



D2.2 Nitrogen removal in HTL trials

Report on the optimization of the N removal from the
feedstock during pretreatment

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SUMMARY

Hydrothermal liquefaction (HTL) is a promising technology for the conversion of wet residues to biofuels, including SAF. However, several challenges remain — one of the main ones being the composition of the feedstock. Since HTL biocrude retains many of the feedstock's characteristics, further catalytic upgrading is required to achieve fuel-grade properties. Nitrogen, in particular, is a challenging heteroatom to remove during the upgrading process. This task aimed to develop pretreatment strategies to reduce the nitrogen content in the feedstock, improving the quality of the resulting biocrude and enhancing the effectiveness of downstream hydrodeoxygenation (HDO). *Spirulina sp.* was selected due to its high nitrogen content, stable properties and availability. A review of relevant literature was conducted, and screening experiments were designed to test various pretreatment methods for nitrogen reduction.

To reduce the nitrogen content of the feedstock, both chemical (acid and base treatments) and physical (heat-treatment, sonication, microwaves) pretreatments methods were investigated. Experimental conditions were selected to prioritize low-cost and straightforward approaches with potential industrial applicability. The experimental procedure evolved dynamically, with early elimination of ineffective methods based on the nitrogen content measured in the supernatant (the aqueous phase remaining after solid-liquid separation). In later stages, TOC measurement was introduced to assess carbon losses alongside nitrogen removal. Microscopy was also used to analyze the slurry, to observe changes in particle size, agglomeration behavior, and possible causes of gel formation.

Base addition enhanced nitrogen removal, particularly when combined with heat-treatment. Higher base concentrations further improved nitrogen transfer to the aqueous phase. However, the base-treated samples exhibited gel formation, which pose challenges in analytical procedures as well as in pumpability on the subsequent HTL operations.

Acid treatments were less effective at removing nitrogen compared to base treatments. However, when combined with heat treatment, they showed improved performance. Additionally, acid-treated samples did not form gels and could be safely neutralized, and the pH could be adjusted to 12 using NaOH without triggering gelling. Among the acids tested, sulfuric acid combined with heat resulted in the highest nitrogen concentration in the supernatant, indicating the greatest nitrogen removal efficiency.

Heat treatment proved effective when used in combination with both acids and bases. In contrast, other physical treatments, such as sonication or microwave irradiation were not effective at reducing nitrogen content neither to disrupt the gelled structure of the samples.

Multiple centrifugation steps following heat treatment did not lead to a significant increase in nitrogen removal, as shown by the nitrogen content of the supernatants over five cycles. However, additional heating did enhance nitrogen removal in both acid-treated and control samples.

Based on the pretreatment results — particularly the nitrogen transferred to the supernatant and the gelling behavior — overnight heating with acid or water was selected for subsequent HTL testing. To reduce energy and time demands at industrial scale, the reheating steps were excluded, and centrifugation was kept to a minimum.

The TOC results of the inorganic acid-treated samples showed that considerable amounts of carbon were also lost in the pretreatment processes. Due to the carbon losses, the real efficiency of the pretreatment cannot be evaluated at this point. The analysis and upgrading activities later in ICARUS project will reveal whether there have been any substantial changes to the algae structure which could affect the reaction pathways to obtain preferred results.

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1 Background

Drop-in style fuels have strong potential to replace fossil fuels, particularly in sectors where the transition to alternatives like batteries, wind, or solar is complex or impractical. Hydrothermal liquefaction (HTL) converts high-moisture feedstocks into biocrude eliminating the need for energy intensive drying steps and thereby reducing processing time, energy use, and infrastructure costs. However, HTL biocrudes are of lower quality than fossil fuels, and are often associated with higher water, oxygen, and nitrogen content which limits their direct application as drop-in fuels. While ideal biofuels should consist primarily of long hydrocarbon chains with minimal oxygen, nitrogen, and sulfur, HTL-derived biocrudes contain significant amounts of these heteroatoms, which must be removed before the fuel can be used for transportation (Ramirez et al., 2015).

Hydrodeoxygenation (HDO) is a catalytic hydrotreatment process, applied to biocrude oils to remove heteroatoms such as nitrogen, sulfur and oxygen. For nitrogen-rich feedstocks, such as HTL biocrude derived from microalgae, nitrogen poses a major challenge during this stage (Haider et al., 2020). Reducing the nitrogen content in the feedstock could affect the reaction pathways during HTL, ideally converting more nitrogen to aqueous forms during the process, reducing the cost time and cost for downstream processing.

Although the complex mechanisms and reaction pathways in HTL are still not fully understood or described, the overall process generally involves decomposition of the biomass feedstock (depolymerization) through hydrolysis reactions and repolymerization/recombination of the different building blocks. In these reactions, water acts as both solvent and reactant to break and reform the bonds in the feedstock molecules, resulting in longer hydrocarbon chains (Ahmad et al., 2025).

1.1 Objectives of Task 2.2.1

The aim of this task was to conduct pretreatment experiments to eliminate or reduce the nitrogen content in the HTL feedstock and thus increase the biocrude quality in subsequent steps. In this work, the available literature was reviewed and evaluated, and studies were designed to investigate pretreatment methods to reduce nitrogen content of HTL biocrude. The screening studies were designed to investigate the efficiency of the different pretreatment methods to reduce nitrogen content of HTL slurry to improve the bio-crude quality as well as allow a more effective HDO treatment. Microalgae (*Spirulina* sp.) was selected as a high nitrogen feedstock due to its stable high nitrogen content, stable properties and availability.

1.2 Reduction of Nitrogen Content in feedstock used for HTL

Feedstocks used for HTL can have a wide range of nitrogen amounts, which is primarily tied to their proteins, as proteins are the macromolecules with the highest nitrogen content (Madsen et al., 2017). The removal of the nitrogen targeting the proteins can involve several processes: removing the entire protein, denaturing the protein into amino acids and removing those, or further breaking down amino acids into smaller molecules. This breakdown isolates the nitrogen from the carbon, allowing the highest possible retention of the carbon content in the feedstock proteins (Prado et al., 2017).

HTL can be performed on a large group of feedstocks with different nitrogen contents. The type of molecules containing nitrogen, as well as the overall percentage of nitrogen by weight are key characteristics in the determination of the pretreatment approaches.

In wood, nitrogen content is quite low, where the fractions with the highest concentration are those which contain more bark. In fir and pine trees, there is more nitrogen in thinner branches than thicker branches, while for oak and beech not much difference is found (Vasileios et al., 2018). The primary form of nitrogen is amino acids, both free and in complete proteins. When nitrogen in the environment

is in excess, nitrogen content (in the form of amino acids like glutamine and arginine) increases in needles and sap, for example with pine trees (Nordin et al., 2001). There are also studies suggesting that the co-liquefaction of microalgae with lignocellulosic biomass can produce higher quality bio-crude oil (Hu et al., 2019).

The following chapters include a short review of available literature, summarizing the knowledge and industrial practices where proteins are treated.

1.2.1 Protein denaturation

Proteins can be denatured in several ways, both physically and chemically (Acharya & Chaudhuri, 2021). Physically, the common approaches include freezing, mechanical rupture, thermal treatment, and sonication. Chemical approaches include treatment with acids or bases, use of surfactants, or introduction of organic solvents. Acids and bases disrupt ionic interactions (salt bridges) which form the tertiary structure of proteins. The cations (e.g. H^+ , Na^+) and anions (e.g. Cl^- , OH^-) interact with the acid groups and ammonium groups of the amino acids, respectively. Strong acids and bases can affect not only the tertiary and quaternary structures (as weak acids do) but also disrupt secondary structures. Hydrogen bonds in the secondary, tertiary, and quaternary structures of proteins can be disrupted by alcohols and other solvents. When placed in an organic solvent, the disruption of the hydrogen bonds allows for new hydrogen bond formation between the amino acids and the organic solvent. Heavy metals can disrupt structure by both acting as salts to disrupt salt bridges as well bonding to sulfur in disulfide bonds – creating insoluble metal-protein salts. These salts would be heavier and could be separated by centrifugation. Ions from acids and bases could then displace the metal to remove it.

In some HTL reaction studies, a pre-processing thermal step was added with the aim of nitrogen reduction. The thermal pretreatment step was reported to result in more water-soluble nitrogen products which remained in the aqueous phase instead of the oil phase after HTL (Yu et al., 2011). They propose that amino acids are deaminated and therefore susceptible to degradation via HTL at high reaction conditions. However, in this context, the temperature considered 'high' was still relatively low compared to the critical point (max conditions discussed were 300C).

