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Wastewater Nutrient Recovery via Fungal and Nitrifying Bacteria Treatment

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Abstract: In efforts to reduce the consumption of fossil fuels and promote recycling biowaste, there is an interest in the production of post-hydrothermal liquefaction wastewater (HTL-AP) from the hydrothermal liquefaction (HTL) process that converts wet biomass into biocrude oil. This study explores ways of transforming potentially toxic HTL-AP into a fertilizer source for hydroponic cropping systems. This study specifically investigates the integration of the white-rot fungus *Trametes versicolor* with nitrifying bacteria (*Nitrosomonas* and *Nitrobacter*) to convert the organic nitrogen compounds into inorganic nitrogen while also producing the enzyme laccase, which has been shown to remove toxic compounds. This study aims to increase the concentration of nitrate-N to valorize wastewater as a suitable fertilizer by measuring several parameters, including laccase activity, pH, nitrate-N, and ammonia/ammonium-N concentrations, and analyzes interactions to optimize the conversion process. The data support the claim that the simultaneous inoculation of *T. versicolor* and nitrifying bacteria significantly increases nitrate-N concentrations in HTL-AP, as it increased by 17 times, or an increase of 32.69 mg/L. In addition, HTL-AP treated with *T. versicolor* and nitrifying bacteria reduced the treatment time by 120 h, highlighting a reduction in personnel time and energy consumption. Therefore, this research accentuates sustainability through fungal and bacterial treatments to develop eco-friendly hydroponic fertilizers. Future research should explore the potential of utilizing the combination of *T. versicolor* and nitrifying bacteria for the treatment of other industrial wastewaters.

Keywords: hydrothermal liquefaction aqueous phase; wastewater; hydroponics; nitrifying bacteria; *Trametes versicolor*

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1. Introduction

Wastewater treatment continues to be an environmental challenge since 80 percent of wastewater in the world flows back into the ecosystem without being treated or reused [1,2]. In addition, with a global effort to reduce reliance on fossil fuels and recycle biowaste, there is an emerging interest in the production of biocrude via processes like hydrothermal liquefaction (HTL), which also generates a greater quantity of the aqueous product called hydrothermal liquefaction aqueous phase (HTL-AP). This is due to the process of HTL, which is the thermal depolymerization of wet biomass under high temperatures and pressures to produce biocrude oil [3,4]. Biocrude is a concentrated, synthetic oil that can be substituted for petroleum crude oil once it has completed its final conversion into renewable gasoline and diesel [4]. There are various biomass feedstocks for HTL that range from biowaste (manure and food processing waste) to industrial processing wastes (wastewater treatment sludge) [4]. The Environmental Protection Agency [5] estimates that 106 million tons of wasted food was generated from the food retail and food and manufacturing sectors in the United States in 2019. Therefore, HTL has become a main asset in decreasing gas emissions and waste, both of which are leading factors in global warming. The byproduct, HTL-AP, is unique in that it may contain heavy metals, depending

on the feedstock source, and high concentrations of nutrients (e.g., nitrogen-containing organic and aromatic compounds) that must be treated before discharge or reuse [6]. More specifically, the process of high temperature and pressure eradicates pathogens and allows for plant nutrients like nitrogen to pass on [7]. However, it must be acknowledged that the nitrogen is in organic forms and, therefore, not accessible to plants. Crops consume nitrogen as nutrients only when it is in an ion state (e.g., ammonium and nitrate-N) [8]. Therefore, an extra step to transform organic nitrogen into inorganic nitrogen must take place. This implementation will make HTL-AP a high potential for food crop production in hydroponic systems, valorizing the HTL overall process [3,8].

The demand for an efficient strategy to treat wastewater has increased as it is the most common byproduct of industrial processes and requires large sums of energy [9]. HTL has been shown to be a promising process for the generation of biocrude oil, hydrochar solids, and CO_2-rich gas [6]. Shen et al. [6] studied how the microbial electrolysis cell (MEC) is an effective approach in treating HTL-AP by removing both organics and nitrogen while still generating a high rate of hydrogen to be recovered and used for onsite HTL biocrude upgrading. This method of microbial electrolysis has attracted attention to its use in biotechnological processes as it employs anaerobic bacteria to an generate electrical current from organic waste [10]. Similarly, Satinover et al. [11] explored the conversion of residual organics into hydrogen and the removal of ammonia using MEC. Satinover was able to develop a circular biofuel production system utilizing the MEC effluent to produce the same microalgal strain [11]. HTL-AP biological treatment has also been explored for its cost-effectiveness and accessibility. Goswami et al. [12] studied the co-cultivation of microalgae strains DBWC2 and DBWC7 and the bacteria strains ORWB1 and ORWB3 as an integrated system. The study demonstrated a sustainable process to produce biocrude oil through the HTL process as there was a high removal efficiency for nitrogen, COD, and phosphate [12]. Xu et al. [13] introduced five Gram-positive strains and five Gram-negative strains of bacteria to mimic a typical pathogenic microorganism in four different HTL feedstocks and discovered that all samples exhibited antibacterial characteristics and mechanisms. The results from this study support previous experimental results that Gram-positive indicators are more sensitive than Gram-negative one to HTL-AP toxicity, which promotes *Nitrosomonas* and *Nitrobacter* as prime candidates for treating HTL-AP as they are Gram-negative bacteria. Chen et al. [14] evaluated the biocrude oil yield and the nitrogen recovery when cultivating a mixed-culture algal biomass. It is evident that microorganisms may flourish and transform compounds within HTL-AP based on the research mentioned.

