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LIST OF ABBREVIATIONS

AMDIS	Automated Mass Spectral Deconvolution and Identification System
B medium	Burk's Medium
BBM	Bold's Basal Medium
BC	Burk's Medium plus Carbenecillin
BPA	Bisphenol A
^{14}C	Carbon-14 Isotope
CARB	Carbenicillin
CARB-R	Carbenicillin Resistant
CAFO	Concentrated Animal Feeding Operations
CF	Ceftiofur
CHO	Chinese Hamster Ovary
CLSC	Continuous Liquid Scintillation Counting
CO_2	Carbon Dioxide
COD	Chemical Oxygen Demand
Da	Daltons
DCM	Dichloromethane
DMSO	Demethylsulfoxide
DNA	Deoxyribonucleic Acid
EBCT	Empty Bed Contact Time
E1	Estrone
E2	Estradiol
EC_{50}	Half Maximal Effective Concentration
FBS	Fetal Bovine Serum
FF	Florfenicol
GAC	Granular Activated Carbon
GC	Gas Chromatography
GC/MS	Gas Chromatography/Mass Spectometry
GC/NPD	Gas Chromatogram with Nitrogen-Phosphorus Detector
HPLC	High Performance Liquid Chromatography
HPLC/PDA	High Performance Liquid Chromatography with Photodiode Array Detector
HTL	Hydrothermal Liquefaction
HTL-WW	Hydrothermal Liquefaction Wastewater
IC_{50}	Half Maximal Inhibitory Concentration
ISTC	Illinois Sustainable Technology Center
LB	Luria-Bertani
LC_{50}	Half Maximal Lethal Concentration
MS	Mass Spectrometry or Mass Spectrometer
NIST	National Institute of Standards and Technology
NOC	Nitrogenous Organic Compounds
NREL	National Renewable Energy Laboratory

NSF	National Science Foundation
OD_{680}	Optical Density at 680 nm
OECD	Organization for Economic Cooperation and Development
PDA	Photodiode Array
SIR	Single Ion Recording
WW	Wastewater

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 (e.g., pharmaceuticals, estrogenic compounds, antibiotic-resistance genes, 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) with a photodiode array (PDA) detector was used to quantify emerging contaminants (florfenicol, ceftiofur, and estrone) before and after HTL treatment showed the removal of tested bioactive compounds to below detection limits when HTL was operated at 250°C for 60 min or at 300°C for ≥ 15 min. Complete breakdown or inactivation of antibiotic-resistance 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 feedstocks such as swine manure or *Spirulina* algae reduced the removal of bioactive compounds and plasmid DNA when HTL was operated at 250°C for a short retention time (15 min). However, this effect was minimal when HTL was operated at 250°C for 60 min or at 300°C for ≥ 15 min.

Detailed analysis of the aqueous product of HTL, also called HTL wastewater (HTL-WW), showed the occurrence of hundreds of nitrogenous organic compounds (NOCs). Reference materials for nine of the most significant NOC peaks were obtained and used to positively identify and quantify their concentrations. The chronic cytotoxicity effects of these NOCs were evaluated using a Chinese hamster ovary (CHO) cell assay, and found that the rank order for chronic cytotoxicity of these NOCs was 3-dimethylaminophenol > 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 concentrations found in 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 a 50% reduction in CHO cell density.

Further testing showed three out of eight tested NOCs could cause 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 NOCs after seven days of operation and 40% of the CHO chronic toxicity. We also found that over 90% of the CHO toxicity could be eliminated by filtering with granular activated carbon (GAC) after algal bioreactor treatment. These post-treatments of HTL-WW synergistically integrate with HTL bioenergy production because both the GAC and the algal biomass from the bioreactor can potentially be fed back into HTL to generate additional biocrude oil, which facilitates beneficial reuse of the nutrient content of HTL-WW. All in all, this novel treatment approach offers significant advantages for reducing the potential toxicity risks associated with byproducts of

HTL bioenergy production and for improving wastewater effluent quality for subsequent water reuse applications.

CHAPTER 1: INTRODUCTION

1.1 Background

Harvesting bioenergy from wastes has been receiving greater attention as a sustainable, secure, and cost-effective domestic energy source, including favorable discussion in various reports by the Department of Energy (Sheehan et al., 1998) and the National Science Foundation (NSF, 2008). Previous work has proposed and studied a highly advantageous integrated wastewater treatment scheme that maximizes biofuel yield and improves water quality to support water reuse applications (Yu et al., 2011a; Yu et al., 2011b; Zhou et al., 2011). This novel approach combines algal bioreactor treatment of various wet biowastes with thermochemical (heat and pressure) treatment of biosolids to produce a valuable biocrude oil product, as well as co-product sidestreams of gas, biochar, and a wastewater, which is heavy-laden with organic compounds. As shown in Figure 1.1, the process diagram begins with a wet biowaste, such as animal manure or domestic wastewater, which is then separated into a dilute liquid fraction and a concentrated biosolids fraction. The concentrated biosolids fraction (70-85% moisture content) is treated using hydrothermal liquefaction (HTL), which produces two potentially valuable products, biocrude oil and biochar. The HTL process also generates a gas product rich in carbon dioxide (CO_2) (> 98%) and an aqueous product 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 and bacterial biomass while cleaning the water. Finally, the algal biomass is separated from the treated water and routed back to the HTL process, where it is converted into more biocrude oil. Our previous research has demonstrated oil conversion efficiencies of 30-75% for algae and biowaste feedstocks as well as a positive energy yield of 3-10 times the input of heat energy (Vardon et al., 2011; Yu et al., 2011a; Zhou et al., 2010). Equally important, our previous work has shown that when the algal biomass undergoes liquefaction, it releases most of the nutrients (50-90%) to the HTL-WW, so multiple cycles of algae growth can occur on the influent wastewater nutrients (Biller et al., 2012; Yu et al., 2011a; Zhou et al., 2011). Thus, this process can multiply the biosolids and the biofuel harvested from the waste treatment process by up to 10 times, which makes the oil potential from biowaste and algae enormous. For example, the municipal and agricultural sectors in the United States produce about 0.2 billion tons per year of organic biowaste solids (ASABE, 2005; EPA, 2008), which could be used to grow up to 2 billion tons per year of additional algal biomass by reusing the waste nutrients as described above. With a typical HTL conversion efficiency of 50%, this process can potentially produce enough biocrude oil to meet the entire US oil demand (1.1 billion tons per year). Meanwhile, the nutrient removal that occurs in this novel process can provide additional treatment not provided by most conventional US wastewater treatment plants, which would generally enhance environmental quality and water reuse potential.

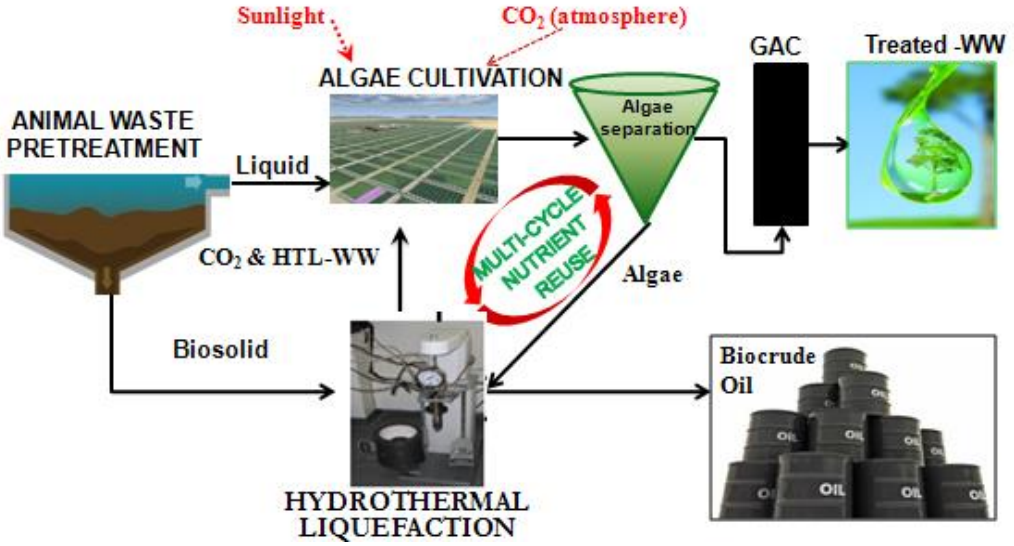


Figure 1.1 Novel integrated process for algal wastewater treatment and bioenergy production.

In this study, we extended our previous research and looked at the effect of the HTL process on emerging contaminants such as pharmaceuticals, steroids, surfactants, plasticizers, and other chemicals that can affect the water reuse potential and that have been detected in wastewater discharges from various human and livestock sources (Brooks and McLaughlin, 2009; Chee-Sanford et al., 2009; Li and Randak, 2009; Watanabe et al., 2008). According to the Union of Concerned Scientists, about 87% of antibiotics (11.2 million kg) used in the United States are administered to livestock as growth promoters, and only 13% (1.4 million kg) are used for human therapeutic and nontherapeutic use (Mellon et al., 2001). It is also important to note that a significant fraction (up to 75%) of administered antibiotics will be excreted in an unaltered state (Elmund et al., 1971). Once these residual antibiotics are released into the environment, they can exert selective pressures on microbial communities and foster the development of antibiotic resistance as a defense mechanism. Evidence of antibiotic resistance associated with antimicrobial chemicals has been emerging over the past decades. For example, the proportion of *Salmonella* isolates exhibiting antibiotic and multiple drug resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline increased from 39% to 97% between 1980 and 1990 (Angulo, 1997; Lee et al., 1994). Chee-Sanford et al. (2001) also reported the detection of tetracycline-resistance genes in swine housing at a concentrated animal feeding operation (CAFO) in the manure lagoon serving that CAFO and in groundwater 250 m downstream of the lagoon. Zahn et al. (2001) observed a three-fold higher concentration of tylosin-resistant bacteria in the exhaust air from CAFOs using medicated feed compared with those using non-medicated feed.

Florfenicol (FF), a synthetic fluorinated chloramphenicol derivate, and ceftiofur (CF), a semi-synthetic β-lactam, are broad-spectrum antimicrobial agents that have been commonly used to treat respiratory diseases in cattle and other animals. After they are administered, only a small

portion of these agents is metabolized, and the majority is excreted in the urine and feces of various animal species. For example, Gilbertson et al. (1995) found that more than 60% of the dose of administrated CF is excreted in urine, and around 10% of the dose was found in feces. These results suggest that land application of agricultural wastes would be a major source of unmetabolized antibiotics to the environment.

In addition to antibiotics, trace levels of natural and synthetic estrogenic hormones have been reported in the environment and are of growing concern due to potential adverse effects on the reproductive biology of vertebrates, even at concentrations below 100 ng/L (Routledge et al., 1998; Schuh et al., 2011). Concentrations of hormones and their partial breakdown products, including 17α-estradiol (α-E2) and estrone (E1), were detected in surface water and well water near a cattle farm in the range of 0.05 to 7.4 ng/L for α-E2 and 4.5 ng/L for E1. 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 groundwater (Finlay-Moore et al., 2000; Hanselman et al., 2004; Raman et al., 2004; Shore et al., 1993). For example, the annual excretion of estrogens from farm 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). Other studies have shown that concentrations of estrogens in wastewater originating from agricultural activities were three to four times higher than from municipal wastes (Gulkowska et al., 2008; Shore et al., 1993; Snyder, 2008; Ternes et al., 2002; Ternes et al., 2003; Zorita et al., 2009). All in all, these facts suggest that estrogens excreted from agricultural activities are a significant concern because of the potential to contaminate water resources and cause adverse effects on the reproductive biology of aquatic vertebrates, even at very low concentrations.

A recent study by Pruden et al. (2006) suggested that antibiotics should be considered emerging contaminants due to their widespread presence in the environment and the potential health and ecosystem risks associated with them. 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 to prevent the spread of antibiotic resistance and feminization among organisms. A significant number of studies have investigated the removal of antibiotics and other pharmaceuticals from drinking water and wastewater (Gulkowska et al., 2008; Snyder, 2008; Ternes et al., 2002; Ternes et al., 2003; Zorita et al., 2009). Although some removal of pharmaceuticals has been observed in conventional wastewater treatment processes, most are not designed to remove micropollutants effectively (Janssens et al., 1997; Suidan et al., 2005; Sumpter and 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.

Extensive work has been done to demonstrate and optimize the HTL conversion of waste organic biosolids into a valuable bio-oil and to characterize the chemical properties of HTL bio-oil for different feedstocks, such as *Spirulina*, swine manure, and anaerobically digested sewage sludge (Anastasakis and Ross, 2011; Vardon et al., 2011; Yu et al., 2011a; Zhou et al., 2010). However, a much smaller amount of information is available from past studies on the aqueous wastewater

product of HTL. Literature data on a range of basic water quality parameters resulting from HTL treatment of swine manure are presented in Table 1.1. The high concentration of biological oxygen demand (BOD), up to 59,000 mg/L, make HTL-WW unsuitable for surface water discharge (Appleford, 2004). Sometimes HTL-WW also has a low pH (< 6), which is most likely attributable to the formation of organic acids and could cause problems for discharge. In addition, high concentrations of ammonia (1,860-7,070 mg/L) were also observed, which results from the breakdown of amino acids and would make HTL-WW unsuitable for direct discharge to the environment. Magnesium, phosphorous, potassium, and in particular, sulfur, were also present at substantial levels. However, because these elements are necessary for plant and algae growth, the high concentrations suggest that the HTL-WW could make a good fertilizer for plants or algae.

Table 1.1 Water quality analysis of HTL-WW (Appleford, 2004).