Sonication can be a promising pretreatment method. Choi et al. (2017) achieved 35-94% improvement in protein yield by using sonication for protein removal from edible insects. Hu et al. (2017) also mentions sonication and provides sources for the proposed mechanisms, which include radiation force, acoustic streaming and cavitation effects. Its primary downfall is the same as for other mechanical methods – namely their high energy costs for industrial scale applications. A study of valorization of tomato waste done in 2017 included a microwave assisted extraction process (MAE) where dried tomato powder was diluted with ethanol and processed with microwave radiation. The procedure started with irradiation of ethanol/tomato mixture at 200W with stirring, then centrifugation, removal of supernatant and evaporation of solvent, and finally resuspension in distilled water (Pinela et al., 2017). The results indicated that this approach used less solvent while increasing yields, making this method in line with green chemistry goals. Product potential was relevant for commercial applications for food and feed.

1.2.2 Breaking peptide bonds

Denaturing proteins changes their secondary, tertiary and/or quaternary structure, but not affecting the primary structure which is held together by peptide bonds. Primary structures are broken down by catabolic reactions in the metabolic system of organisms to yield ammonia which is converted to another molecule for excretion (e.g. urea, uric acid). This process is enzymatic inside living organisms and can be emulated outside the cell with bacterial enzymes like sortase which cut at specific sites and is thus highly useful for the protein engineering industry (Popp & Ploegh, 2011).

1.2.3 Microalgae as feedstock for HTL - Breaking the cell wall

Microalgae are considered a highly promising feedstock for biofuel applications due to its rapid growth and flexible cultivation conditions which are not dependent on seasonal variations. One of the biggest drawbacks to utilizing this feedstock is the breaking of the cell wall to release the lipids. Current technologies reviewed by Onumaegbu et al. (2018) for pretreatment aimed at breaking the cell wall of microalgae were similar to techniques that denature proteins. Techniques include, for example, mechanical (high-pressure homogenizer and bead mills), physical (ultrasonic and microwave methods), thermal (autoclave and steam explosion), chemical (catalytic and enzymatic), and combinations.

1.2.4 Removal of intact proteins - Value added products

Extracting proteins is especially relevant to industries producing proteins as a product, or those which have products containing protein isolates. Relevant industries include food production, medicine and medical research, value-added chemicals production, genetic analysis, and many more. Each of these industries isolate proteins for their products either from a feedstock containing a high amount of protein within its biological composition or use microorganisms to create said proteins, which must then be separated from those microorganisms. In these applications, the extracted protein is treated with more care and not denatured in the process of extraction, meaning they must be separated without disturbing the peptide bonds between amino acids. Hassan Kamani et al. (2024) studied the separation of proteins using an alkaline solution, for example addition of NaOH to a 10% w/w feedstock slurry, followed by centrifugation, resuspension and another centrifugation. They found that this method isolates the proteins into the supernatant, which is also beneficial for the application in this work. Then they pooled the supernatants followed by reprecipitation at a lower pH (around pH 4-6 using HCl) and a centrifugation step to collect the proteins. Carbohydrase enzymes were shown to be an effective technique for protein extraction from tomato pulp and peels. Extractions were followed by protein concentration assays (e.g. Bradford assay) and the proteins were separated via SDS-PAGE. However, the study notes that the techniques are not economically feasible due to high production costs of carbohydrases, because the improvement in the amount was relatively low (Baker et al., 2022). Protein stabilization with glycerol has been simulated and is concluded to prevent aggregation during extraction (Vagenende et al., 2009).

1.2.5 Use of NaOH as pretreatment for HTL feedstock

The use of sodium hydroxide before HTL is especially advantageous if the HTL experimental plan includes NaOH addition for pH increase before the HTL reaction.

There are also pretreatment protocols which remove the NaOH before the HTL reactions. For example, a cold pretreatment protocol using a solution of NaOH, urea and water (7:12:81 ratio) showed a marked decrease in protein and carbohydrate content of the feedstock (Hu et al., 2017). In this process the feedstock is treated with said solution after it has been frozen (-5 to -10C) overnight. The solution was stirred with biomass (microalgae; *C. vulgaris*) for 5 minutes before a quench with 1.0 M HCl, the centrifugation and vacuum filtration to obtain the pre-treated feedstock. In this case, the HTL reactions were performed in small scale (100mL) and under batch conditions of 250°C and approx. 45 bar. Although their crude oil had higher HHV values and lower nitrogen values, the change (7.49% N to 6.39% N) was not significant enough to assure the lack of further upgrading. Furthermore, the relative high volumes of NaOH are not cost effective at higher scales without recycling steps added. The mechanism proposed is that changes to chemical composition favor different reaction pathways during HTL. A work on alkaline pretreatment of cypress wood before HTL further explains this concept (Liu et al., 2014).

2 Materials and Methods

2.1 Feedstock

The spirulina powder was purchased from an online commercial supplier, naturalis.no. The order consisted of 11 kg, divided into 44 packages of 250 g each, all from the same batch. The feedstock analysis was conducted by a commercial laboratory Eurofins, which included analyses for moisture, ash content, elemental composition (CHONS), selected inorganics (Cl, Al, Ca, K, Mg, Na, Si), as well as crude fat and protein contents (see Table 1).

Table 3. Composition of spirulina powder.

Analysis	Result	Unit	Uncertainty
Moisture content	9.5	%	10%
Ash content 550 oc incl. ash contamina	6.59	% dw	11%
Ash 550 oc incl ash contaminants, as r	5.97	%	11%
Sulfur S	0.794	% dw	26%
Sulfur S as received	0.719	%	26%
Chlorine Cl	0.137	% dw	25%
Chlorine Cl as received	0.124	%	25%
Carbon C	51.4	% dw	5%
Carbon C as received	46.6	%	5%
Hydrogen H	6.7	% dw	13%
Hydrogen H as received	7.1	%	13%
Nitrogen N	11.49	% dw	12%
Nitrogen N as received	10.4	%	12%
Oxygen O (calculated)	22.9	% dw	
Oxygen O as received (calculated)	29.1	%	
Aluminium (Al)	260	mg/kg matter	dry 61
Calcium (Ca)	2600	mg/kg matter	dry 310
Potassium (K)	13000	mg/kg matter	dry 1900
Magnesium (Mg)	3800	mg/kg matter	dry 750
Sodium (Na)	4200	mg/kg matter	dry 930
Silicon (Si)	1100	mg/kg matter	dry 340
Crude fat	5.28	g/100 g	± 10%
Crude Protein Kjeldahl (Nx6,25)	64.4	g/100 g	± 10%

Some spirulina of the same type from the same producer was available from prior projects and was utilized in some of the screening tests to develop experimental procedures and to avoid shortages during the HTL experiments. As pretreatment studies progressed, differences were noted between older and newer batches of Spirulina, therefore subsequent work was completed on the new batch. The work on older batches was repeated for protocols capable for nitrogen removal.

2.2 Screening of the nitrogen removal methods

The experimental procedure in this task has been developed in a dynamic way. The efficiency of the pretreatment was evaluated based on the nitrogen content of the supernatant (aqueous phase after the separation of solid algae). The inefficient pretreatment methods were abandoned rather early, while other approaches were tested. During the last phases of the study, TOC measurement was introduced to assess the carbon losses along with the nitrogen removal. In addition to the nitrogen content measurement of the supernatant, the slurry was studied with a microscope to reveal changes in particle sizes, agglomeration behavior and reasons for gelling.

To reduce the nitrogen content of the feedstock, both physical and chemical pretreatment approaches were studied. Experimental parameters were selected with the aim of identifying a low cost and simple approach that could be relevant in a commercial context. Figure 1 summarizes the different pretreatment methods investigated. The tests included chemical treatments (acids and bases), physical treatments (sonication and heat) and combinations of those. The combinations of chemical and physical treatments were adjusted as the work progressed, and not all combinations were investigated.

