. Of these tested compounds, phenol and 2,6 dimethyl-3-pyridinol showed the highest removal after 1 day of algal treatment (98.8% and 100%, respectively). No removal of ε-caprolactam was observed after 1 day of algal treatment. However, all tested compound achieved nearly 100% percent removal after 7 days of treatment. Complete removal of all tested NOCs to below detection was obtained with algal bioreactor treatment after 14 days of treatment. Based on the difference in removal rates for the various tested NOCs, we hypothesize that some NOCs such as phenol, 1-methyl-2-pyrrolidinone, and 2,2,6,6-tetramethyl-4-piperidinone are more favorable for algal uptake than others. Once these compounds have been degraded, algae would utilize the rest of the NOCs available in the medium. These data also in agreement with the data obtained for algae growth inhibition of nitrogen compounds presented in a previous chapter, where we found that phenol, 1-methyl-2 pyrrolidinone, 2-pyrrolidinone, and 2,2,6,6 tetramethyl-4- piperidinone are the least toxic NOCs to algae. Looking in to the literature, Scragg found that the initial growth of *Chlorella vulgaris* (*C. vulgaris*) and *Chlorella VT-1* was inhibited to varying degrees by 100-400 mg/L phenol. *Chlorella VT-1* was found to be more tolerant being able to grow albeit slowly in the presence of

400 mg/L phenol whereas this concentration inhibited the growth of *C. vulgaris* (Scragg, 2006). Klekbner and Kosaric also found that phenol can be easily degraded by different algae (*Chlorella* sp., *Scenedesmus obliquus* and *Spirulina maxima)* at a concentration of 1000 mg/L (Klekner & Kosaric, 1992). Mann and Florence reported that presence of 1-methyl-2pyrrolidinone would enhance instead of inhibit growth of *Nitzschia closterium*, a common Great Barrier Reef diatom (Mann & Florence, 1987). Similarly, the OECD Guideline 201 for algal growth inhibition tests suggested that the EC_{50} value of 1-methyl-2-pyrrolidinone is >500 mg/L. In a previous chapter, we showed that most NOCs are not toxic to algal growth at their detected concentration in HTL-WW. The data presented here further confirm that algae can effectively remove NOCs in HTL-WW and use them as nutrients for their growth. All in all, our data showed that algal bioreactor can be an effective and essential component of an integrated wastewater treatment-bioenergy production system. Integration of algal bioreactors into this system allows simultaneous production of HTL feedstock and improvement of HTL-WW quality.

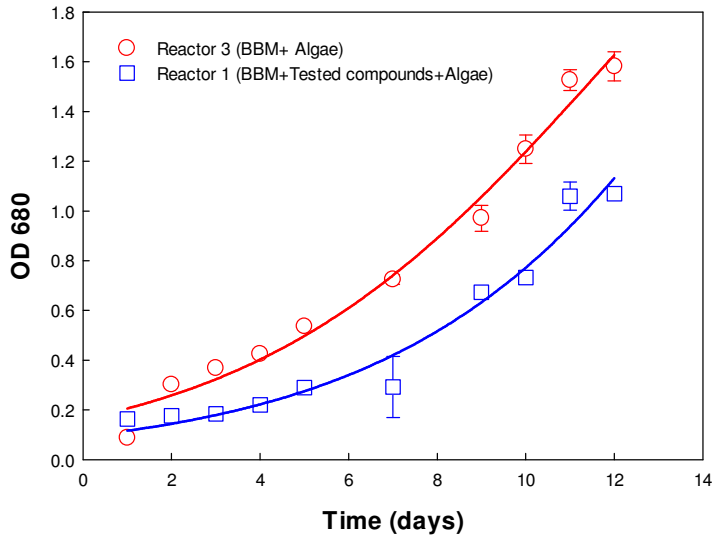

Figure 6.1 Comparison of algal growth in BBM medium (Reactor 3) and BBM medium spiked with nitrogen based compounds (Reactor 1)

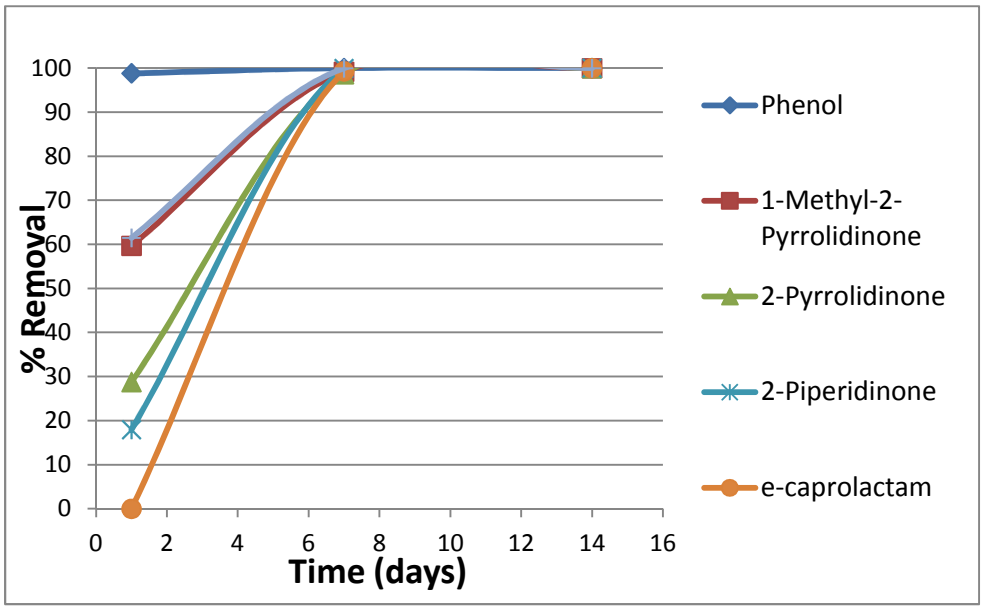

Figure 6.2 Removal of Nitrogen containing compounds by batch-algal bioreactor

6.3.2 Algal Growth in Hydrophobic and Hydrophilic Fractions of HTL-WW

Figure 6.3 compares the growth of algae and biomass in hydrophilic and hydrophobic HTL-WW. Clear differences in the bioavailability of hydrophobic and hydrophilic fractions were evidenced in our algal bioassay. Specifically, algae did not grow in hydrophobic fraction during the first 5 days of operation (Figure 6.3 B) suggesting that the presence of hydrophobic compounds in HTL-WW can inhibit algal growth. Thus, it requires a certain time for algae to adapt with the hydrophobic HTL-WW enhanced medium. From day 6, algae started to grow faster for about a week. Then, algal growth and biomass production decreased, suggesting that the nutrients in growth medium had been completely consumed by the algae and bacteria. Beside the nutrients from BBM medium, the only remaining source of nutrient is from added hydrophobic HTL-WW. However, algae could not effectively utilize this nutrient source to stimulate their growth. That is because hydrophobic compounds are often more recalcitrant to microbial transformation and can persist for long periods in the aquatic environment. On the other hand, we observed stimulated algal growth and biomass production in hydrophilic fraction. Exponentially growth of algae in the hydrophilic fraction during the first 12 days of operation and then the growth remained stable for another week (Figure 6.3 A). Continuous growth of algae and production of biomass in the hydrophilic fraction suggested that the hydrophilic compounds in HTL-WW are more readily utilized by bacteria and algae to stimulate their growth.

