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Culture de microalgues sur phase aqueuse issue de liquéfaction hydrothermale

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Microalgae cultivation on recycled hydrothermal liquefaction aqueous phase

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A *mis padres*,

A *Andreas*,

Por su amor y apoyo incondicional.

Summary

Microalgae are a promising source of biomass to produce renewable energy as an alternative to fossil fuels. Hydrothermal liquefaction (HTL) is a route for converting wet algal biomass into biofuel. HTL produces biocrude oil next to an aqueous phase (AP). This effluent represents a rich source of nutrients for microalgae cultivation that remains limited by its toxicity. Recycling the AP as a substrate is proposed to recover nutrients and close the process loop, produce more algal biomass, and turn this biomass into a new biocrude.

This thesis was conducted within the framework of the ANR project RAFBIOALG. The project studies each stage and overall microalgae-to-biofuel conversion through HTL. The specific objective of this thesis was to investigate the potential and limitations of AP recycling for the production of new microalgal biomass. The first step was to select an appropriate strain to produce high-quality feedstocks for HTL (**Paper I**). This first study compared the biomass quality and lipid content of six *Chlorellaceae* strains under balanced nutrient limitation to promote storage-compound accumulation. *Chlorella vulgaris* NIES 227 stood out as the most promising candidate for lipid-based biofuels and produced 54% lipids in phototrophic conditions with the highest energy content.

To successfully incorporate the AP during biomass production, the microalgae tolerance and the AP impact on biomass quality were assessed (**Paper II**). The microalgae's response to the AP was monitored over five cycles of growth under nutrient-replete conditions. The last cycle was prolonged to reach nutrient-limiting conditions to assess the AP effect on lipid accumulation. The results demonstrated that a few cycles of acclimation to the AP were effective in improving the microalgal growth rate. Using AP as a substrate reduced the need for external N, S, and P, while producing 2.95–4.27 g/L of biomass with 16–36% lipid content. However, the presence of the AP overall reduced biomass production and it slowed down the accumulation of lipids. This resulted in biomass with suboptimal characteristics, as it reduced the overall energy content of the biomass in all strains. Nevertheless, *C. vulgaris* NIES 227 maintained the best lipid productivity and lipid profile for HTL among the tested strains.

It was identified that recycling AP supported microalgal growth, yet further measures were necessary to alleviate its inhibitory effects: reduced photosynthetic performance, compromised growth rate, and lower-quality biomass for HTL. To mitigate this inhibition, adaptive laboratory evolution (ALE) was studied via batch transfers and turbidostat mode on *C. vulgaris* NIES 227 (**Paper III**). Both methods effectively counterbalanced the AP toxicity observed in the wild-type (WT) and produced two distinctive ALE strains. The resulting ALE strains, grown in AP, maintained competitive productivities (0.310 and 0.258 g/L/d) of lipid-rich biomass, 37% and 56% respectively. These results showed the potential of AP-grown biomass for HTL conversion, which was similar to the WT in the control medium at 0.261 g/L/d and 60% of lipids. Interestingly, the acquired tolerance of the adapted strains persisted after the removal of AP and under axenic conditions.

Overall, this thesis stresses the importance of strain selection and optimization, which are essential for any microalgal application yet remain strategic for the production of sustainable energy sources. The promising results of *C. vulgaris* NIES 227 for lipid-based biofuels further highlight the need for continuous research and development in this field. Additionally, ALE emerges as a promising approach to improving the tolerance of microalgae to AP toxicity, which can be implemented to manage other toxic compounds or effluents containing growth inhibitors. This thesis provides valuable insights and recommendations toward the successful recycling of the AP, ultimately contributing to the transition toward sustainable energy sources.

Keywords:

Adaptive laboratory evolution; Aqueous phase recycling; Biofuels; Biomass; Hydrothermal liquefaction; Microalgae; Lipids.

Résumé

Les microalgues représentent une source prometteuse de biomasse pour la production d'énergie renouvelable comme alternative aux combustibles fossiles. La liquéfaction hydrothermale (HTL) est un moyen de convertir la biomasse algale humide en biocarburant. L'HTL produit un bio-brut et un sous-produit, la phase aqueuse (AP). Cet effluent représente une riche source de nutriments pour la culture des microalgues, mais sa toxicité limite son recyclage.

Cette thèse a été réalisée dans le cadre du projet ANR RAFBIOALG, qui étudie chaque étape de la conversion globale de la microalgue en biocarburant par HTL. L'objectif spécifique de cette thèse était d'étudier le potentiel et les limites du recyclage de l'AP pour la production de nouvelle biomasse microalgale. Pour ce faire, la première étape a consisté à sélectionner une souche de microalgue permettant la production d'un intrant de haute qualité pour l'HTL. Cette première étude a comparé la qualité de la biomasse et la teneur en lipides de six souches de *Chlorellaceae*. *Chlorella vulgaris* NIES 227 s'est distinguée comme une souche idéale pour les biocarburants à base de lipides. Elle a produit 54% de lipides en conditions phototrophiques et avait le contenu énergétique le plus élevé.

Pour réussir à utiliser l'AP pour la production de biomasse, il a fallu évaluer la tolérance des microalgues et l'impact de l'AP sur la qualité de la biomasse. La réponse des microalgues à l'AP a été suivie au cours de quatre cycles de croissance, suivis d'un cinquième cycle prolongé. Les résultats ont montré que quelques cycles d'acclimatation à l'AP permettaient d'améliorer le taux de croissance des microalgues. L'utilisation de l'AP comme substrat a réduit les besoins des souches en nutriments (N, S et P) tout en produisant entre 2,95 et 4,27 g/L de biomasse ayant une teneur en lipides entre 16% et 36%. Cependant, la présence d'AP a donné lieu à une biomasse aux propriétés non optimales, car elle a réduit la production de biomasse, ralenti l'accumulation de lipides et donc réduit le contenu énergétique global de la biomasse dans toutes les souches. *C. vulgaris* NIES 227 a conservé la meilleure productivité lipidique et le meilleur profil lipidique pour l'HTL parmi les souches testées.

Le recyclage de l'AP a donc permis la croissance des microalgues, mais d'autres mesures ont été nécessaires pour atténuer ses effets inhibiteurs : réduction des performances photosynthétiques et du taux de croissance, et biomasse de moindre qualité pour l'HTL. Pour atténuer cette inhibition, l'évolution adaptative en laboratoire (ALE) a été étudiée via des dilutions successives et en mode turbidostat sur *C. vulgaris* NIES 227. Ces deux méthodes ont permis de compenser efficacement la toxicité de l'AP observée chez la souche sauvage. Les deux souches ALE produites, cultivées en AP, ont maintenu des productivités (0,310 et 0,258 g/L/j) de biomasse riche en lipides (37% et 56%, respectivement) pour la conversion par HTL, proche de la souche sauvage en milieu contrôle avec 0,261 g/L/j et 60% des lipides. Il est intéressant de noter que la tolérance acquise par les souches adaptées a persisté après l'élimination de l'AP et dans des conditions axéniques.

Cette thèse souligne l'importance de la sélection et de l'optimisation des souches, qui restent stratégiques pour la production de sources d'énergie durables. Les résultats prometteurs de *C. vulgaris* NIES 227 pour la production de biocarburant à partir de lipides soulignent la nécessité d'une recherche et d'un développement continus dans ce domaine. En outre, l'ALE apparaît comme une approche prometteuse pour améliorer la tolérance des microalgues à la toxicité de l'AP, qui peut être mise en œuvre pour gérer d'autres composés toxiques ou des effluents contenant des inhibiteurs de croissance. Cette thèse fournit des informations et des recommandations précieuses pour le recyclage réussi de l'AP, contribuant ainsi à la transition vers des sources d'énergie durables.

Mots-clés :

Biocarburants ; Biomasse microalgale ; Évolution adaptative en laboratoire ; Liquéfaction hydrothermale ; Lipides ; Microalgues ; Recyclage de la phase aqueuse

List of Publications

I. **Ramírez-Romero, A.,** Da Costa Magalhães, B., Dimitriades-Lemaire, A., Sassi, J.-F., Delrue, F., Steyer, J.-P., (2022). *Chlorellaceae* Feedstock Selection under Balanced Nutrient Limitation. *Fermentation* 8, 554. https://doi.org/10.3390/fermentation8100554

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Other publications from the author not presented in this thesis:

IV. Chambonniere, P., **Ramírez-Romero, A.**, Dimitriades-Lemaire, A., Sassi, J.-F., Delrue, F., (2022). Photosynthetic Carbon Uptake Correlates with Cell Protein Content during Lipid Accumulation in the Microalga *Chlorella vulgaris* NIES 227. Fermentation 8, 614. https://doi.org/10.3390/fermentation8110614

V. da Costa Magalhães, B., Matricon, L., **Ramírez Romero, A.**, Checa, R., Lorentz, C., Chambonniere, P., Delrue, F., Roubaud, A., Afanasiev, P., Laurenti, D., Geantet, C., (2023). Catalytic hydrotreatment of bio-oil from continuous HTL of *Chlorella sorokiniana* and *Chlorella vulgaris* microalgae for biofuel production. Biomass and Bioenergy 173, 106798. https://doi.org/10.1016/j.biombioe.2023.106798

VI. Geantet C., **Ramírez-Romero, A.,** Matricon L., da Costa Magalhães, B., Ruben C., Lorentz C., Delrue F., Roubaud A., Afanasiev P., Laurenti D., Brockmann D., From growth to fuel: an overview of algal biofuels HTL pathway with the case study of *Chlorella sorokiniana. Manuscript in preparation for resubmission* (2023).

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Abbreviations

ALE: Adaptive Laboratory Evolution
AP: Aqueous Phase
BA: Batch-Adaptation/Batch-Adapted
CCALA: Culture Collection of Autotrophic Organisms (Czech Republic)
DW: Dry Weight
FAME: Fatty Acid Methyl Esters
FM: Maximum Fluorescence
FV: Variable Fluorescence
GC-FID: Gas Chromatography with Flame Ionization Detection
GHG: Greenhouse Gas Emissions
HTL: Hydrothermal Liquefaction
IC: Inorganic Carbon
IN: Inorganic Nitrogen
MUFA: Monounsaturated Fatty Acids
NIES: National Institute for Environmental Studies (Japan)
OD: Optical Density
PBR: Photobioreactor
PSI: Photosystem I
PUFA: Polyunsaturated Fatty Acids
QY: Quantum Yield
SFA: Saturated Fatty Acids
TA: Turbidostat-Adaptation/Turbidostat-Adapted
TC: Total Carbon
TOC: Total Organic Carbon
TN: Total Nitrogen
WT: Wildtype (i.e. an organism or strain that has not undergone genetic modification or other intentional alterations)

1. Introduction

1. 1. Need for an Energy Transition

The climate crisis is among the greatest challenges of our era, as it impacts natural ecosystems and as a consequence, human systems as well, with observed problems in water security and food production, health and well-being, and cities, settlements and infrastructure, population displacement among others (Kemp et al., 2022). At the same time, the global human population is ever-growing, and it is estimated to reach up to 9.7 billion by 2050, which implies a greater demand for resources such as food, water, and energy (United Nations, 2022). The reliance on fossil fuels represents a pressing issue. Fossil fuels are the largest contributor to global greenhouse gas (GHG) emissions accounting for 64% of total emissions. It has been estimated that limiting global warming to around 1.5°C requires a 43% reduction in GHG emissions by the year 2030 (Pörtner et al., 2022)

To mitigate the cascading effects of climate change (**Figure 1. 1**), the IPCC proposes an energy transition toward sustainable and carbon-neutral sources as one of several solutions that must be implemented. Additionally, the volatility of oil prices and geopolitical issues (i.e., the Russian-Ukrainian conflict) have intensified the need to look for alternative energy sources. For instance, the recent surge in international fossil fuel prices will raise European households' cost of living in 2022 by close to 7% of consumption on average (Ari et al., 2022). Altogether, it is required to decrease the use of and dependence on fossil fuels. Now more than ever, we need to explore alternative ways of generating energy while minimizing negative effects on the environment and society. Bioenergy, or renewable energy produced from natural sources, is becoming increasingly popular as a good alternative to fossil fuels (Oncel, 2013). However, one of the greatest challenges lies in providing enough water and raw materials without affecting the food supply, or increasing the water and carbon footprints. Additionally, new technologies must be able to compete with the price of conventional fossil fuels. These challenges highlight the need to explore potential pathways to obtain bioenergy in the most sustainable and cost-effective way.