Water Quality Parameter	**Mean**	**Lowest value**	**Highest value**
Biochemical oxygen demand (mg/L)	35,240	420	59,000
Total suspended solids(g/L)	33	21	105.52
pH	5.52	4.86	7.98
Ammonia (mg/L)	3,413	1,860	7,070
Chloride (mg/L)	667	84	1,378
Nitrate (mg/L)	0.87	0.06	2.21
Phosphate (mg/L)	921	66	1,436
Sulfate (mg/L)	427	142	971
Chromium (mg/L)	0.27	0	1
Iron (mg/L)	28	0	78
Magnesium (mg/L)	242	0	579
Manganese (mg/L)	2	0	5
Nitrogen (mg/L)	6,360	4,752	8,651
Phosphorus (mg/L)	434	3	1,068
Potassium (mg/L)	1,482	56	2,411
Rubidium (mg/L)	0.58	0	1
Sulfur (mg/L)	9,651	0	35,326
Zinc (mg/L)	1.67	0	12

A variety of organic compounds have been previously reported 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). Table 1.2 compares previously reported organic compounds in HTL-WW generated from different feedstocks and one gasification process. In addition, Elliot (1992) reported a list of over 60 organic compounds that are commonly detected in wastewater generated from thermochemical conversion of biomass feedstocks. However, these 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.

The human and animal toxicity of HTL-WW is also a concern. Elliot (1992) summarized a list of 48 hazardous constituents likely to be found in post-HTL wastewater, several of which have reported toxic effects, including phenol, toluene, benzene, 2-methylarizidine, and aziridine (Netzeva et al., 2004; Verschaeve and Kirschvolders, 1990; Weisburger et al., 1981; Yardleyjones et al., 1991; Zhao et al., 2009). The high concentration of ammonia found in HTL-WW is also likely to be toxic to many aquatic organisms (Camargo and Ward, 1995; Scott and Crunkilton, 2000). For example, Tsukahara et al. (2001) found that the high concentration of ammonia (16.62 g/L) in a condensate solution recovered from gasification was toxic to *Chlorella vulgaris*. These results suggest that HTL-WW is likely to have toxic effects in natural ecosystems and require treatment prior to discharge.

Table 1.2 Organic compounds found in the aqueous phase of thermochemical processes.

HTL conversion of Macro-alga *L.saccharina* (Anastasakis and Ross, 2011)	**HTL conversion of *E.prolifera* (Zhou et al., 2010)**	**Coal Gasification (Condensate) (Gangwal, 1981)**	**HTL conversion of swine manure (Appleford, 2004)**
Dianhydromannitol 1-(2-furanyl)-ethanone (acetylfuran) Isosorbide 2-cyclopenten-1-one Pyrrole derivatives Indole 3-amino-phenol	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	Phenol Naphthalene Fibenzofuran Fluorene Pyrene Benzo[α]fluorence Bisphenyl 1-Methylnaphthalene 2-Methylnaphthalene Pyridine Methyl-,dimethyl-,and ethyl-substituted pyridines Quinolone Acridine Benzoacridine and methyl derivatives	Dianhydromannitol 1-(2-furanyl)-ethanone (acetylfuran) Isosorbide 2-cyclopenten-1-one, Carboxylic acids Alcohols, Ketones Cyclic hydrocarbons Amides Azines, Pyrroles 3-amino-phenol Indole

Assuming that treatment of HTL-WW is needed prior to environmental discharge, we considered what treatment could be provided by algal cultivation reactors fed with recycled HTL-WW. The ability of algae to remove, transform, and degrade a wide range of organic pollutants including phenolic, aromatic, and steroid compounds; oil contaminants; and agrochemicals has been summarized in numerous studies. For example, the microalgae *Chlorella vulgaris* and *Coenochloris pyrenoidosa* have been shown to remove many contaminants, including phenols, nitrophenols, chlorophenols, and bisphenol A (BPA) (Hirooka et al., 2003; Hirooka et al., 2006). Algal species such as *Oscillatoria salina, Plectomena terebrabs, Aphanocapsa spp.*, and *Synechococcus spp.* have been shown to bioremediate oil contaminants (Cerniglia et al., 1980; Raghukumar et al., 2001). Alternatively, the use of granular activated carbon (GAC) to remove various organic contaminants of concern by adsorption is also well documented (Aksu, 2005; Tryba et al., 2003). In addition, GAC has been widely used in conjunction with microbial biodegradation processes, and the biological removal of adsorbed organics can provide continuous *in-situ* regeneration of GAC adsorption capacity (Suidan et al., 1983). Finally, both activated carbon made from coal and any biomass grown during treatment of HTL-WW can be recycled back to the HTL process to produce more bio-oil (Hartman and Hatcher, 2014; Zhou et al., 2011).

The aim of this research was to investigate the effects of thermochemical treatment on emerging bioactive contaminants commonly present in wet wastes. Our working hypothesis was that the elevated temperature and pressure of HTL processes (250-350°C and 10-20 MPa) will degrade and deactivate a wide range of organic compounds as well as antibiotic-resistant genetic materials. Thus, the health and ecosystem risks associated with pharmaceuticals and the development of antibiotic resistance from human and livestock wastewaters can be mitigated, and the potential for water reuse can be enhanced. Furthermore, costs associated with antibiotic-resistant infection 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 alternatives, improving water quality, and enhancing the potential for beneficial reuse of wastewaters.

1.2 Research Objectives and Approaches

The specific objectives and approaches for this research are summarized below:

1. Characterize the effect of HTL on the fate of bioactive compounds in biowastes under different operating conditions. 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 biocrude 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 wastes. High-performance liquid chromatography with a

photodiode array detector (HPLC/PDA) was the primary method used to measure emerging contaminants before and after HTL treatment.

2. Investigate the effect of HTL on antibiotic-resistant genes and gene transfer under different operating conditions. High-efficiency electroporation transformation and natural transformation of HTL-treated DNA were simulated. Particularly, plasmid DNA extracted from pure *E. coli* culture and manure slurry samples was transferred into common environmental bacteria, such as *E. coli* and *Azotobacter vinelandii*.

3. 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 GC/MS measurements with known analytical standards used for quantification.

4. Investigate the toxicological risks associated with NOCs and the whole organic mixture in HTL-WW. Bioassays with CHO cells were used to quantify cytotoxicity of NOCs identified by GC/MS and the complex organic mixture extracted from HTL-WW. Algal growth/inhibition tests with NOCs and the complex organic mixture were also conducted using *Chlorella protothecoides.*

5. Assess the use of algal bioreactor treatments and adsorptive treatments to improve the chemical and biological quality of HTL-WW. Algal wastewater treatment bioreactors were operated to measure algal uptake of NOCs over time. Semi-batch algal bioreactors and GAC columns were used to treat HTL-WW and then quantify the reduction in toxicity and other water quality impacts.

1.3 Benefits of the Research

This study offers the advantages of enhancing wastewater reuse knowledge and capabilities for simultaneous wastewater treatment and bioenergy production in one integrated process. The HTL process can effectively convert biowaste into valuable biocrude oil while breaking down emerging contaminants such as pharmaceuticals and antibiotic-resistant genes to inactive compounds. As a result, the quality of wastewater effluents can potentially be improved for human health benefits and reduced ecosystem risks. However, the HTL process can also produce some new chemical compounds with potentially negative impacts and health risks. Thus, it is important to assess the new compounds and the potential to mitigate them. Our proposed integrated system for wastewater treatment and bioenergy production can potentially simultaneously improve the quality of wastewater and expand bioenergy production from wastes. This study also provides pioneering work on the effects of HTL on emerging contaminants and toxicity, which is useful for adjusting current HTL operating conditions to enhance environmental benefits.

CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals and Reagents

BPA labeled with ^{14}C was purchased from American Radiolabeled Chemicals (Saint Louis, MO). Carbenicillin disodium (CAS# 4800-94-6), 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), 1-methyl-2-pyrrolidinone (CAS# 872-50-4), E1 (CAS# 53-16-7), FF (CAS# 73231-34-2), CF (CAS # 80370-57-6), and solvents (chloroform [CAS# 67-66-3], ethanol [CAS# 64-17-5], ethyl acetate [CAS# 141-78-6]) were purchased from Sigma Aldrich (Milwaukee, WI). Phenol (CAS# 108-95-2), 2-picoline (CAS# 109-06-8), and 2-pyrrolidinone *(*CAS# 616-45-5) were purchased from Alfa Aesar (Ward Hill, MA). All chemicals and solvents were purchased at the highest level of purity available.

2.2 Feedstocks for Hydrothermal Liquefaction (HTL) Experiments

Spirulina biomass (solids content of 95%) was obtained as a dry powder from Cyanotech (Kailua-Kona, HI) and stored at 4°C prior to processing. Fresh swine manure was collected from the grower-finisher pen floors at the Swine Research Center at the University of Illinois at Urbana-Champaign. The manure sample was blended with tap water using a commercial Waring® 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 hrs in a convection oven (DKN 400, Yamato Co.). The volatile solid content was measured by burning the swine manure in a muffle furnace (Barnstead Thermolyne Co.) at 600°C for 3 hrs or until the weight became stable. The volatile fraction of the total solids was 80-85%.

2.3 Microbial Strains, Culture Conditions, and Growth Conditions

2.3.1 Escherichia coli (E. coli) and Azotobacter vinelandii (A. vinelandii)

Plasmid-encoded resistance to carbenicillin (CARB) was maintained in *E. coli* strain S17-1 λpir. This strain is referred to as CARB-R- *E. coli* S17-1 λpir throughout this report to distinguish it from the unmodified strain of *E. coli* S17-1 λpir, which is not resistant to CARB. 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 Laboratories, Sparks, MD) containing 5μg/mL of CARB and incubated overnight at 37°C while being shaken at 300 rpm. After the incubation time had passed, the pure culture was streaked onto an LB plate containing CARB and incubated overnight at 37°C to create single colonies. This plate was then kept at 4°C and used for inoculation in subsequent experiments. Subculturing onto new plates was performed every two weeks to keep the culture pure and active. In high-efficiency electroporation transformation experiments, *E. coli* strains DH5α and S17-1 λpir were used as recipient cells. These strains were also stored at -80°C and then subsequently revived and maintained using the same techniques as described above for CARB-R-*E. coli* S17-1 λpir. The competent cells were then prepared following a 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 while being shaken at 170 rpm for 18-20 hrs to grow a culture of competent cells (Lu et al., 2010).

2.3.2 Chinese Hamster Ovary Cells

Chinese hamster ovary (CHO) cell line AS52, clone 11-4-8, was used for the cytotoxicity assay (Hsie et al., 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, and 0.25 µg/mL amphotericin B in 0.85% saline), and 1% glutamine at 37°C in a humidified atmosphere of 5% CO_2.

2.3.3 Algal Culture

Chlorella protothecoides (*C. protothecoides*) was provided from the Culture Collection of Alga at the University of Texas (Austin, TX). Stock cultures of *C. protothecoides* were maintained routinely on both agar slants and liquid media 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 ± 1°C with continuous illumination. The purity of the culture was established by repeated streaking and microscopic examination. An inoculum culture was prepared four days prior to the start of inhibition tests to obtain algae in the exponential growth phase. The growth of algae was monitored spectrophotometrically by measuring absorbance at 680 nm.

2.4 Hydrothermal Liquefaction Experiments

Breakdown of bioactive compounds by HTL treatment was studied using custom HTL bomb reactors manufactured by Swagelok Co. (Solon, OH). Each reactor included a cap (Part # 1 SS-600-C), SS tubing (Part # SS-T6-S-049-20), and an N-series needle valve (Part # SS-6NBS6-G). A set of four reactors was run in parallel to simultaneously test the effect of different operating conditions (temperature and reaction time) on the breakdown of bioactive compounds. The tested HTL operating conditions are summarized in Table 2.1. The starting test solution was prepared in DI water at the following concentrations: CF (50 mg/L), FF (50 mg/L), and E1 (5 mg/L). Before each HTL experiment, 2.7 mL of the starting solution was added into each reactor. The last reactor was loaded only with 2.7 mL of DI water and used as the blank control. The reactors were then loaded into a preheated furnace at the desired reaction temperature and maintained for a desired reaction time. After the reaction time had passed, the reactors were rapidly cooled down by submerging them in a water bath. Samples from each reactor were then collected into separate HPLC vials for later analysis as described in Section 2.8.

Table 2.1 Summary of HTL operating conditions.

Retention time (min)	Temperature (°C)	
	250	300
15	250-15	300-15
30	250-30	300-30
60	250-60	300-60

To study the effect of HTL feedstock on the removal of bioactive compounds, the tests described above were repeated with a gradually increasing amount of *Spirulina* feedstock (from 0.1 to 20% solids concentration) added into the HTL reactor, which also contained 50 ppm CF, 50 ppm FF, and 2 ppm E1. As the solids content of the *Spirulina* feedstock increased to 10% or more, we encountered analytical problems due to sample matrix interference. Thus, results reported only the percentage of the removal of tested compounds in the presence of 0.1-5% solids content.

Because of the problems with detecting CF, FF, and E1 after HTL treatment when more than 5% solids content of *Spirulina* feedstock was used, we developed a new HPLC-CLSC technique that allows the detection of bioactive compounds at trace levels in the presence of higher amounts of biosolids feedstock. This alternative approach was used for HTL tests with swine manure at a 20% solids content that was spiked with radio-labeled ^{14}C-BPA. Three HTL operating conditions were investigated, all of which used a temperature of 300°C. The three different reaction times were 15, 45, and 60 min.

To test the effects of HTL operating conditions on the breakdown of 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 (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 three times with pure nitrogen. The initial pressure was set at 88-92 PSI (607-635 kPa) 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. The HTL aqueous product was collected and then used for DNA extraction.

To characterize the HTL wastewater (HTL-WW), we used a larger 2 L stainless steel batch reactor with a coupled magnetic stirrer to generate a larger sample of HTL-WW. This reactor was loaded with 600 g of feedstock (*Spirulina*) at a 20% dry solids content (80% water), sealed, and then purged three times with pure nitrogen. The initial pressure was set at 88-92 PSI (607-635 kPa) to prevent water from boiling during the experiment. Finally, the reactor was heated to the desired reaction temperature of 300°C, and the reaction temperature was maintained for a reaction time of 30 min. Subsequently, the reactor was rapidly cooled and the gaseous product was vented. Crude oil, solid residue, and wastewater were collected for subsequent separation.

HTL-WW was separated from the crude oil and solid residue with a 0.2 μm pore-size glass fiber filter. Filtered HTL-WW was used for organic compound extractions and gas chromatography analysis.