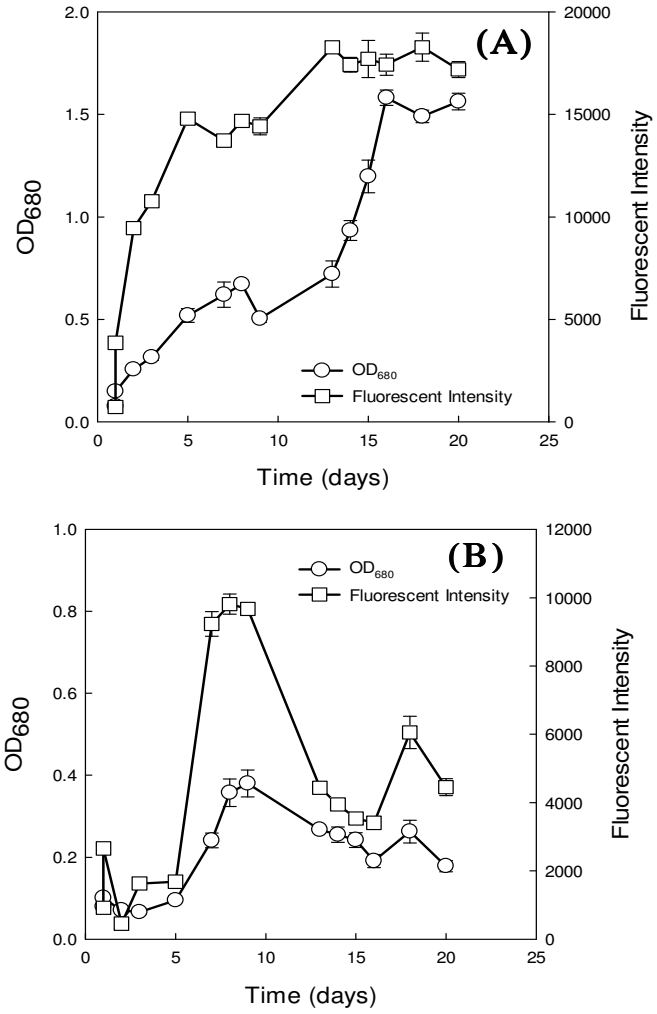

Figure 6.3 Algal growths in hydrophobic and hydrophilic HTL-WW fractions.

6.3.3 Removal of Inorganic and Organic Nitrogen in Fractionated HTL-WW

Figure 6.4 shows the removal of inorganic nitrogen, organic nitrogen, and total nitrogen in hydrophobic and hydrophilic HTL-WW by algal bioreactors. As demonstrated in Figure 6.4 A, total nitrogen was significantly removed in the hydrophilic reactors. Specifically, more than 90% of total nitrogen in hydrophilic HTL-WW was removed after 3 weeks of operation. Removal of total nitrogen accounted for removed inorganic and organic nitrogen in each fraction. Our data showed that inorganic nitrogen was readily utilized by algae and bacteria in

the reactor. Up to 97% of inorganic nitrogen was removed by the time the reactors were stop. Meanwhile, only 82% removal of organic nitrogen was obtained. That is because organic nitrogen is not directly bioavailable to algae. These macromolecules have to be transformed into inorganic nitrogen by bacteria before they can stimulate algae growth (Pehlivanoglu-Mantas & Sedlak, 2006). It is also important to note that organic nitrogen was only utilized by algae by the end of our operation. During the first two weeks of operation, we observed a negative removal of organic nitrogen. This was attributable to organic nitrogen released from dead algae when their cells ruptured. At beginning, nutrients from BBM medium and inorganic nitrogen are more favorable to algae and bacteria. Once the readily available nutrients were depleted, bacteria started to convert organic nitrogen into inorganic elements that can be used by algae. Thus, we only observed removal of organic nitrogen during the last week of our operation.

Significant lower removal of total nitrogen (<20%) was obtained in the hydrophobic fraction (Figure 6.4 B). Specifically, total nitrogen concentration stayed nearly constant during the first week of operation. During the next two weeks, total nitrogen was slowly removed and reached to 16% removal by the time the reactors were stopped. Looking into removal of inorganic and organic nitrogen, we noticed that only 32% of inorganic nitrogen was removed after 3 weeks of operation. No removal was obtained for organic nitrogen in hydrophobic fraction suggesting that hydrophobic organic nitrogen is more recalcitrant than hydrophilic organic nitrogen with respect to microbial transformation. The removal of total nitrogen in hydrophobic fraction was mostly contributed by removal of inorganic nitrogen. These data demonstrated differences in the characteristics of hydrophobic and hydrophilic nitrogen in HTL-WW. Past studies demonstrated that marine dissolved organic nitrogen includes two groups with distinct bioavailability: a biodegradation resistant group mostly made up of high molecular

weight compounds with amide functional group (Aluwihare et al., 2005; McCarthy et al., 1997) and a labile fraction that includes urea, combined with amino acids and nucleic acids (Bronk et al., 2007; Bronk et al., 2010). Like marine dissolved organic nitrogen, HTL-WW organic nitrogen has diverse chemical composition that determines its bioavailability. Research confirms that a large fraction of the dissolved organic nitrogen includes truly recalcitrant components that persist in the environment for months to hundreds of years (Benner, 2002; Bronk et al., 2007). This fact combined with our experimental data suggests that the hydrophobic fraction of HTL-WW contains mostly recalcitrant components. On the other hand, the hydrophilic fraction of HTL-WW contains most of labile materials that can be converted into inorganic forms by biological treatment provided in algal reactors (Krasner et al., 2009; Westgate & Park, 2010). Distinct difference between two classes of dissolved organic nitrogen in HTL-WW also suggests that conventional biological treatment process may not be effectively used to treat HTL-WW. The use of alternative biological treatment system such as membrane biological reactors or employing physical removal process such as reverse osmosis or activated carbon my reduce the concentration of hydrophobic dissolved organic nitrogen (Krasner et al., 2009).