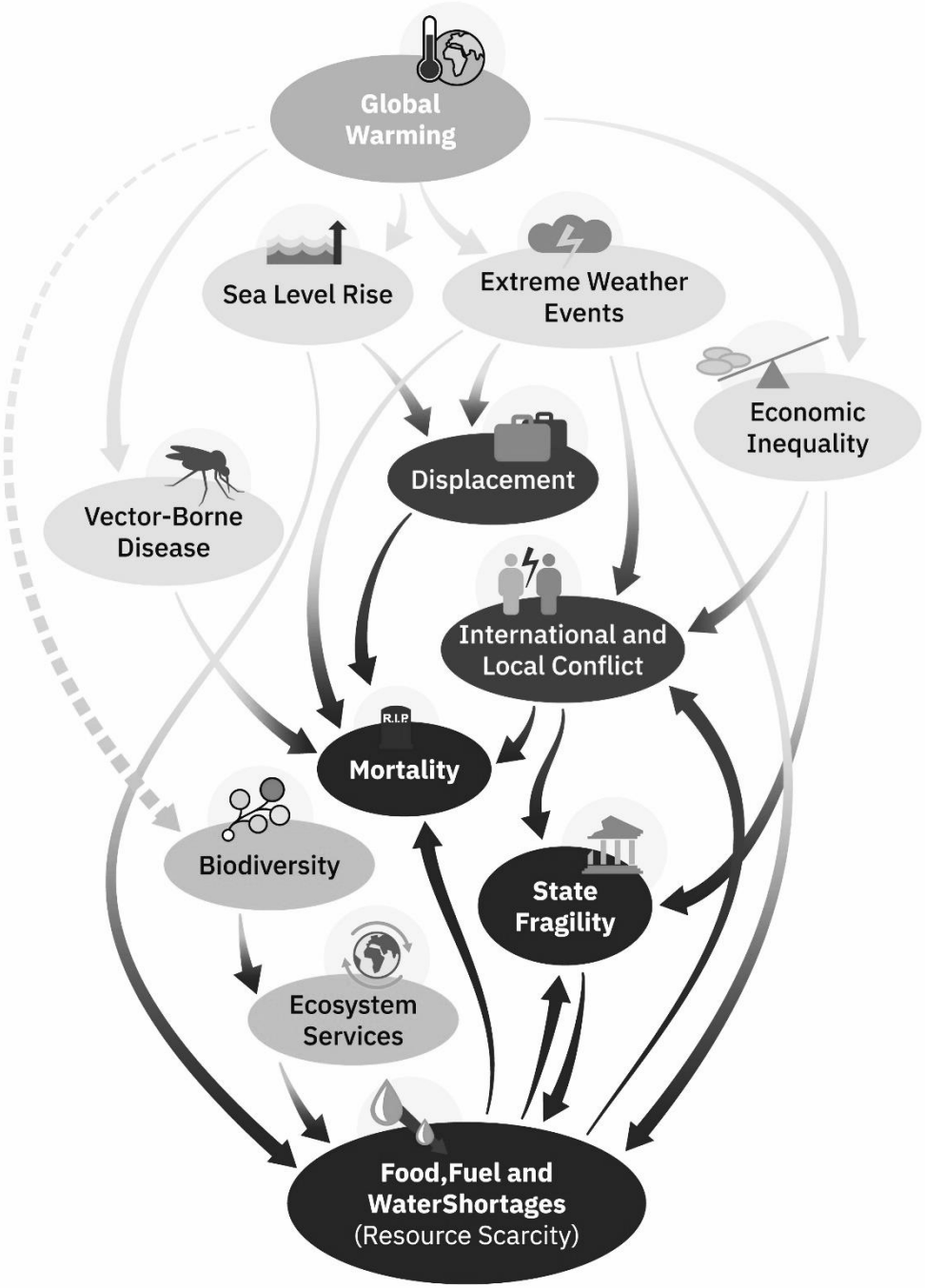


Figure 1. 1. Cascading effect of the climate crisis. Scheme sourced from (Kemp et al., 2022).

In order to successfully transition toward sustainable bioenergy, some critical considerations should be applied to any source of energy: low-cost, sustainable, and environmentally friendly (Athar and Zaidi, 2020). To make the process economically viable and competitive with petroleum prices, raw materials should be low-cost and widely available. Furthermore, raw material production should have a low environmental impact and should not compete with food supply or land use.

1. 2. Microalgae for Bioenergy Production

Microalgae are an attractive source of biomass for fossil fuel alternatives (Khan et al., 2018). These microorganisms have been studied as energy sources since the 1950s at the University of California, Berkeley (Meier, 1955). Oswald and colleagues proposed an integrated system of microalgae ponds followed by anaerobic digestion units for the production of methane as a source of fuel (Oswald and Golueke, 1960). During the 1970s oil crisis, the Aquatic Species Program (1978–1996) was implemented in the USA to explore and develop renewable energy sources using microalgae as raw material (Sheehan et al., 1998). This project initially aimed at using microalgae to produce hydrogen, but later shifted its focus toward biodiesel production. A major limitation that slowed the scalability of the research was the high production cost and necessary selling price compared to petroleum oil.

Forty-five years later, as climate change has become a more pressing issue and geopolitical crises put energy supplies at risk, the need to find new alternatives has become a priority. Interestingly, microalgae, among the remains of plants and animals, contribute to the carbon sources that form fossil crude oil (Vandenbroucke and Largeau, 2007). The organic matter of these remains has been subjected to high pressure and heat over millions of years. These conditions promote the breakdown of biomass, which is transformed into the hydrocarbons found in conventional petroleum oil (Zhang, 2011).

1. 3. Potential of Microalgae Biomass

Microalgae are microscopic aquatic microorganisms found in a wide range of aquatic habitats, including lakes, ponds, rivers, oceans, and even wastewater. These microorganisms perform photosynthesis similarly to plants, consuming carbon dioxide (CO_2) and releasing oxygen (O_2). Hence, these microorganisms convert solar energy, their primary energy source, into chemical energy and produce biomass in the form of numerous compounds. With an estimate of more than 1 million species, of which only 50,000 have been described to date, microalgae have enormous potential to be exploited due to their unique characteristics and ability to grow in different environments (Guiry, 2012).

Microalgae have numerous characteristics that make them a promising source of bioenergy. Microalgae can have a higher growth rate and photosynthetic efficiency than most terrestrial plants (Borowitzka and Moheimani, 2013). It is projected that lipid productivity of microalgae could be three to eighteen times higher than that of agricultural crops. For instance, microalgae's lipid productivity has been estimated to be of 18 tons/ha/year compared to palm with 6/tons/ha/year, which its harvest has lead to massive deforestation (Barbosa et al., 2023). Additionally, the versatility of microalgae cultivation represents a major advantage. These photosynthetic microorganisms can indeed grow and adapt to a wide range of environmental conditions; thrive in several water sources ranging from seawater to wastewater; and can be cultivated in very simple to more sophisticated systems (Khan et al., 2018). Furthermore, unlike other biomasses, such as crops destined for food and feed, the cultivation of microalgae for bioenergy does not compete for arable land.

1. 3.1. Microalgae Carbon and Nutrient Requirements for Biomass Production

Microalgae can thrive in almost any place where light, CO_2, and water are available. Furthermore, they can grow using several types of carbon sources, including i) phototrophic growth: atmospheric inorganic sources (i.e., CO_2) as the sole carbon source in the presence of light; ii) heterotrophy: organic carbon sources (such as glucose, acetate, and glycerol) in the absence of a light source; or iii) mixotrophy: a combination of both inorganic and organic sources (Barbosa et al., 2023).

This ability to grow under different carbon sources can influence the yield of microalgae biomass. Uptake of a dual carbon source during mixotrophy enables the microalgae to overcome the limitations of autotrophic growth, such as dependence on light intensity and quality, and the limitations of heterotrophic growth, such as the requirement for additional carbon sources and dependent on the capacity of the strains to live in the dark (Castillo et al., 2021). When grown under mixotrophic conditions, microalgae generally present improved growth rates, biomass and lipid accumulation (Marchello et al., 2018; Shen et al., 2019).

In addition, microalgae require a supply of nutrients to grow. Macronutrients such as nitrogen, phosphorus, sulfur, and potassium are needed for the synthesis of cell structural components (Markou et al., 2014). Microalgae also need other minerals in smaller quantities, such as iron, zinc, copper, and magnesium, to perform various metabolic processes (Procházková et al., 2014). These micronutrients play a crucial role in different cellular functions such as energy production, enzyme activity, and pigment synthesis.

1. 3.2. Microalgae Biomass Composition for Biofuel Production

Microalgae biomass has a wide range of applications, including food for human consumption, animal feed, biofuel, and bioplastics (**Figure 1. 2**). Microalgae convert CO_2 during photosynthesis into organic compounds such as carbohydrates, lipids, proteins, and nucleic acids (Calijuri et al., 2022). These macromolecules are essential to various cellular processes in microalgae for growth and survival, and they can serve to produce a wide range of energy sources. Microalgae also contain secondary metabolites responsible for cellular functioning, including pigments, vitamins, and anti-inflammatory and antioxidant compounds (Markou and Nerantzis, 2013). These compounds are attractive high-value products for the cosmetic and nutraceutical markets (Abu-Ghosh et al., 2021). Microalgae extracts can also be used as plant stimulants or even as herbicides (Costa et al., 2019), providing an environmentally friendly alternative to conventional agricultural products such as glyphosate, which is banned from use in Europe as of 15th December 2022 (Commission Implementing Regulation (EU) 2017/2324) .

The composition of microalgae biomass is defined by their genetics and influenced by the cultivation conditions. For instance, under the same conditions, some microalgae naturally produce higher amounts of lipids while others produce higher amounts of carbohydrates (Takeshita et al., 2014). Likewise, one microalga can present varying biochemical compositions under different cultivation conditions (Qiu et al., 2017). Environmental factors can steer the microalgae's metabolism toward the production of certain macromolecules; these include light (duration, intensity, and spectrum), nutrient supply, and culture conditions such as pH and temperature (Markou, 2020). When these environmental conditions are not optimal for microalgae, this can trigger a shift in the metabolism of microalgae and thus its biochemical composition.

1. 3.3. Metabolic Triggers for Energy-Rich Biomass

Microalgae cultivated under conditions of nutrient deficiency accumulate considerable amounts of lipids or carbohydrates (Markou and Nerantzis, 2013). Nitrogen enables cellular division when sufficient and triggers carbon accumulation when deficient in the form of carbohydrates or lipids (Di Caprio, 2021). Thus, nutrient supply can define both the microalgae composition and the amount of produced biomass. The biosynthesis of lipids and carbohydrates serves as a survival mechanism for microalgae under stress conditions, such as nutrient deficiencies (Zhu et al., 2018). The specific preference of each strain toward lipid or carbohydrate storage under nitrogen stress depends on each microalgae's metabolic capacities and energy requirements for the biosynthesis of these molecules (Li et al., 2015). Storage of starch is an immediate response to N deficiency, and later carbohydrate degradation and lipid biosynthesis take place after prolonged stress for long-term energy storage (Nordin et al., 2020).

1. 4. Conversion of Biomass into Biofuels

Microalgae's ability and versatility to store energy-rich compounds make them a promising source of bioenergy (Mondal et al., 2017). At the same time, these compounds serve as energy carriers during biomass conversion into biofuels (Biller and Ross, 2011). Understanding the influence of the strained nature in conjunction with the cultivation conditions is essential for microalgae applications. Depending on the composition of the biomass, this

can have preferred applications, and depending on the biofuel of interest, different biomasses would be better suited.

Several pathways exist today to convert biomass into a variety of energy sources (Box 1). These pathways can be defined based on: (i) the type of biomass or raw material used (commonly referred to as feedstock), (ii) the biomass compound(s) of interest, (iii) the end product, and (iv) their conversion method.