2.5 DNA Extraction/Purification

The extraction of plasmid DNA from pre- and post-HTL treatment of the CARB-R- *E. coli* S17-1 λpir culture was conducted following previously reported methods (Sambrook and Russell, 2001). Extraction of plasmid DNA from swine manure spiked with CARB-R- *E. coli* S17-1 λpir both pre- and post-HTL treatment was conducted followed another previously published method (Trochimchuk et al., 2003). The DNA concentration and size distribution were determined by Nanodrop® ND-1000 (Thermo Scientific, Waltham, MA) and gel electrophoresis, respectively. The DNA samples were divided into aliquots and stored at -20°C until used.

2.6 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 following a published method (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). Specifically, 40 μL of competent *E. coli* DH5α or S17-1 λpir was mixed with 2 μL of the DNA to be transformed and then pipetted into a plastic cuvette containing electrodes. A short electric pulse was applied to the cells, causing small holes in the membrane through which the DNA enters. The cells were then resuspended immediately in a medium of super-optimal broth with catabolite repression (SOC) and incubated at 37°C for 1 hr. After the incubation time had passed, the cells were plated on selective medium containing 5 μg/mL of carbencillin and incubated at 37°C overnight. The transformation frequency was determined by dividing the number of colony-forming units on selective medium (transformant) by the total number of colony-forming units as measured on LB medium. 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, nor when DNA and cells were mixed but not subjected to an electric pulse.

2.7 Natural Transformation of *Azotobacter vinelandii*

Natural transformation processes were simulated by exposing plasmid DNA extracted from the pre-and post-HTL treatment of CARB-R- *E. coli* S17-1 λpir to *A. vinelandii* strain DJ (wild type). Transformations were conducted by mixing 200 μL of competent cell suspension and various volumes of plasmid DNA (target mass of 2 μg of DNA). After incubating at room temperature for 20-30 min, the transformation mixtures were then diluted with sterile phosphate buffer and spread onto plates that contained only B medium and plates that contained B medium and 5 μg/mL of CARB (BC plates). The plates were incubated at 30°C for three to five days until the growth of colonies could be seen. Transformation frequencies were calculated by dividing the number of colony-forming units (CFUs) on selective B medium with CARB (transformants) by the total number of CFUs on B 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 of the detection limit, providing an upper limit of gene transfer frequency.

2.8 HPLC Analyses

2.8.1 HPLC Analysis for 14C-labeled BPA

Detection of ^{14}C-labeled BPA was performed with an HPLC (LC-20, Shimadzu Scientific Instruments, Inc., Columbia, MD) with a continuous liquid scintillation counter (CLSC) (β-RAM Model 2, IN/US Systems, now LabLogic, Brandon, FL) connected directly after the PDA detector. IN-Flow 2:1 scintillation cocktail (IN/US Systems) was used as the mobile phase for the CLSC detector to make radioactive decay events measurable as fluorescent emissions. Two columns connected series, a Waters 4 μm Nova-Pak® C18 guard column (3.9 × 20 mm) and a Waters 4 μm Nova-Pak® C18 analytical column (3.9 × 150 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 the gradient elution method are shown in Table 2.2, which resulted in a retention time of 33 min and a detection limit of 203 ng/L for BPA.

Table 2.2 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

2.8.2 HPLC Analysis of Florfenicol, Ceftiofur, and Estrone

Concentrations of FF, CF, and E1 in post-HTL wastewater were analyzed at the Illinois Sustainable Technology Center (ISTC) using a Waters 2695 Separations Module HPLC equipped with a Waters 996 PDA detector. Separations were performed using a Hypersil C18 column (250 mm × 4.6 mm i.d.; 3 μm particle size; Keystone Scientific, Bellefonte, PA). 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, 224, and 205 nm, respectively, and the corresponding retention times for CF, FF, and E1 were 4.6, 5.5, and 16.4 min, respectively.

2.9 Liquid-Liquid Extraction of Heteroaromatic Compounds from HTL-WW

Heteroaromatic compounds in HTL-WW were extracted using a previously published method (Johansen et al., 1996). Dichloromethane (DCM; 50 ml), HTL-WW (2.5 mL), and potassium hydroxide (5 mL; 5 M) were added to a 250 mL separatory funnel and slowly inverted several times. The emulsion was released 2-5 min after being mixed, and the DCM layer was collected as extraction part 1. The remaining layer was recovered and adjusted to pH 5 with 6 M 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 parts 1 and 2 were concentrated to 1 mL with the aid of a TurboVap concentrator, transferred to a GC vial, and stored at -20°C prior to GC/MS analysis.

2.10 Gas Chromatography and Mass Spectrometry Analyses

Specific NOCs were profiled with an Agilent Technologies 7820A gas chromatogram with thermionic detection (GC/NPD) of nitrogen and phosphorus. Separations were achieved with a Varian CP-sil 8 column (30 m × 0.25 mm × 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 split ratio of 1:100. The column was initially held at 35°C for 5 min, then increased (25°C/min) to 130°C and held for 4 min, then increased at a rate of 25°C/min to 240°C and held for another 4 min, and finally increased at 25°C/min to 280°C and held for 7 min.

A general scan of organic compounds in HTL-WW was performed under the same conditions as the GC/NPD analysis. The magnetic sector mass spectrometer (Ultima, Waters, Milford, MA) collected and measured all masses ranging from 35 to 350 Da. The data were 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 were performed by calibration with reference materials procured from commercial sources. Identical instrumental conditions were used 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 the injection of reference compounds. Analysis with SIR programs can greatly enhance instrument sensitivity and reduce matrix interference since only ions of interest are collected and measured.

2.11 Extraction of Organics from HTL-WW

Organic compounds in HTL-WW were extracted on XAD-2 and XAD-8 resins (Richardson, 2011). The XAD-2 (CAS# 10357) and XAD-8 (CAS# 20278) resins were purchased from Sigma-Aldrich. The resins were prepared by consecutively washing them with 0.1 N NaOH, distilled water, and methanol for 30 min each. Next, XAD resins were further washed by Soxhlet extraction for 24 hrs using each of the following chemicals: methanol, ethyl acetate, and methanol again. Washed XAD resins were stored in methanol at 4°C prior to use. 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 600 ml of ultrapure water, 400 ml of 0.1 N HCl, 200 ml of 0.1 N NaOH, and a single rinse of ultrapure water. Because of the high concentration of organic

compounds 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 HCl, 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 aqueous layer was discarded. The remaining ethyl acetate was passed through organic free sodium sulfate to remove water and concentrated to 1 mL with the aid of a RotoVap concentrator. The extracts were transferred to 1 mL conical vials and stored at -20°C before processing.

2.12 CHO Cell Chronic Cytotoxicity Assay

The CHO cell chronic cytotoxicity 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 cultivation period of 72 hrs (~3 cell cycles) (Plewa et al., 2002; Plewa, 2009). Various test sample dilutions in demethylsulfoxide (DMSO) were further diluted with F12 + FBS cultivation medium to analyze a range of concentration factors. This assay was calibrated following previously published procedures (Plewa et al., 2002; Plewa and Wagner, 2009). For each NOC or HTL-WW sample concentration factor, 8-16 replicate wells were analyzed, and the experiments were repeated at least two times. A concentration-response curve was generated for each sample, and a regression analysis was conducted for each curve. The LC_{50} (% C½) values were calculated from the regression analysis and represent the sample concentration factor that induced a 50% reduction in cell density 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).

2.13 Algal Growth Inhibition Assays

Algal growth inhibition assays were performed in sterile 24-well polystyrene microplates (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 two replicate cultures per concentration. Two plates were prepared for each tested compound to provide four replicates of each test concentration and eight 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, 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 hrs 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 hrs after the beginning of the exposure. Average algal growth rate for a period was calculated as the logarithmic increase in biomass using equation 1:

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_i - t_j} \quad (1)$$

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 equation 2:

$$I_\mu = \frac{\mu_c - \mu_\tau}{\mu_c} \times 100 \quad (2)$$

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.

2.14 Batch and Semi-Batch Algal Bioreactor Tests for Removal of NOCs and Toxicity

A set of three batch algal bioreactors was operated in parallel to investigate the removal of NOCs 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 set up to evaluate the reduction of tested compounds due to volatility or other non-biological mechanisms. Reactor 3 contained only medium and algae and was used as a negative control of the experiment. Samples from these reactors were collected daily for 2 weeks and subjected to liquid-liquid extraction prior to GCMS analysis.

A culture of *C. protothecoides* was grown in a mixture of HTL-WW diluted with municipal wastewater from primary effluent (Urbana-Champaign Sanitary District). We started with diluted HTL-WW at approximately 1% strength and periodically added aliquots of HTL-WW to gradually expose the culture to increasing amounts. The growth of algae was monitored regularly by measuring the absorbance at 680 nm. The improvement of wastewater quality was examined by measuring chemical oxygen demand (COD) with APHA's standard method (APHA, 1995). We stopped adding HTL wastewater into the reactor once we observed stagnant algae growth. Algae-treated HTL wastewater was then extracted for organic compounds and tested for mammalian cytotoxicity using CHO cells according to the methods described earlier.

2.15 Adsorptive Treatment with 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 at 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 through the GAC. Algae-treated HTL-WW (as described in section 2.14) was fed into the GAC column upward at a constant flow rate of 1.0 mL/min using a high-performance liquid chromatography pump. The effluent after GAC treatment was collected, and organic compounds were extracted for toxicity testing.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Breakdown of Pure Bioactive Compounds Subjected to HTL Treatment Conditions

Figure 3.1 presents the percent removal of FF (Panel A), CF (Panel B), and E1 (Panel C) at six different HTL operating conditions when processed without any other HTL feedstock added to the reaction mixture. When the concentration of tested compounds was below the detection limit, we used half of the detection limit of each compound to calculate the percent removal. Thus, the maximum removal given by our method was 99.95% for both CF and FF and 99.2% for E1. As demonstrated in Figure 3.1, under the same HTL operating conditions, the descending rank order for bioactive compound removal was CF > FF > E1. Better removal of tested compounds was generally observed with increasing reaction time or reaction temperature. For example, with a reaction time of 30 min, the percent removal of E1 was 88% and 99.2% at 250°C and 300°C, respectively (Figure 3.1 C). Removal of FF increased from 94.2% to 99.3% as the temperature increased from 250°C to 300°C (Figure 3.1 B). Looking at the effect of retention time, we observed that the removal of E1 at 250°C increased from 72.7% to 99.8% as the reaction time increased from 15 to 60 min (Figure 3.1 C). Similarly, removal of FF at 250°C increased from 94.2% to more than 99.9% as the reaction time increased from 15 to 60 min. More than 99.7% removal of CF was obtained at all tested operating conditions (Figure 3.1 A). It was noteworthy that when the reaction temperature was ≥ 300°C, increasing the reaction time did not significantly improve the removal of bioactive compounds because the removal was essentially complete. 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 250°C, it required 60 min of reaction time to achieve maximum removal of bioactive compounds.

3.2 Breakdown of Bioactive Compounds by HTL in the Presence of Feedstock

To study the effect of HTL feedstock on the removal of bioactive compounds, we gradually added a different amount of *Spirulina* feedstock (from 0.1 to 20% solid content) into the starting solution, which contained 50 ppm CF, 50 ppm FF, and 5 ppm E1. As the solid content of the *Spirulina* feedstock increased to 10% and 20%, we encountered issues with matrix interference and were unable to accurately quantify the concentration of tested compounds post-HTL treatment. Thus, we report only the percent removal of tested compounds in the presence of 0.1 to 5% solid content feedstock in the following figures. Figure 3.2 presents the percent removal of E1 in the presence of 0.1, 0.5, 1, 2, and 5 % *Spirulina* feedstock. As demonstrated in Figure 3.2 A for HTL conditions of 250°C and 15 min retention time, the removal of E1 generally decreased as the feedstock solid content increased. In this case, the removal of E1 decreased from 76% to 61% as the solid content increased from 0.1% to 5%. This suggests that at some HTL operating conditions, the presence of sample matrix could interfere with the removal of bioactive compounds. However, as the retention time increased to 30 min and above, the effect of the sample matrix on removal of E1 was not significant (Figure 3.2 B). Additionally, when the HTL was operated at 300°C, variations in the solid content of HTL feedstock did not significantly affect the removal of E1.

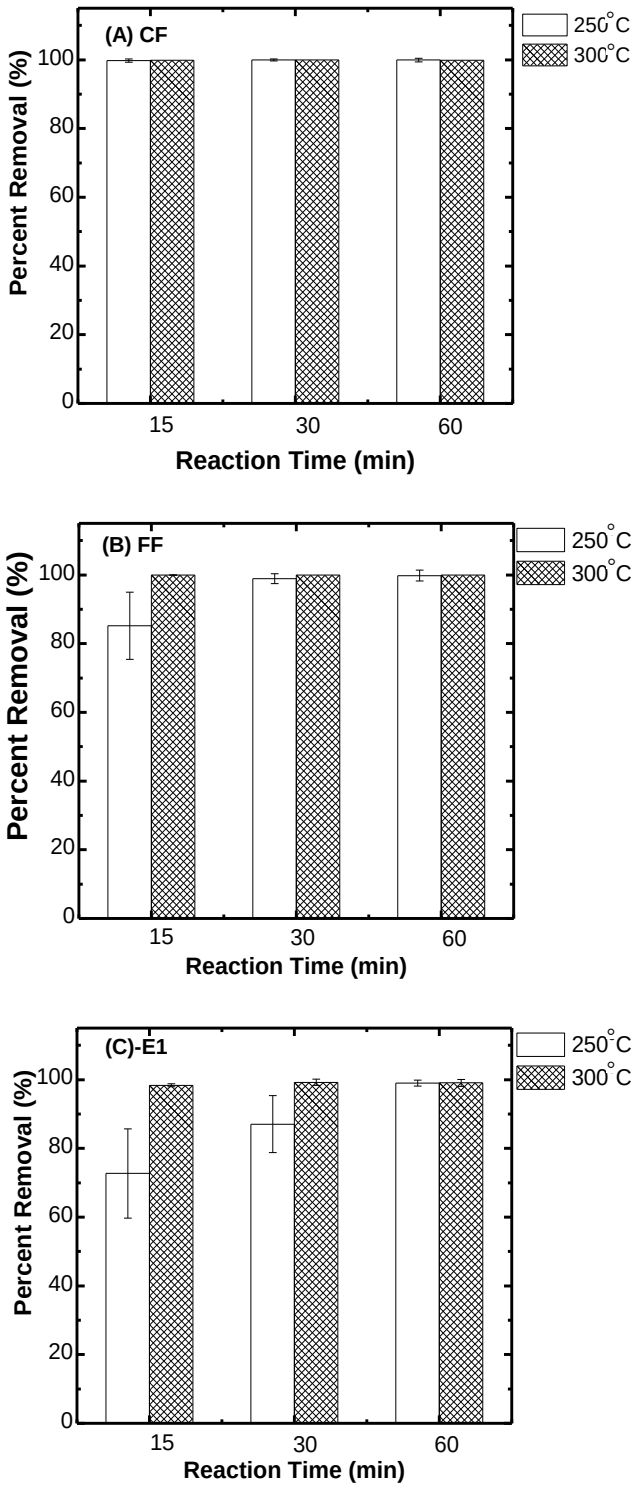


Figure 3.1 Percent removal of ceftiofur (CF; panel A), flofernicol (FF; panel B), and estrone (E1; panel C) under different HTL operating conditions.