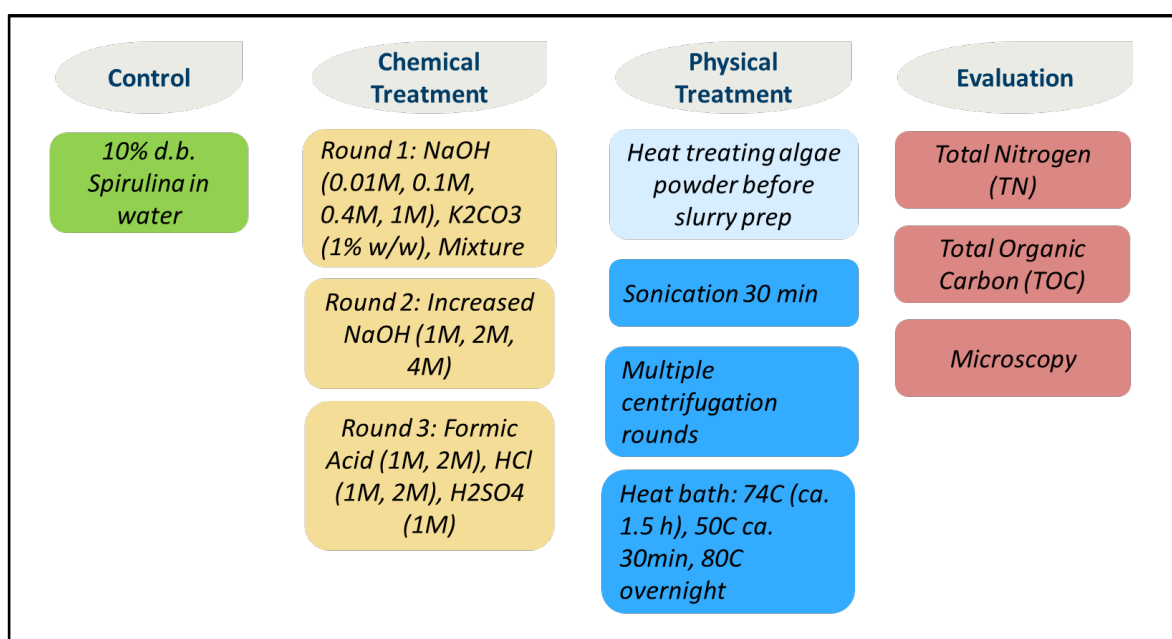


Figure 1. Overview of experimental approach for pretreatment methods.

In all pretreatment experiments 20g of 10wt% slurry was prepared, where 2.1g algae (including its 5.5% moisture) was added to 17.9g tap water or indicated acid/base solution in a test tube and mixed well.

After pretreatment, the solids and supernatant were centrifuged and the supernatant analyzed.

2.2.1 Pretreatment with base

For the base pretreatment tests the reagents were purchased from VWR Chemicals (NaOH, Analytical, 98.5-100.5%, AnalaR NORMAPUR® pellets Reag. Ph. Eur.; K₂CO₃, ≥99.0%, AnalaR NORMAPUR® analytical reagent sodium-free)

For pretreatment testing with base, the initial tests were conducted using low concentrations (0.01M -0.4M) of sodium hydroxide (NaOH) base solutions for slurry preparation. After the first tests, higher NaOH concentrations were used as well as potassium carbonate (K_2CO_3) solution was prepared for slurry. Tests were also performed with a combination of sodium hydroxide and potassium carbonate. For heat treatment, when indicated, the samples were treated at 74°C for a period of 1.5 hours. A number of initial tests were conducted with an older batch of algae of the same type and supplier to save the newer batch for HTL studies. However, microscopy revealed differences between the old and new batches, so further studies were done with the new batches.

2.2.2 Pretreatment with acid

The acid pretreatment tests contained slurries were prepared with the following acid solutions: 1M hydrochloric acid (HCl), 2M hydrochloric acid (HCl), 1M sulfuric acid (H_2SO_4), 1M formic acid (FA), 2M formic acid (FA) where the acids were purchased from VWR Chemicals (Hydrochloric acid 35%, GPR RECTAPUR®; Sulfuric acid, 95.0-97.0%, AnalaR NORMAPUR® analytical reagent; formic acid 80% technical).

2.2.3 Sonication and heat treatment

Sonication and heat treatment was done in a VWR USC-TH Ultrasonic cleaner bath with a non-adjustable speed of 45 kHz and adjustable heating up to 80C. The bath was heated when the heat treatment is indicated, the maximum temperature reached was 74°C. The residence time for sonication was 30 minutes.

2.2.4 Solid / Liquid separation

Centrifugation was done in 50 mL centrifuge tubes in a VWR MegaStar 3.0 OR centrifuge at 4000 rpm for 30 min. Repeated rounds of centrifugation were also tested. In the repeated centrifugation tests, centrifugation was done 5 times where each time the supernatant was removed and replaced with the same weight value of water/pretreatment agent and mixed thoroughly before a new centrifugation. The first, third, and fifth supernatant were analyzed for TN values to identify trends.

2.2.5 Analysis of the samples

The nitrogen content of the supernatant was identified as indicator of nitrogen removal, the higher the nitrogen content of supernatant, the more efficient is the pretreatment. At the same time, for the most promising pretreatments, the total organic carbon was also monitored to track carbon losses during the tests. Pretreatment protocols were evaluated by total nitrogen (TN) content and total organic carbon (TOC) content of the supernatant. The TN content and TOC were analyzed using a HACH LCK 338 (20-100 mg/L TN) and HACH LCK 387 (300-3000 mg/L TOC) cuvette test kits respectively, with a HACH DR 3900 spectrophotometer. The supernatant samples were diluted to match the ranges of the cuvette kits before the analysis. Microscopy (Keyence VHX-600 digital microscope) was used to investigate the effects on cellular structure under different conditions and to confirm centrifugation settings were sufficient for removal of cell debris.

3 Results and Discussions

3.1 Feedstock Pretreatment

As mentioned, combinations of chemical and physical treatments were adjusted as the work progressed, and not all combinations were investigated. The primary example of this was found during the initial phase of base testing, where heat treatments showed greater success than sonication, as showed by higher nitrogen content in the removed supernatant. Consequently, sonication as pretreatment method was discarded early and was not assessed for many of the subsequent chemical treatments.

3.1.1 Pretreatment with Base

The first part of the current study investigated the effect of two bases, K_2CO_3 and NaOH in combination with heat treatment and sonication. The base treatments resulted in considerable gelling in almost all samples, which made the sampling and analysis difficult, therefore the base treatment results should be treated with care. Therefore, the reporting focuses on the major trends, while irregularities and uncertainties are explained with sampling and/or analysis errors. Also, due to the gelling tendency, base treatments were not suggested for HTL slurry preparation.

Figure 2, shows the results from the very first tests to tune base concentrations and to investigate whether heat alone makes a difference.

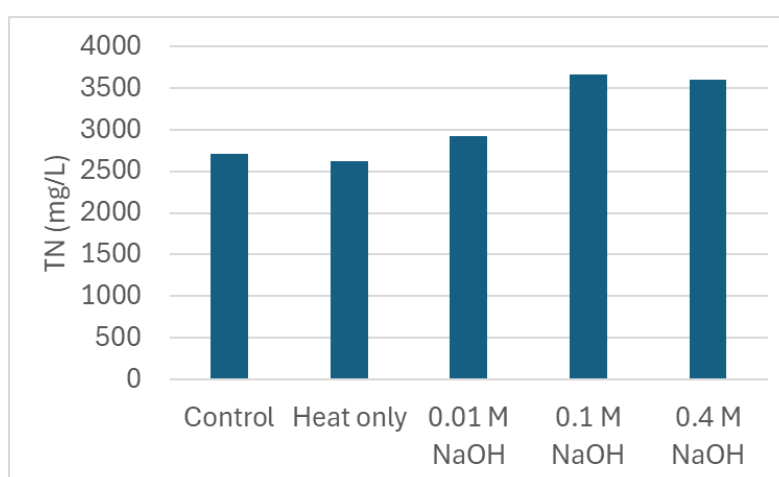


Figure 2 Pretreatment with heat and weak bases.

The tests with weak base (NaOH) showed slight improvements over the control. Increasing the base concentration increased the nitrogen removal, however, no greater removal of nitrogen was achieved with the 0.4M NaOH compared to 0.1M, therefore even higher concentrations (1-4 M) were applied in the subsequent investigations.

Figure 3 shows the results obtained after a more comprehensive experimental campaign involving two different bases (NaOH and K_2CO_3), as well as heat and sonication treatments.