Box 1. Biomass-to-biofuel pathways.

i) Biofuels can be classified by the nature of the feedstock, typically identified by generations (Mat Aron et al., 2020). First-generation biofuels are made from food crops, including corn, barley, sugarcane, and wheat. Second-generation biofuels avoid using food crops by shifting to lignocellulosic biomass and agricultural residues, while third-generation biofuels use microalgae and other microorganisms as a source of biomass. Fourth-generation biofuels are made from metabolically modified microorganisms, including microalgae.

ii) The main component of interest can be processed by different routes and influence the final product. For instance, lipid-rich biomass is better suited for producing bio-oil, biocrude, and biodiesel. In the production of biodiesel, lipids are directly extracted from biomass for further conversion (Patel et al., 2020). Similarly, carbohydrate-rich biomass can be transformed into bioplastics or bioethanol (de Carvalho Silvello et al., 2022).

iii) Microalgae biomass can be converted into liquid biofuels, gaseous biofuels (biogas and hydrogen), and solid biofuels (biochar) (W.-H. Chen et al., 2015). Among the liquid biofuels, microalgae can be used to produce biodiesel, bioethanol, and bio-oil through different conversion processes.

iv) Various biomass conversion methods exist, including thermochemical, biological, and chemical conversion of biomass. Each method has different advantages and disadvantages and results in different end products. For instance, biodiesel is obtained through the extraction of lipids from the biomass, followed by their chemical conversion (transesterification); bio-oil is obtained via thermochemical conversions like hydrothermal liquefaction and pyrolysis; and bio-ethanol, hydrogen, and methane are produced by biological pathways (Peng et al., 2020).

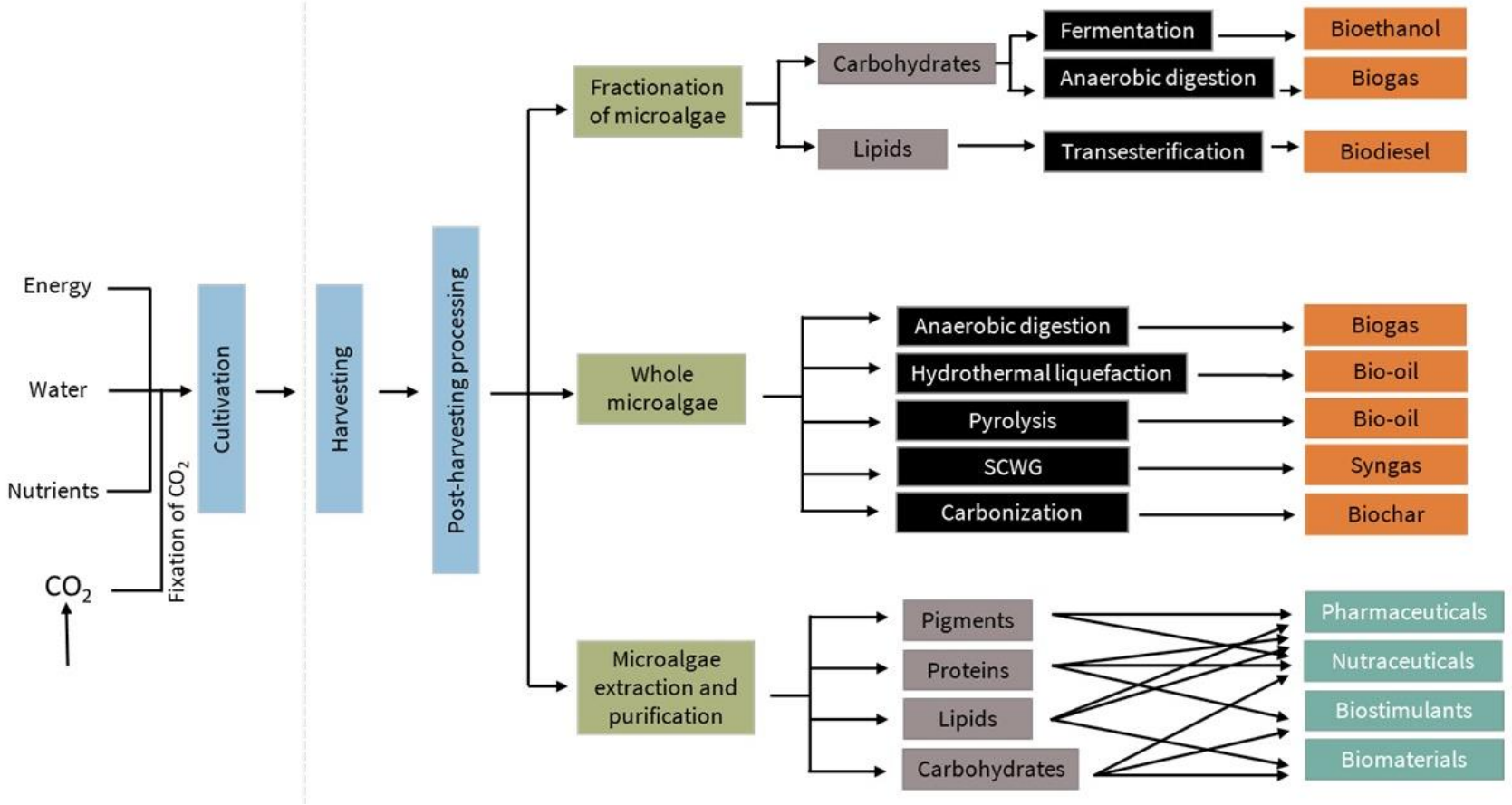


Figure 1. 2. Overview of the products derived by microalgae biomass and the conversion pathway(s) associated to each product. Microalgal-based systems begin from the production of microalgal biomass and are followed by a series of downstream stages after recovery and processing of the biomass. This biomass can then serve for a plethora of applications through different conversions pathways. SCWG: Super Critical Water Gasification.

1. 4.1. Thermochemical Conversions

Over millions of years, fossil organic matter has naturally undergone thermochemical reactions in the Earth's subsurface, decomposing into hydrocarbons and giving origin to petroleum crude (Venkatachalam et al., 2021). Current technologies are based on this natural thermochemical conversion and are among the most promising biomass-to-energy pathways (Table 1. 1). During this type of conversion method, the feedstock is exposed to heat and pressure, causing the breakage of chemical bonds among the biomass components and the creation of new chemical compounds, including biofuel hydrocarbons (Castello et al., 2018). These technologies provide several advantages over chemical and biochemical methods. Remarkably, the ease and efficiency with which a wide variety of biomasses can be converted into liquid biofuels (Grande et al., 2021). While the economy of these processes is questionable, due to the utilization of higher pressure conditions, biomass can be processed with a high conversion rate and a high energy output (Gollakota et al., 2018).

Table 1. 1. Biomass conversion processes and main products obtained.

Conversion	Process	Biofuel
Thermochemical	Gasification	Syngas
	Hydrothermal Liquefaction	Bio-oil
	Pyrolysis	Biodiesel
Biological	Anaerobic Digestion	Biogas
	Fermentation	Bioethanol
	Water photolysis and anaerobic fermentation	Hydrogen
Chemical	Transesterification	Biodiesel

1. 4.2. Microalgae-to biofuel via Hydrothermal Liquefaction

In particular, hydrothermal liquefaction (HTL) is a promising route for biofuel production from wet microalgae. This technology is based on the thermochemical conversion principle of applying heat and pressure to the biomass, but in the presence of water. Beyond the enormous potential of microalgae biofuel, its production faces several major bottlenecks, including the high cost of microalgae cultivation, the energy and cost required for drying the microalgae biomass, as well as the energy-intensive and time-consuming solvent-based oil extraction techniques. (Bagchi et al., 2021). Coupling HTL with microalgae may address some of these issues. One of the most appealing advantages of this process is the use of water as the main reactant to convert biomass. Producing the biocrude oil directly from the wet microalgal slurries, which contain 70–90% water, reduces the energy intensity required to dry the high-moisture content of microalgae biomass (Djandja et al., 2020). Additionally, HTL processes the entire microalgae biomass, removing the need for prior biomass treatment.

Microalgae-based hydrothermal liquefaction (HTL) is a multi-stage process that requires several steps to convert biomass into biofuels. The process typically begins with microalgae cultivation, where the biomass is grown under specific conditions to maximize lipid content. The biomass is then harvested and undergoes feedstock preparation, where the biomass is de-watered by centrifugation, filtration or flocculation, to adjust it to a certain water content in the HTL reactor (typically 80%).

Next, the biomass is subjected to HTL reaction, which occurs in a reactor under high temperatures and pressures in the presence of water. The reaction converts the biomass into biocrude, which is a mixture of the produced crude oil and reactant water. The biocrude is then separated from the aqueous phase using various techniques such as decantation or filtration. The separated biocrude is further processed through bio-oil upgrading, which involves removing impurities and stabilizing the bio-oil. Finally, the bio-oil undergoes refining to produce a finished biofuel product.

Microalgae as an HTL feedstock

After microalgae cultivation, the obtained biomass can serve as feedstock for biofuel production. Microalgae biomass is among the most studied feedstocks for HTL (Prestigiacomo et al., 2022). In combination with the HTL process conditions (i.e., temperature, biomass loading, reaction time), the feedstock composition can have an important effect on the success of the HTL to obtain a high-quality biocrude. Furthermore, a biocrude of lower quality requires more intensive or additional upgrading steps, which results in a less cost-efficient process. These biochemical characteristics of the feedstock, in combination with the process reacting conditions, determine the properties, and yield of the biofuel (Nguyen et al., 2020; Ratha et al., 2022). Therefore, specific attributes of the biomass, presented in **Box 2**, are necessary to target high-quality HTL biofuels.

HTL conversion and by-products

During HTL, biomass is exposed to temperatures ranging between 250°C to 450°C and a pressure going from 100 to 350 bar (Castello et al., 2018). Under these conditions, macromolecules are broken down into simpler compounds and undergo further chemical reactions (**Figure 1. 3).** Biomass components are decomposed by a series of hydrolysis, decarboxylation, and deamination reactions, and a final repolymerization of the reactive intermediate (Hietala and Savage, 2021). The oil fraction is then recovered in the biocrude, along with several by-products that are further separated by their physicochemical properties. Various methods such as filtration, centrifugation, or decantation are used for product separation.

Box 2. Ideal characteristics of HTL feedstocks

- High energy content in the feedstock is desirable to maximize the energy yield from the HTL process. Typically, carbon-rich biomasses promote a higher energy content (Yoo et al., 2015).

- High lipid content in the feedstock is desirable to produce HTL bio-oil, as lipids are easily converted into the bio-oil. Moreover, HTL biocrude originates predominantly from the conversion of lipids, followed by proteins, and lastly by carbohydrates (Biller and Ross, 2011).

- Next to the lipid quantity, the lipid quality can also influence the efficiency of the process and the biofuel properties. Certain fatty acids could require further upgrading. For instance the presence of double bonds can result in molecules that are harder to process, affecting the quality of the resulting bio-oil and its economic cost (Bezergianni et al., 2018).

- Low oxygen and nitrogen content are also advantageous. Low levels of these elements minimize the presence of N- and O- heterocycles in the biofuel. These recalcitrant compounds must be removed from the bio-oil during the upgrading process, which otherwise can result in NOx emissions, a higher risk of oxidation, and lower energy efficiency (Li et al., 2019; Obeid et al., 2019)

- Low sulfur content is also desirable as it also results in recalcitrant compounds in the biofuel, causes engine corrosion, and promotes SOx emissions. This element is highly regulated in biofuels (Obeid et al., 2019).

- A feedstock with a low ash content is preferable because it reduces the amount of ash that must be removed from the biocrude before and during the upgrading process. Moreover, minerals can also disrupt the HTL reaction, resulting in a decrease in the biocrude yield (Liu et al., 2020a).