Similar results were also obtained for FF removal in the presence of *Spirulina* feedstock. At 250°C and a 15 min retention time, the removal of FF also decreased as the feedstock solid content increased (Figure 3.3 A). For example, the removal of FF decreased from 87% to 76% as the solid content increased from 0.1% to 5%, suggesting that the presence of feedstock provided some protective effect for bioactive compounds. However, when the reaction time was extended to 30 min and longer, the presence of feedstock did not have a significant effect on FF removal (Figure 3.2 B). When HTL was operated at 300°C, the percent removal of FF was nearly constant as the solid content and the reaction time increased, suggesting that the feedstock protective effect could be overcome by increasing the reaction temperature. HTL at 300°C and above was sufficient to provide removal of FF to below detection limits.

Figure 3.4 presents the percent removal of CF in the presence of various amounts of *Spirulina* feedstock. Unlike FF and E1, removal of CF was not significantly affected by the presence of *Spirulina* feedstock. Nearly complete removal of CF was obtained at all tested HTL operating conditions. This could result from the fact that CF is more soluble in water and would be 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 the complete removal of CF was achieved for all tested conditions. The behavior of CF compared with the other tested compounds also highlighted that the effectiveness of HTL for destroying bioactive compounds can be specific to the chemical characteristics of each compound, particularly at lower temperatures and reaction times.

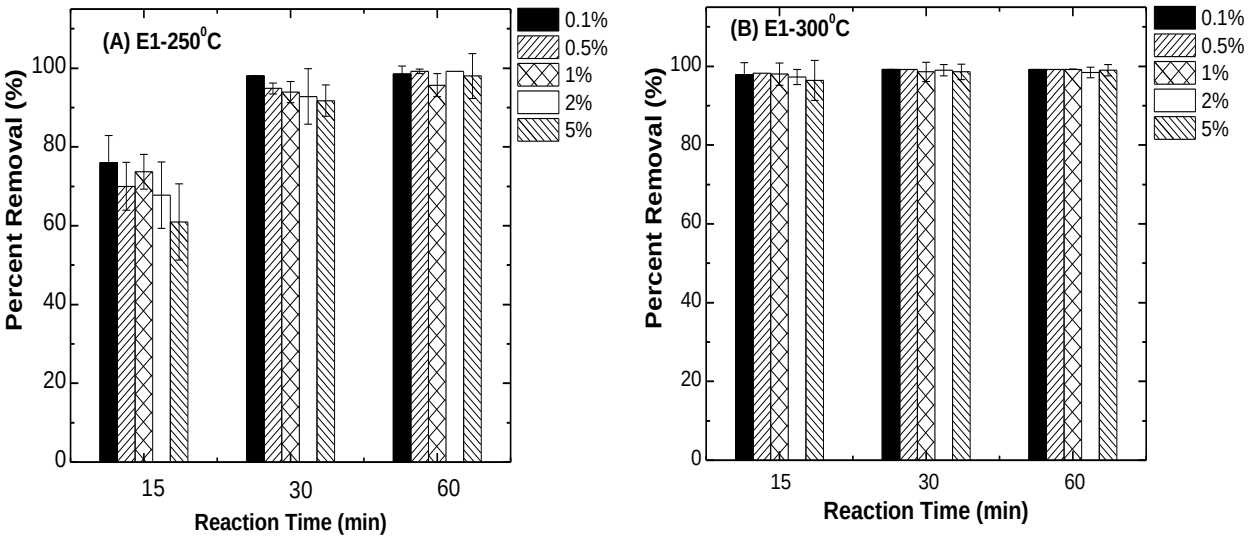


Figure 3.2 Percent removal of estrone (E1) in the presence of *Spirulina* feedstock during HTL at 250°C (panel A) and 300°C (panel B).

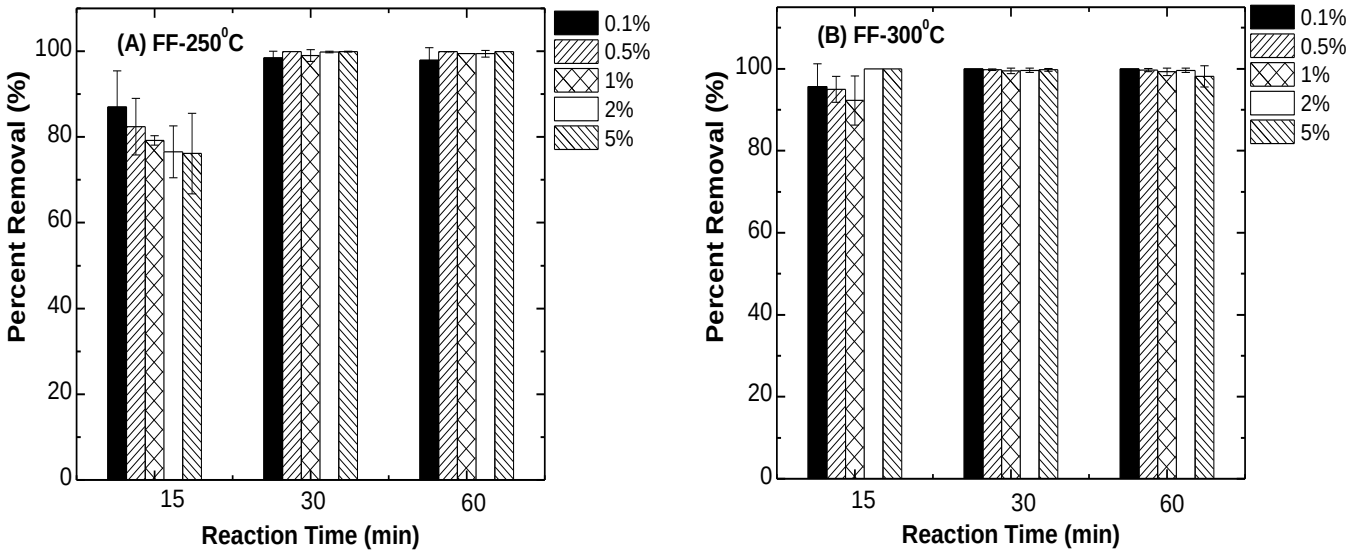


Figure 3.3 Percent removal of florfenicol (FF) in the presence of *Spirulina* feedstock during HTL at 250°C (panel A) and 300°C (panel B).

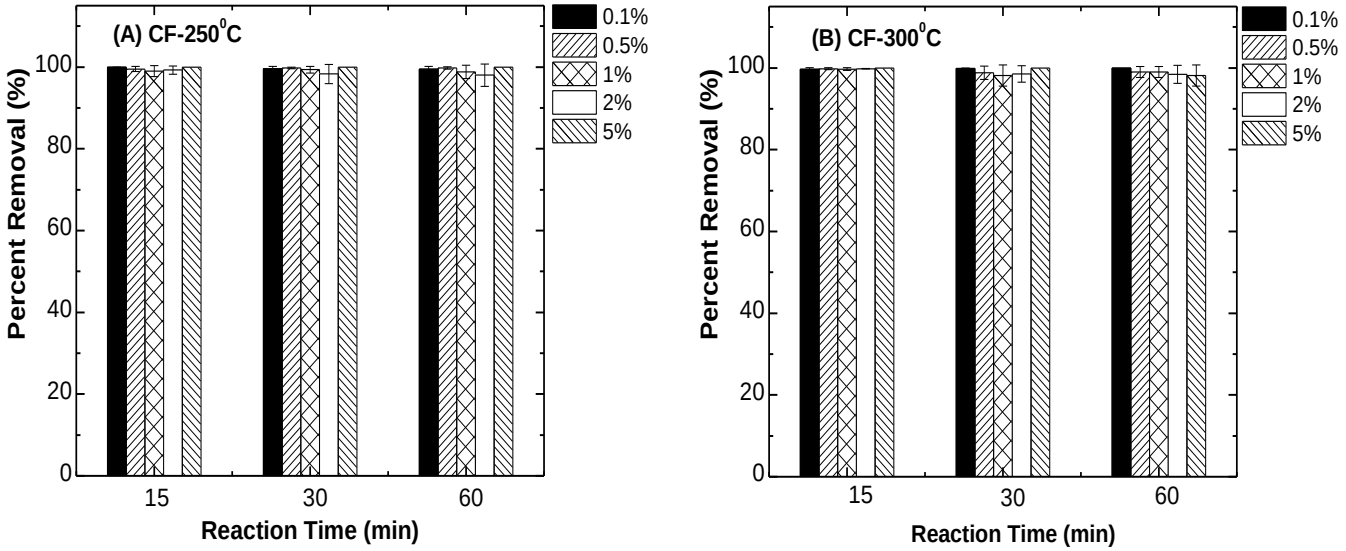


Figure 3.4. Percent removal of ceftiofur (CF) in the presence of *Spirulina* feedstock during HTL at 250°C (panel A) and 300°C (panel B).

3.3 Breakdown of Bisphenol A by HTL Treatment

As mentioned in Section 3.2, we were not able to detect CF, FF, and E1 after HTL treatment when a high level of sample matrix was present (more than 5% solid content of the feedstock). However, the typical solid content of HTL feedstocks is usually around 20%. Thus, it was desirable to develop a more sensitive analytical method that would detect trace concentrations of bioactive compounds in the presence of typical HTL feedstocks. The HPLC method developed for detection of ^{14}C-labeled BPA allowed us to detect BPA at concentrations as low as 203 ng/L. Figure 3.5 presents the HPLC chromatograms of BPA in DI water, tap water, and 1% swine manure before and after HTL treatment at 300°C and a 30 min retention time. BPA was not detected after HTL treatment in all three samples. Instead, we found that there was a consistent breakdown product that eluted at approximately 15 min of retention time for all samples after HTL treatment. To study the effect of the sample matrix on the removal of BPA, we spiked BPA directly into swine manure (20% solid content) and ran this sample with HTL under the same temperature (300°C) at three different reaction times: 15, 45, and 60 min. Once again, no BPA was detected after HTL treatment of swine manure feedstock (Figure 3.6), and the BPA breakdown product had a similar retention time to the BPA breakdown product detected in HTL wastewater of DI water, tap water, and 1% swine manure (Figure 3.5). Variations in the HTL reaction time did not cause a significant difference in the retention time of BPA breakdown products, which indicates that the breakdown of BPA occurred quickly, and the breakdown product was relatively stable. These results also suggest that the operation of HTL at a temperature of 300°C or more would be sufficient to eliminate the protective effect of HTL feedstock and ensure the removal of these bioactive compounds. Figure 3.7 shows the percent distribution of ^{14}C from BPA to HTL-WW as the reaction time was varied. The percentage of ^{14}C for 60, 45, and 15 min of reaction time was 22%, 18.5%, and 18.3%, respectively, and the differences between these values were not statistically significant.

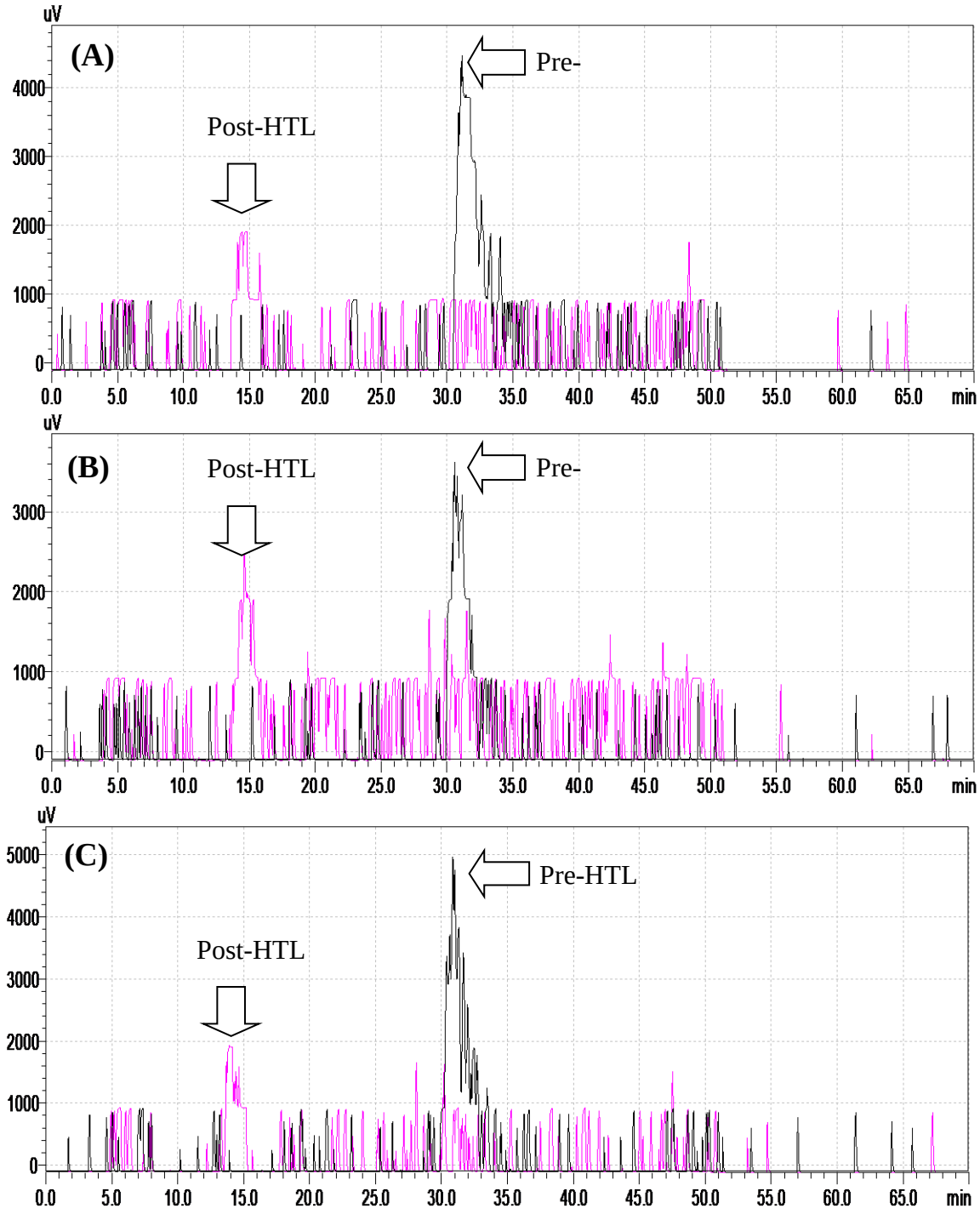


Figure 3.5 Detection of BPA and its breakdown product before and after HTL treatment at 300°C and 30 min of reaction time: (A) DI water; (B) Tap water; and (C) 1% swine manure in DI water.