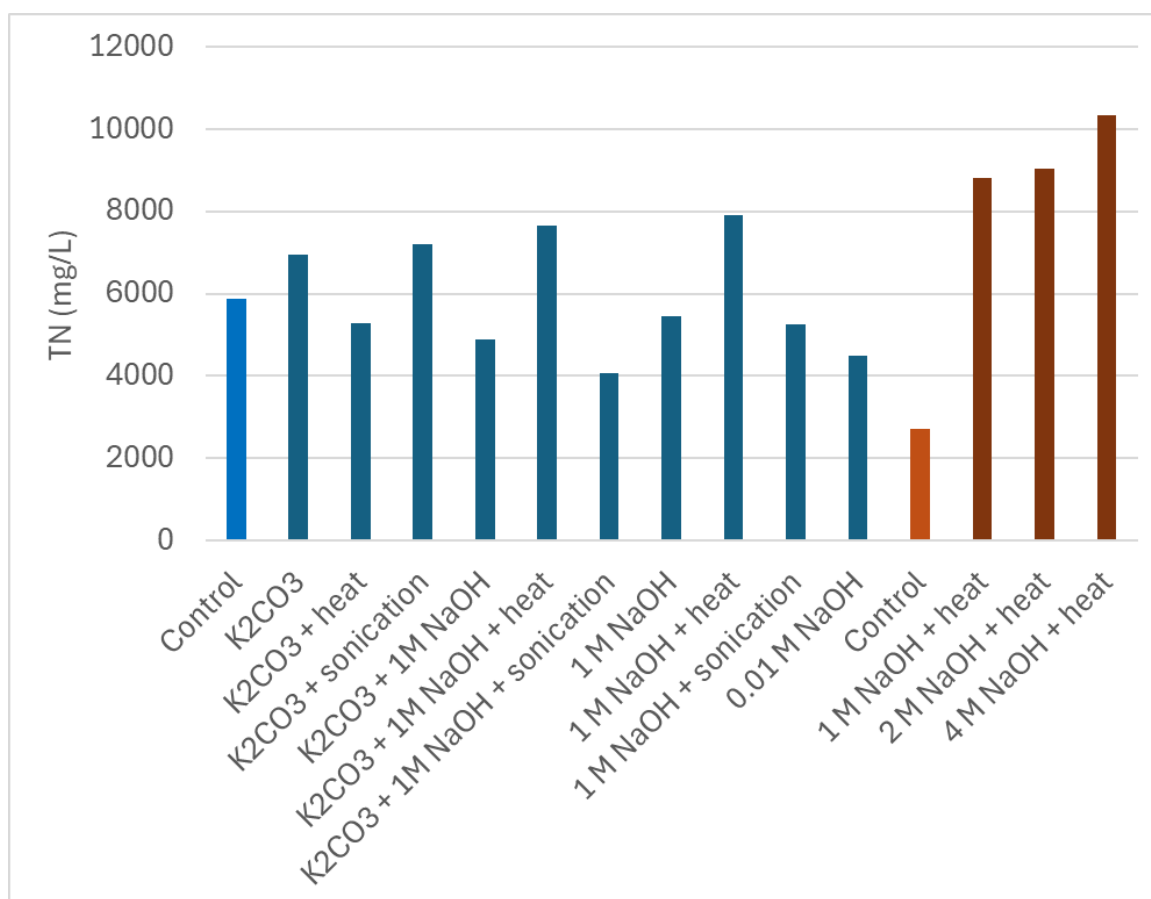


Figure 3. Base pretreatment studies comparison. The data shows the measured values of first centrifuged supernatant. The high concentration (1, 2 and 4M NaOH indicated with red-brown color) samples were re-tested on the other (new) Spirulina powder.

Increasing base concentration raises the nitrogen transfer to the liquid phase, which is further boosted with heat treatment. Heat treatments in combination with the base treatment showed greater success than sonication in combination with base treatment, as indicated by higher nitrogen content in the removed supernatant. Consequently, sonication as pretreatment method was not assessed further. The separation of the supernatant during centrifugation stages was not efficient due to gelling in case of the higher concentrations of NaOH (1M, 2M, 4M), therefore the resulting TN readings might have larger uncertainties for those samples, for example it appears too low for the $K_2CO_3 + heat$ sample. Gelling was observed with both NaOH and K_2CO_3 , therefore the observed irregularities are explained with sampling from a gelled sample. K_2CO_3 did not seem to be more efficient than NaOH therefore it was excluded from further studies. In the base tests two different spirulina batches were used (both from the same producent) during the first investigations, as marked with different colors on Figure 3. Nonetheless, the results revealed significant differences between them. Therefore, in continuation, only the newly purchased spirulina batch was used.

Filtration was also tested during the initial base pretreatment testing methods. The filtration tests were done with a vacuum flask and a Büchner funnel with filter paper. Figure 4 shows some of the difficulties encountered.

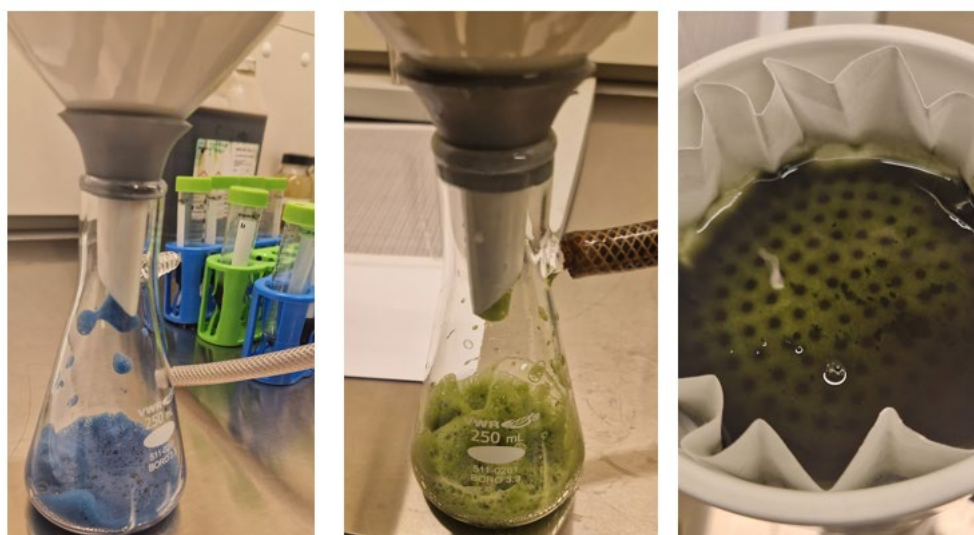


Figure 4 Difficulties during sample filtration, foaming and pore clogging on the filter paper. Note the color differences, the blue color is typical of living cyanobacteria such as spirulina, and the treated or dead cells indicated by green color.

During filtration, the liquid/solid separation was not achieved due to blockage of the filter paper. In addition, the liquid phase foamed. Thus, the liquid was difficult to collect from the vacuum flask. Therefore, centrifugation remained the sole solid-liquid separation method.

Centrifugation worked generally well for liquid/solid separation, if the samples did not gel but additional rounds of centrifugation had very limited additional nitrogen removed. During base testing, 5 rounds of centrifugation were performed with replacement of supernatant by the respective base of the sample. Figure 5 shows the nitrogen content of the supernatants after multiple (1, 3 and 5) rounds of centrifugation.

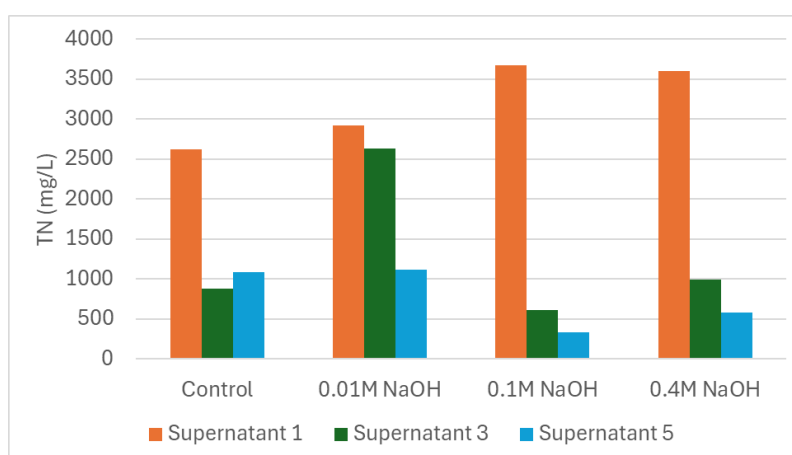


Figure 5 The total nitrogen content of supernatants after multiple (1, 3 and 5) centrifugations

During the five rounds of base centrifugation, the amount of TN in the supernatant samples decreased significantly. The largest fraction of nitrogen was removed in the first round of centrifugation, with subsequent rounds removing small additional amounts. In case of 0.01M NaOH the high reading for the supernatant 3 was probably caused by sampling error.

Table 4 shows the pH values of the obtained supernatants after various treatments.

Table 4 pH values of the supernatants obtained after the various treatments

	Room Temp	50C, 30 min	74C, 1.5 h	Sonication 30 min
Algae and water	7.51	7.32	6.81	7.65
+ NaOH	12.81	12.33	12.03	12.80
+ K ₂ CO ₃	10.15	9.59	9.06	10.17
+ K ₂ CO ₃ + NaOH	12.95	12.56	12.44	12.85

The table shows that the pH is decreasing with heat treatment, but the sonication does not alter that. Heat treatment probably induces chemical or cell degradation reactions, which is also confirmed by the increased nitrogen content of the heated supernatants.