Nitrate-N is the most optimal fertilizer for food crop production in hydroponic systems. HTL-AP is known to contain heavy metals and organic molecules, which is why biological treatment, more specifically fungal treatment, has high potential for this wastewater. The literature suggests that there is advanced technology that is costly and produces hazardous waste when treating wastewater. However, a sustainable approach for contaminated water is mycoremediation because it is cost effective and utilizes fungi as an agent to treat heavy metals due to its high adsorption and heavy metal tolerance [15]. There are various mechanisms in fungi to remove heavy metals such as enzymatic detoxification, exclusion via a permeability barrier, adsorption on extracellular structures, efflux pumps, and methylation [16]. Various strains of microorganisms, specifically fungi, can produce active laccases, which are polyphenol oxidases that catalyze the oxidation of various aromatic compounds [17]. Furthermore, the enzymes excreted are capable of degrading aromatic derivatives; white-rot fungus can produce ammonium/ammonia through ammonification by intaking organic nitrogen compounds [8]. The presence of ammonium allows for the possibility of nitrification to occur. The biochemistry of nitrification consists of a biological process in which nitrite and nitrate ions are generated from ammonium [18]. The integration of mycoremediation and bioremediation appears to have the potential to deplete the toxic environment while converting organic nitrogen into inorganic nitrogen, particularly nitrate, transforming this waste stream into a fertilizer for hydroponic systems.

Previous studies have reported the efficiency of treating wastewater using fungal species; the most investigated species has been white-rot fungi. This strain has been heavily implemented as an alternative treatment due to its capacity to transform compounds and tolerate toxins through its versatile enzymatic machinery [19]. The white-rot fungi strain, *Trametes versicolor*, has been studied extensively and reported to produce more than 20 times the laccase activity of other strains when under adverse conditions [19]. The high rates of extracellular enzymes produced by *T. versicolor* have made this strain an attractive candidate to treat HTL-AP and generate ammonium/ammonia. In order for HTL-AP to be used as an alternative hydroponic nutrient solution, it is necessary for the organic forms of nitrogen to be converted to ammonia, which can then be converted to nitrate; this has been previously accomplished by nitrifying bacteria [20]. The most studied nitrifying bacteria consist of *Nitrosomonas* and *Nitrobacter* since the other reported genera of nitrifying bacteria have not been well characterized and many of them have been unable to carry out nitrification successfully [18]. The nitrification process is initiated by *Nitrosomonas* as it oxidizes ammonium and is followed by the oxidation of nitrite to nitrate by *Nitrobacter* [21]. Therefore, this study aims to deplete the toxic environment and increase inorganic nitrogen in HTL-AP, with the overall goal of recycling this water as a fertilizer for hydroponic systems through the treatment of HTL-AP using co-cultivation of *T. versicolor*, as well as *Nitrosomonas* and *Nitrobacter*.

2. Materials and Methods

2.1. Hydrothermal Liquefaction Aqueous Phase (HTL-AP)

The HTL-AP utilized in this study was from the hydrothermal liquefaction process of food waste (240 °C to 280 °C, 1600 psi to 1800 psi, and 0.14 gpm to 0.18 gpm) and was supplied by the Environment-Enhancing (E2-E) Laboratory of the University of Illinois at Urbana-Champaign. The wastewater was kept in storage until use at 4 °C. For this study, HTL-AP was diluted with deionized water to create a 5% mixture and was stored at 4 °C. Jesse and Davidson [7] tested physical treatment methods of several mixtures of HTL-AP, including 2.5% and 5%. They went on to discover that lettuce was capable of growing, to some extent, in a 2.5% mixture, but they did not test lettuce growth using the 5% mixture. Therefore, the 5% mixture was chosen in this study as it aligns closely with the approach used by Jesse et al. [3] while making an incremental advancement in the concentration of HTL-AP that may be used for future use in hydroponic cropping systems. For consistency across experiments, all HTL-AP used for the samples in this study came from the same batch. The characterization of the raw 5% HTL-AP is described in Table 1.

Table 1. HTL-AP characteristics. NH_3/NH_4^+-N: ammonia/ammonium-nitrogen, pH, NO_3-N: nitrate-nitrogen, and Lacc: laccase activity.

HTL-AP	NH_3/NH_4^+-N (mg/L)	pH	NO_3-N (mg/L)	Lacc (U/L)
5%	4.41 ± 0.11	4.33 ± 0.10	2.07 ± 0.14	−0.012 ± 0.002

2.2. Fungal Strain

The most effective fungal treatment executed in wastewater is the implementation of white-root fungi due to their ability to transform compounds via enzymatic machinery [19]. According to Leme [8], *Trametes versicolor* is the most promising strain in transforming wastewater, hence its use in this experiment. *T. versicolor* was provided by the Miller Mycology Lab—Illinois Natural History Survey at the University of Illinois at Urbana-Champaign. The cultivation of this fungal species required a 5-day process in which the fungi were frequently cultivated in sterile potato dextrose agar (PDA) medium at 28 °C and kept at 4 °C until further use. The PDA was prepared and sterilized via an autoclave at 15 psi and 121 °C for 15 min. The conditions of the autoclave were consistent throughout the

duration of the experiment and will be referenced through its parameters (15 psi, 121 °C, 15 min).