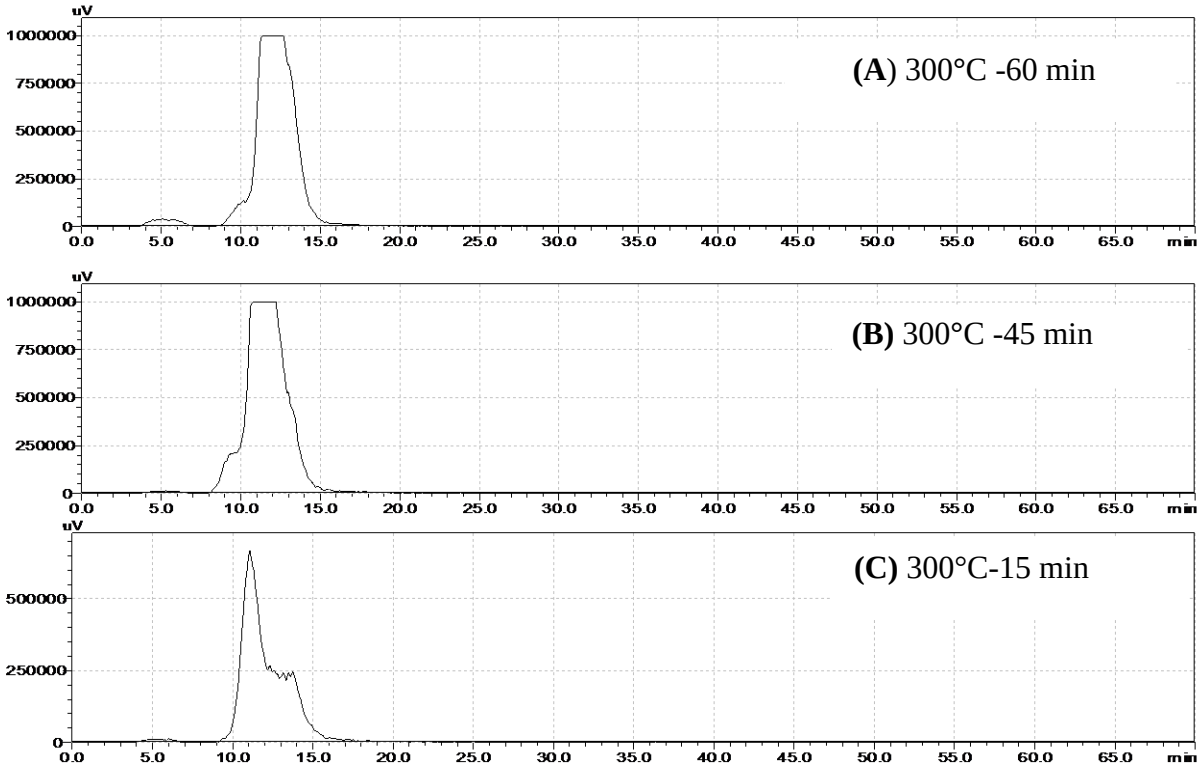


Figure 3.6 Detection of BPA and its breakdown products after HTL treatment of swine manure at 300°C and three different reaction times: (A) 60 min, (B) 45 min, and (C) 15 min.

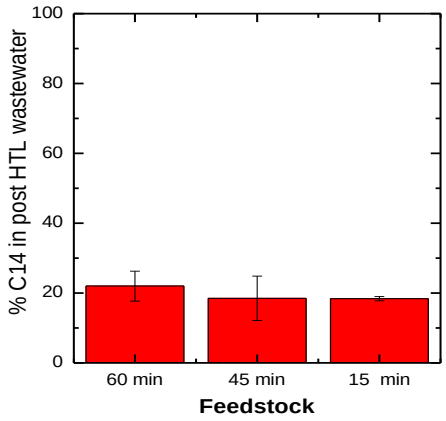


Figure 3.7 Percentage of ^{14}C in HTL wastewater after HTL treatment at 300°C and three different reaction times.

3.4 Breakdown of Plasmid DNA in *E. coli* Culture via HTL Treatment

Figure 3.8 shows the size and yield of plasmid DNA before and after HTL treatment by running extracted DNA samples on an electrophoretic agarose gel. As this figure shows, Plasmid DNA was successfully extracted from a fresh liquid culture of CARB-R-*E. coli* S17-1 λpir and appeared as a crisp, well-defined band in wells 2 and 3. In contrast, the DNA extracted after HTL treatment of CARB-R-*E. coli* S17-1 λpir culture did not show any crisp bands in the gel for all tested HTL treatment conditions (wells 4 to 9). Data from the agarose gel suggested that plasmid DNA had been broken down into small fragments that were mostly below the detection limit (506 base pairs). Figure 3.9 presents the concentration of plasmid DNA extracted from CARB-R-*E. coli* S17-1 λpir culture pre- and post-HTL treatment. This experimental data showed more than 99% removal of plasmid DNA after HTL treatment under all tested conditions. In general, the concentration of post-HTL DNA decreased with increasing reaction temperature and time. For example, at 250°C the DNA concentration decreased from 262 to 1.52 ng/μL as the reaction time increased from 0 to 60 min (Figure 3.9, open circles). Similarly, at 300°C, the DNA concentration decreased from 285 to 1.13 ng/μL as the reaction time increased from 0 to 60 min (Figure 3.9, open triangles). Our experimental data also demonstrated that when the HTL was operated at 250°C, increasing the reaction time could result in slightly better removal of DNA. However, when the HTL temperature was 300°C, the DNA concentration after treatment was nearly constant as the reaction time increased from 15 to 60 min. For relatively short reaction times (< 30.0 min), the HTL operating temperature was the predominant factor affecting the breakdown of DNA. However, the effect of the operating temperature had less of an impact as reaction time increased, as shown by the data for 250°C and 300°C, which had only minor differences when the reaction time was 60 min. All in all, our experimental data demonstrated that HTL treatment could effectively break down genetic materials (DNA) in animal waste while producing valuable biocrude oil. The breakdown efficiency can be maximized either by extending the reaction time if the HTL is operated at low temperatures (≤ 250°C) or by increasing the operating temperature to at least 300°C if a short reaction time is used (≤ 30 min).

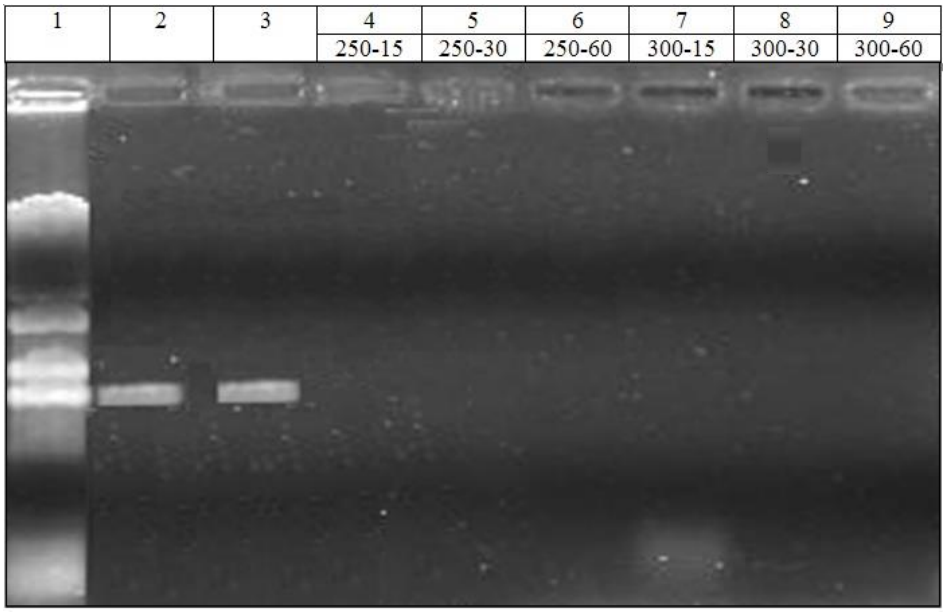


Figure 3.8 Agarose gel of plasmid DNA extracts from pure CARB-R-*E. coli* S17-1 λpir culture before HTL treatment (wells 2 and 3) and after various HTL treatments (wells 4-9) versus size standards (well 1).

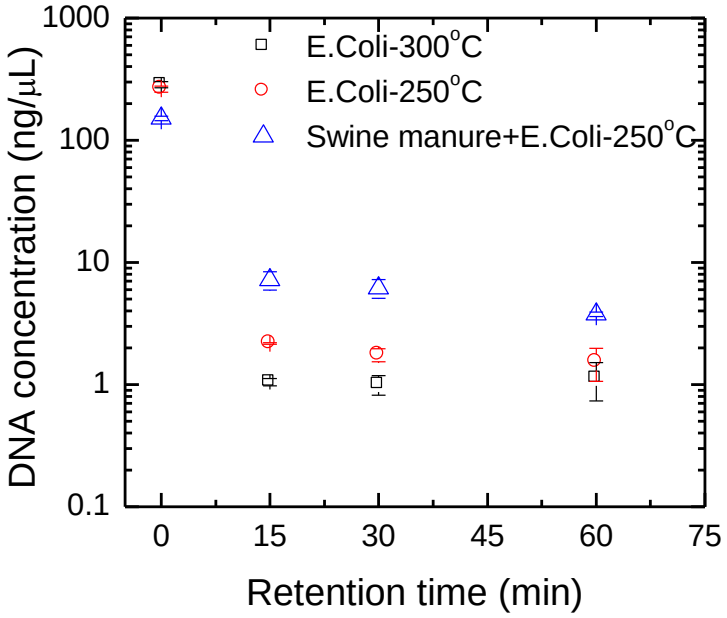


Figure 3.9 Concentration of plasmid DNA extracted from fresh liquid culture and post HTL treatment at different operating conditions.

3.5 Breakdown of Plasmid DNA in the Presence of Swine Manure Matrix

Figure 3.9 (open squares) shows the removal of plasmid DNA extracted pre- and post-HTL treatment of swine manure spiked with CARB-R- *E. coli* S17-1 λpir culture. As demonstrated in Figure 3.9, the DNA concentration decreased with the increasing reaction time, which is consistent with what we observed for HTL treatment of pure CARB-R-*E. coli* S17-1 λpir culture. For example, as the reaction time increased from 15 to 60 min, the remaining DNA concentration in HTL wastewater decreased slightly from 7.2 to 3.8 ng/μL. It is also notable that, under the same operating conditions, the concentration of DNA extracted from post-HTL treatment of the swine manure sample was significantly higher than that of pure CARB-R-*E. coli* S17-1 λpir culture. For example, under the HTL conditions of 250°C and 15 min reaction time, DNA concentrations in post-HTL treatment of swine manure and pure CARB-R-*E. coli* S17-1 λpir culture were 7.2 and 2.1 ng/μL, respectively (Figure 3.9). This observation suggests that a swine manure matrix can provide a protective effect for genetic materials and make them less accessible during HTL treatment. Based on our previous observations about the protective effect of HTL feedstock on the removal of bioactive compounds, we expect that the protective effect of HTL feedstock on genetic material would also become less effective when the HTL was operated at 250°C for a longer reaction time or at higher reaction temperature (≥ 300°C).

3.6 High-Efficiency Electroporation Transformation of *E. coli*

As discussed in section 3.5, there was a low level of DNA remaining in post-HTL wastewater after the HTL treatment. If the remaining DNA was still active and released into the environment, it could potentially be transferred into other bacteria. The concentration of extracellular DNA (plasmid and chromosomal DNA) in the aquatic environment varies from 0.2-25.6 μg/L, and the transformation frequencies of most prokaryotic species vary from 10^{-7} to 10^{-2} (Lorenz and Wackernagel, 1994). Thus, it is important to confirm if the residual DNA was completely deactivated after HTL treatment. In order to do that, we conducted a series of electro-transformation experiments where DNA extracted from pre- and post-HTL treatment of different feedstocks was transferred into two different recipient strains of *E. coli* (DH5α and S17- 1λpir).

Figure 3.10 presents the transformation frequencies of different host bacteria with plasmid DNA extracted from pre- and post-HTL treatments of CARB-R *E. coli* S17-1 λpir culture. Pre-HTL treatment DNA was successfully transferred to *E. coli* strain DH5α and S17- 1 λpir at the frequency of 1.4×10^{-6} and 1.65×10^{-4}, respectively (Figure 3.10). Transformation frequencies of different *E. coli* strains using an electroporation method 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* (Dower et al., 1988). Our transformation frequencies are in the middle to lower range found in previously published work. The transformation frequencies obtained for *E. coli* S17-1 λpir were about two orders of magnitude higher than *E. coli* DH5α. This was expected as the *E. coli* S17-1 λpir strain has chromosomally integrated conjugal transfer functions (RP4 transfer functions). Thus, when it is used as a specific host strain into which the transposon vector DNA is transformed, the transfer occurs by biparental mating, without the need for a helper strain. In contrast, post-HTL DNA was not transferable to either DH5α or S17- 1 λpir, indicating that post-HTL DNA had been completely deactivated.