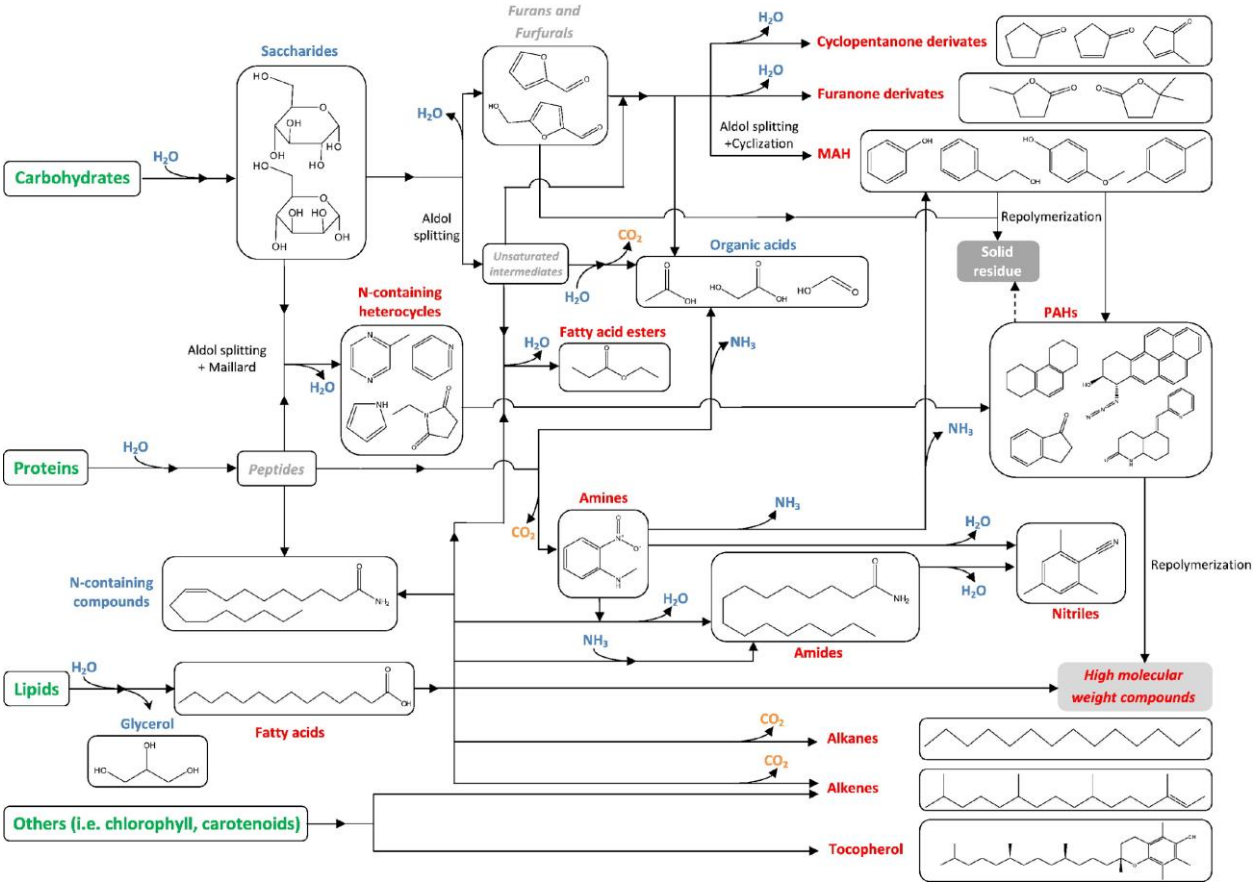


Figure 1. 3. Scheme of biomass decomposition during HTL conversion (López Barreiro et al., 2015).

After the separation process from the biocrude, the bio-oil is obtained, along with a solid fraction known as biochar, gas exudes mainly consisting of CO_2, and an aqueous phase (Y. Chen et al., 2017). This bio-oil is the main product of interest and contains a significant portion of the energy content of petroleum oil, ranging from 70% to 98%. For instance, the energy content of bio-oil usually falls within the range of 30 to 50 MJ/Kg, which is comparable to that of petroleum oil at around 43 MJ/Kg (Brown et al., 2010). The bio-oil is a complex mixture of organic compounds including acids, alcohols, aldehydes, esters, ketones, phenols, and guaiacols. This composition results in a mixture with undesired properties, such as high viscosity, high corrosiveness, and low heating value, and thus, requires upgrading to reduce the levels of undesirable O- and N-containing molecules, improve quality, and enhance energy content (Saber et al., 2016; Zhao et al., 2017). Lastly, the upgraded oil must pass through distillation, similarly to petroleum fuels, to recover different fractions of liquid biofuels such as diesel and jet fuel.

1.5. Microalgae Biofuels Current Bottlenecks

The cost of microalgae biomass production is a major bottleneck and the most significant contributor to the total porduction cost of biofuel. Microalgae biomass production faces technical challenges, mainly due to a lack of energy-efficient cultivation systems and due to their high demand for nutrients. This translates into very high costs that have constrained microalgae biofuels' race against fossil fuels. For instance, the production cost of microalgal biodiesel has been estimated at $7.50/GGE (Gallons of Gasoline Equivalent), but still remains much higher than that of petroleum diesel at $3.08/gallon (Patnaik and Mallick, 2021). Actual costs of microalgae biomass production are still too high for low-value applications like biofuel production, ranging from more than 5€ to 20€ per kilo of biomass, costs that are only acceptable for high-value products including nutraceuticals or cosmetics (Fernández et al., 2021). The latter study suggests that this cost could be lowered from 1.6 €/kg to as low as 0.6 €/kg if maximum biomass productivities were attained all throughout the year and if the cost of water and nutrients were eliminated by sourcing them from wastewater and CO_2 from flue gases.

Microalgae also face important challenges regarding their environmental impact, as they require a high supply of macronutrients that limit their environmental and economic viability (Delrue et al., 2012). Life cycle analyses of microalgal biofuel production systems give a large variety in GHG emissions depending on the hypothesis taken (Garcia et al., 2020). However some conclude that microalgae biofuel are two to ten times more GHG emissions than petroleum fuels (Garcia et al., 2020). Furthermore, microalgae cultivation competes with other crops for resources, such as mineral fertilizers and water. At the same time, the supply of fertilizers used for their cultivation has a significant impact on climate change. For instance, nitrogen fertilizers are produced through the Haber-Bosch process, responsible for 1.44% of global CO_2 emissions and consume up to 2% of global energy (Kyriakou et al., 2020). Similarly, phosphorus supply depends on the exploitation of a finite resource (Cordell et al., 2009). This poses a threat to its availability for use in the production of edible crops and raw materials for bioenergy. The extensive and unconscious use of these fertilizers has also affected their natural biogeochemical cycles, and their relocation is a major cause of eutrophication (Fowler et al., 2013).

1.6. Toward Circular and Sustainable HTL Processes

Aiming to close the gap between microalgae-based biofuels and fossil fuels, various strategies have been considered to improve the economic value and environmental footprint of biofuel production. Microalgae cultivation in wastewater, for example, has been recognized as a win-win scenario for water treatment and nutrient recovery (Plöhn et al., 2021). The integration of wastewater and process effluents could counterbalance the water and nutrient footprints (Acién Fernández et al., 2018). Simultaneously, microalgae have been proven to help in recovering a significant part of the nutrients found in various types of wastewater, including sewage, centrate, and manure (Farooq, 2021). For biofuels, in particular, considered a low-value product, this approach could push forward the sustainability and economic feasibility of the process (Hwang et al., 2016).

Similarly, significant effort has been put toward the use of by-products generated during the HTL process, which would otherwise be considered waste or process effluents. This represents both a key advantage and a key challenge for the success of microalgal HTL: the potential recirculation of the process effluent to recover nutrients, reducing the environmental impact of waste disposal (Marangon et al., 2022), and decreasing the economic impact of mineral fertilizers needed for microalgae production (Das et al., 2020). Recycling process effluents, which may include wastewater generated during biomass harvesting or conversion into biocrude, is one of several proposed strategies for achieving resource sustainability (Yang et al., 2011). Using HTL process effluents or wastewater as a nutrient supply for microalgae cultivation represents an important opportunity for saving costs in biomass production while maximizing environmental benefits (Osundeko et al., 2019).

The aqueous phase (AP) is the major by-product of HTL of microalgae, which contains the water-soluble molecules. The reintegration of this effluent has been studied at different stages of the microalgae-to-bio crude HTL process (Leng et al., 2021). Reincorporating the AP during the HTL reaction, for example, has been proposed to use this effluent as an HTL reactant to recover organic matter while increasing the biocrude yield (Zhang et al., 2019). Additionally, significant effort has been put toward the re-integration of the AP in the biomass production step as a substrate to grow new

microalgae. However, this effluent has also been identified to be toxic for microalgae, limiting its recycling for microalgae cultivation.

Other than the process effluents, it is also interesting to use HTL by-products including the gaseous fraction that is produced during HTL, as it is primarily composed of CO_2, followed by H_2, and may account from 5% and up to 20% of the original organic content of the feedstock (Leow et al., 2015; Garcia Alba et al., 2012). The gaseous phase has been recommended as a source of CO_2 to support photosynthesis in microalgae (Yu et al., 2011), while the H_2 can be used during the upgrading stage to improve the quality of the bio-oil through the process of hydrotreating (Xu et al., 2018).

HTL also produces biochar having a high content of ash, corresponding typically to less than 10% of the feedstock (López Barreiro et al., 2013). This solid residue contains an important fraction of the initial mineral input used for microalgae biomass production (Dandamudi et al., 2021). Dandamudi et al. showed the potential recovery of N (61%) and P (32.5%) from the biochar at pH 2.5 in the form of ammoniacal nitrogen and phosphates to grow *Galdieria sulphuraria*. Similarly, some effort has been put into the precipitation of P for struvite formation ($NH_4MgPO_4{\cdot}6H_2O$), which is attractive as a slow-release fertilizer (Ovsyannikova et al., 2020).

1.6.1. Process Waste to Biomass Substrate

The AP contains a large part of the nutrients originally used for microalgae biomass production (Biller et al., 2012). Interestingly, the nutrients that are found back in the AP could contribute up to 50% of the macronutrients needed to produce new microalgae biomass (Das et al., 2020). That is the case specifically of macronutrients like N and P, and dissolved trace minerals. Additionally, carbon sources are also found in the AP, such as short-chain organic acids, acetate, glycerol, and simple sugars (Gai et al., 2015). The rich composition of the AP could support mixotrophic growth in microalgae (Stablein et al., 2021). This nutrient supply has also been proven to support microalgae growth without the addition of extra nutrients (Jena et al., 2011).

AP Two-Faced Composition

As a result of HTL reactions, hundreds of different molecules also accompany the nutrient sources. Many studies have found that this mix of compounds inhibits microalgae growth. Stablein et al. reported diminished microalgal growth in AP, species- and concentration-dependent, in parallel to an inhibited photosynthetic performance (Stablein et al., 2021). This inhibition has been linked to both the concentration and the variety of compounds, some of which include N-organic compounds, phenolic compounds, but also unidentified compounds (Gai et al., 2015; Pham et al., 2013). Numerous studies have shown the ambivalent potential of AP recycling as a substrate for microalgae cultivation due to its complex composition, presented in **Box 3**.

Accordingly, previous studies have emphasized the importance of diluting the AP to counterbalance its toxicity. Biller et al. observed microalgal growth comparable to the control medium (BBM) at a dilution of 1/400, where the number of inhibitory compounds (TOC and phenols) was not as high as at 1/50 (Biller et al., 2012). Similarly, Jena et al. observed a significantly higher amount of biomass produced with a 1/500 AP-based medium compared to lower AP dilutions (Jena et al., 2011). In contrast, microalgal monocultures were reported to be killed or severely inhibited at lower AP dilutions (1/100 and 1/50) (Godwin et al., 2017).

Factors affecting the growth of microalgae in AP from HTL

The potential of using AP as a nutrient source lies in finding a balance between its nutrient-rich nature and its inhibitory effect. At the same time, this complex mix contains a wide array of molecules that are inhibitory to microalgae and that combined have a synergistic inhibitory effect. For instance, the presence of a high diversity of heterocyclic N-containing organic compounds has been found to have a higher toxicity effect when combined than when individually tested (Alimoradi et al., 2020). It is important to emphasize that each AP may have unique inhibitory effects on microalgae, as AP composition varies depending on the feedstock composition and the HTL reacting conditions. Microalgae-based HTL can result in AP with a TN ranging from 4 to 15 g/L, and a TOC ranging from 10 to 50 g/L. At the same time phenol and derived compounds exhibit varying levels of toxicity in microalgae, with EC_{50} values ranging from 2.10 to 200 mg/L (Aruoja et al., 2011).

Box 3. Historical view of studies on AP for microalgae cultivation

Minowa and Sawayama, 1999	The recovered process solution (AP) could be recycled as an N source for microalgae cultivation.
Tsukahara et al., 2001	AP must be diluted 300-fold for microalgal survival.
Jena et al., 2011	AP can support microalgae growth without the addition of extra nutrients.
Garcia Alba et al., 2013	The lack of nutrients caused by high dilutions of the AP is a major cause of growth reduction.
Pham et al., 2013	Identification of N-organic compounds in AP as potential inhibitors.
Leng et al., 2018	Different approaches could be implemented to alleviate the AP inhibitory effects including pre-detoxification steps, strain improvement, and co-cultivation.
Das et al., 2020	The AP can contribute up to 50% of the nutrient requirement for microalgal biomass production.
Watson et al., 2020	AP has a rich and complex composition that could be valorized through different pathways to recover value-added products, including anaerobic digestion and bioelectrochemical systems.
Stablein et al., 2021	Microalgae show mixotrophic growth under AP cultivation.