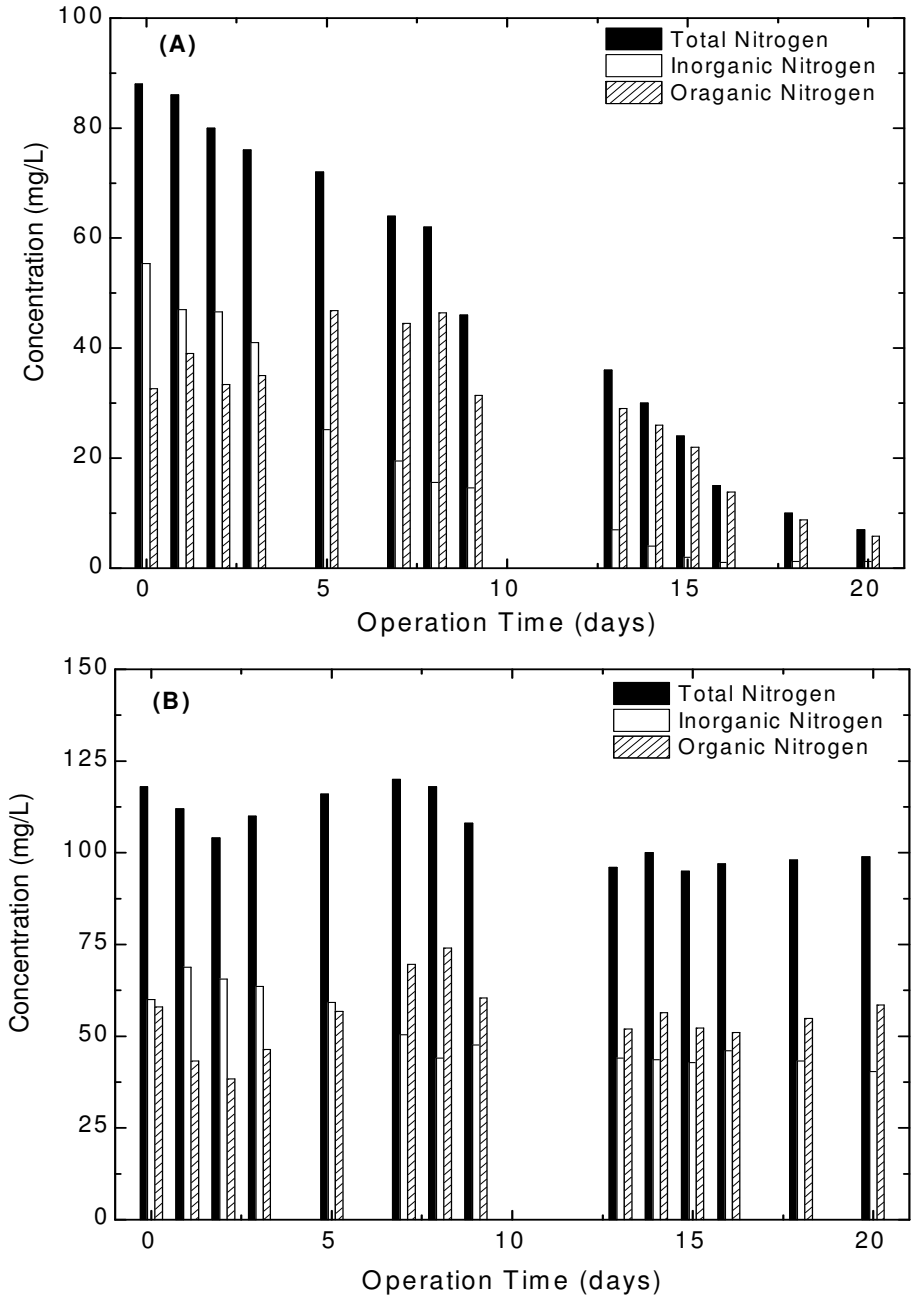

Figure 6.4 Removal of dissolved organic nitrogen, inorganic nitrogen, and total nitrogen in hydrophilic (A) and hydrophobic (B) HTL-WW fractions

6.3.4 Reduction of HTL-WW Cytotoxicity via Sequencing Batch Algal Bioreactor

Figure 6.5 presents data from a semi-batch algal bioreactor treatment of HTL-WW, in which 1% aliquots of HTL-WW were added every few days for 2 weeks. This graph shows continuous growth of algal biomass, quantified as $OD_{680.}$ It also shows stepwise removal of a fraction of the organic compounds, quantified as chemical oxygen demand (COD), after each addition of HTL wastewater. However, there was also an organic fraction that was not biologically assimilated, and thus, the COD level gradually increased over the course of the test. The increase in biomass indicated that algae and bacteria can successfully utilize the organics and nutrients in HTL-WW. These data agree with previous batch studies in our research group showing that algal growth was enhanced by addition of HTL-WW at less than 5% of the growth medium (Zhou, 2010). Other previous research has shown that HTL-WW contains a significant amount of small-molecule breakdown products of biomass macromolecules (Anastasakis & Ross, 2011; Appleford, 2004) and these small molecules are more favorable for algal uptake (Neilson & Lewin, 1974). Our data showed that approximately half of the COD was removed during by algal bioreactor treatment. The algal treated HTL-WW was then subjected to extraction of the remaining organics, which were tested for toxicity with the same CHO test described earlier. As presented in Figure 6.6, the LC_{50} value of algal-treated HTL-WW increased to a concentration factor of 0.113×(11.3% HTL-WW), demonstrated that this treatment removed 40% of the induced toxicity. In this case, algae used some organic compounds in HTL-WW as their carbon source and reduced the toxicity. Algal treated HTL-WW, however, is still very toxic and would likely require further treatment before it can be released into the environment.

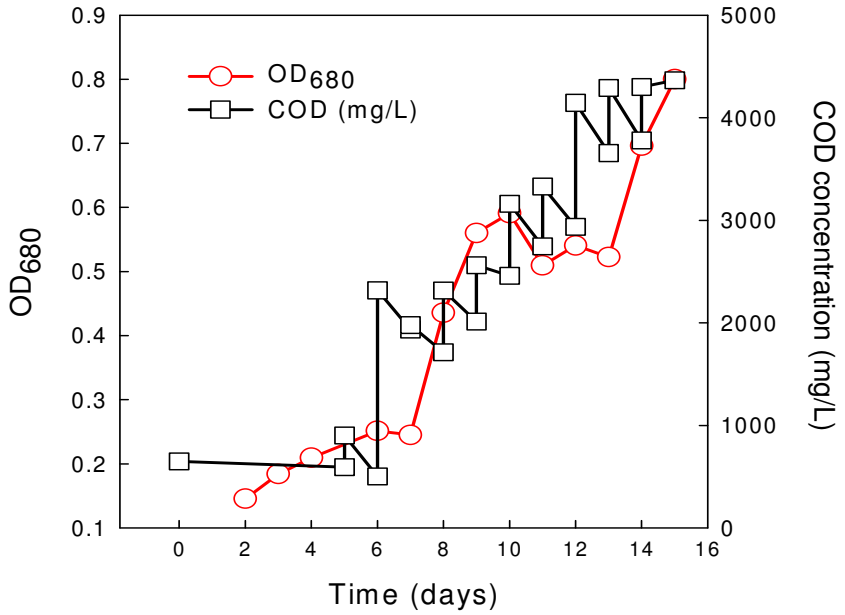

Figure 6.5 Biomass growth and removal of organic pollutants in a semi-batch algal bioreactor fed with routinely with 1-3% HTL-WW from *Spirulina* (10% total HTL-WW).

6.3.5 Reduction of HTL-WW Cytotoxicity via Adsorptive Treatment with GAC

After treatment with an algal bioreactor, HTL-WW was further treated by passing it through a GAC column. Figure 6.6 compares the concentration-response curves for *Spirulina* HTL-WW before and after treatment with algal bioreactor and GAC. As demonstrated in Figure 5.6, the LC_{50} value of GAC treated HTL-WW increased as EBCT increased. Specifically, the LC_{50} value of GAC treated HTL-WW increased from a concentration factor of 0.4×(40% HLT-WW) to 1×(100% HTL-WW) as EBCT increased from 5 min to 20 min, respectively. These data suggest that elimination of HTL-WW toxicity may be achieved by increasing the amount of GAC used per volume of treated HTWW and/or increase the EBCT. Figure 6.7 presents the cytotoxicity index values of HTL-WW before and after algal bioreactor and GAC treatment. As demonstrated in Figure 6.7, the cytotoxicity index value of HTL-WW reduced 30% after algal

bioreactor treatment and 92.5% after subsequent GAC treatment. Further investigation and optimization of GAC system design is needed to provide highest toxicity removal, and determine systems costs.