The total organic carbon content (TOC) of some of the supernatants were analyzed but due to gelling, the results were not conclusive, therefore not presented.

3.1.1 Gelling and Clumping

As pointed out previously, gelling was observed particularly following base treatment with heating, with the strongest concentrations of base (up to 4M) showing the most significant gelling. This phenomenon could pose a challenge to the HTL experiments in the continuous reactor flow reactor. To address this, microscopy and observations were employed to monitor and potentially mitigate gelling. Ideally, breaking down the cellular structure more thoroughly would reduce clumping. However, proteins or complex molecules released from the cell wall could aggregate, forming even more problematic gels, as suspected in the NaOH heat treatment results.

As part of the various attempts to break down the cellular structure of dry spirulina powder, heating the dry powder at 100°C and 150°C was tested. This approach aimed to avoid boiling off liquid from a slurry or using a sealed vessel. After heating, the powders were resuspended in water, sodium hydroxide (NaOH), and some in potassium carbonate (K₂CO₃) to compare the rehydrated structure against the control. More rounded shapes were observed for the 150°C treatment compared to the 100°C treatment, but neither appeared significantly more broken down by this pretreatment.

Microwave treatment was also tested to see if it could help break down the outer wall structure. However, no noticeable changes were observed by microscopy, which indicates that no noticeable changes were observed from the microwave treatment.

To gain more understanding of the underlying causes of gelling, microscope images were taken. Figure 6 shows the microscope images for the untreated control and 1 M NaOH slurry samples after various treatments. The slurries were centrifuged but not dried prior imaging.

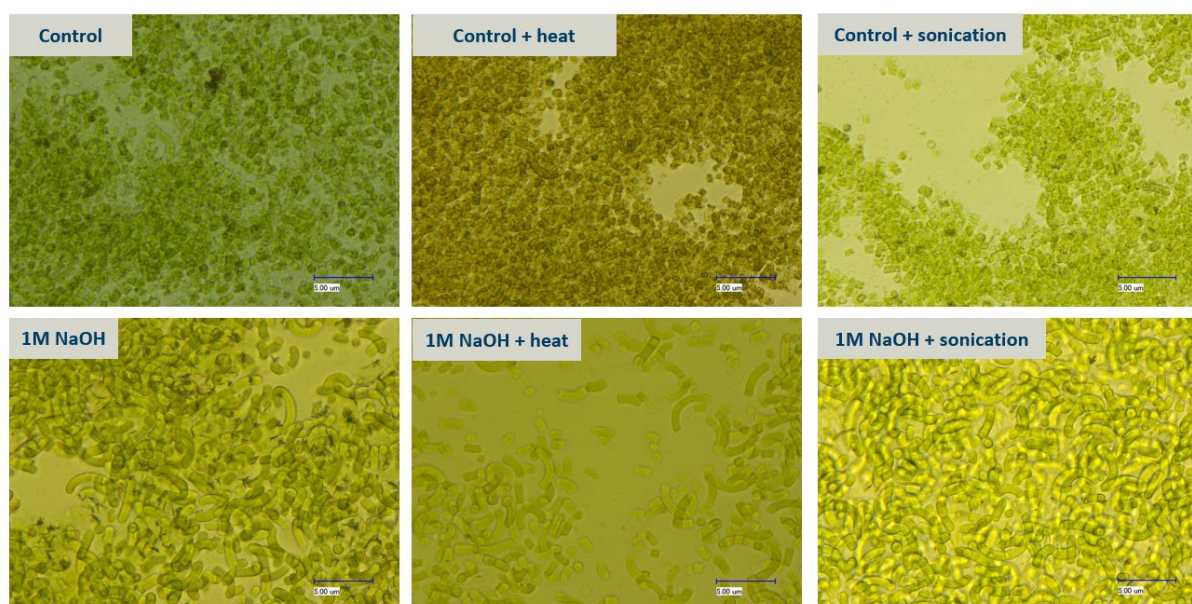


Figure 6 Microscope images of control and 1M NaOH treated spirulina samples with additional heat- and sonication treatments. The size bar is 50 µm long

As the images indicate, the gelled samples have different particle forms. In native spirulina, the cells can aggregate to form macaroni-like spirals (Nicoletti, 2016). Although the aggregation is neither seen nor experienced as gelling by adding water, base treatment seems to bring a similar structure back. This also means that, after these treatments, the cell wall structure is not completely fragmented. Compared to the image of living spirulina presented by Nicoletti (2016) the macaroni-like structure in this work in case of the NaOH treated samples appear less segmented. It is speculated that the possible macaroni shape consists of swollen single cells rather than agglomerates.

As mentioned earlier, gelling was most severe with the highest concentrations of base. Heating and centrifugation at higher temperatures (40°C) were attempted for the gelled samples, but this was insufficient to separate the 2M and 4M samples appropriately. The supernatant was also gelling after separation during the 1M test. Higher concentrations of NaOH exacerbated this undesired effect of gelling, making this tests unsuitable for HTL studies. Total nitrogen (TN) was measured as best as possible directly from the top layer right after centrifugation while samples rested in a heat bath to avoid gelling as much as possible. This TN value should be assumed to have higher uncertainty since the solids and supernatant were not easily separated and easily solidified. Hot filtration was considered to obtain more accurate values for TN/TOC of the higher base with heat treatment condition, but the supernatant itself was already gelling after separation from the solid residue for the 1M test.

As part of the various attempts to break down the cellular structure of dry spirulina powder, heating the powder at 100°C and 150°C was tested. This approach aimed to avoid boiling off liquid from a slurry or using a sealed vessel. After heating, the powders were resuspended in water, sodium hydroxide (NaOH), and some in potassium carbonate (K₂CO₃) to compare the rehydrated structure against the control. As Figure 7 shows, more rounded shapes were observed for the 150°C treatment compared to the 100°C treatment, but neither appeared significantly more broken down by this pretreatment.

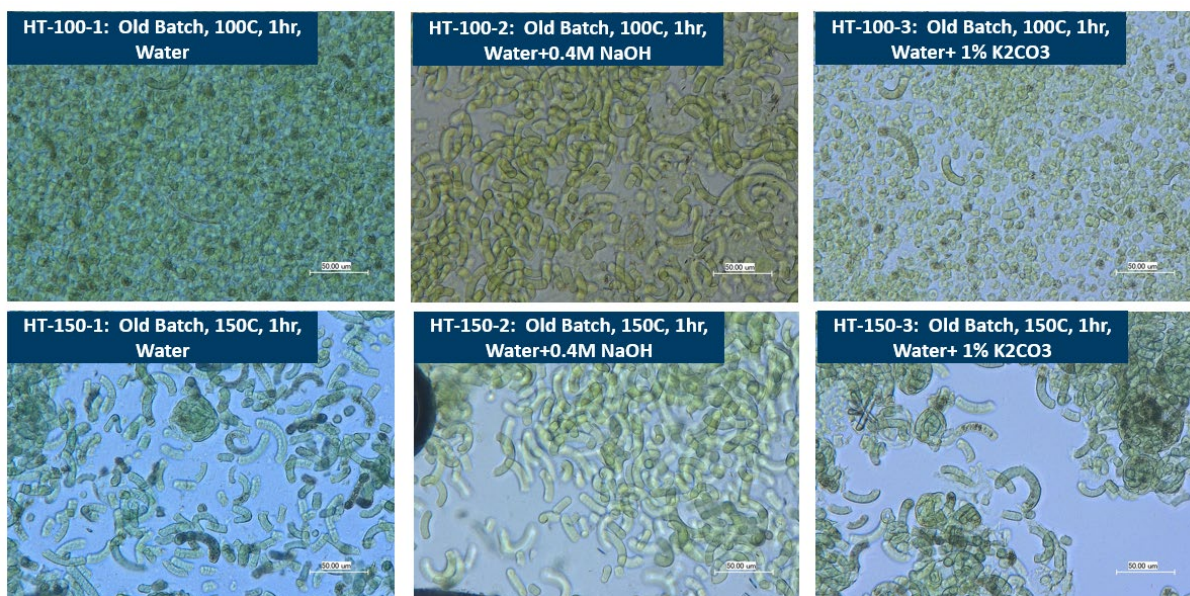


Figure 7 Microscopy results of the trial aiming to heat-treat algae powder to avoid gelling

Microwave treatment was also tested to see if it could help break down the outer wall structure. However, no noticeable changes were observed by microscopy.