1.7. Current AP Challenges and Opportunities

To compete with fossil fuels, it is essential to consider and optimize every step of the process, from the strain selection and production of biomass to its conversion into biofuels. All this while aiming for sustainable and circular processes. Regarding the biomass production stage, a lot of attention has been given to the yield of compounds of interest such as lipids, carbohydrates, pigments, or high-value metabolites. To move toward more environmentally friendly energy sources, strains must be chosen that take into account not only the yield and productivity of the compound of interest but also the implications of the overall feedstock composition throughout the several stages of the HTL process. Furthermore, it is essential to work with a conscious and efficient use of nutrients while considering the microalgae metabolism and downstream stages of the biomass, from biomass harvesting until obtaining the final biofuel product.

Moreover, when recycling AP to produce HTL feedstocks, it is critical to understand how the AP affects not only microalgae growth but also biomass yield and composition. Even though several studies have been conducted, less attention has been targeted toward studying the impact of the AP on biomass quality for biofuel production via HTL. The quality of the feedstock has a direct impact on the yield and quality of the biocrude (Cheng et al., 2017). For instance, a high-lipid biomass is advantageous to produce biocrude and results in an upgraded bio-oil of higher quality (da Costa Magalhães et al., 2022; Yoo et al., 2015).

Although growth using AP has been reported, productivities are typically beyond those achieved in control media (Watson et al., 2020). Additional steps to pre-treat the AP are necessary to diminish the AP inhibition when aiming for its use for microalgae cultivation. With this purpose, multiple approaches have been explored, such as high dilutions of the AP of up to 500 times, detoxification steps including activated carbon and adsorbing resins, use of microalgae polycultures and nutrient replenishment (Box 3). Optimizing microalgae growth in AP requires implementing one or more of these strategies. These strategies should be employed without adding up to the complexity and cost of the process and while maintaining microalgae productivity and biomass quality.

1. 7.1. Strategies for Microalgal Growth Improvement: Acclimation and Adaptive Laboratory Evolution

Acclimation and adaptive laboratory evolution are two distinct methods for improving microalgal growth under harsh conditions. Acclimation is a short-term, reversible response to sudden environmental changes. It involves adjustments in protein activity or gene expression that are necessary to thrive in the new environment (Borowitzka, 2018). In contrast, adaptive laboratory evolution (ALE) is a long-term exposure in a controlled laboratory setting to a selective pressure (e.g., inhibitory compounds). This process can lead to complex changes, including alterations in metabolic pathways and genetic mutations, that result in the selection of advantageous traits (LaPanse et al., 2020). While acclimation is often reversible, this is usually not the case in adaptive laboratory evolution, where genetic changes occur and are passed on to the next generations.

Furthermore, several studies have reported significant improvement after adapting or acclimating microalgae to toxic compounds or effluents with inhibitory effects (

Table 1. 2). Wang et al. reported the adaptation of a *Chlorella* sp. to high concentrations of phenol (700 mg/L) (L. Wang et al., 2016). The resulting strain doubled its maximal biomass concentration, in contrast to the wild type, while removing up to 500 mg/L of phenol in seven days. Two more studies were conducted particularly for microalgae cultivation in AP, with reported information on acclimation to AP. Hognon et al. reported improved growth on *C. reinhardtii* after six cycles of acclimation to AP with a final biomass concentration higher than in the control medium (Hognon et al., 2015). Similarly, *Picochlorum* sp., acclimated to 1/1000 AP, was able to maintain photosynthesis and respiration even at a dilution as low as 1/50, compared to the control cells that were highly inhibited at a dilution of 1/100 (S. Wang et al., 2016). Further studies on this approach could represent a viable opportunity to reduce inhibition and improve microalgae cultivation in AP toward circular and more sustainable processes.

Table 1. 2. Microalgae adaptation studies to toxic effluents or compounds.

Microalgae starting strain	Environmental stress	Operation mode	Main result	Total time (d)	Reference
• *Chlorella sp.* AE10	10% CO_2, 200 ppm NOx, 100 ppm Sox	Batch	Adapted strain resistant to elevated levels of flue gas	138	Cheng et al., 2019
• *Chlorella sp.*	Phenol 700 mg/L	Semi-continuous	500 mg/L phenol could be fully removed in 7 days by the resulting strain	n.d.	L. Wang et al., 2016
• *Chorella sorokiniana* *Coelastrella sp.* *Acutodesmus nygaardii*	Swine wastewater	Chemostat	Adapted microalgae were able to efficiently treat high concentrations of organic and inorganic matter of undiluted piggery wastewater	n.d.	Lee et al., 2021a
• *Chlorella sp.* *Scenedesmus sp.*	Textile wastewater	Fed-batch	Color removal efficiency improved and increased TN and TP removal	95	Kumar et al., 2018
• *Chlamydomonas luteoviridis* *Chlorella vulgaris* *Desmodesmus intermedius* *Hindakia tetrachotoma* *Parachlorella kessleri*	Wastewater	Batch	Enhanced N, P, BOD removal efficiency and improved lipid productivity	8 rounds of 7 days	Osundeko et al., 2014
• *Chlorella pyrenoidosa*	NH_4 4.7 g/L	Serial transfers	Increased NH_4 tolerance and uptake	7	Wang et al., 2021
• *Nostoc ellipsosporum*	Municipal wastewater	Inoculum acclimation	Acclimation enhanced the biomass yield to 2.9 g/L	15	Devi and Parthiban, 2020
• *Scenedesmus intermedius* *Microcystis aeruginosa* *Dunaliella tertiolecta*	Petroleum and diesel oil	Serial transfers	After 300 generations of artificial selection, strains resistant to low doses of petroleum were obtained	60	Romero-Lopez et al., 2012
• *Chlamydomonas reinhardtii*	HTL-AP	Batch	Increased biomass production compared to the control in AP	6 rounds of 10 days	Hognon et al., 2015

1.8. Aim of the work

This thesis was conducted within the framework of the French ANR project RAFBIOALG. This project explores HTL for converting microalgae biomass into biofuels, using a two-step approach that involves HTL to produce bio-oil followed by catalytic upgrading to meet transportation fuel standards. The project also targets to develop a fully-integrated process that includes microalgae cultivation, all subsequent steps, and a life cycle assessment.

With the aim to work toward circular processes, this thesis focuses specifically on the **potential and limitations of recycling the aqueous phase (AP) as a substrate for microalgae biomass production**. Thus, I performed several experiments which I discuss in detail in the section of **Materials & Methods**. The results of these experiments are presented in **Papers I** to **III** that address the following research questions:

Paper I. Strain selection targeting high-quality HTL feedstocks:
Among a small study group of promising candidates, given their productivity, robustness, and potential to accumulate lipids: which strain would be better suited for HTL feedstocks?

Paper II. Impact of the AP on microalgae growth:
How do these strains perform under AP cultivation? How is the quality of the biomass affected by the presence of the AP?

Paper II & III. Acclimation and adaptive laboratory evolution to the AP:
Can adapting the microalgae to the mix of compounds present in the AP reduce this inhibition? How far can this adaptation be directed toward a higher AP tolerance?

Paper II & III. Cultivation of high-lipid content microalgae in AP:
Is it possible to recycle the AP for microalgae cultivation while maintaining good biomass and lipid productivity? Which microalgae can produce a high lipid content and tolerate high concentrations of AP?

To work toward environmentally friendly energy sources, it is critical to select strains while taking into account not only the yield and productivity of the compound of interest but also the implications of the feedstock throughout the HTL process. Therefore, the first research objective of this thesis was to identify a suitable strain for HTL. In **Paper I**, six strains from the family *Chlorellaceae* were studied under nutrient-limited conditions aiming to produce biomass with a high-lipid content. Due to its high lipid content and overall composition, *Chlorella vulgaris* NIES 227 was identified as the most promising candidate feedstock for lipid-based biofuels with the best energy-content biomass.

To successfully incorporate the AP during biomass production, the microalgae AP tolerance and its impact on biomass quality were assessed. In **Paper II**, as the biomass composition is highly influenced by the choice of strain, in combination with the environmental conditions, this second study aimed to identify a robust strain capable of adjusting to the effects of the AP while maintaining a high-quality HTL feedstock. Among the tested strains, *Chlorella vulgaris* NIES 227 maintained the best lipid productivity and lipid profile for HTL. It was identified that recycling AP supported microalgal growth, yet further measures were necessary to alleviate its inhibitory effects For these reasons, *Chlorella vulgaris* NIES 227 was chosen for further optimization of AP cultivation.

AP-supported cultivation was proven to be a feasible step toward circular processes, however, the addition of this effluent has an impact on the microalgae's growth rate and overall biomass quality. For this, ALE was considered to be a potential solution to mitigate AP inhibition. In **Paper III**, the objective was to study whether a prolonged period of adaptation to the AP could offset the negative effects of this effluent on microalgae growth. The adaptation of *Chlorella vulgaris* NIES 227 aimed to i) offset the negative effect of the AP on the growth rate and biomass productivity; ii) maintain the capacity of this strain to accumulate a high amount of lipids; and iii) increase the AP concentration in the medium, and with this, its contribution to the nutrient content of the medium.

2. Materials & Methods

This chapter describes the materials and methods used in this study of microalgae cultivation under HTL-AP. It begins by detailing the microalgae strains and methods used for microalgal cultivation, the cultivation conditions, and the methods used for monitoring and analyzing the microalgal growth and biomass production. The chapter concludes with the description of the analytical techniques used to characterize the AP and biomass qualities. The next section includes an overview of the HTL process that was used to process the biomass and produce biofuel and the AP. The third section briefly describes the main experimental designs that are presented in this thesis. Further details can be found in the results section in their respective chapters.

2. 1. Microalgae cultivation

2. 1.1. Microalgae strains

Considering the potential inclusion of effluent from the HTL process, which often contains high levels of organic compounds, ammonium, and some heavy metals that could inhibit microalgal growth (Gai et al., 2015; Pham et al., 2013), it is necessary to focus on microalgae strains that can thrive in harsh conditions. *Chlorellaceae*, divided into two sister clades, *Chlorella*, and *Parachlorella*, are attractive for the production of biomass for biofuel purposes (Krienitz et al., 2004). This microalgae family is known for its fast growth rate and resistance to adverse growth conditions (Ummalyma et al., 2019). Additionally, different studies have demonstrated the potential of *Chlorella* sp. to produce biomass with a high content of carbohydrates or lipids when grown under stress conditions, such as nutrient limitation (Li et al., 2013; Přibyl et al., 2012; Shen et al., 2015).

With this in mind, six strains from the family *Chlorellaceae* were chosen as interesting candidates to study during this work (**Figure 2. 1**). The strains were acquired from the National Institute for Environmental Studies (NIES, Japan), and the Culture Collection of Autotrophic Organisms (CCALA, Czech Republic). The study group consisted of one *Chlorella sorokiniana* (NIES 2173), three *Chlorella vulgaris* (NIES 227, CCALA 256 and CCALA 269), and two *Parachlorella kessleri* (CCALA 251 and 253). Further, referred individually by their respective identifier number (e.g., NIES 227).



Figure 2. 1. Transfer of mother cultures to liquid cultures of the strains of interest.

2. 1.2. Cultivation conditions

Stock cultures were kept in an automated incubator (ZWYC-290A, Labwit Scientific, Australia), set to a light intensity of 20 µmol/m^2/s and with other environmental conditions (T: 25°C, agitation: 130 rpm, CO_2: 2% v/v) kept constant (Figure 2. 2). Culture experiments were conducted in the same incubator and other than the light, environmental conditions remained identical. The light intensity (100–230 µmol/m^2/s) and photoperiod were adjusted according to the objective and duration of each experiment. The volume of the cultures was equally adapted to each experiment according to the number and frequency of the sampling. All cultures were kept in sterile Erlenmeyer flasks with autoclaved and/or filtered-media using a sterile 0.2 µm PES membrane (Steritop 45 mm-Millipore Express Plus, Millipore, USA).