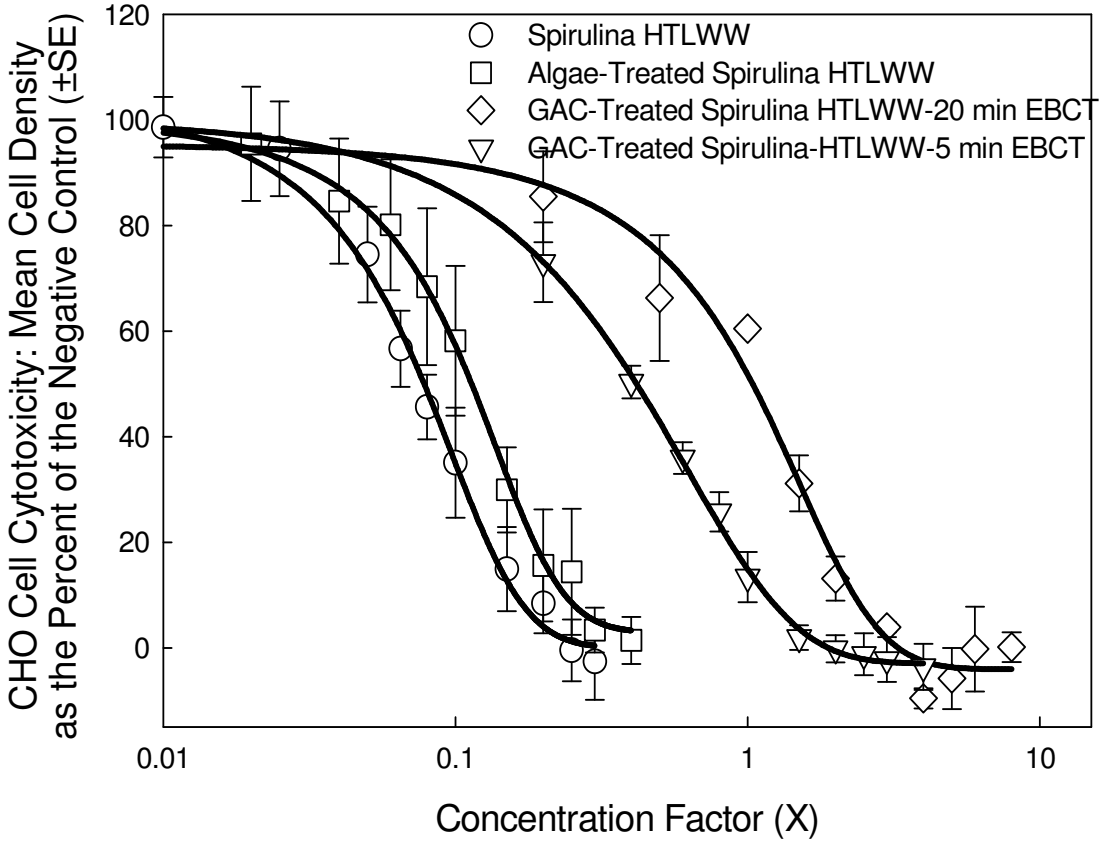

Figure 6.6 CHO cytotoxicity concentration response curves for organic extract from HTL-WW generated from hydrothermal liquefaction of *Spirulina* before and after treatment with algal bioreactor and GAC.

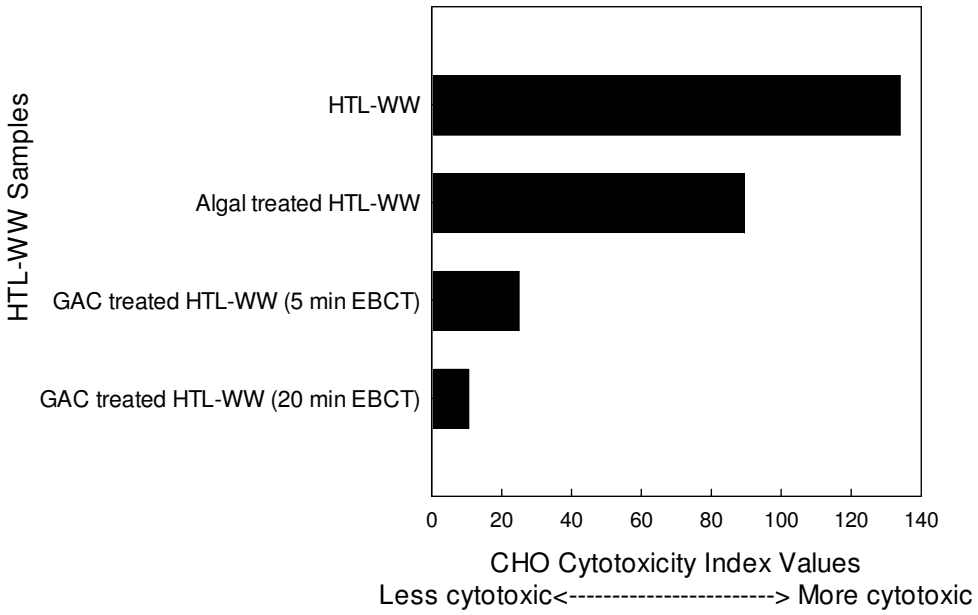

Figure 6.7 Comparison of the CHO cell cytotoxicity index values for HTL-WW before and after treatment with algal bioreactor and GAC. Index values are expressed in dimensionless units.

6.3.6 Reduction of HTL-WW Cytotoxicity via Catalytic Hydrothermal Gasification Treatment

Organic compounds in HTL-WW were successfully gasified into gases. The gas product was 64.7% methane, 4.1% carbon dioxide, and 31.2% hydrogen (by volume). COD concentration of HTL-WW decreased from 88,492 mg/L to 2767 mg/L, corresponding to 96.7% COD in HTL-WW. Figure 6.8 shows the concentration response curves (A) and the cytotoxicity index (B) of HTL-WW pre-and post-CHG treatment. As demonstrated in Figure 6.8, the LC_{50} of HTL-WW increased from 0.75% to 1.2% and the corresponding cytotoxicity indexes of pre- and post-CHG HTL-WW were 1333 and 833 respectively. In other words, CHG can remove 37.5%

of the HTL-WW cytotoxicity (based on LC_{50} values) . The nutrients retained in HTL-WW post-CHG treatment can be recycled to the algal bioreactor to produce additional algal biomass for biocrude production with HTL.

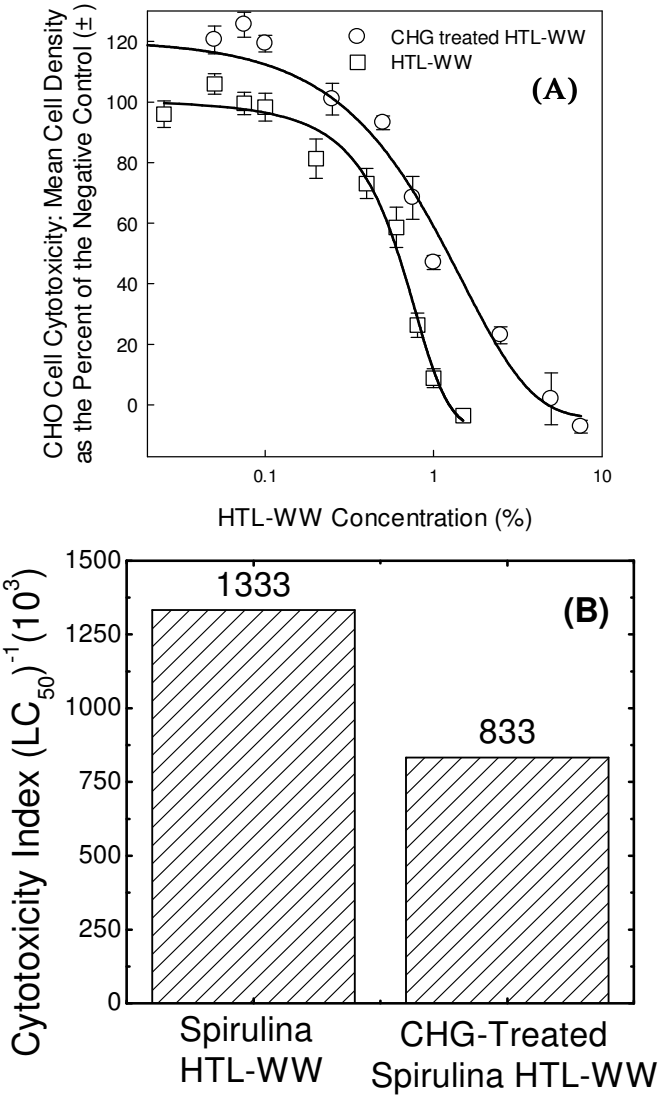

Figure 6.8 Comparison of CHO cell cytotoxicity concentration response curves and CHO cell cytotoxicity index values for HTL-WW before and after catalytic hydrothermal gasification treatment