2.3. Mycelial Suspension

The mycelial network is the root-like structure of a fungus that consists of branching hyphae and are cultivated to obtain pellets via an orbital shaker. The preparation of the mycelial suspension was completed following previously reported procedures with few alterations [22]. A total of two 250 mL Erlenmeyer flasks filled with 75 mL sterile malt extract medium (ME, 2% w/v, 15 psi, 121 °C, 15 min) were inoculated with two fungal plugs (1 cm diameter) in each from the growing region on the PDA dish. Each flask was then sealed using a gauze filter and a second layer of KC100 sterilization wrap. The previous steps were executed in a biosafety cabinet and the sterile technique was implemented. The flasks were then incubated in an orbital shaker for four days at 22 ± 1 °C and 135 rpm, to allow for aeration to take place through convection. The now-larger fungal plugs were rinsed with sterile deionized water (15 psi, 121 °C, 15 min) and submerged in sterile 0.85% NaCl (15 psi, 121 °C, 15 min) for storage purposes, within which they remained active and lost their morphology. The final step included utilizing a bead beater homogenizer through the addition of 6 matrix M beads (MP biomedicals; Irvin, CA, USA; 60 s at 6.5 m/s) to suspend the mycelial plugs. A visualization of this process may be observed in Figure 1. Once again, the mycelial suspension was stored at 4 °C until further use.

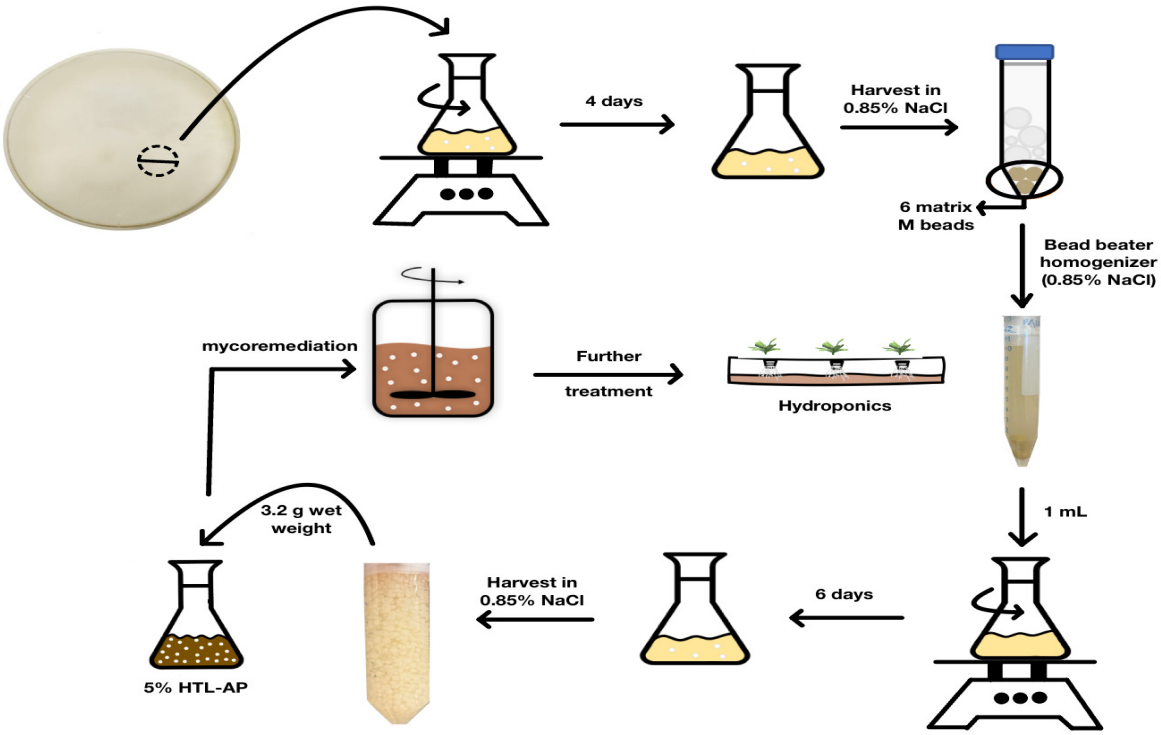

Figure 1. A visual depiction of the process of transforming mycelial suspension and the generation of fungal pellets.

2.4. Fungal Pellets Production

To produce the fungal pellets, the methodology of Blánques et al. [23] was implemented with some alterations. A total of eight 1 L Erlenmeyer flasks filled with 250 mL of sterile malt extract medium (2% w/v) were inoculated with 1 mL of the mycelial suspension. Similar to previous procedures, the flasks were place in an incubator at 22 ± 1 °C and 135 rpm for a total of six days. The inoculation of fungal pellets has been shown to increase the release of ammonia/ammonium in HTL-AP and prevent dispersed mycelium from growing over bioreactor walls, which impacts mixing and broth oxygenation [8]. Using

the bead beater homogenizer, as viewed in Figure 1, provided fungal pellets smaller in diameter. The increased quantity of smaller fungal pellets increased convection and the chance for ammonia/ammonium to be released. However, although fungal pellets allow for easier fungal biomass separation from liquid sources compared to other shapes, the smaller pellets are more challenging to remove than larger pellets. The following procedure was executed to properly conserve the *T. versicolor* until further use. In a biosafety cabinet and via the use of the sterile technique, the pellets were transferred aseptically to 50 mL centrifuge tubes after being washed twice with deionized water. Each centrifuge tube was filled with a sterile saline solution (0.85% NaCl) for storage.