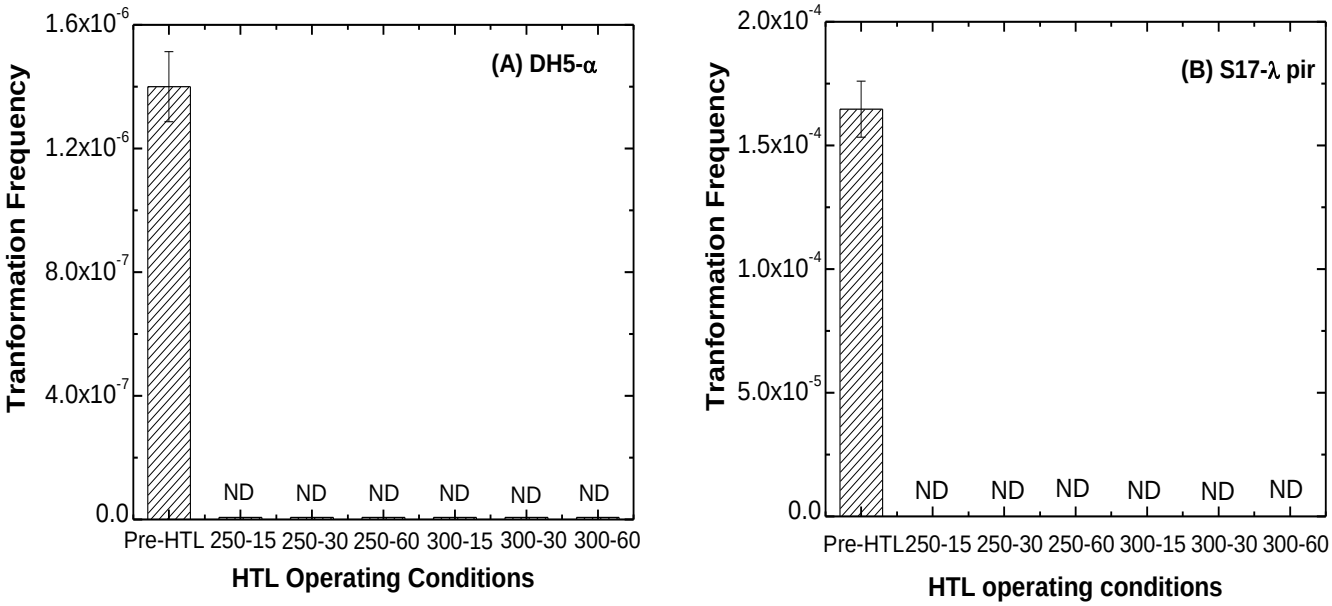


Figure 3.10 Electro-transformation frequency of *E. coli* DH5α (panel A), and electro-transformation frequency of *E. coli* S17-1 λpir (panel B) (ND=not detected).

We also did one set of electro-transformation experiments with the DNA extracted from pre- and post-HTL treatment of swine manure spiked with CARB-R- *E. coli* S17-1 λpir . For this experiment, the HTL process was operated at 250°C with 15, 30, and 60 min reaction times. As shown in Figure 3.11, pre-HTL DNA extracted from the swine manure sample was successfully transferred into *E. coli* (strain S17-1 λpir) at the transformation frequency of 1.57×10^{-5}, but post-HTL DNA was not transferable to *E. coli* S17-1 λpir. This was consistent with our observations from the electro-transformation experiment of post-HTL DNA without swine manure present in the HTL reaction mixture. The effects of HTL operating conditions on the breakdown of DNA and transformation frequency might be observed at lower operating temperatures and shorter reaction times. However, we did not investigate those conditions, as we are currently focused on the range of HTL operating conditions that provide reasonable biocrude oil yield. Data from transformation tests provide additional information to confirm that HTL treatment can effectively destroy genetic materials (DNA) in biowaste samples and eliminate the potential for the transfer of antibiotic resistance from biowastes such as manure into the environment.

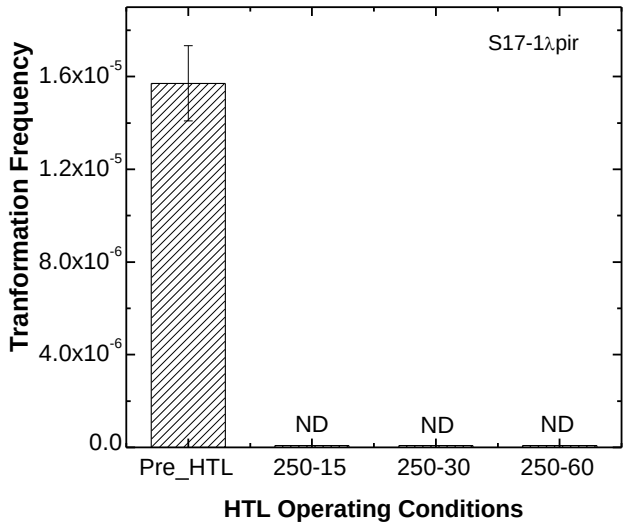


Figure 3.11 Electro-transformation of *E. coli* S17-1 λpir with plasmid DNA extracted from pre- and post HTL treatment of swine manure (ND=Not detected).

3.7 Natural Transformation of *Azotobacter vinelandii*

Figure 3.12 presents natural transformation frequencies of *A. vinelandii* where plasmid DNA extracted from pre-HTL *E. coli* culture was successfully transferred into *A. vinelandii* at the frequency of 7.1×10^{-6}. After HTL treatment, we did not observe any natural transformation, which represents more than a 98.6% reduction in the ability to transfer plasmid DNA with antibiotic-resistant genes from *E. coli* to *A. vinelandii*. It is also important to note that although the natural transformation frequency of *A. vinelandii* is two orders of magnitude lower than the electro-transformation frequency of *E. coli* S17-1 λpir, natural transformation still occurs at a detectable level. Therefore, it is beneficial to remove and/or deactivate the DNA in animal waste prior to releasing it to the environment. Our preliminary data with both electro-transformation and natural transformation consistently showed more than a 98.6% reduction in transformation frequencies after HTL treatment. Thus, we concluded that the genetic material (DNA) in biowastes can be significantly removed and completely deactivated by HTL treatment. As a result, the potential for transfer of antibiotic resistance from animal waste into the environment can be effectively eliminated during HTL conversion of animal waste to biocrude oil.

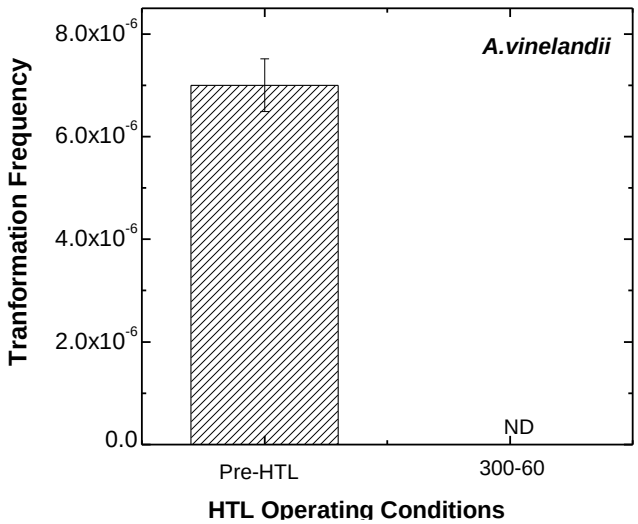
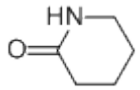
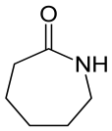
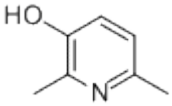
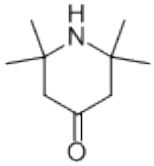
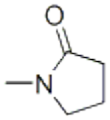
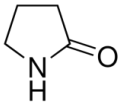
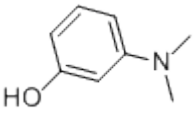
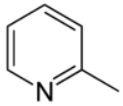


Figure 3.12 Natural transformation of *A. vinelandii* with plasmid DNA extracted from pre- and post-HTL treatment (ND=not detected).

3.8 Detection and Quantification of NOCs

The GC/NPD and GC/MS data collected for this study indicate that many classes of nitrogen compounds were present in the DCM extracts of HTL-WW samples resulting from liquefaction of *Spirulina* at 300°C with a 30 min retention time. Nine of these compounds showed relatively large chromatogram peaks, and are quantified in Table 3.1. Quantification of these nine NOCs showed a wide range of concentrations, from 139 mg/L (δ-Valerolactam) to 0.052 mg/L (2-Picoline). Prevalent peaks included δ-valerolactam followed by ε-caprolactam, 2,6-dimethyl-3-pyridinol, and 2,2,6,6-tetramethyl-4-piperidinone. Indole, pyrrole derivatives, and 3-amino-phenol have been reported previously in the HTL-WW resulting from liquefaction of the macro-alga *Laminaria saccharina* (Anastasakis and Ross, 2011). In other past research, GC/MS general scan analysis of wastewater from HTL conversion of *Enteromorpha prolifera* also showed the occurrence of 3-aminophenol, 2-piperidione, and 2-pyrrolidinone (Zhou et al., 2010). Other NOCs detected included acetamide, 2-aminophenol, propanamide, 3,6-dimethyl-2(1H)-pyridinone, 4-aminophenol, and N-methyl-acetamide. GC analysis of raw wastewater from coal gasification demonstrated that the majority of nitrogen heterocyclical compounds are pyridine and methyl-, dimethyl-, and ethyl-substituted pyridines. Other nitrogenous compound peaks noted in the literature include quinolone, acridine, benzoacridine, and methyl derivatives of these compounds (Gangwal, 1981). Although the chemical composition of HTL-WW was dependent on both the specific feedstock and the operating conditions (Anastasakis and Ross, 2011; Eager et al., 1981; Jena et al., 2011), we found that wastewater from HTL conversion of algal biomass most often contains amino-phenol, 2-piperidione, 2-pyrrolidinone, and pyridine. Information on HTL-WW characteristics remains limited mainly due to the difficulty of sample analysis, as it contains high levels of complex matrix effects. The systematic method for characterizing nitrogen-containing compounds in very complex matrices as presented in this study provides an effective analytical tool to characterize and quantify different types of HTL-WW.

Table 3.1 Characteristics of nitrogen-based compounds detected in HTL-WW generated from HTL conversion of *Spirulina* at 300°C and 30 min retention 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

3.9 Cytotoxicity of HTL-WW

3.9.1 Cytotoxicity of NOCs Detected in HTL-WW

A CHO chronic cytotoxicity analysis for the nine nitrogen-based compounds detected and quantified in HTL-WW is presented in Figure 3.13. This plot shows average toxicity data points for each concentration (8-16 independent clones). LC_{50} was calculated as the concentration that induced a 50% reduction of cell density as compared to the concurrent negative control, and the LC_{50} values are reported in Table 3.2. 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. The cytotoxicity index value was determined as $(10^3)*(LC_{50})^{-1}$, where a larger value represents a greater toxic potency (Figure 3.14).

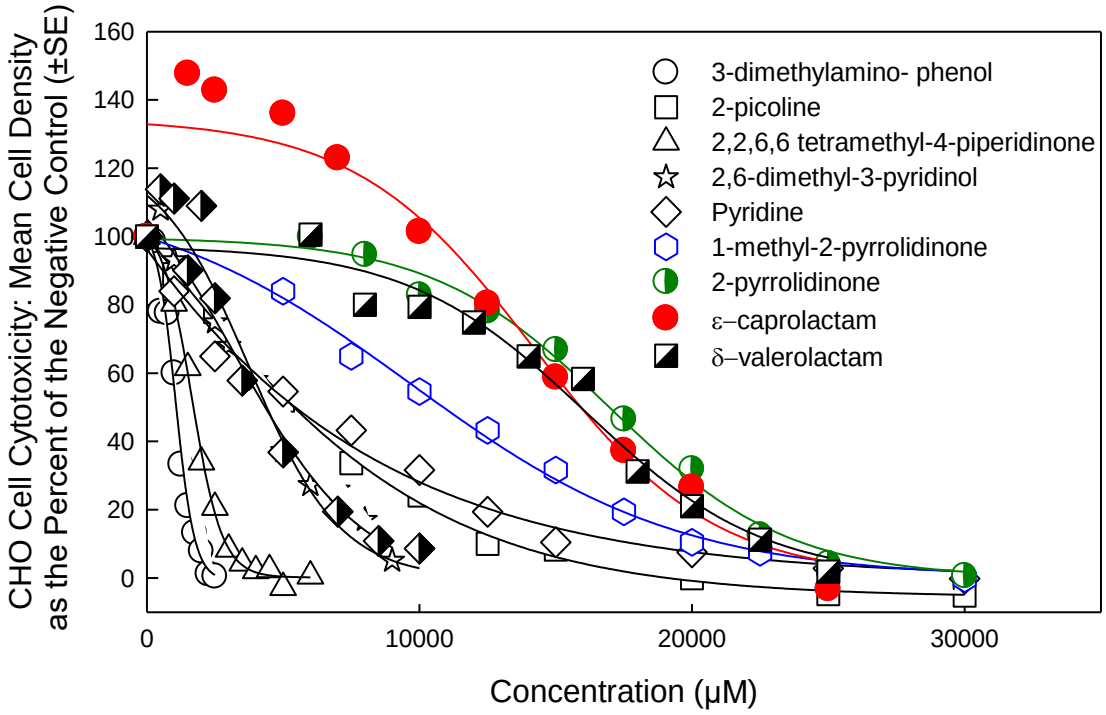


Figure 3.13 Comparison of the CHO cell cytotoxicity concentration-response curves for individual NOCs detected in HTL-WW.