6.3.7 Comparison of Cytotoxicity of Treated HTL-WW and Municipal Wastewater

Figure 6.9 compares the concentration-response curves for municipal wastewater from different treatment stages and algal-GAC treated HTL-WW. As demonstrated in Figure 6.9, the toxicity of municipal wastewater was gradually reduced after each treatment stage. Specifically, the LC_{50} values of the centrate, raw municipal wastewater, primary effluent and secondary effluent were 1.2×, 2.7×, 3× and 4×, respectively. The 10% HTL-WW is more toxic than the centrate of municipal wastewater with a LC_{50} value of 0.79 × vs. 1.2×. The LC_{50} values of tested samples were converted from concentration factor × to the percent of the sample in medium (%) and used to calculated cytotoxicity indexes. Figure 6.10 presents the cytotoxicity index values calculated based on LC_{50} values as the percent of the sample in medium of treated HTL-WW and municipal wastewater from different treatment stages. As demonstrated in Figure 6.10, the primary treatment of municipal wastewater removed about 10% of wastewater toxicity since this treatment step only removed heavy solids and floating materials. The secondary treatment, which involves removal of dissolved and suspended solids, provided about 32% removal of the wastewater cytotoxicity. The combination of primary and secondary treatment resulted in about 40% removal of wastewater toxicity. Meanwhile, a combination of algal bioreactor and GAC treatment could provide 92.5% removal of HTL-WW toxicity and bring it LC_{50} to 12×, which is much higher than that of secondary effluent from the wastewater treatment plan. These results suggested that the conventional wastewater treatment was not effectively designed to remove cytotoxicity of the wastewater.

One major process component in the E2-Energy approach is a wastewater treatment step that is synergistically integrated with the bioenergy production process. In this step, the wastewater co-product from bioenergy production (HTL-WW) is diluted with the liquid fraction

of the original wastewater and used as the influent for algal cultivation. Our data shows that this influent is significantly more cytotoxic than the influent of a municipal wastewater treatment plant. However, algal uptake of nutrients and organics followed by adsorptive treatment with GAC has effectively improved the chemical and biological quality of this influent. The effluent of E2-Energy system has a much better biological quality than the secondary effluent of conventional wastewater treatment plan with a LC_{50} of 10× vs. 4×. In addition, the algal biomass grown in the wastewater treatment step is then separated from the cleaned water and fed back into the thermochemical conversion reactor to produce more bioenergy products. All in all, this data demonstrates the outperformance of our wastewater-bioenergy production system compared to the conventional wastewater treatment schemes and provides essential evidence to support the feasibility of this system.

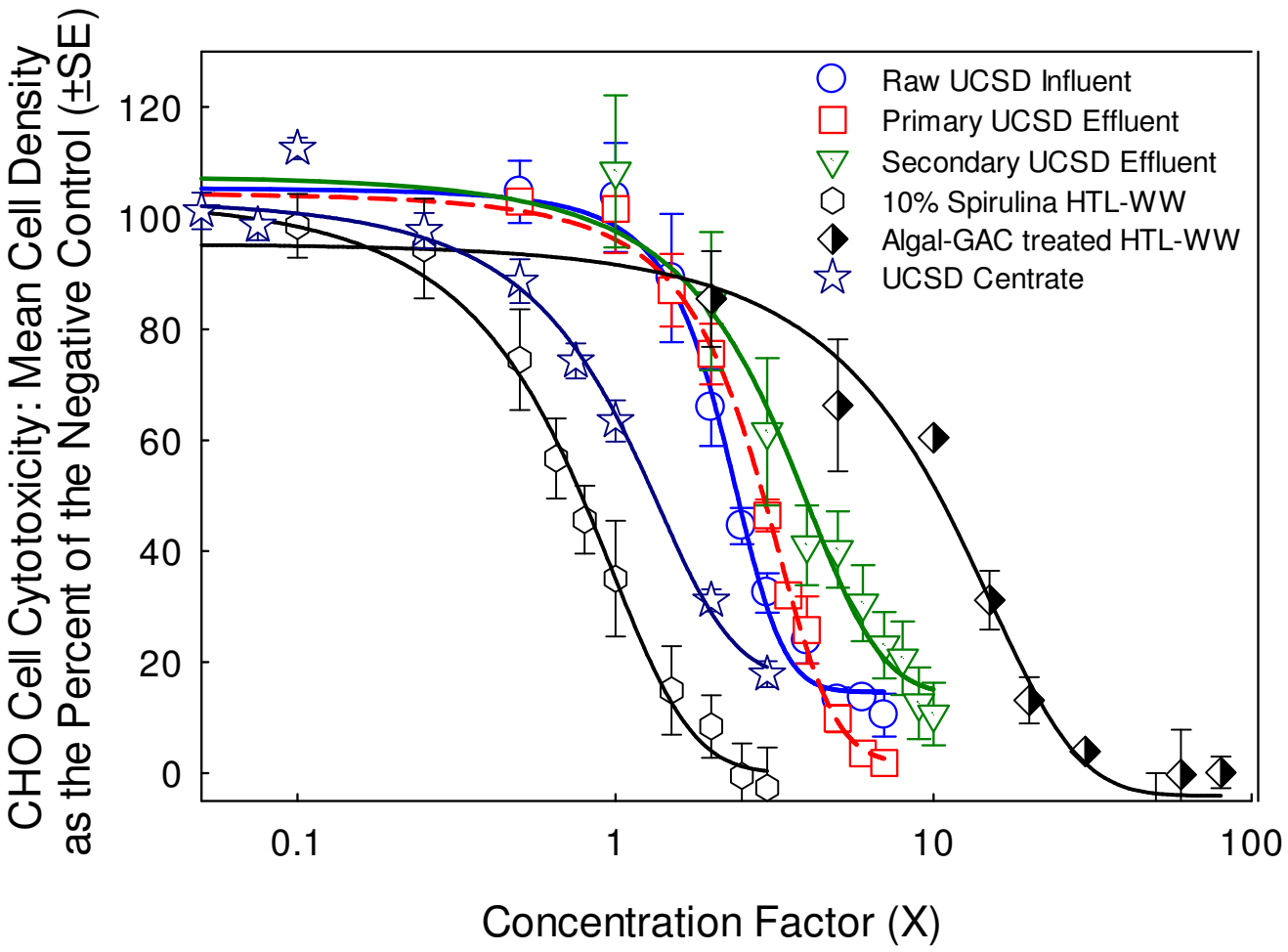

Figure 6.9 Comparison of the CHO cell cytotoxicity index values for HTL-WW before and after treatment with algal bioreactor and GAC and municipal wastewater from different treatment stages. Index values are expressed in dimensionless units.