2.5. Wastewater Nitrifying Experiment

This study was conducted using 250 mL flasks in which each flask had 50 mL of 5% sterile HTL-AP (15 psi, 121 °C, 15 min). To ensure sterility, some samples were inoculated with 3.2 g (6.4% *w*/*v*) of wet fungal pellets (TV-5HTL-AP) in the biosafety cabinet and had a cultivation time of 72 h in an incubator at 28 °C and 135 rpm. Other samples contained 190 µL ATM Aquarium Products Colony Nitrifying Bacteria (ATM Aquarium Products, Las Vegas, NV, USA), which consisted of the strains *Nitrosomonas* and *Nitrobacter*. The value, 190 µL, was determined from the label recommendations of the product. The addition of nitrifying bacteria was completed within a biosafety cabinet to ensure a sterile transfer; however, it must be acknowledged that the product itself was not sterile since it is an off-the-shelf product. The functionality of this product has been shown by Jesse et al. [7], where nitrification by the nitrifying bacteria was observed in HTL-AP. In addition, various combinations of treatments were also tested, such as B+TV-5HTL-AP, TV->B-5HTL-AP, and B-TV-5HTL-AP (Table 2), to discover which treatment scheme would generate the highest concentration of inorganic nitrogen. The incubator orbital shaker was kept at a constant temperature and speed, which was 28 °C and 135 rpm, respectively, to eradicate any external variables. Negative controls were produced (5HTL-AP) for future comparison analysis with the treated samples. Once the incubation times were completed, each sample was filtered (0.45 µm) and then stored at 4 °C until analyzed. Three trials were implemented for each method (Table 2).

Table 2. Description and label of each treatment executed in this study. The mention of *T. versicolor* and *Nitrosomonas*/*Nitrobacter* refers to the addition of 3.2 g (6.4% *w*/*v*) and 190 µL, respectively. The orbital shaker operated at 28 °C and 135 rpm.

Sample Label	Details of Treatment
5HTL-AP	HTL-AP was diluted with deionized water to create a 5% mixture.
TV-5HTL-AP	5HTL-AP was inoculated with *T. versicolor* and had a cultivation time of 72 h in an orbital shaker.
B+TV-5HTL-AP	*T. versicolor* and *Nitrosomonas*/*Nitrobacter* were introduced to the 5HTL-AP at the same time and incubated for 72 h in the orbital shaker.
B-TV-5HTL-AP	5HTL-AP was inoculated with *T. versicolor* and had a cultivation time of 72 h in an orbital shaker; it was then filtered and *Nitrosomonas*/*Nitrobacter* were introduced. The sample was then placed in the orbital shaker for another 5 days.
TV->B-5HTL-AP	*T. versicolor* was cultivated in 5HTL-AP for 48 h in the orbital shaker, and then *Nitrosomonas*/*Nitrobacter* were introduced and placed back into the orbital shaker for another 24 h.
B-5HTL-AP	Addition of *Nitrosomonas*/*Nitrobacter* in 5HTL-AP and incubated in the orbital shaker for 72 h.

2.6. Wastewater Characterization

2.6.1. Nutrient Analysis

The concentrations of ammonia/ammonium-nitrogen (NH_3/NH_4^+-N) and nitrate-nitrogen (NO_3-N) were measured using Hach methods 8038 and 8039, respectively. To achieve an accurate analysis, at least a quintuplicate of each measurement was collected. This collection of data was made possible using the Hach DR/2010 spectrophotometer (Loveland, CO, USA). The average and standard deviations for each analysis were calculated.

2.6.2. Enzyme Assay

Laccase activity in the samples was assessed using the methodologies of Majcherzyk et al. [24] and Boujeblen et al. [25]. The procedure consisted of the addition of the samples to a solution of 1 mM 2,2-azino-bis-[3-ethylthiazoline-6-sulfonate] in 0.1 M Na-tartrate buffer at a pH value of 4.5. The solution mixture was then poured into a 1 cm pathlength cuvette and analyzed at 510 nm of absorbance. The unit of the values collected for the laccase activity was defined as the rate at which 1 µmol of ABTS was oxidized per minute (1 U = 1 µmol ABTS oxidized min^{-1}, ε 420 = 36,000 M^{-1} cm) [25].

2.6.3. Statistical Analysis

An analysis of variance (ANOVA) was used to validate that each treatment was unique and would produce values significantly different from one another. ANOVA is a type of hypothesis test that compares the means of variables in two or more independent population groups [26]. Particularly, a one-tailed test was employed to calculate the possibility of deviation from the null hypothesis, in which the null hypothesis assumes there is no difference between the number of interventions—in this case, the five sample means of each treatment [27]. Specifically, the post hoc Tukey Honestly Significant Difference (HSD) test was used to compare each treatment to one another. The Tukey–Kramer test was implemented when the sample sizes were unequal.