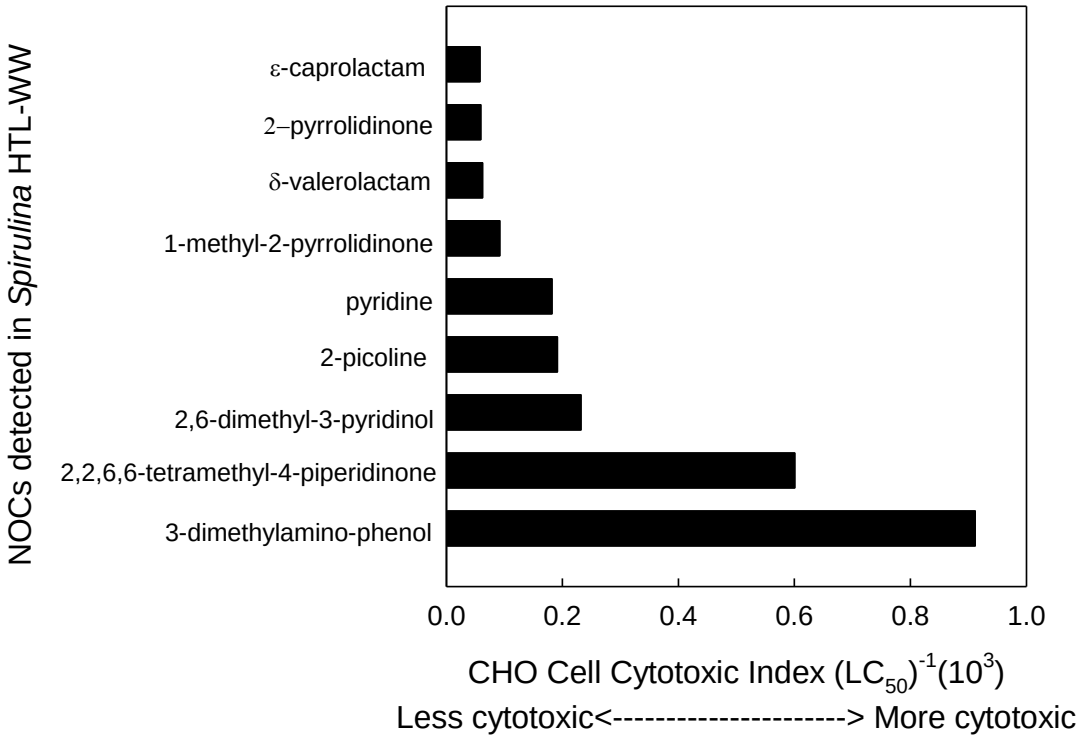


Figure 3.14 Comparison of the CHO cell cytotoxicity index values for NOCs detected in HTL-WW. Index values are expressed in dimensionless units.

CHO cell cytotoxicity responses varied significantly among the nine 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 nine 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 cells. 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 increases the resistance to retardation, decreases the solubility, and increases the toxicity of the compound. Heteroatom substitution into or onto the ring also alters both toxicity and solubility. Our data showed that the three most cytotoxic NOCs contained methyl groups and heteroatoms on their rings.

Table 3.2. Induction of chronic cytotoxicity in CHO cells by NOCs detected in *Spirulina* HTL-WW.

Nitrogenous organic compounds	Lowest cytotoxic conc. (μM)[a]	R^2 [b]	LC_{50}(μM) ± SE [c]	ANOVA test statistics
3-dimethylamino phenol	500	0.99	1100±11.9	$F_{10,77} = 124.11$; $P \leq 0.001$
2,2,6,6-tetramethyl-4-piperidinone	1000	0.99	1670±10.1	$F_{10,77} = 83.33$; $P \leq 0.001$
2,6-dimethyl-3-pyridinol	2500	0.99	4310±3	$F_{10,77} = 54.12$; $P \leq 0.001$
2-picoline	1000	0.99	5230±8.0	$F_{10,77} = 146.76$; $P \leq 0.001$
pyridine	1000	0.99	5500±8.0	$F_{10,77} = 111.66$; $P \leq 0.001$
1-methyl-2-pyrrolidinone	5000	0.98	10900±7.8	$F_{10,77} = 120.51$; $P \leq 0.001$
σ-valerolactam	8000	0.98	16100±11.9	$F_{10,77} = 58.53$; $P \leq 0.001$
2- pyrrolidinone	10000	0.99	16900±11.4	$F_{10,77} = 72.24$; $P \leq 0.001$
ε-caprolactam	12500	0.91	17300±12.0	$F_{10,77} = 130.91$; $P \leq 0.001$

[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 LC50 value (%C1/2 value) was calculated.
[c]The LC50 is the sample concentration that induced a cell density that was 50% of the negative control. The estimated SE of the LC50 was derived as the average SE of all the data points in the concentration-response curves. NOCs were listed according to their descending CHO cell cytotoxicity.

3.9.2 Cytotoxicity of Organic Extract from HTL-WW

Figure 3.15 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 the concentration factor compared with the original sample (i.e., 1 × = 100% HTL-WW), and the plotted data are the average of 8-16 independent clones plus or minus their standard error. These results show that the organic mixture in HTL-WW was highly cytotoxic to CHO cells (Figure 3.15). The LC_{50} value of raw *Spirulina* HTL-WW and swine manure HTL-WW was 0.075 and 0.074 times the concentration factor (or 7.5% and 7.4% HTL-WW), respectively, and full-strength HTL-WW would have induced a 100% reduction in CHO cell density. 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, causing chromosome aberrations and sister chromatid exchanges in human cells (Verschaeve and Kirschvolders, 1990). The compound 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 with sufficient evidence of carcinogenicity in humans and laboratory animals (Yardleyjones et al., 1991).

Although the acute toxicity threshold of several components in Elliot's list have been tabulated (Elliot, 1992), 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 are sufficient to prove that the organic mixtures 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 levels of toxicity in HTL wastewater.

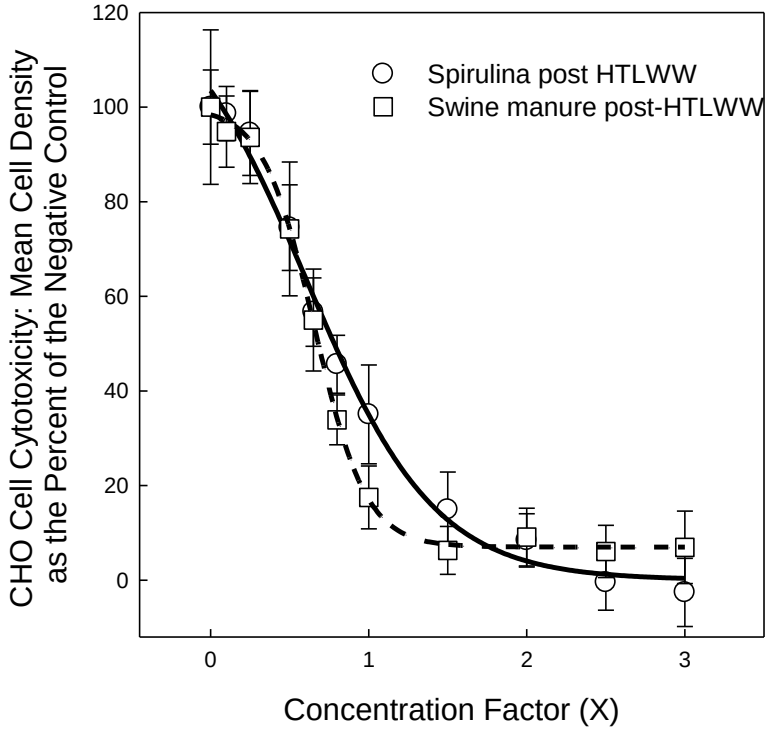


Figure 3.15 A comparison of cytotoxicity concentration response curves for HTL-WW generated from hydrothermal liquefaction (HTL) of *Spirulina* and swine manure.

3.10 Algal Growth Inhibition Effect of NOCs

Algal inhibition tests were conducted with seven of the NOCs detected in *Spirulina* HTL-WW and phenol, which was also detected in the HTL-WW. Two of the seven NOCs tested, ε-caprolactam and δ-valerolactam, did not show any algal inhibition effects at their maximum

solubility in BBM medium. Table 3.3 presents the inhibition data for phenol and the other five NOCs that had inhibitory effects on *C. protothecoides,* with all concentrations presented in ppm. In this table, the IC_{50} value was the concentration of tested NOCs that inhibits 50% of cell growth as compared with the concurrent negative control. The R^2 refers to the goodness of fit of the regression analysis from which the IC_{50} was calculated. The IC_{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 IC_{50} value was 3-dimethylamino-phenol > 2,6-dimethyl-3-pyridinol > methyl-2-pyrrolidinone > 2-pyrrolidinone > 2,2,6,6-tetramethyl-4-piperidinone.

A comparison of the relative inhibition effects of the NOCs analyzed in this study is presented in Figure 3.16. Our data were in agreement with the ecological information reported for several of the same compounds in the literature. For example, Scragg (2006) found that the initial growth of *Chlorella vulgaris* and *Chlorella* VT-1 was inhibited to varying degrees by 100-400 mg/l phenol. In the presence of 400 mg/L phenol, growth of *C. vulgaris* was inhibited, and growth of the more tolerant *Chlorella* VT-1 continued, albeit slowly. Klekbner and Kosaric (1992) reported that high concentrations of phenol (1000 mg/L) can be easily degraded by different algae (*Chlorella sp., Scenedesmus obliquus,* and *Spirulina maxima*). Megharaj et al. (1991) reported that p-aminophenol, which has a similar structure to 3-dimethylamino-phenol, inhibited growth of *C. vulgaris* at a concentration of 5-50 mg/L. Our data showed an IC_{50} value of 10.2 mg/L for 3-dimethylamino-phenol. Mann and Florence (1987) reported that the presence of 1-methyl-2-pyrrolidinone would enhance rather than inhibit growth of *Nitzschia closterium,* a common Great Barrier Reef diatom. Similarly, the Organization for Economic Cooperation and Development (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. prothecoides* at a concentration of 945 mg/L. Of all tested compounds, 3-dimethylamino-phenol and 2,6-dimethyl-3-pyridinol were the most toxic, with an IC_{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 caused inhibition effects on algal growth at concentrations much higher than their detected concentration in HTL-WW (Table 3.3). Although most of the individual nitrogen-based compounds did not inhibit algal growth, significant algal growth inhibition still occurred when more than 5% of HTL-WW was present in the algal culture medium (Zhou et al., 2011). This is because NOCs are only one class of compounds present in the HTL-WW with potential inhibitory effects. Thus, the observed inhibition effects might result from other types of compounds that have not yet been investigated or from synergistic interactions between multiple compounds that cause more inhibition than the individual compounds cause on their own.

Table 3.3 Algal inhibition analysis of nitrogen-based compounds detected in HTL-WW.

Nitrogen-based compounds	Range of significant concentration-response (ppm)	R^2 of regression analysis	IC_{50} (ppm)[a]	Detected Conc. in HTL-WW (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] Regression was used to determine the IC_{50} value from the inhibition concentration-response

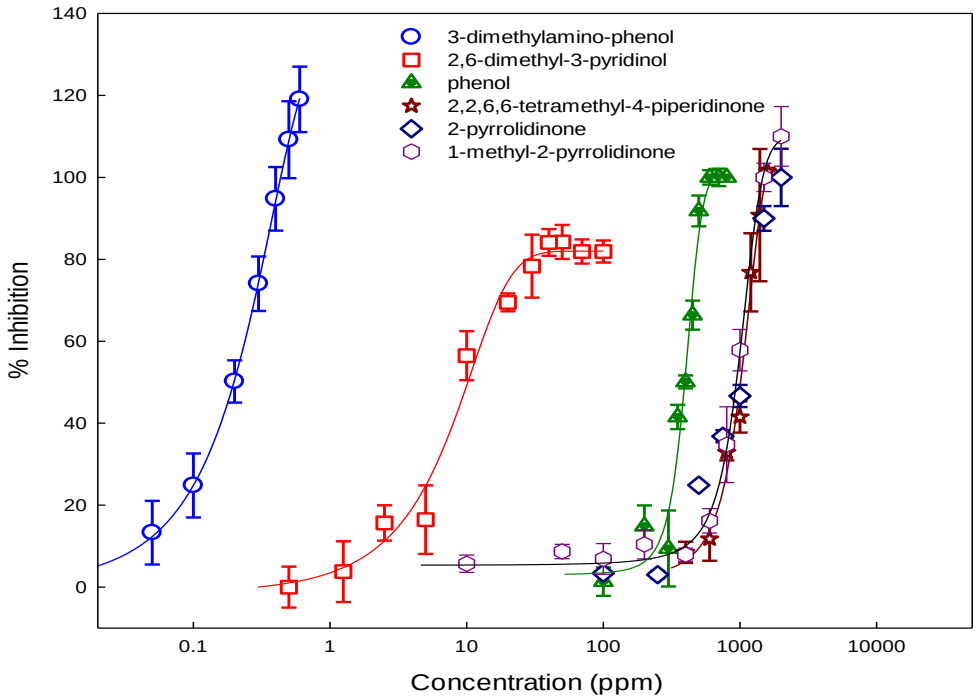


Figure 3.16 Comparison of the concentration response curves for the algal inhibition test of six nitrogen-based organic compounds.

3.11 Removal of NOCs via Batch-Algal Bioreactor

Figure 3.17 compares algal growth of *Chlorella protothecoides* in reactor 1 (BBM and NOCs) and reactor 3 (BBM only). The NOC concentrations dosed into reactor 1 were below the levels shown to cause algal inhibition individually in the tests reported earlier in section 3.10. The batch-algal bioreactor results indicate that algae was able to grow well in the presence of relatively low NOC concentrations (Figure 3.17). However, the density of algae in reactor 1 was much lower than in reactor 3 (negative control). This observation supports our hypothesis that algal growth can be affected by synergistic effects between multiple organic compounds in HTL-WW because none of these compounds caused algal inhibition individually at the concentrations used in this test.