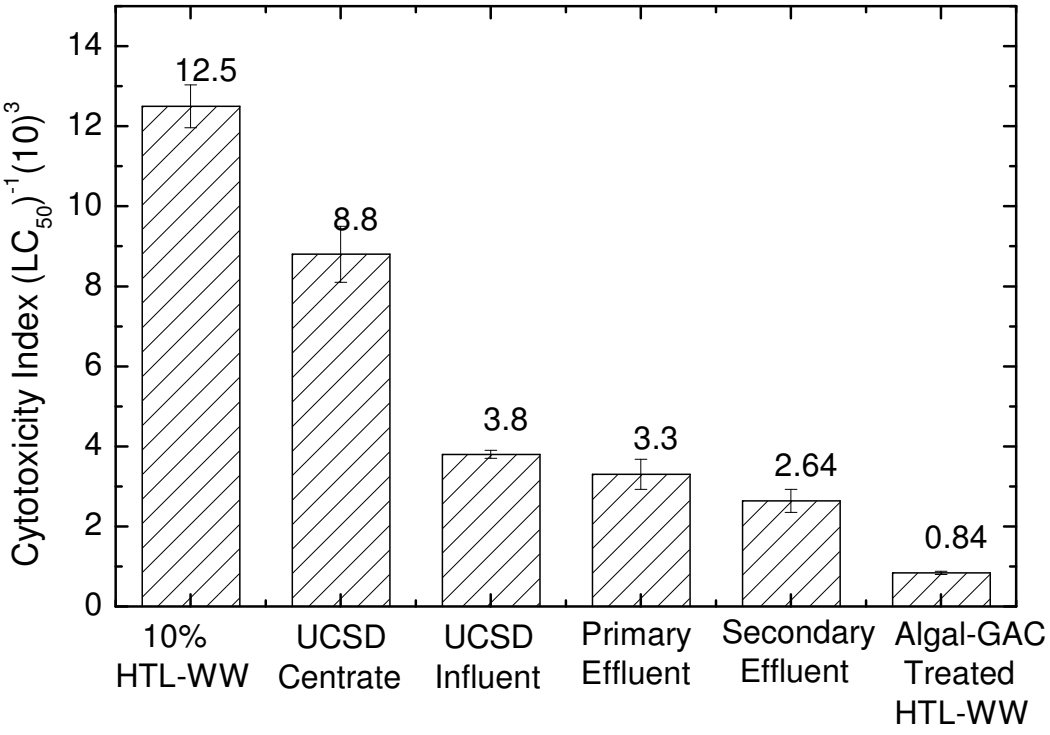

Figure 6.10 Comparison of the CHO cell cytotoxicity index values for HTL-WW before and after treatment with algal bioreactor and GAC and municipal wastewater from different treatment stages. Index values are expressed in dimensionless units.

6.4 CONCLUSIONS

In conclusions, batch-algal bioreactor can effectively remove NOCs and reduce the toxicity of HTL-WW. Completely removal of NOCs was obtained after 7-14 days of operating algal bioreactor. Hydrophobic and hydrophilic fractions of HTL-WW showed a distinct effect on stimulating algal growth. Addition of hydrophilic HTL-WW into growth medium can strongly enhance algal growth as compare to the positive control. Meanwhile, the presence of hydrophobic HTL-WW was found to inhibit algal growth. The percent removals of total nitrogen in hydrophilic and hydrophobic fractions by algal bioreactors were 90% and 20%, respectively.

The distinct difference in performance of algal bioreactors suggested that hydrophobic fraction of HTL-WW contains recalcitrant compounds that are not biodegradable by algae and bacteria. In contrast, hydrophilic fraction contains biodegradable compounds and could be effectively utilized to stimulate the growth of algae.

Treatment of 10% diluted HTL-WW with semi-batch algal bioreactor provided 30% reduction of HTL-WW toxicity of organic extract. Subsequent treatment with GAC provided 92.5% removal of HTL-WW toxicity (data related to LC_{50}). On the other hand, conventional wastewater treatment can only provide 32% removal of the wastewater toxicity. Treatment of the whole strength HTL-WW with CHG removed 37.5% of the HTL-WW. CHG-treated HTL-WW can be fed back to the algal bioreactor for additional removal of toxicity. Effluent from our wastewater- bioenergy production system has a better biological quality than that of conventional wastewater treatment plant. The data also demonstrate the feasibility of our novel integrated bioenergy production and wastewater treatment where HTL-WW was recycled to algal cultivation bioreactors followed with a GAC treatment to provide beneficial use of chemicals in HTL-WW and provide substantial removal of organic toxicants. The biomass from algal bioreactor and the GAC used to treat HTL-WW can be fed back to HTL process to generate additional bio-crude oil. All in all, the studied wastewater bioenergy system offers potential advantages for reducing the potential toxicity risks associated with byproducts of HTL bioenergy production and improving wastewater effluent quality.

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CHAPTER 7: SUMMARY AND FUTURE RESEARCH

7.1 SUMMARY

This study has investigated the impacts of a novel integrated process combining algal wastewater treatment with hydrothermal liquefaction (HTL) on the fate of emerging bioactive contaminants and the potential for wastewater reuse. The feasibility of this novel integrated process was demonstrated and some potential negative impacts of the system on wastewater effluent quality were also identified and addressed with potential post treatments. The integrated process for algal wastewater treatment-bioenergy production (E2-Energy) with proposed improvements is presented in Figure 7.1. In this improved system, we proposed to add a GAC treatment step post- algal bioreactor and/or a CHG treatment pre- algal bioreactor. Addition of these treatments would enhance the growth of algae and improve the chemical and biological quality of the wastewater effluent from our system. The proposed integrated system also offers other advantages that will be discussed in the following sections.

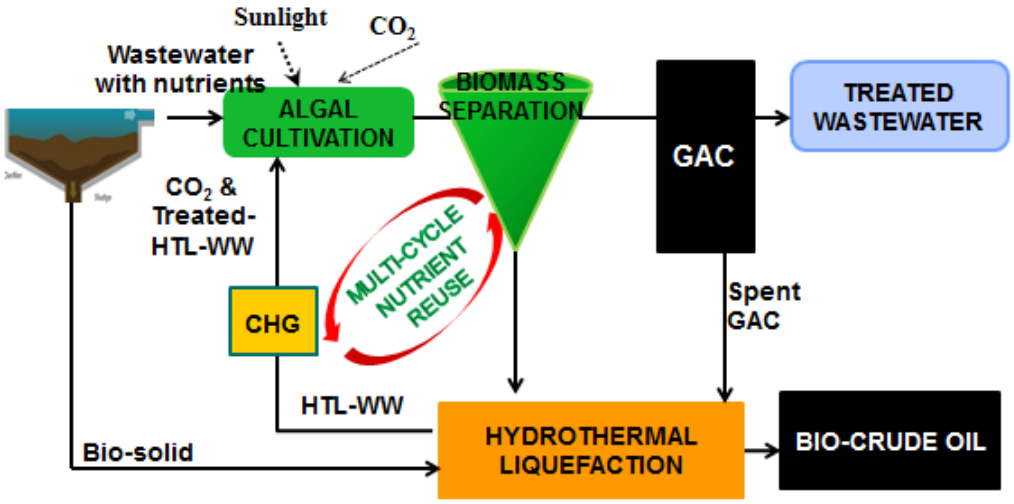

Figure 7.1 Improved integrated process for algal wastewater treatment-bioenergy production, which is referred to as Environment-Enhancing Energy (E2-Energy)