3. Results and Discussion

The main objective of this study was to increase the concentration of nitrate-N (NO_3-N) in HTL-AP through the application of fungal treatment and nitrifying bacteria. Briefly, biological nitrification of ammonia occurs in two distinct steps—oxidation of ammonia to nitrite and nitrite to nitrate, via the activities of *Nitrosomonas* and *Nitrobacter* bacteria, respectively [3]. This reaction can be described as follows:

$$2\,NH_4^+ + 3\,O_2 + \textit{Nitrosomonas} \rightarrow 2\,NO_2^- + 4\,H^+ + 2\,H_2O$$

$$2\,NO_2^- + O_2 + \textit{Nitrobacter} \rightarrow 2\,NO_3^-$$

For nitrification to occur it is vital for ammonium to be present. As is known, HTL-AP contains organic compounds including amino groups (NH_2). *Trametes versicolor* is capable of secreting enzymes to perform ammonification within this wastewater [17]. Ammonification refers to the chemical reaction in which amino groups that are associated with organic forms of nitrogen are converted into ammonia (NH_3) or ammonium (NH_4^+-N) [23]. The final product will act as a substrate for the nitrification processes. The basic chemical equation of the process can be described as follows:

$$R\text{-}NH_2 + H_2O \rightarrow NH_3 + R\text{-}OH$$

$$NH_3 + H_2O \rightarrow NH_4^+ + OH^-$$

These processes are vital to hydroponic systems since plants will only uptake nitrogen as nutrients if it is in an ion state, i.e., NH_4^+ and NO_3^-. The literature states that the byproduct, HTL-AP, does not contain pathogens but instead vital nutrients needed for crop growth, but it may contain heavy metals [3]. The inoculation of *T. versicolor* was intended to reduce the toxic environment while increasing ammonium in HTL-AP. This fungal mycelium has

the capacity to excrete laccase enzymes to diminish a range of organic nitrogen pollutants and resolve heavy metals through sorption [10]. These laccases are polyphenol oxidases that may act as catalysts in the oxidation of various aromatic compounds such as phenols (-OH), which are often a part of the molecular structure of heavy metals. Specifically, fungal laccases use molecular oxygen as an electron acceptor and have been observed to catalyze the oxidation of pharmaceuticals and biocides for several substances, such as endocrine compounds, anti-inflammatory drugs, antibiotics, and various halogenate pesticides [22]. An increase in laccase activity provides a biocatalyst to promote the biodegradation of micropollutants in wastewater in a complementary treatment step because of their sole need for oxygen as the co-substrate and their wide range of substrates. Concurrently, it can take in small organic nitrogen compounds and return their excess as ammonium via ammonification. The availability of inorganic nitrogen will provide the nitrifying bacteria with a stable foundation to perform oxidation and produce nitrate, which is the most optimal form of nutrients within hydroponic systems. Table 3 shows a comparison of the different treatments based on pH and laccase activity, as well as their corresponding NH_3/NH_4^+-N and NO_3-N concentrations after treatment.

Table 3. NO_3-N, NH_3/NH_4^+-N concentrations, laccase activity, and pH values along with their respective Tukey HSD statistical analysis values.

Sample Name	NO_3-N	NH_3/NH_4^+-N	pH	Laccase Activity
	mg/L Tukey HSD	mg/L Tukey HSD	Tukey HSD	U/L Tukey HSD
5HTL-AP	2.07 ± 0.14 a	4.40 ± 0.10 a	4.33 ± 0.10 a	−0.012 ± 0.00 a
TV-5HTL-AP	16.04 ± 1.49 b	21.80 ± 1.60 b	6.12 ± 0.42 b	2.251 ± 0.316 b
B+TV-5HTL-AP	34.76 ± 2.14 c,d	25.00 ± 1.50 b	6.95 ± 0.26 b,c	4.419 ± 0.212 c
B-TV-5HTL-AP	27.87 ± 3.20 d,e	26.00 ± 7.70 b	7.53 ± 0.14 c	*
TV->B-5HTL-AP	22.58 ± 3.71 b,e	21.00 ± 9.40 b	6.74 ± 0.73 b,c	2.342 ± 0.535 b
B-5HTL-AP	4.27 ± 0.93 a	1.30 ± 0.50 a	4.24 ± 0.09 a	−0.011 ± 0.004 a

Note: Mean values that are not significantly different ($p > 0.05$) are followed by the same letter. Each letter is unique to its measurement and should not be compared to other columns. * The laccase activity was not measured because debris was observed in this sample, which could cause an obstruction in analyzing enzyme activity using spectrophotometry.

In a similar experiment, Jesse et al. [7] also induced nitrification in an untreated 5% HTL-AP mixture and observed a 1.75 mg/L increase in the nitrate-N concentration. The miniscule change is most likely due to the acidic conditions of untreated HTL-AP. These two circumstances further imply the success of combining mycoremediation to create a viable environment for bioremediation to take place. The most prominent sample was B+TV-5HTL-AP, in which all organisms were inoculated into the wastewater at once with a nitrate-N increase of 32.69 mg/L.