Figure 2. 2. Experimental microalgae cultures grown in a Labwit incubator.

Cultivation media

Fresh Water Medium (FWM) was used as the reference medium for all experiments. This medium was designed to cover the nutrient requirements of 4 g/L of biomass of a *Chlorophyceae*-type microalga without nutrient deficiencies. The calculations were made based on elemental analyses of the biomass of *Parachlorella kessleri* NIES 2152 with the following elemental composition: 50.1% C, 7.21% H, 6.68% N, 1.33% P, 0.465% S, 0.848% K, 0.428% Mg and 0.0045% Ca. Analyses were performed by SOCOR (France).

FWM is composed of: 1459 mg/L $NaNO_3$, 102 mg/L NH_4Cl, 233 mg/L. KH_2PO_4, 143 mg/L $MgSO_4 \cdot 7H2O$, 1.5 mg/L, and $CaCl_2 \cdot 2H_2O$ (Table 2. 1). One mL per liter of Hutner's trace element solution (Hutner et al., 1950) was added per liter of medium. The media were adjusted to pH 7.5 by addition of NaOH 2.5 M and autoclaved for 20 min at 121°C.

Table 2. 1. Mineral composition of FWM.

FWM	mg/L	mM
$NaNO_3$	1459	19.1
NH_4Cl	102	1.9
KH_2PO_4	233	1.7
$MgSO_4 \cdot 7H_2O$	143	0.6

2. 1.3. Microalgae growth monitoring

Microalgae growth can be determined by measuring the abundance and size of the cells, increase in biomass in terms of dry weight (DW), or absorbance in terms of optical density (OD). Additionally, measurements related to the microalgae photosynthetic activity can serve as a proxy of the physiological status of the cells under stress conditions such as nutrient deficiency or the presence of inhibitory compounds (e.g., AP). The strain and cultivation conditions can greatly influence these measurements. Thus, they were measured for all strains and in each experiment and no correlations between OD and DW were used.

Optical density

Optical density is widely used as a quick method for measuring microalgal growth. To minimize interference from chlorophyll and other photosynthetic pigments, wave lengths of 550 or beyond 700 nm are typically employed, as these are wavelengths where the absorption of pigments is minimal (Moheimani et al., 2013). Biomass growth was then determined by OD measurements at 880 nm, taken in 1-mL cuvettes with an Epoch2 UV-Vis spectrophotometer (BioTek Instruments, USA). Samples were diluted to obtain an OD ranging between 0.2 and 0.8.

Cell counting

Microalgae growth was also monitored via cell counting using the Multisizer 4 Coulter Counter (Beckman Coulter, USA), equipped with a 70 µm aperture tube. A culture aliquot from 20 to 100 µL (depending on the cell density) was diluted in 10 mL of filtered (0.2 µm) isotonic water (Isoton II Diluent, Beckman Coulter, USA). The following parameters were determined: number of cells, cell volume, and mean diameter of the population.

Biomass concentration

Dry weight was determined with 2 to 10 mL of culture. Samples of a volume higher than 2 mL were centrifuged for 10 min at 4500 rpm (4410× *g*) in an Allegra X15R (Beckman-Coulters, Brea, CA, USA), equipped with an SX4750A Swinging Bucket Rotor. Samples of 2-mL were centrifuged at 14500 rpm (14100× g) for 10 minutes in a Mini Spin Plus (Eppendorf, Germany). The supernatant was collected to determine nutrient consumption by ion chromatography (see nutrient analysis), while the pellet was washed with distilled water and centrifuged again. The pellet was recovered in pre-weighed aluminum plates and dried at 105°C for at least 24 h. The aluminum plates were then cooled in a desiccator and weighed again.

Photosynthetic activity

The maximum quantum efficiency of photosystem II is an indicator of how much energy in the form of light a particular microalgae is able to capture and use for photosynthesis (Schreiber, 2004). The maximum quantum efficiency can be measured by calculating the Fv/Fm ratio, the ratio of variable fluorescence (Fv) to maximum fluorescence (Fm). This ratio, also referred to as the maximum quantum yield (QY), is obtained by comparing the fluorescence of an illuminated sample to that of a dark-adapted sample (Eq. 1). A higher QY indicates a more efficient photosynthetic system.

In green microalgae, the QY ranges from 0.7 to 0.8, while lower values can suggest that microalgae are under physiological stress (e.g., nutrient deficiency or presence of inhibitory compounds) (Masojídek et al., 2013). To measure the Fv/Fm, samples of 3 mL were diluted to an OD around 0.2 at 880 nm. The samples were then dark adapted for 5 to 10 minutes before being exposed to a light pulse (620 nm). The emission was measured in the range of 665 to 750 nm using the OJIP and QY protocols of an AquaPen-C AP 100-C fluorometer (Photon Systems Instruments, Czech Republic).

The parameters of the QY protocol were set based on pre-tests carried out before the experiments: Flash pulse: 30%, Super Pulse: 60%, Actinic light: 100 μmol/m^2/s and 10%.

$$QY = (Fv/Fm) = (Fm - Fo/Fm) \quad (1)$$

where:

Fo is the minimum fluorescence in a dark adapted sample

Fm is the maximum fluorescence in a dark adapted sample

Fv is the variable fluorescence between Fo and Fm

2. 1.4. Growth parameters

This section discusses several parameters that were determined to compare the growth and productivity of the research strains.

Biomass concentration

Microalgal biomass concentration was determined by measuring the dry weight (DW) of the samples and calculated by differential weight as follows:

$$DW\ (g/L) = \frac{W_0 - W_f}{V} \quad (2)$$

where:

W is the weight (g) of the empty aluminum plate (W_0) and after (W_f) biomass drying;

V is the volume of the sample (L).

Biomass productivity

Biomass productivity was calculated using DW at a given growth time ($t_{j,i}$):

$$P\ (g/L\ /d) = \frac{DW_{ti} - DW_{tj}}{t_i - t_j} \quad (3)$$

Growth rate

The tolerance and acclimation of each strain to the AP was monitored during each cycle and measured based on their growth rate and AP inhibition. These parameters were calculated according to the Alga Growth Inhibition Test No. 201 (OECD, 2011), considering biomass as OD at 880 nm or number of cells:

$$\mu\ (d^{-1}) = \frac{lnX_j - lnX_i}{t_j - t_i} \quad (4)$$

where:

μ is the average specific growth rate during the exponential timepoints i, j

X_i is the biomass at time i;

X_j is the biomass at time j

The specific growth rate is reported as the average of several timepoints during the exponential growth (typically between day 0 and day 3). Maximum growth rate refers to the highest value observed during the exponential phase.

AP inhibition

$$AP\ inhibition\ (\%) = \frac{\mu_c - \mu_{AP}}{\mu_c} \times 100 \quad (5)$$

where:

μ_c is the growth rate in AP-free control media;

μ_{AP} is the growth rate measured in AP-based media

2. 2. Analytical techniques

2. 2.1. Nutrient and media characterization

Different techniques were implemented and developed to characterize the media and AP samples. These analyses followed nutrient consumption during the experiments to determine nitrogen depletion in the media and overall nutrient recovery.

Nutrient consumption and AP nutrient content

Ion chromatography (Model 940 Professional IC Vario, Metrohm, Switzerland) was used to measure nutrients in the APs and culture media. For anions (acetate, chloride, nitrate, phosphate, and sulfate), a Metrosep A column was employed with an elution solution based on sodium carbonate (3.6 mM) and an injection flow of 0.7 mL/min. For cations (ammonium, magnesium, potassium, and sodium), a Metrosep C4 column was used with an elution solution based on nitric acid (1.7 mM) and dipicolinic acid (0.7 mM), and injected at 0.9 mL/min. Before injection, the recovered supernatants were filtered with a 0.2-µm PES filter (Puradisc 25, Whatman, UK) and diluted as needed to be within the calibration range (2–40 ppm). Certified calibration solutions from Sigma-Aldrich were used for calibrating each ion, respectively.

Total carbon and total nitrogen

A total organic carbon analyzer (TOC-LCSH, Shimadzu, Japan), equipped with an infrared detection system (NDIR), was used to determine the inorganic carbon (IC) and total carbon (TC) of APs and culture media. Total organic content (TOC) was calculated as the difference between the measured TC and IC. Total nitrogen (TN) was measured in parallel in the same apparatus coupled with a chemiluminescence detection module for TN (TNM-L, Shimadzu, Kyoto, Japan). Ion chromatography measured inorganic N sources ($N\text{-}NO_3^-$ and $N\text{-}NH_4^+$) as previously described. The difference between the inorganic nitrogen (IN) and TN was considered as the total organic nitrogen (TON). Filtered samples were diluted with distilled water to be within the following calibration ranges: TC: 1–100 ppm (Potassium hydrogen phthalate, ref. P1088, Sigma-Aldrich, USA), IC: 1–100 ppm ($NaHCO_3$, ref. S5761, and Na_2CO_3, ref. S7795, Sigma-Aldrich, USA), and TN: 0.5–20 ppm (KNO_3, ref. 105063, Supelco, USA).

2. 2.2. Biomass characterization

Several techniques were implemented to assess the quality of the biomass among strains, experiments, and HTL feedstocks. First a general characterization was performed of each of the major biomass constituents: lipids, carbohydrates, and proteins. The percentage of each biomass constituent contributed to the overall mass balance of the samples, with any remaining amount identified as others. Typically, mass balances in microalgae biomass range from 70% to 90%, where mass balances of 100% are rarely achieved (Laurens et al., 2014).

Sample preparation

The final biomass was washed with distilled water to remove excess minerals from the media by the two-step centrifugation protocol described for DW determination. The washed pellets were freeze-dried (COSMOS 20K, Cryotec, France), and stored at -20°C until further analyzed. Before measurements, the samples were manually grinded and homogenized using a porcelain mortar and pestle, and placed in a desiccator before weighing (Figure 2. 3).



Figure 2. 3. Grinding and homogenization of microalgal biomass.

Elemental composition

As important as the biochemical composition is, the elemental composition has become one of the most effective determinations to estimate energy generation of any biomass (Hossain et al., 2019). Lyophilized biomass was analyzed by Bruno da Costa Magalhães at IRCELYON for its CHONS content, using a FLASH 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Approximately 1 mg of microalgae was weighed and injected into the equipment. The C, H, N, and S analysis was performed via the analysis of the CO_2, H_2O, N_2, and SO_2 gasses formed during the combustion of the samples at 900°C, respectively. On the other hand, the oxygen analysis was performed by quantifying CO issued from the pyrolysis (inert atmosphere) at 1000°C. The formed gasses were analyzed with a TCD detector.

Lipid quantification and fatty acid characterization

When it comes to lipid-based biofuels, it is crucial to determine the exact fraction and specific fatty acids profile of the biomass. The amount and quality of the lipids will directly affect the bio-oil produced, its properties, and the economics of the HTL process. For this, two methods were employed to determine the lipid content of microalgal biomass and to characterize the fatty acids profiles.

In this work, lipid content was quantified using an adapted gravimetric method adapted from Axelsson and Gentili (2014). Briefly, the lipids of biomass samples of 10–20 mg were extracted with the addition of 2.5 mL of methanol, followed by the addition of 5 mL of chloroform. Samples were incubated for 10 min in an ultrasonic bath and homogenized with a vortex. When the samples were well suspended, 1 mL of a 0.73% NaCl solution was added to the mix and the sample was centrifuged at 1500 rpm (1470× g) for 10 min. Then, the top layer was removed and the hydrophobic phase was manually recovered with a Pasteur pipette. The leftover biomass and top layer were suspended with 2 mL of chloroform for a second lipid recovery and the hydrophobic phase was recovered again after centrifugation.

A second method was implemented to quantify the total fatty acid content and to characterize the fatty acid composition of the microalgae. Samples of 5–20 mg of lyophilized biomass were subjected to transmethylation to obtain fatty acid methyl esters (FAMEs). The total FAMEs and fatty acid (FA) profiles were determined using a gas chromatography-flame ionization detection (GC-FID) system. To characterize the final fatty acid profile in the microalgae, samples of 5–20 mg of lyophilized biomass were subjected to transmethylation to obtain fatty acid methyl esters (FAMEs). The samples were incubated for 1 h at 85°C with 3 mL of the transesterification agent (1.25M hydrogen chloride in methanol, Ref: 17935, Supelco) and 0.2 mL of the 15:0-Me internal standard (prepared at 3 mg/mL in anhydrous hexane). After cooling down the samples at room temperature, 3 mL of hexane were added to recover the lipid fraction, followed by 1 mL of distilled water. Finally, the hexane was recovered after centrifugation at 1500 rpm for 5 min.