3.1.2 Pretreatment with Acids

The acid pretreatment conditions involved various acids and concentrations: 1M hydrochloric acid (HCl), 2M hydrochloric acid (HCl), 1M sulfuric acid (H_2SO_4), 1M formic acid (FA), 2M formic acid (FA), and the control. Results from this campaign are shown in Figure 8.

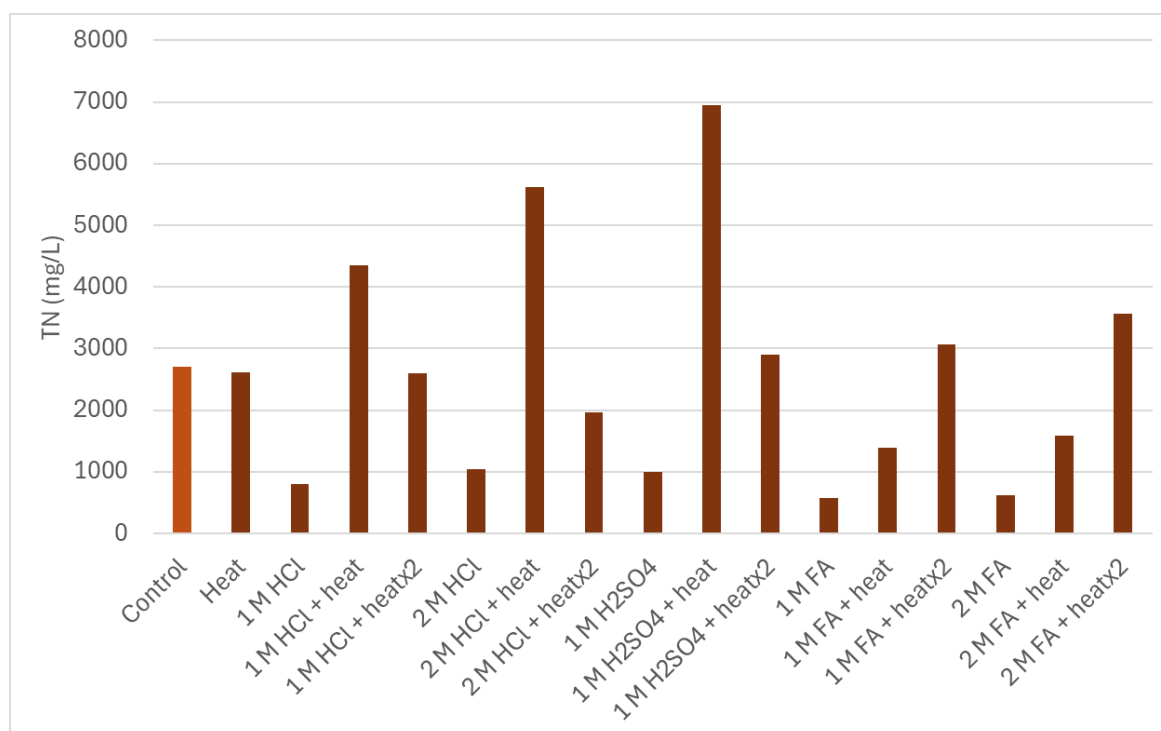


Figure 8 Results from acid pretreatment studies.

Nitrogen removal was minimal with acids alone, it even appears that acid aids keep the nitrogen in the solids, when compared to the control sample. Heating alone did not enhance the nitrogen transfer to liquid phase. However, heating in combination with acids on the other hand, significantly enhanced nitrogen removal. The nitrogen transfer to the liquid phase increased with increasing acid concentration especially inorganic acids compared to the control. Organic acids are generally weaker, but they do not introduce heteroatoms into the system, therefore, formic acid was investigated, but the results did not show satisfactory results. Based on the results it appears that formic acid needs more time and longer heat treatment to remove nitrogen, and the nitrogen removal in case of formic acid was the weakest of all acids investigated. Compared to the other samples, sulfuric acid treatment in combination with heat appeared to be superior to remove nitrogen from spirulina slurries.

The effect of additional centrifugation and additional heat treatment was also investigated, as shown in Figure 9. The protocol consisted of an initial mix of slurry (10% algae), heated for 12 hours, followed by five rounds of centrifugation, and then reheated.

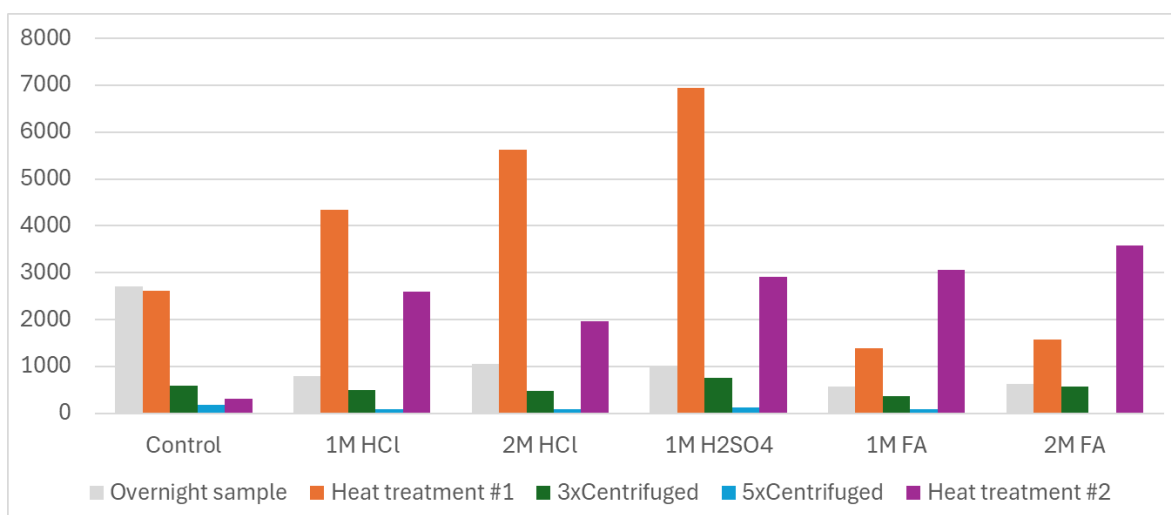


Figure 9. Acid pretreatment studies comparison. Centrifugation testing was completed on a single sample with 5 total rounds of centrifugation, and the TN was analyzed on supernatant collections 1, 3, and 5 to assess if a trend of increasing or decreasing nitrogen removal occurred.

The figure shows that additional centrifugation after heating did not significantly increase nitrogen removal, as evidenced by the nitrogen content of the supernatants. However, further heating did improve nitrogen removal in both acid-treated and control samples. Multiple heat rinses with 1M sulfuric acid showed the highest overall nitrogen removal. In case of formic acid treatment, the as mentioned additional heat treatment, removed more nitrogen than the first heat treatment, but this was the opposite for the inorganic acid treatments.

The TOC results for the samples treated with inorganic acids are shown in Figure 10. Formic acid introduces organic carbon into the mixture, and since the conversion rate of the acid is not known, the analysis cannot be done without error. Figure 11 shows the nitrogen removal as measured by TN and carbon removal as measured by TOC compared to the nitrogen and carbon present in the feedstock.

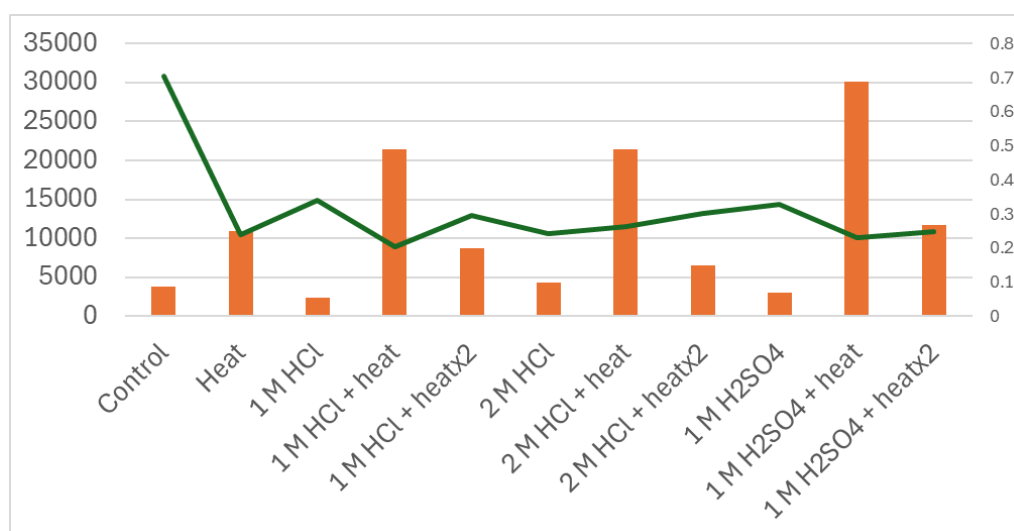


Figure 10 TOC analysis results for the acid treated sample. The TN/TOC ratio is shown as a green line on the diagram