According to the Tukey test, this value is not significantly different from the final nitrate-N concentration of B-TV-5HTL-AP; however, it must be acknowledged that B+TV-5HTL-AP may be viewed as a superior method as this treatment requires less time, equipment, and procedures. These factors are vital within industry as they all promote a cost-effectiveness and eco-friendly process. The other treatments with a Tukey p-value > 0.05 are TV-5HTL-AP and TV->B-5HTL-AP. When considering the insignificant difference between these values, the treatment only utilizing *T. versicolor* may be preferred as it utilizes less resources and processes.

There was no significant difference in any of the values measured between the negative control group and B-5HTL-AP, including nitrate-N; however, *Nitrosomonas* and *Nitrobacter* may have performed some nitrification since the total value of ammonia-N decreased while the total value of nitrate-N increased, implying nitrification. Figure 2 provides a clear visualization of the increase in both NH_3/NH_4^+-N and NO_3-N concentrations after each treatment, which highlights the rank of success for each treatment and its residual ammonium.

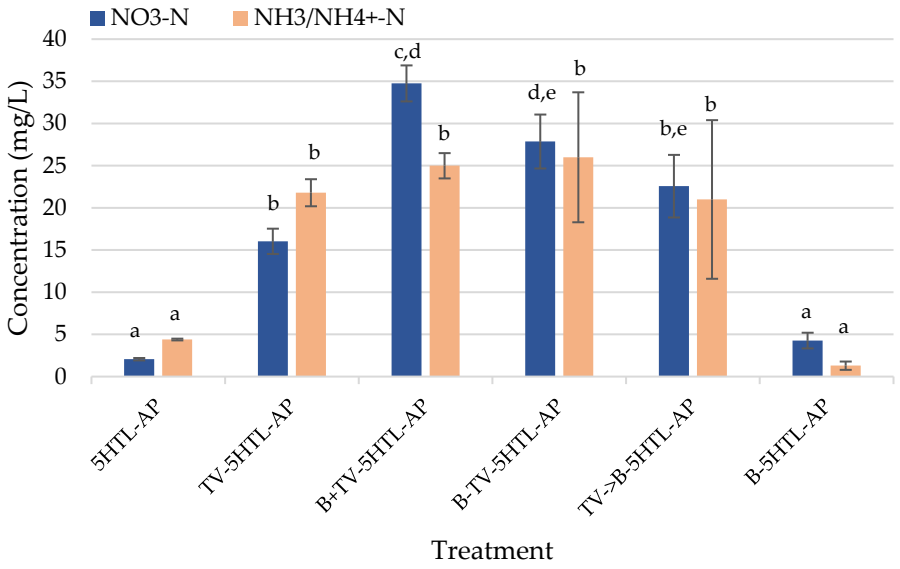

Figure 2. The concentration of NO_3-N and NH_3/NH_4^+-N plotted according to the corresponding treatment executed. Mean values that are not significantly different ($p > 0.05$) are followed by the same letter. Each letter is unique and the NO_3 and NH_3/NH_4^+-N concentrations should not be compared.

The treatments inoculated with both nitrifying bacteria and *T. versicolor* had a significant increase in concentration of NO_3-N and NH_3/NH_4^+-N. The capabilities of the nitrifying bacteria in HTL-AP can be supported by the trend observed in Figure 2, that every sample inoculated with nitrifying bacteria has a higher concentration of nitrate-N rather than ammonia/ammonium-N. This finding suggests that conversion took place; however, high residuals of ammonium were seen for treatments B+TV-5HTL-AP, B-TV-5HTL-AP, and TV->B-5HTL-AP with a NO_3-N:NH_3/NH_4^+-N ratio of 1:0.714, 1:0.929, and 1:0.913, respectively. It is demonstrated that the sample B+TV-5HTL-AP had the greatest amount of conversion when compared to B-TV-5HTL-AP and TV->B-5HTL-AP. This indicates that inoculating *T. versicolor*, *Nitrosomonas*, and *Nitrobacter* at the same time improves the conversion of NH_3/NH_4^+-N to NO_3-N. There are other possible factors that may enhance the amount of conversion, such as the concentration of the nitrifying bacteria added to the samples. Future research should explore the inoculation of various concentrations of *Nitrosomonas* and *Nitrobacter* to optimize the amount of conversion. A limitation that may have impacted the capabilities of the nitrifying bacteria is the temperature in the environment during treatment. The temperatures that are most optimal for nitrification range from 30 °C to 36 °C; at 49 °C oxygen consumption rates reach a maximum [18]. The temperature conditions for *T. versicolor* and the nitrifying bacteria differ; however, executing this experiment at 28 °C was determined as the best fit for the success of both microorganisms. Future research should focus on altering the temperatures to analyze the effects on functionality and conversion.

The purpose of hydroponics is vital in creating a sustainable and eco-friendly industry. The process of valorizing this wastewater using mycoremediation, integrated with bioremediation, allows for responsible consumption and production and maintains a circular economy. Optimizing nutrient analysis for the treated wastewater will procure an effective fertilizer for hydroponic systems in lettuce production. The inorganic form of nitrogen is the preferred form for plant uptake and the recommended rate of nitrogen needed for optimal lettuce production is 150 mg/L [7]. As seen in Table 3, the treatment with the greatest amount of inorganic nitrogen is B+TV-5HTL-AP with a total value of 59.76 mg/L. This means that only 39.84% of the recommended rate of nitrogen is available for plant uptake. As previously mentioned, further studies may be done to manipulate various factors to optimize conversion and achieve a higher concentration of NH_3/NH_4^+-N to NO_3-N.