After transesterification, FAMEs were quantified using a Gas Chromatography equipped with a Flame Ionization Detector (GC-FID, GC-2010 Pro AOC-20i/AOC-20s, Shimadzu, Japan). The FAME samples were injected with a volume of 0.5 or 1 μL in split mode by a Shimadzu AOC-20i-s autosampler. The chromatograph was equipped with a 30 m × 0.32 mm ID FAMEWAX capillary column (Restek, Bellefonte, PA, USA), filled with a 0.25 μm Crossbond polyethylene glycol stationary phase (polar phase). The column temperature was increased from 150°C to 240°C at a rate of 5°C/min. For quantitative analysis of total FAMEs, tripentadecanoin (TAG C15:0, ref. T4257, Sigma Aldrich, USA) was used as an internal standard (prepared to 3 mg/mL in 95% anhydrous hexane). Samples were incubated at 85°C for 1 hour with 0.2 mL of the internal standard and 3 mL of the transesterification agent (1.25 M HCl–methanol, ref. 17935, Supelco, USA).The GC-FID data acquisition was carried out using LabSolutions software (Shimadzu, Kyoto, Japan). The compound identification was performed by the retention indices method and confirmed by two sets of standards: a mix of six FAMEs (Ref. 07631-1AMP, Supelco), and a mix of 37 FAMEs (Ref: CRM47885, Supelco, St. Louis, MO, USA). With these standards, 95% of the fatty acid profile was validated. To further validate the identification of some specific FAMEs (C16:2 and C16:3), samples were sent to an external laboratory to confirm compounds by a GC-mass spectroscopy (MS) detection system.

Total sugars

The measurement of carbohydrate content in microalgal biomass is commonly performed using a colorimetric phenol-sulfuric acid method (DuBois et al., 1956). Carbohydrates are hydrolyzed into simple sugars, which are then dehydrated to form furfural and hydroxymethyl furfural in this method. These compounds react with phenol to form an aromatic complex, resulting in a color change. If the colorimetric response of the sugars is not uniform, this method can overestimate or underestimate carbohydrate content (Laurens et al., 2012). Despite this limitation, the phenol-sulfuric acid method serves as a reliable proxy to estimate carbohydrate content.

Following the DuBois protocol, carbohydrate content was determined as glucose equivalents. Briefly, 0.5 mL of 1.25 M H_2SO_4 was added per mg of freeze-dried biomass (2–6 mg). The vortexed samples were incubated at 100.5°C for 3 hours. After cooling, 50 µL aliquots were resuspended in 450 µL distilled water and mixed with 2.5 of mL concentrated H_2SO_4 (95%) and 500 µL of phenol solution (5% w/v). Total carbohydrate content was determined colorimetrically by measuring the absorbance of the sample at 483 nm next to a calibration curve of glucose standard solutions (0.02–0.1 g/L).

Protein content

Protein content is typically measured using colorimetric assays such as the Lowry or Bradford tests, but these methods can be subject to interferences and provide inconsistent results. A more reliable alternative is elemental N conversion to protein, which involves using a conversion factor to estimate protein content. Nitrogen-to-protein conversion factors, traditionally 6.25 for any biomass, need to be adapted to the specific type of biomass (Van Wychen et al., 2021). For microalgal biomass, a conversion factor of around 5 has been found to be more appropriate while distinguishing between specific proteinaceous N and non-proteinaceous N found in microalgae (Templeton and Laurens, 2015). This method is insensitive to non-nitrogenous interferences such as lipids and carbohydrates and is therefore considered a good proxy for biomass protein content (Laurens et al., 2014).

In this work, the final protein content was estimated based on an N-to-protein conversion factor of 5.04 determined for a *Chlorella vulgaris* at later stages of cultivation (Templeton and Laurens, 2015). Nitrogen content in biomass was determined using the described TOC/TN analyzer (Shimadzu TOC-L, Japan). Briefly, approximately 5 mg of freeze-dried biomass were weighed into 50-mL Falcon tubes and filled with distilled water to a volume of 50 mL. Biomass suspensions were homogenized in one-minute cycles in an automated bead mill (MM400, Retsch, Germany). The system was set at a vibrational frequency of 30 Hz with 30-second breaks between cycles. To avoid precipitation during analysis by the TOC, samples were kept under agitation in 50-mL glass bottles with a magnetic stirrer.

Ash content

The measurement of the ash content of a biomass shows its inorganic and mineral content contained in the sample. Ash content in the microalgal biomass was determined according to Van Wychen and Laurens (Van Wychen and Laurens, 2016). Briefly, biomass samples were weighed into ceramic crucibles and dried overnight in a drying oven at 105°C. After 24 hours, the crucibles were transferred to a desiccator and cooled to room temperature before being weighed to determine the moisture content. To determine the final ash content, the dried samples were placed in a muffle furnace and heated to 575°C until a constant weight was reached.

Others: the unaccounted fraction

The percentage of each determined biomass constituent (i.e., lipids, carbohydrates, and proteins) contributed to the overall mass balance of the samples, with the remaining fraction further referred to as others. For a complete understanding of any biomass, this non-null fraction (10–30%) must be characterized. This fraction contains various compounds that are not accounted for in macromolecule measurements, such as nucleic acids, pigments, secondary metabolites, inorganic salts, organic vitamins, and algaenans (Van Wychen et al., 2021). Moreover, the remaining unaccounted fraction may also include parts of the biomass that were not completely measured during analyses.

Biomass energy content

Empirical correlations are typically used to calculate the higher heating value (HHV) from the biomass elemental composition. This parameter is an important fuel property that defines the energy content of fuels. HHV was then estimated, based on the final elemental composition according to the following equation (Channiwala and Parikh, 2002) where C, H, O, N, S and A are the mass percentages of carbon, hydrogen, oxygen, nitrogen, sulfur, and ash respectively:

$$HHV(MJ/Kg) = 0.3491C + 1.1783H + 0.1005S - 0.1034O - 0.0151N - 0.0211A \quad (5)$$

2. 2.3. Data analyses

Figure conception and the statistical data analyses were carried out with Excel Microsoft and GraphPad Prism (version 9.5.0). In **Paper II**, a linear mixed effects model was used to assess the effect of cultivation time (day 4, 11, 18, 25), strain (NIES 227, CCALA 256, NIES 2173, CCALA 251, CCALA 253), and their interaction on SFA, MUFA, and PUFA. A post-hoc Tukey's test was performed to evaluate the differences among biomasses at the final day of cultivation (day 25).

2. 3. HTL and AP production

As part of the project RAFBIOALG, numerous HTL conversions were performed in CEA, Grenoble by Lucie Matricon and Anne Roubaud to optimize the biocrude yield from microalgae biomass. Different HTL conversions were performed to study the dynamics of the process under different conditions (temperature, reaction time, and biomass loading) and with feedstocks with varying protein, lipid, and carbohydrate contents from two different strains (Figure 2. 4). Several APs from these HTL reactions were used during this work (see HTL aqueous phase.

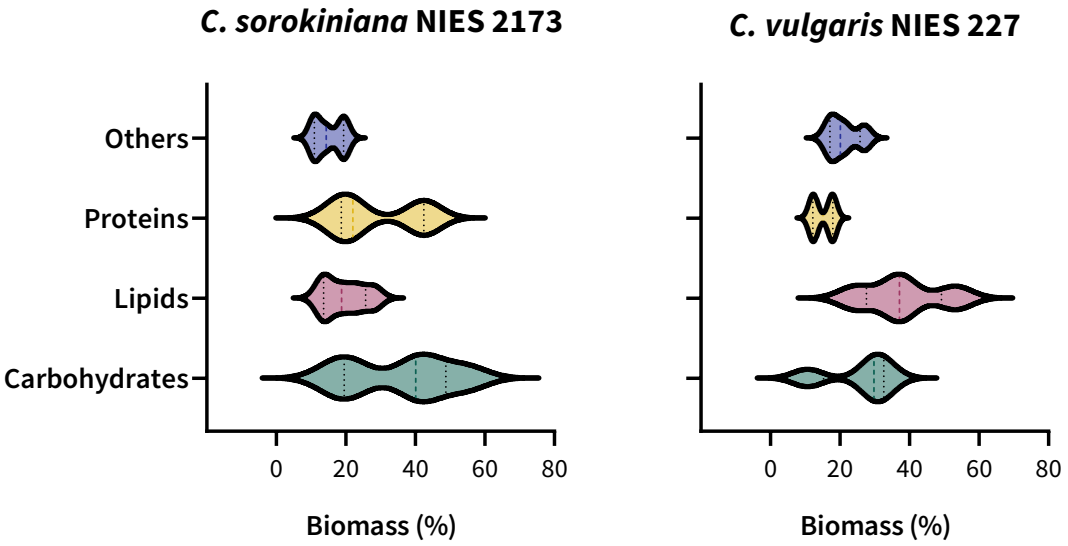


Figure 2. 4. Biomass composition of RAFBIOALG feedstocks from *C. sorokiniana* NIES 2173 (n=5) *and C. vulgaris* NIES 227 (n=4). Violin plots illustrate the distribution and incidence of the data, by length and width, respectively.

2. 3.1. Feedstock: microalgal slurry

The microalgae biomass was obtained from CEA, Cadarache. Briefly, *C. sorokiniana* NIES 2173 was cultivated in a 25-liter PBR, and scaled up into two 180-liter Flat Panel Airlift (FPA) photobioreactors (PBR) from Subitec Gmbh (Stuttgart, Germany). *C. vulgaris* NIES 227 was grown in a 285-liter tubular PBR (Jumbo XL, Synoxis, France) (Chambonniere et al., 2022). The microalgae were grown with natural sunlight inside a greenhouse in Saint-Paul-Lez-Durance, France. The pH of the cultures in all PBRs was set and maintained at 7.0–7.5 by CO_2 addition. All biomass was harvested and concentrated by centrifugation. Table 2. 2 shows the biomass composition of the numerous feedstocks produced for the HTL reaction through different culture batches.

C. sorokiniana NIES 2173 was pre-selected by the RAFBIOALG project to start biomass production and HTL conversion. This strain was attractive for its high productivity and robustness previously identified (Li et al., 2013). However, as observed in Figure 2. 4 and Table 2. 2, this strain produced predominantly carbohydrates rather than lipids. These first biomass batches and further lab-scale results (see 4. Preliminary assays) underscored the need to look for a more suitable strain for HTL conversions (see **Paper I**). *C. vulgaris* NIES 227 was identified as an ideal strain for lipid-based biofuels, and therefore it was used to produce further HTL feedstocks.

Table 2. 2. Microalgal biomass composition used for different HTL reactions.

		Biomass composition (%)								
Microalgae	**Feedstock ID**	**Carb.**	**Lipids**	**Proteins**	**Others**	**C**	**H**	**N**	**S**	**O**
C. sorokiniana NIES 2173	2020RAFCS0	16.9	18.8	45.4	18.9	52.2	7.2	8.6	0.1	24.0
	2020RAFCS1	22.1	23.2	43.9	10.8	52.6	7.4	8.3	0.1	31.6
	2020RAFCS2	54.6	13.3	17.7	14.4	50.3	7.4	3.3	0.1	33.6
	2020RAFCS3	43.0	14.0	23.1	19.9	52.1	7.3	4.4	0.3	35.9
	2020RAFCS4	40.1	28.2	20.6	11.1	52.1	7.9	4.1	0.7	35.2
C. vulgaris NIES 227	2021RAFCV1	29.2	37.0	12.1	21.7	64.3	8.1	2.4	0.3	24.9
	2021RAFCV2	10.7	53.2	17.6	18.5	58.3	6.9	3.5	0.3	31.0
	2021RAFCV3	33.4	37.3	12.6	16.7	64.0	10.9	2.5	0.4	22.2
	2021RAFCV5	30.3	24.5	18.1	27.1	54.0	8.1	3.6	0.4	33.9

2. 3.2. HTL process: Batch and continuous tests

Microalgal paste was diluted before each test in water to the desired concentration of solids (10–20 wt.%). The HTL batch reactions were performed in a 0.6-liter stainless steel autoclave (Parr Instrument Company, St. Moline, IL, USA). An initial pressure was set at 10 bar for tests performed at ≤300°C and at 8 bar for tests carried out at ≥300°C. The stirring speed was set to 385 rpm, with a temperature ramp of 15°C/min. TOP Industrie (Vaux-le-Pénil, France) created a custom-built, pilot-scale HTL reactor. This reactor was used for continuous HTL tests. The continuous tests were performed at 300°C and 340°C and at a pressure of 150 and 180 bar, respectively. The microalgal slurry was injected into the HTL reactor at a fixed flow rate, between 1.5 and 2 L/h. The residence time was in the order of 15 min. The agitation was fixed at 250 rpm.