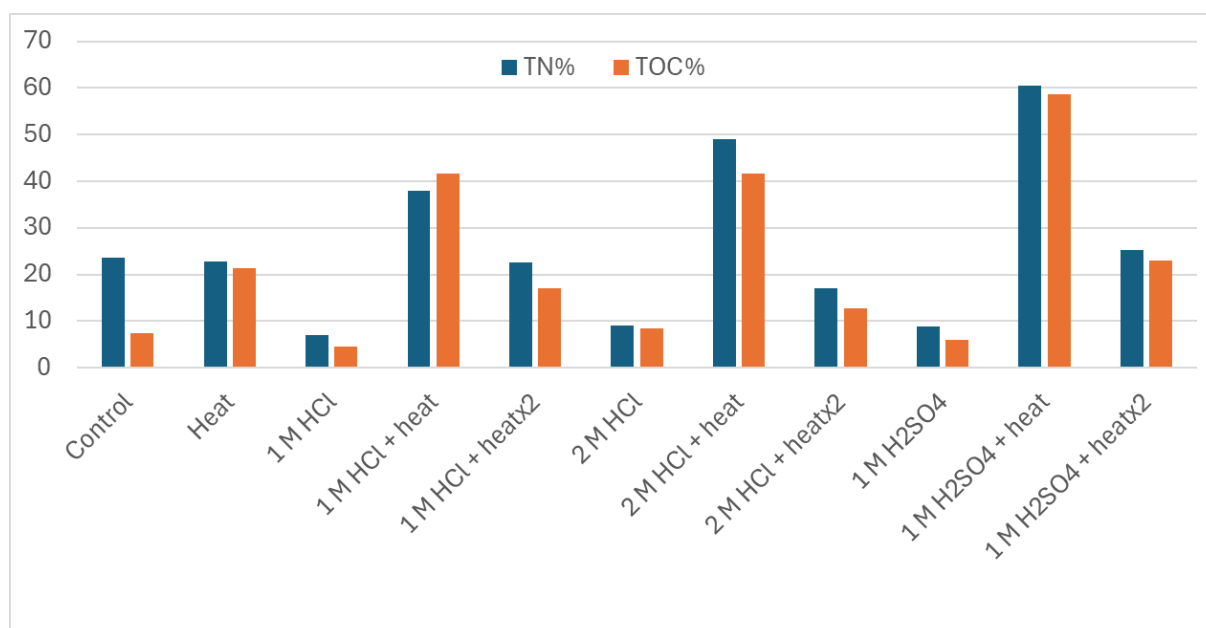


Figure 11 Nitrogen removal (TN) and carbon removal (TOC) efficiencies

The TOC results indicate that together with nitrogen, organic carbon is also removed. The more nitrogen is removed the more carbon is lost. Based on the diagram above, it is difficult to argue for the efficiency of the most efficient pretreatment approaches for nitrogen removal. However, pretreatment can alter the algae structure (Deepika et al., 2024) and the altered structure can behave differently, triggering other reaction routes. If nitrogen removal remains crucial for the subsequent catalytic upgrading processes, one must accept that considerable amounts of carbon are also lost in the pretreatment processes.

Microscopy images were also taken for the acid treated samples to investigate how the algae particles interact in the slurry. Acid treatments initially showed agglomeration or clumping before heat treatment as shown in Figure 12. The image shows round agglomerates rather than a spaghetti-like shape.

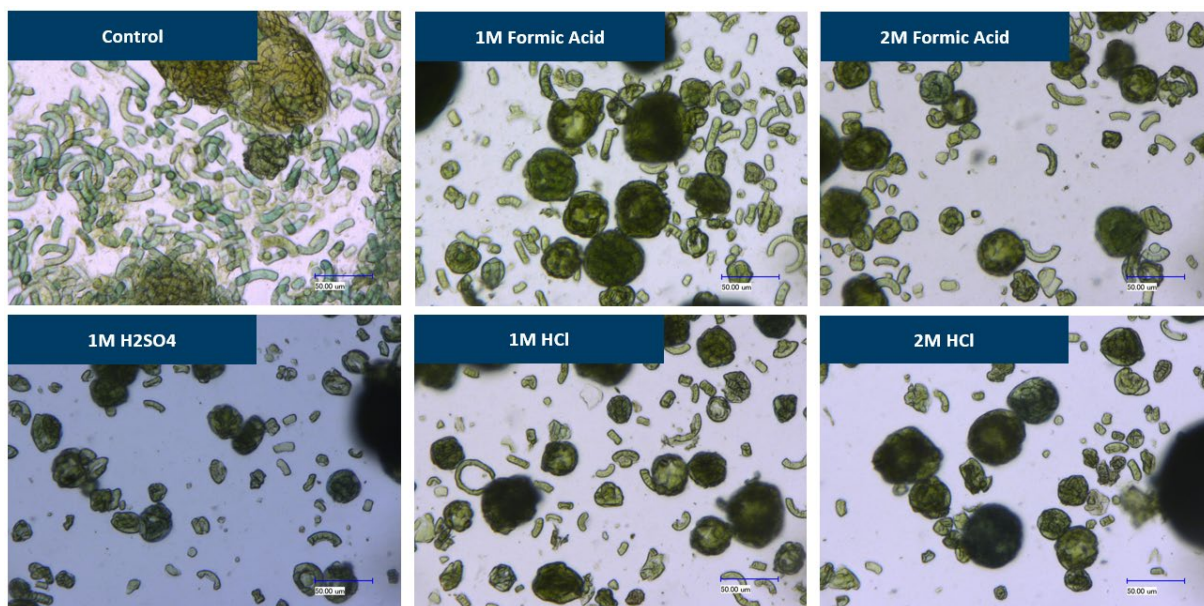


Figure 12 Microscopy images of acid treated samples

The clumping observed after acid treatment diminished already after the first heat-treatment (see Figure 13), as well as with further reductions observed after additional heat-treatment as presented on Figure 14.

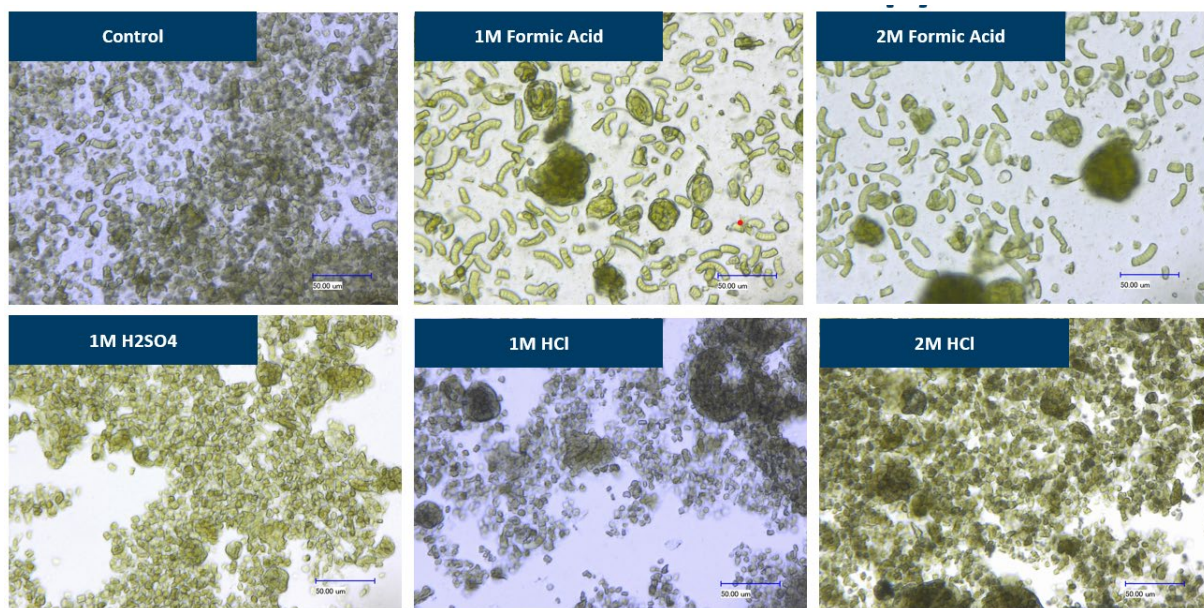


Figure 13 Microscopy images of the acid-treated algae samples after one round of heat treatment