7.1.1 Effect of HTL Operating Condition on Destruction of Bioactive Compounds and Antibiotic Resistant Genes

The current study investigated the impacts of a novel integrated process combining algal wastewater treatment with hydrothermal liquefaction (HTL) on the fate of bioactive contaminants and the potential for wastewater reuse. One important topic of interest is the effect of HTL on the fate and transport of antibiotic resistance genetic material. We confirmed with a variety of experiments that HTL treatment can effectively destroy genetic materials (plasmid DNA) by breaking them up into small, inactive fragments that are not active if transferred to other bacteria. Removal of DNA from the biowastes by HTL treatment was in the range of 95% to 99.8% for all tested HTL operating conditions. At lower operating temperatures (250°C or less), extending the retention time from 15 minutes up to 60 minutes enhanced the destruction of DNA in biowastes. However, at an operating temperature of 300°C and above, the effect of extending retention time was insignificant. Since most HTL treatments would be conducted at a temperature above 250°C or at a retention time of 60 min or longer for optimal oil yield, we expect that genetic materials in the biowastes are expected to be below analytical detection limits. The complex organic matrix of HTL feedstocks did provide some protection for genetic materials in biowastes during HTL treatment. This protective effect, however, could be reduced or eliminated by extending the HTL retention time and/or increasing the operating temperature. Although there was a certain amount of DNA found remaining in the post HTL wastewater, this DNA was completely deactivated, as demonstrated in a variety of transformation experiments. Natural transformation and high efficiency electro-transformation experiment of post-HTL DNA consistently showed 98.6% and 100% reduction in transformation frequencies, respectively, suggesting that the DNA was completely deactivated by HTL treatment. Thus, we conclude that

HTL treatment can effectively deactivate genetic materials in biowastes and prevent the potential of transferring antibiotic resistant materials from biowastes into the environment.

HTL processing of livestock manure can also effectively destruct a broad range of bioactive compounds under practical operating conditions (>250°C and 60 min retention time). Extending HTL reaction time from 15 to 60 minutes provided some additional removal of bioactive compounds when HTL was operated at temperature ≤250°C. However, when HTL was operated at a temperature of 300°C and above, the effect of HTL reaction time on bioactive compounds removal was minimal. The presence of HTL feedstock lowered the removal of bioactive compounds by 5−10%, especially when the HTL was operated at a lower temperature (≤250°C) and shorter retention times (≤15 min). Experimental results also showed essentially complete removal of all tested compounds in presence of *Spirulina* (up to 5% solids content) or swine manure (20% solids content) when HTL was operated at 300°C and ≥30 min reaction time. These HTL operating conditions are also practical HTL operating conditions for providing good oil yield. Thus, HTL process can be successfully utilized to simultaneously produce valuable bio-crude oil while destructing bioactive compounds in animal waste. As a result, health and ecosystem risks associated with bioactive compounds in biowastes can be mitigated via HTL treatment. In addition, the costs associated with antibiotic resistant treatments and the development of new antibiotics could be reduced over the long run.

7.1.2. Chemical and Biological Characterization of HTL Wastewater

We identified 9 specific NOCs found to be in HTL-WW. CHO chronic cytotoxicity assay showed that all 9 tested NOCs in HTL-WW are not cytotoxic to mammalian CHO cells at their detected concentration. Inhibition of algal growth was also measured for 6 of these compounds. Of all tested NOCs, 3-dimethylamino phenol and 2,6-dimethyl-3-pyridiniol caused 50% algal

growth inhibition at their detected concentration in HTL-WW. It is noteworthy that NOCs with methyl groups (3-dimethylamino phenol, 2,2,6,6, tetramethyl-4 piperidinone, and 2,6-dimethyl-3-pyrrolidinol) were more toxic to mammalian cells and caused more algal inhibition than those without them. Comparison of LC_{50} and I_{50} values of the same NOCs indicated that LC_{50} was consistently higher than I_{50} values, suggesting that algae are more sensitive to NOCs than mammalian cells.

Although the detected NOCs showed minimal mammalian toxicity effects, the full organic mixture extracted from HTL-WW, was found to be highly toxic to mammalian cells. At a concentration factor of only 0.075× (i.e., 7.5% HTL-WW), a 50% reduction in CHO cell density was observed. Similarly, algal growth would be significantly inhibited by the addition of HTL-WW at more than 5% of the growth medium. Since organic compounds in HTL feedstocks and HTL-WW exist as a complex mixture, there is significant potential for synergistic and antagonistic effects of multiple compounds on the toxicity or growth inhibition with various living organisms. Thus, it is important to ensure that HTL-WW is treated before releasing it into the environment.

7.1.3 Effects of HTL Operating Condition of HTL-WW Quality

HTL operating conditions can strongly affect the chemical and biological characteristics of HTL-WW. The initial solids concentration of the feedstock has the most dominant effect on the water quality parameters in HTL-WW. The higher the solids concentration, the more nutrients, organics and solid residues were found in HTL-WW. Meanwhile, reaction time and reaction temperature did not show a consistent effect on concentration of nutrients and organics (COD) HTL-WW.

The cytotoxicity of HTL-WW was also strongly affected by HTL operating conditions. Of the three tested operating parameters, solids concentration showed a strong effect on cytotoxicity of HTL-WW. Increasing solids concentration cause increase in HTL-WW toxicity. Once the maximum cytotoxicity has reached, which happened when 25% solids concentration was used, further increase in solids concentration did not affect the toxicity of HTL-WW. Increase in reaction temperature generally enhances HTL-WW toxicity and oil yield. On the other hand, prolonged reaction time would reduce the toxicity of HTL-WW. There is a moderate association between HTL-WW cytotoxicity and the concentration of COD in HTL-WW. We also noticed that operating condition that provides high bio-oil yield usually results in more cytotoxic HTL-WW. For the purpose of optimizing HTL process for maximal oil yield and less cytotoxic wastewater, HTL of C.*pyrenoidosa* should be conducted at 280°C for 60 min using 35% solids concentration. Under this condition, we obtained highest oil yield (43%) and lower cytotoxicity index. It is important to note that this condition is specific for *C.pyrenoidosa* as the element composition of the feedstock would strongly affect bio-oil yield and quality of HTL-WW.

7.1.4 Removal of Nitrogen from Hydrophobic and Hydrophilic Fractions of HTL-WW by Algal Bioreactors

Hydrophobic and hydrophilic HTL-WW fractions show a distinct behavior when subjected to algal bioreactors. More than 90% of total nitrogen in hydrophilic HTL-WW was removed after 3 weeks of operation. Inorganic nitrogen was better removed by algal reactors than organic nitrogen. Up to 97% of inorganic nitrogen was removed by the time the reactors were stop. Meanwhile, only 82% removal of organic nitrogen was obtained. Significant lower removal of total nitrogen (<20%) was obtained in the hydrophobic HTL-WW fraction. The removal of total nitrogen in hydrophobic fraction was mostly contributed by removal of

inorganic nitrogen. No removal was obtained for organic nitrogen in hydrophobic fraction suggesting that hydrophobic organic nitrogen is more recalcitrant than hydrophilic organic nitrogen with respect to microbial transformation. Distinct difference in bioavailability of dissolved organic nitrogen in hydrophobic and hydrophilic HTL-WW fractions suggests that conventional biological treatment processes may not effectively be used for treatment of HTL-WW. Alternative biological treatment system such as membrane biological reactors or employing physical removal process such as reverse osmosis or biological activated carbon may be better options for removing recalcitrant dissolved organic nitrogen in HTL-WW.