Parameters that can affect ammonification and nitrification include, but are not limited to, laccase activity and pH. The concentration of NO_3-N and NH_3/NH_4^+-N were measured to determine which combination of organisms would increase the level of nitrate-N the most. To discover the variables that could help advance the levels of nitrate-N and ammonium-N in the wastewater, pH levels and laccase activity were monitored and evaluated at the end of each sample run.

The value of pH is an important factor in the success of this experiment. In reference to the negative control group, the level of pH increased for all samples containing *T. versicolor*, which supports the fungi's ability to perform ammonification. Figure 3 shows a correlation between high levels of pH and high concentrations of NH_3/NH_4^+-N and further illuminates that the value of pH most likely increased due to the presence of ammonia.

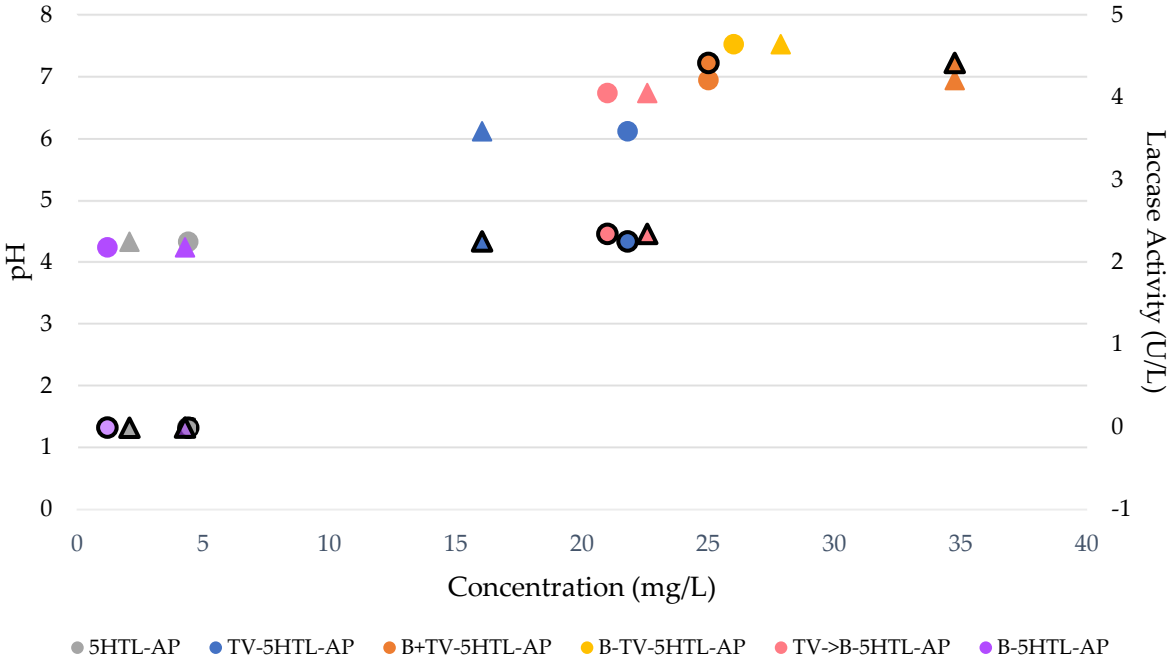

Figure 3. The values of pH and laccase activity plotted according to the corresponding concentration of NO_3-N and NH_3/NH_4^+-N of the respective treatment. The triangles represent the concentrations of NO_3-N and the circles represent the concentrations of NH_3/NH_4^+-N. The black outline around the correlated shape indicates laccase activity values and should be measured using the secondary vertical axis.

It is important to acknowledge that the methodology used for the analysis of the wastewater measures both ammonium-N and ammonia-N. The main element that determines the ratio of ammonia to ammonium in water is the pH value of the water. Since the experiment was executed at room temperature to align with previous research [7,8] and in an environment where the pH was less than 6.0, the proportion of ammonium-N (NH_4^+) in the water is significantly higher than ammonia (NH_3). At a pH slightly above 9.0, the proportion of ammonia and ammonium is about 50 percent [28]. Therefore, the data shown in Figure 3 further supports the claim that ammonia was produced since the pH and concentration of NH_4^+ and NH_3 increased, implying that the proportion of ammonia expanded since the wastewater was becoming more basic. On the contrary, the sample inoculated with only *Nitrosomonas* and *Nitrobacter* saw a decrease in pH. This observation can be accepted as it is known that these organisms require a pH of 5 or higher to survive. The failure of nitrifying bacteria to cope with acidic conditions is mostly due to the unavailability of a substrate. The substrate of *Nitrosomonas*, NH_3, will become increasingly protonated while the substrate of *Nitrobacter*, NH_2, will undergo protonation to nitric acid, creating a more acidic environment and, therefore, decreasing the pH [29].

The nitrate-N and ammonium concentration values after each treatment are reported in Table 3. The negative control group, 5HTL-AP, can be viewed as the reference point to verify that the methodology produced unique and successful results. As expected, the negative control group had relatively small values for both nitrate-N and ammonium with 2.07 mg/L and 4.40 mg/L, respectively. There was not a significant difference between the nitrate-N concentration of the negative control group and the B-5HTL-AP sample. This observation was anticipated because the functionality of the nitrifying bacteria was restricted as the pH growth-limiting value of *Nitrosomonas* is 5. Most of the nitrifying bacteria more than likely died since the 5HTL-AP had a pH of 4.33.