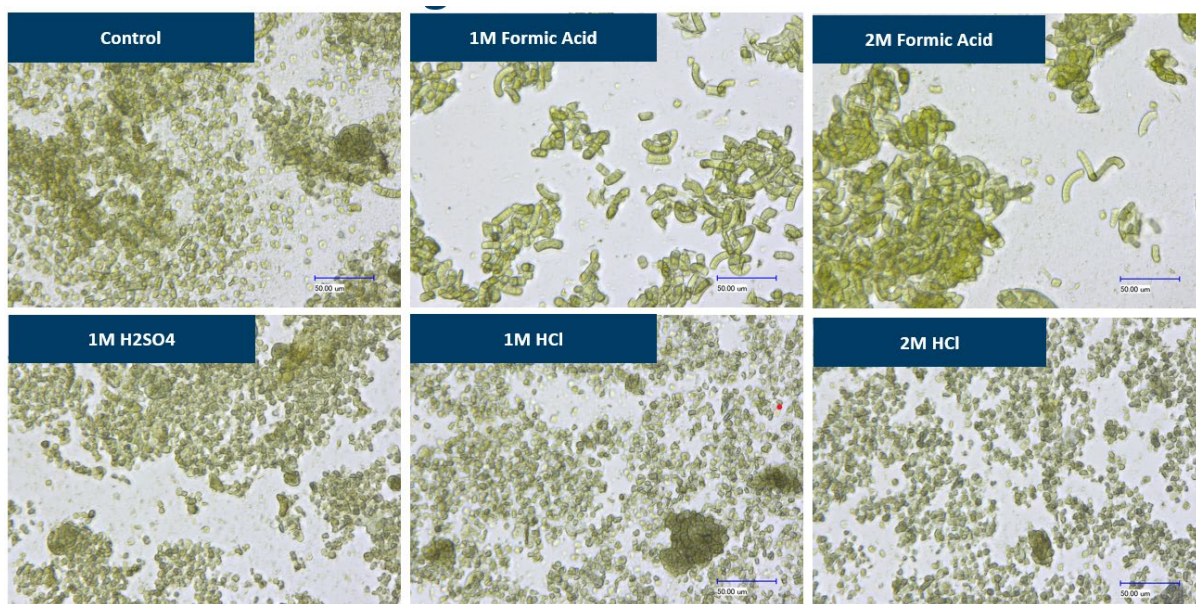


Figure 14 Microscopy images of the acid-treated algae samples after the second round of heat treatment

As the HTL process is preferably carried out under basic reaction regime, the acid-treated samples need to be neutralized, and the pH adjusted to 12 to accompany the needs of the optimal feedstock slurry properties for HTL. The process was carried out in three phases, after the last centrifugation, water was added to the samples, and the pH was adjusted using NaOH in two rounds once to 7 and thereafter to 12. The microscopy images taken along the process are shown in Figure 15.

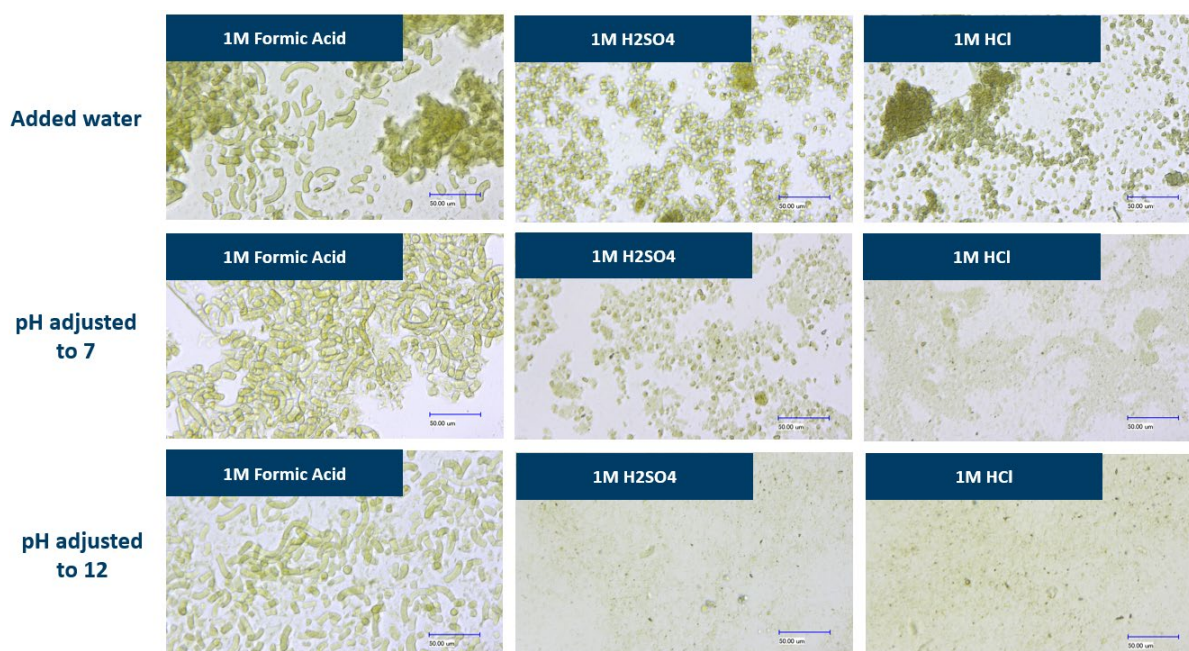


Figure 15 Microscopy images of the acid-treated algae samples during the pH adjustment process.

When the supernatant was removed and water was added to re-suspend the solid residue, it was evident that cells had broken down well with acid and heat-treatment. Neutralizing the supernatant with NaOH further improved the breakup and distribution of cell materials, resulting in a highly

pulverized state. The images also confirm that NaOH addition to acid treated samples does not cause particle swelling and gelling.

4 Conclusions

The objective of this task was to perform pretreatment experiments investigating how the feedstock nitrogen content can be effectively reduced by various pretreatment methods. Microalgae (*Spirulina sp.*) was selected as a nitrogen containing feedstock due to its high nitrogen content, stable properties and availability.

Base addition increased the nitrogen removal in an efficient manner, especially when combined with heat-treatment. However, the base-treated samples formed gels, which pose challenges both for analysis as well as pumpability in a subsequent HTL process. As base treatment was more efficient, compared to acid treatment, additional efforts were made to break the gelled structure, but the gelling could not be resolved in the course of this work. Physical treatments, such as sonication or microwave treatment did not seem effective for breaking up the gelled structure of the samples or remove nitrogen.

Acid treatment shows weaker nitrogen removal than base treatment, but in combination with heat-treatment acids seemed also effective. In addition, acid samples did not gel and could be pH adjusted to 12 with NaOH without gelling. Of the investigated acids, sulfuric acid treatment in combination with heating had the highest nitrogen concentration in the supernatant compared to the other acid-treated samples.

Additional centrifugation after heating did not significantly increase nitrogen removal, as evidenced by TN testing over five rounds. However, further heating did improve nitrogen removal in both acid-treated and control samples.

Based on the results from the pretreatment tests showing the nitrogen removed to the supernatant in addition to observed gelling issues, the overnight heating with acid and with water were selected for HTL testing. Due to the energy and time requirements also at industrial scale and to maximize efficiency, it was decided that no reheating will be employed as well as the centrifugation will be kept minimal.

The TOC results of the inorganic acid-treated samples showed that considerable amounts of carbon are also lost in the pretreatment processes. As no information on how the removal of the nitrogen and carbon containing components affects the HTL reaction pathways, more information is expected during consecutive activities to reveal the real effect of the investigated pretreatment methods.

Based on the results from the pretreatment experiments as well as the uncertainties regarding the carbon loss, multiple HTL experiments with the following slurry treatments were recommended.

- Baseline: untreated, 10% DM algae-water slurry feed directly to HTL
- Water-washed: 10% DM algae washed suspended in cold water overnight and centrifuged. 10% DM slurry prepared with water and fed into HTL
- Heat-treated: 10% DM algae-water slurry heated overnight, centrifuged, 10% DM slurry prepared with water and fed into HTL
- Sulfuric acid + heat treated: Heated overnight heat in 1M sulfuric acid, centrifuged, neutralized with NaOH and centrifuged again. 10% DM slurry prepared with water and fed into HTL

The detailed slurry preparation methods, recipes and analysis of the feedstock in addition to the results from the HTL experiments will be presented in D2.3.

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