7.1.5 Mitigation of HTL-WW Cytotoxicity

The feasibility of using batch and semi-batch algal bioreactors to removed remove NOCs and reduce the toxicity of HTL-WW was demonstrated in several experiments. More than 99.6 % removal of NOCs was obtained after 7 days of operating algal bioreactor. 30% reduction of HTL-WW toxicity of organic extract was obtained with algal bioreactor treatment. Subsequent treatment with GAC provided 92.5% removal of HTL-WW toxicity (based on LC_{50} values). These data show the benefits of recycling HTL-WW back to algal cultivation bioreactors in the novel integrated process for bioenergy production and wastewater treatment that was investigated in this study. However, it also highlights that significant toxicity remains after algal bioreactor treatment and that GAC treatment and/or other treatment methods are needed to provide further removal of organic toxicants resulting from HTL treatment of wastes. The biomass from an algal bioreactor and the GAC used to treat HTL-WW can both be fed back to HTL process to generate additional bio-crude oil. Treatment of the full strength HTL-WW with CHG provided 96.7% removal of COD and 37.5% of cytotoxicity in HTL-WW. CHG-treated

HTL-WW can also be recycle to algal bioreactor for additional removal of cytotoxicity and production of algal biomass.

All in all, this study enhanced our knowledge and capabilities for producing bioenergy from wastewater. The HTL process can effectively convert biowastes into valuable bio-crude oil while destructing emerging contaminants such as pharmaceuticals and antibiotic resistant genes. As a result, the quality of wastewater effluents can be improved for human health benefits and reduced ecosystem risks. However, the HTL process also generates some toxic and inhibitory compounds that are not originally present in the feedstock. We have demonstrated that these deleterious compounds in HTL wastewater can be taken up in part by recycling water to algal bioreactors, which is integrated into the novel treatment process proposed in this study. Further removal of toxic compounds can be provided by GAC adsorptive treatment of HTL wastewater. Our proposed integrated system has the potential to simultaneously improve the quality of wastewater and significantly expand bioenergy production from wastes. Information provided by this research is useful for managing HTL processes to make them more effective for both producing bio-crude oil and increasing environmental benefits.

7.2 FUTURE RESEARCH

7.2.1 Characterizing the Effect of Thermochemical Bioenergy Production Processes on the Fate of Bioactive CECs

The present study showed that significant removal of CECs was achieved by HTL treatment, but the number of operating conditions was limited and the focus was on processing settleable manure solids. In the future, we would like to expand on our recent work to study more operating conditions as well as comparing the relative performance of HTL and other bioenergy

conversion processes such as catalytic hydrothermal gasification (CHG). Specifically, we can expand our current range of operating conditions to cover least 18 different combinations of temperature (range 200–350°C) and reaction time (range 5–60 min). Relative performance of HTL and CHG on removing CECs under these operating conditions can also be compared. Past work has demonstrated the ability of CHG to convert various organic compounds into a biogas with methane, hydrogen and carbon dioxide. For instance, 99.9% COD reduction with CHG of olive processing wash water using a Raney nickel catalyst (350°C, 21 MPa) has been achieved (Elliott and Sealock, 1996). Hydrothermal gasification processes have been shown to degrade 90−100% of specific organic compounds such as phenol, analine, tetrahydrofuran, toluene, and cyclohexanol using a Raney nickel catalyst or a Sn-modified Raney nickel catalyst (Li et al., 2008). However, no work has been reported to date with the potentially problematic CECs that are common in manure liquids. Thus, it is desirable to have better understanding on the effect of HTL and CHG operating conditions on the fate of CECs. Sequential conversion of biowaste feedstock by HTL first and CHG second is also an attractive combination for bioenergy production and removal of CECs from biowastes that requires further investigation. While HTL provides a more valuable bioenergy product (oil), the CHG can harvest residual aqueous organics from HTL into a useful biogas and reduce the load going to the wastewater treatment process.

7.2.2 Identification of Breakdown Bioactive Compounds Post HTL Treatment

The current study has shown that thermochemical waste-to-energy can effectively remove or deactivate bioactive CECs in manure under a practical operating condition. The major mechanism for removing CECs by HTL is breaking them into compounds of smaller molecular weight. We also confirmed with variety of experiments that antibiotic resistant genes in animal

manure could be completely deactivated with HTL treatment. However, the number of tested bioactive compounds is limited to only two antibiotics and two estrogens. In addition, the breakdown products of tested bioactive compounds after HTL treatment have not been identified. Therefore, it is desirable to expand the list of bioactive compounds as well as identify breakdown products of these compounds after HTL treatment. Even though substantial removal of all tested bioactive compounds was achieved under practical HTL operating conditions, the bioactivity of their breakdown products has not been verified. Different bioassays can also be conducted to verify the activity of breakdown CECs after HTL treatment. The activity of HTL treated antibiotics can be evaluated by exposing these compounds to environmental bacteria such as *E. coli* to see if they still exert selective pressures on microbial communities. For estrogenic compounds, we can compare their estrogenic activity pre- and post-HTL treatment using an *in vitro* proliferation assay with estrogen receptor-positive human breast cancer MCF-7 cells. This data will provide supporting information to confirm that our waste to energy system can simultaneously provide significant environmental benefits and valuable bioenergy products.

7.2.3 Optimization of Granular Activated Carbon Treatment

The current GAC treatment setup has not been optimized and only provided 20 min EBCT for the HTL-WW and 92.5% removal of HTL toxicity. Our aim is to design a practical treatment scheme that would provide complete removal of HTL-WW toxicity before it is released into the environment. Thus, further investigations in optimization of GAC system design and operating condition is needed to provide highest toxicity removal at a reasonable cost. Our preliminary results suggest that complete removal of HTL-WW toxicity would be obtained if we increased the amount of GAC used/volume of HTL wastewater or extended the retention time by adjusting the flow rate of HTL-WW passing through the GAC column. A series of GAC

column tests where same volume of HTL-WW will be treated with different amount of GAC (20g, 40g, and 50 g) and a fixed EBCT should be conducted to optimize the design of GAC column. The effect of EBCT on toxicity removal by fixing the amount of GAC used to treat HTL-WW should also be investigated. Variation of EBCT can be obtained by adjusting the flow rate of HTL-WW passing through GAC column. The ultimate goal is to balance between amount of GAC and EBCT and provide highest toxicity removal, and determine systems costs.

7.2.4 Fractionation of HTL-WW

The current work had fractionated the hydrophobic portion of HTL-WW into hydrophobic basic, hydrophobic acidic and hydrophobic neutral fractions and evaluated the cytotoxicity of these fractions. It would be interesting to fractionate the hydrophilic portion of HTL-WW into acidic, basic and neutral fractions and perform the cytotoxicity tests on these fractions as well. The complete cytotoxicity data set of fractionated HTL-WW will provide useful information for future researches in chemical characterization of HTL-WW.

7.2.5 Development of a Predictive Model for HTL-WW Cytotoxicity

The current work collected a good cytotoxicity data set for developing a mathematic model that could be used to predict the toxicity of HTL-WW based on the process operating parameter. This model can be used to determine the dilution factor of HTL-WW in municipal wastewater for algal cultivation purpose or for environmental discharge. Validity of the model can be verified by comparing experimental and predicted values of HTL-WW. Similar model can also be developed for optimization of bio-crude oil yield. A combination of these two model will allow us to identify HTL operating conditions that can provide reasonable bio-crude oil yield and low cytotoxic HTL-WW.

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