Heavy metals can be a concern with HTL-AP as they are toxic to the environment and create an undesirable environment for nitrifying bacteria to flourish; however, no measurements of these compounds were recorded in this study as the focus was on optimizing the levels of nitrate-N and its conversion, in addition to heavy metals not likely to be present in a food waste derived HTL-AP. Further studies should follow to observe the capabilities of nitrifying bacteria in HTL-AP with an altered pH through acid–base titration to see its compatibility with the effects of fungal treatment. As pH imposes one of the greatest limitations on this experiment, it may be advantageous to explore how nitrifying bacteria would interact in an HTL-AP environment with a suitable pH and no fungal species. This revelation may also be useful in measuring heavy metals and their effects.

The symbiotic component of the functionality of *T. versicolor* and nitrifying bacteria is enzyme activity. These organisms secrete enzymes that can perform oxidation, which lead to the processes known as ammonification and nitrification. *T. versicolor* secretes laccases, which are enzymes that are responsible for catalyzing the oxidation of electron-donating groups such as anilines (-NH_2) and phenols [17]. *Nitrobacter* and *Nitrosomonas* can utilize the product of this reaction, ammonium, to produce nitrate. Nitrifying bacteria secrete two enzymes, ammonia monooxygenase and hydroxylamine oxidoreductase, that use ammonium as a substrate to complete nitrification [30]. It must be acknowledged that the sample, B-TV-HTL-AP, has been removed from this part of the study. This is because this sample was observed to have debris that was presumed to be *T. versicolor*. This obstruction could cause an error when analyzing the laccase activity since it is measured using spectrophotometry. Considering this exclusion, the sample with the most optimal conditions was B+TV-5HTL-AP and could be the reason why it had the highest laccase activity. It is possible that the nitrifying bacteria and fungi had more time to work interchangeably in the same environment, therefore provoking higher enzyme secretion. As expected, the sample only containing nitrifying bacteria produced no enzymes due to the acidic environment and lack of ammonium. A trend is visible in Figure 3 that is like that of pH in the aspect that there is a correlation with higher levels of laccase activity and high concentration values of nitrate-N and ammonia.

As stated above, ammonia monooxygenase and hydroxylamine oxidoreductase use ammonium as a source of substrate to produce nitrate. This explains why TV-5HTL-AP had a higher concentration of NH_3/NH_4^+-N as the sample did not contain nitrifying bacteria that could secrete the enzymes to perform nitrification. The relationship between laccase activity and concentrations of NO_3-N and NH_3/NH_4^+-N prove that *T. versicolor* released these enzymes and ammonification took place.

4. Conclusions

This study aimed to enhance the potential of hydrothermal liquefaction aqueous phase (HTL-AP) as a valuable resource for downstream hydroponic crop production. The subsequent HTL-AP, while rich in organic nutrients, requires treatment due to its potentially high heavy metal concentration (depending on source of feedstock) before it can be reused or discharged. A promising solution to this challenge was explored in this study using the novel approach of combining mycoremediation using *Trametes versicolor* with the activity of nitrifying bacteria (*Nitrosomonas* and *Nitrobacter*) to increase the concentration of nitrate-N in HTL-AP, thus transforming it into a suitable hydroponic fertilizer, as detailed in

Table 3. The integration of these biological agents focused on simultaneously eradicating the toxic environment, promoting ammonification, and initiating nitrification, resulting in the transformation of organic nitrogen compounds into inorganic nitrogen (nitrate)—the preferred form for plant nutrient uptake in hydroponic systems. There are various factors that affect the success of these microorganisms such as the toxicity and pH levels of the environment they are in. The samples had the highest increases in both nitrate-N and ammonia when the microorganisms were subjected to water with a pH range of 6–7.5. There was a correlation observed in which a sample within this pH range had higher laccase activity and, therefore, the concentrations of ammonia and nitrate-N were also significantly higher. The experimental analysis performed exemplifies the success of this integrated approach, as demonstrated by the significant increase in nitrate-N concentration in the treated HTL-AP samples. The use of *T. versicolor* led to an amplification in ammonification, providing a substrate for the nitrifying bacteria to perform nitrification. The amalgamation of fungi and nitrifying bacteria produced the most notable increase in nitrate-N levels, illustrating the synergistic effects of the dual treatment method and explaining the success of the B+TV-5HTL-AP sample. Specifically, an increase of 32.69 mg/L was observed in this sample; however, the nitrate-N concentration is not significantly different from the B-TV-5HTL-AP treatment. Acknowledging this factor, B+TV-5HTL-AP can still be viewed as a more suitable treatment as it requires less equipment, and its treatment time is 120 h less. This study also acknowledged the significance of pH and enzyme activity in propelling these processes. The combination of fungal and bacterial treatment shows promise for treating HTL-AP wastewater and increasing its value as a fertilizer, all while reducing waste, greenhouse gas emissions, and overall time and cost to perform the treatment. It is possible this research can be extrapolated to other types of industrial wastewater, providing a cost-effective treatment option while advancing sustainability and environmental conservation efforts.

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