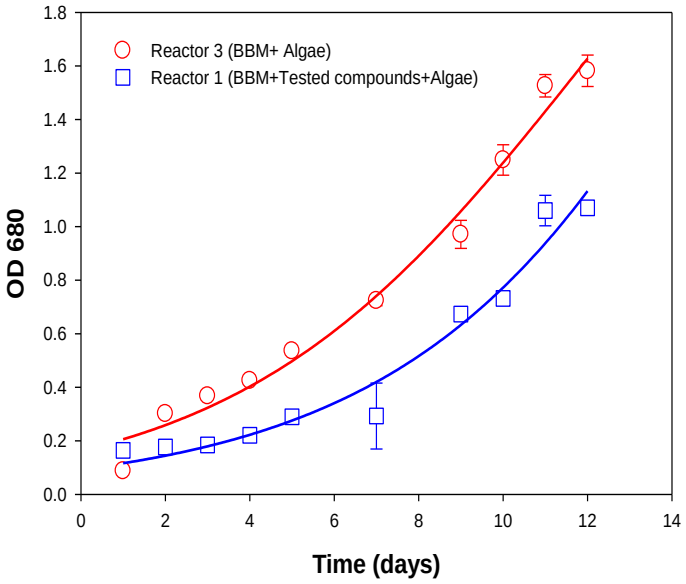


Figure 3.17 Comparison of algal growth in BBM medium (Reactor 3; open circles) and BBM medium spiked with nitrogen-based compounds (Reactor 1; open squares).

Figure 3.18 illustrates the removal of phenol and several nitrogenous organic compounds (NOCs) detected in *Spirulina* HTL-WW by the algal bioreactor run in a batch mode. Of the tested compounds, phenol and 2,6 dimethyl-3-pyridinol showed the fastest removal after one day of algal treatment (98.8% and 100%, respectively). ε-caprolactam showed the least after one day of algal treatment. However, all tested compounds achieved nearly 100% removal after seven days of treatment, and removal to below detection limits was observed in the algal bioreactor after 14 days of treatment. Based on the difference in removal rates of tested NOCs, we hypothesized that some NOCs, including 1-methyl-2-pyrrolidinone and 2,2,6,6-tetramethyl-4-piperidinone, are more favorable than others for uptake and incorporation in algal biomass. Once these compounds were used up, the algae utilized the rest of the NOCs available in the medium.

These data also agree with the data presented in Figure 3.16, which showed that 1-methyl-2 pyrrolidinone, 2-pyrrolidinone, and 2,2,6,6 tetramethyl-4- piperidinone were the least inhibitory NOCs to algal growth. The data presented here confirm that algae can effectively remove NOCs in HTL-WW and use them as nutrients for their growth. All in all, these data showed that an algal bioreactor is an advantageous component of integrated systems for wastewater treatment and bioenergy production, as shown in Figure 1.1, because it can simultaneously produce HTL biofuel feedstock and improve effluent water quality.

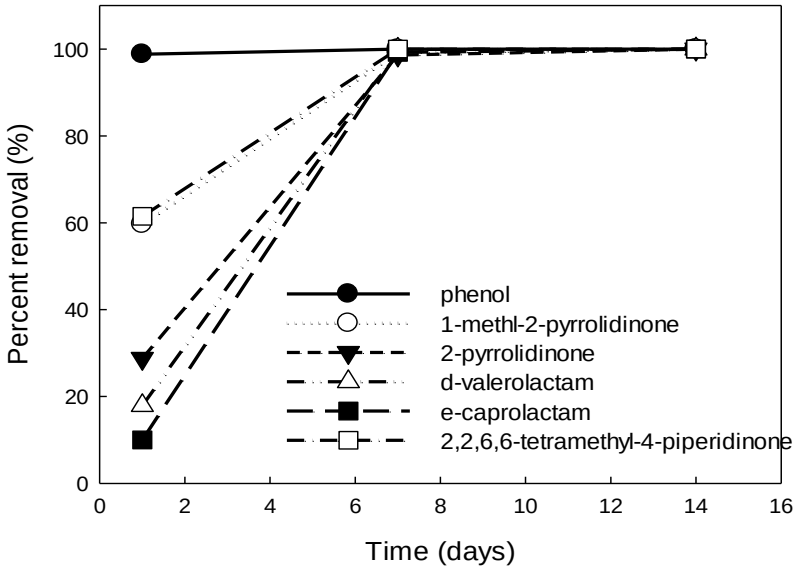


Figure 3.18 Removal of nitrogenous organic compounds by batch-algal bioreactor.

3.12 Reduction of HTL-WW Toxicity via Sequencing Batch-Algal Bioreactor

Figure 3.19 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 two weeks. This figure shows continuous growth of algal biomass, quantified as optical density at 680 nm (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 OD_{680} over time in Figure 3.19 reflects increasing biomass, and shows that algae and bacteria can successfully utilize the organic compounds and nutrients in HTL-WW. These data agree with previous batch studies in our research group showing that algal growth was enhanced by the addition of HTL-WW at less than 5% of the growth medium (Zhou et al., 2010). Other previous research has shown that HTL-WW contains a significant amount of small-molecule breakdown products of biomass macromolecules (Anastasakis and Ross, 2011; Appleford, 2004), and these small molecules are more favorable for algal uptake (Neilson and

Lewin, 1974). Our data showed that approximately half of the COD was removed during algal bioreactor treatment.

The algal-treated HTL-WW was subjected to extraction of the remaining organics, which were tested for toxicity with the same CHO test described earlier. As presented in Figure 3.20, the concentration response curve of algal-treated HTL-WW was shifted to the right of the untreated HTL-WW curve. This shift corresponds to an increased LC_{50} value for algal-treated HTL-WW to a concentration factor of 0.113 × (11.3% HTL-WW). In this case, algae used some of the organic compounds in HTL-WW as their carbon or energy source and reduced the induced toxicity by 40%. Algal-treated HTL-WW, however, is still very toxic and would likely require further treatment before it can be released into the environment.

After treatment with an algal bioreactor, the HTL-WW was further treated by passing it through a GAC column. Figure 3.20 compares the concentration-response curves for *Spirulina* HTL-WW before and after treatment with an algal bioreactor and GAC. Two different EBCTs were used, 5 min and 20 minutes, which correspond to an increasing level of GAC treatment. The LC_{50} value of GAC-treated HTL-WW increased as EBCT was increased (Figure 3.19). 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 might be achieved by increasing the amount of GAC used per volume of treated HTL-WW. Figure 3.21 presents the cytotoxicity index values of HTL-WW before and after the algal bioreactor and GAC treatment. The cytotoxicity index value of HTL-WW was reduced 40% after algal bioreactor treatment and 92.5% after subsequent GAC treatment (Figure 3.21). Further investigation of the GAC system design, performance, and cost is needed to provide the optimum removal of HTL-WW toxicity and determine the economic feasibility.

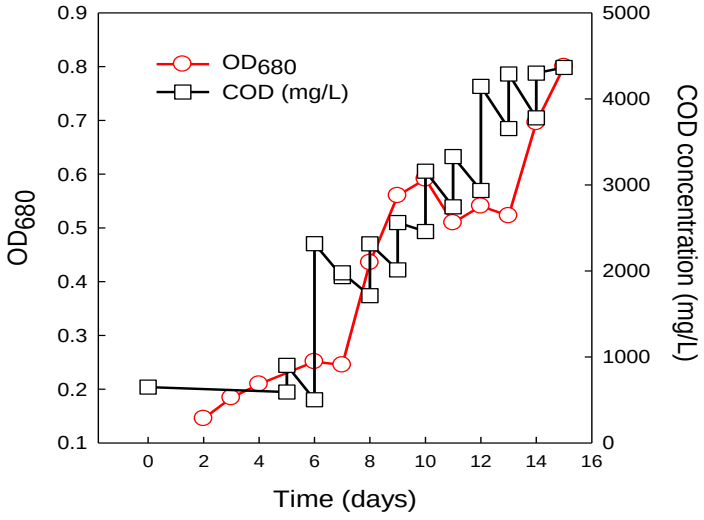


Figure 3.19 Removal of organic pollutants in 10% post-HTL wastewater from *Spirulina* by semi-batch-algal bioreactor.

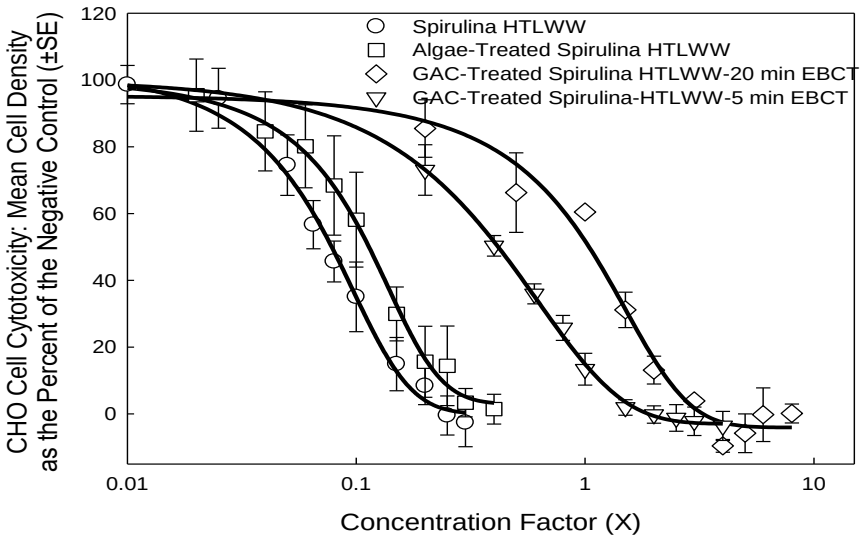


Figure 3.20 CHO cytotoxicity concentration response curves for organic extract from HTL-WW generated from hydrothermal liquefaction of *Spirulina* before and after treatment with algal bioreactor and GAC.

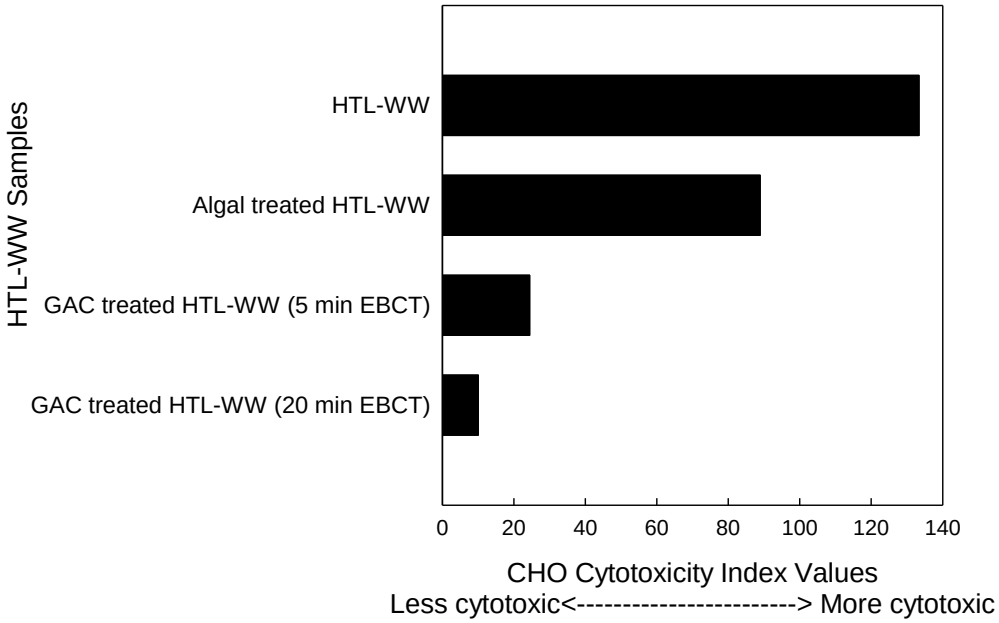


Figure 3.21 Comparison of the CHO cell cytotoxicity index values for HTL-WW before and after treatment with algal bioreactor and GAC. Index values are expressed in dimensionless units.

CHAPTER 4: CONCLUSIONS

4.1 Effect of HTL Operating Conditions on the Breakdown 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-resistant 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 various biowastes, such as animal manure, 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 breakdown of DNA in biowaste. However, at an operating temperature of 300°C and above, the effect of extending retention time was insignificant. Because 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 biowaste are likely to be well removed. The complex organic matrix of HTL feedstocks did provide some protective effect for genetic materials in biowastes during HTL treatment. However, this protection could be reduced by extending the HTL retention time and/or increasing the operating temperature. Although there was a certain amount of DNA 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 experiments with post-HTL DNA consistently showed more than a 98.6% reduction in transformation frequencies. All of the HTL-treated samples had no detectable transfer of antibiotic resistance, suggesting that the DNA was completely deactivated by HTL treatment. Thus, we conclude that HTL treatment can effectively deactivate genetic materials in bio-wastes and prevent the potential of transferring antibiotic-resistant materials from bio-wastes into the environment.

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

4.2 Chemical and Biological Characterization of HTL Wastewater

We identified nine specific NOCs found to be prevalent in HTL-WW. CHO chronic cytotoxicity assays showed that none of the nine tested NOCs in HTL-WW were cytotoxic to mammalian CHO cells at their detected concentrations. However, some of these NOCs were found to at least partially inhibit algal growth. Specifically, 3-dimethylamino phenol and 2,6-dimethyl-3-pyridiniol caused at least 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 generally more toxic to mammalian cells and caused more algal inhibition than those without them. Comparison of LC_{50} and IC_{50} values of the same NOCs indicated that LC_{50} values were consistently higher than IC_{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. Due to the fact that 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 toxicity or growth inhibition for various living organisms. Thus, it is important to ensure that HTL-WW is treated before releasing it into the environment.

4.3 Mitigation of Aqueous Toxicants Produced During the HTL Conversion of Algae to Biofuels via HTL with Algal Bioreactor and Adsorptive Treatments

The feasibility of using batch and semi-batch algal bioreactors to remove NOCs and reduce the toxicity of HTL-WW was demonstrated in several experiments. More than 99.6% removal of NOCs was obtained after seven days of operating the algal bioreactor. A 40% reduction in the toxicity of organics extracted from HTL-WW was obtained with the algal bioreactor treatment. Subsequent treatment with GAC provided up to 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 (See Figure 1.1). However, it also highlights that significant toxicity remains after algal bioreactor treatment and that GAC treatment 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 the HTL process to generate additional biocrude oil. Therefore, this novel treatment system offers several advantages for reducing the potential toxicity risks associated with the byproducts of HTL bioenergy production and improving wastewater effluent quality.

To conclude, this study enhanced our knowledge and capabilities for producing bioenergy from wastewater. The HTL process can effectively convert biowaste into valuable biocrude oil while destroying emerging bioactive 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 biocrude oil and providing environmental benefits.

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