2. 3.3. Product separation

After HTL, the processed biomass is distributed into different fractions: the biocrude (consisting of a mix of the biochar, or solid fraction, and the bio-oil), the gaseous fraction, and the aqueous phase (AP) (**Figure** 2. 5). During the batch reactions, two sieves (200 and 50 µm) were placed inside the recovery tank to separate the biocrude from the AP. The products were recovered after cooling of the reactor. During the continuous tests, separation by decantation was carried out to recover the aqueous phase from the bio-oil, as no biochar was obtained under these conditions. The collected APs from each HTL were stored at -20°C until further use.

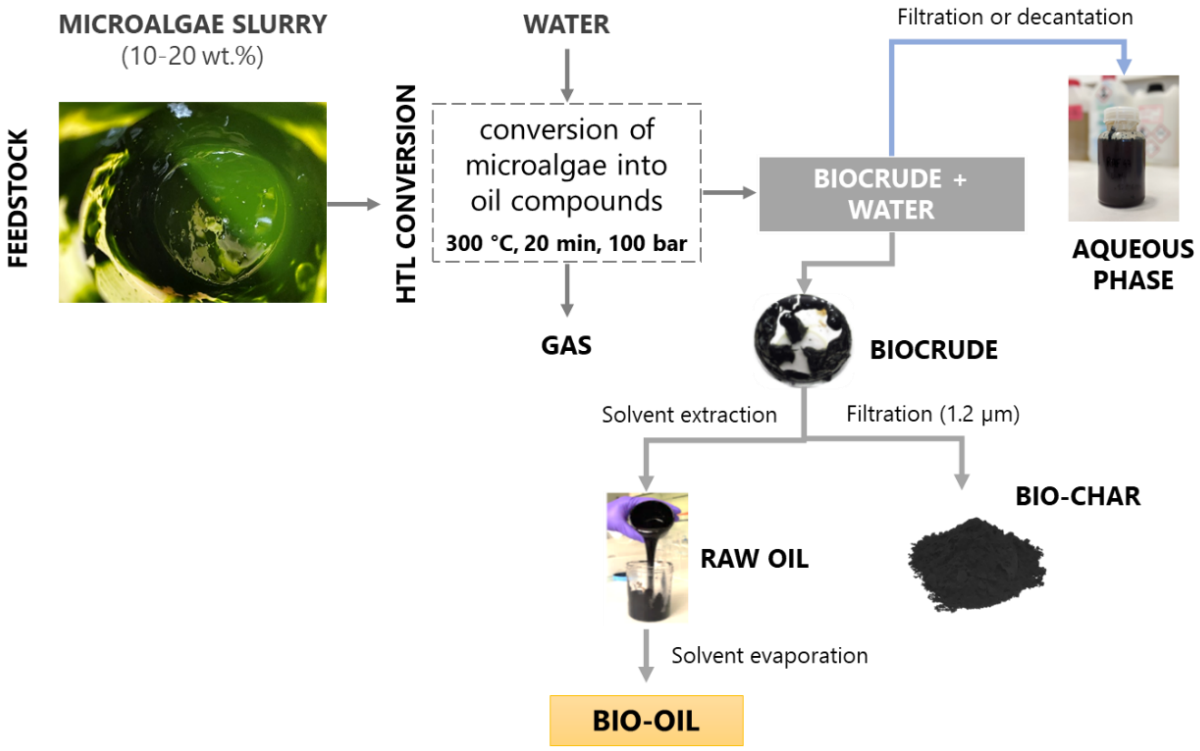


Figure 2. 5. Product separation during the hydrothermal liquefaction process. Adapted from Matricon et al. (Matricon et al., 2023).

2. 3.4. HTL aqueous phase

Numerous APs from individual HTL reactions were received from CEA Grenoble and kept at -20°C until further use (Figure 2. 6). The samples were identified by the HTL reaction number (1-57), and by the feedstock batch (Table 2. 2). Different APs were used during this work depending on their availability, quantity needed, and purpose of the experiment.

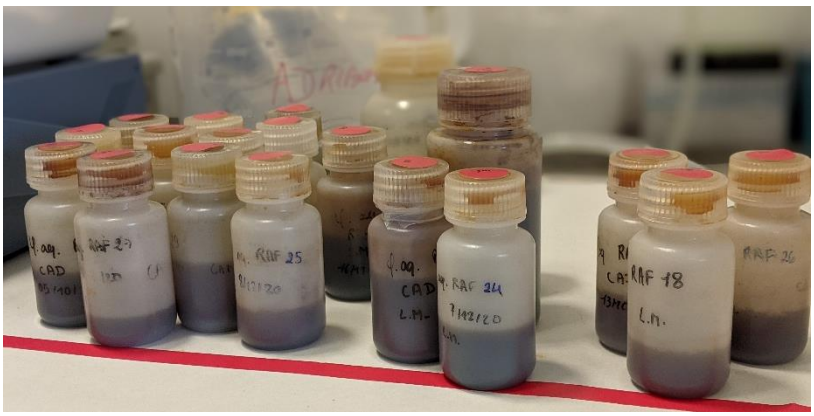


Figure 2. 6. Samples of AP received from different HTL reactions.

Furthermore, to provide a sufficient amount of the same AP for long-term experiments, the available APs (corresponding to 26 individual HTL reactions) were mixed in equal volumes (Table 2.3). This AP mixture is further referred to as AP_{mix}.

Table 2.3. AP samples used to formulate the AP_{mix}, obtained after HTL under different reacting conditions for varying algal feedstocks.

Feedstock ID	AP ID	HTL process	Temp. (°C)	Time (min)	Biomass load (wt.%)
2020RAFCS0	AP 07	Batch	300	30	11
	AP 09	Batch	300	30	9
2020RAFCS1	AP 12	Batch	270	5	10
	AP 13	Batch	270	5	17
	AP 14	Batch	270	20	10
	AP 15	Batch	270	20	17
	AP 21	Batch	270	20	10
	AP 22	Batch	270	5	10
	AP 23	Batch	270	5	17
	AP 24	Batch	270	20	17
	AP 29	Batch	350	5	10
	AP 30	Batch	350	20	10
	AP 31	Batch	350	5	17
	AP 40	Batch	350	20	17
2020RAFCS2	AP 11	Continuous	300	15	10
	AP 17	Continuous	300	15	10
2020RAFCS3	AP 16	Batch	270	20	10
	AP 18	Batch	270	5	17
	AP 19	Batch	270	5	10
	AP 20	Batch	270	20	17
	AP 25	Batch	270	5	10
	AP 26	Batch	270	20	10
	AP 27	Batch	270	5	17
	AP 28	Batch	270	20	17
	AP 33	Batch	350	5	10
	AP 34	Batch	350	20	10

2. 4. Preliminary assays

2. 4.1. Balanced nutrient limitation for lipid accumulation

Preliminary assays were performed to establish the nutrient supply that would better induce accumulation of lipids in *C. sorokiniana* NIES 2173. This strain was grown under different concentrations of nitrate to induce several levels of N deficiency. Nitrogen is indeed known to allow cellular division when sufficient, and to induce compound accumulation when deficient as lipids or carbohydrates (Figure 2. 7) (Nordin et al., 2020).

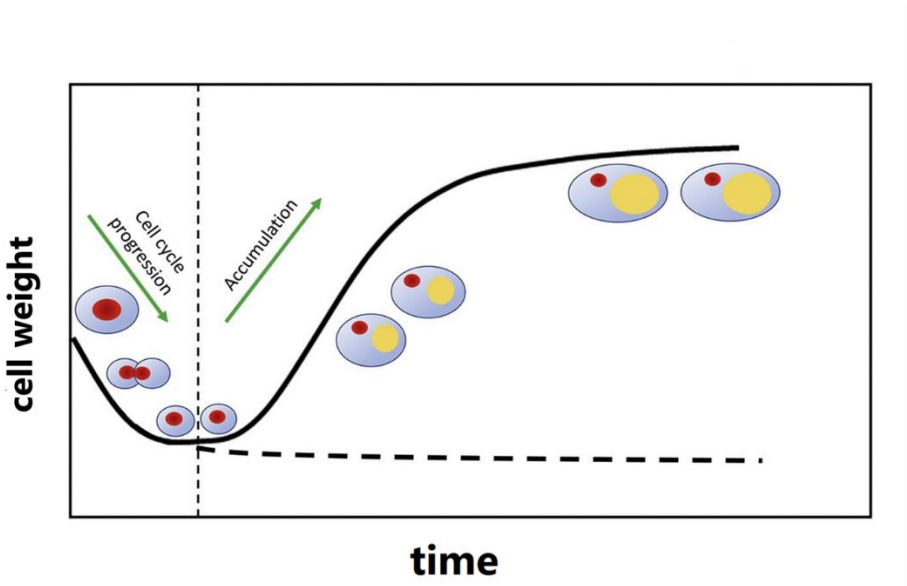


Figure 2. 7. Qualitative microalgal weight under N-deficiency divided in a period of cellular division, followed by compound accumulation after N-depletion. Modified from (Di Caprio, 2021).

Modified FWM media were prepared with varying N-NO_3^- concentrations corresponding to 20% (96 mg/L), 40% (144 mg/L) and 100% (240 mg/L) of the original FWM recipe. The rest of the nutrients was not modified for these tests. Cultivation was maintained in flasks with triplicates at constant environmental conditions. The light intensity was set to 100 µmol/m^2/s, with a photoperiod of 20 h light and 4 h darkness. Growth, biomass and nutrient consumption were monitored by OD, DW and IC, respectively.

The media with 40% N resulted in better growth and lipid content (Figure 2. 8). After six days of cultivation, it produced a maximum lipid content of 29% (N 40%), compared to 24% in FWM and 21% in N 20%. At the end of the cultivation, the medium with 40% N resulted in the highest biomass and lipid concentration. A noteworthy decrease in the lipid content was observed from day six to the last day of cultivation, and no increase in the other two media. A second test demonstrated that a higher level of N deficiency did not result in higher lipid accumulation in this strain. On the contrary, a lower supply of N resulted in less biomass and a significant decrease in lipid accumulation over time (Figure 2. 9). These preliminary findings emphasized the need for identifying strains with a high and consistent lipid content.

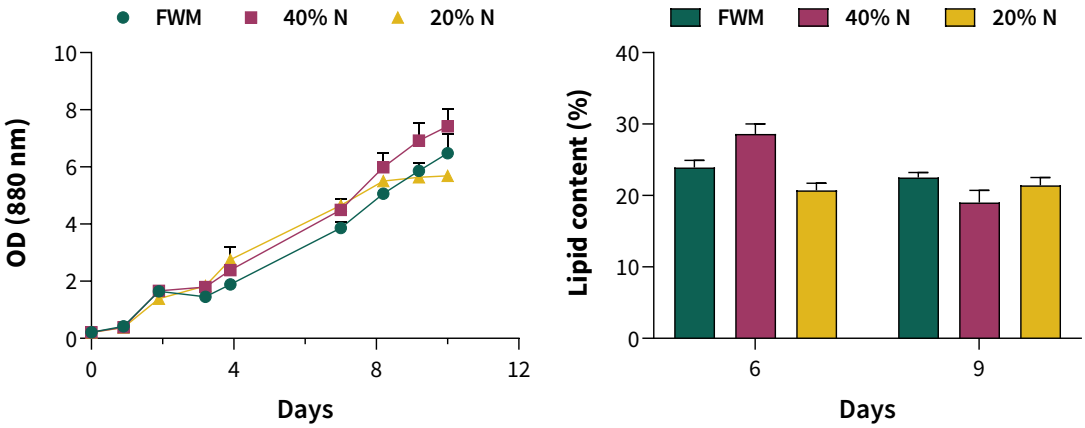


Figure 2. 8. Growth and lipid accumulation of *C. sorokiniana* NIES 2173 under different levels of N supply.

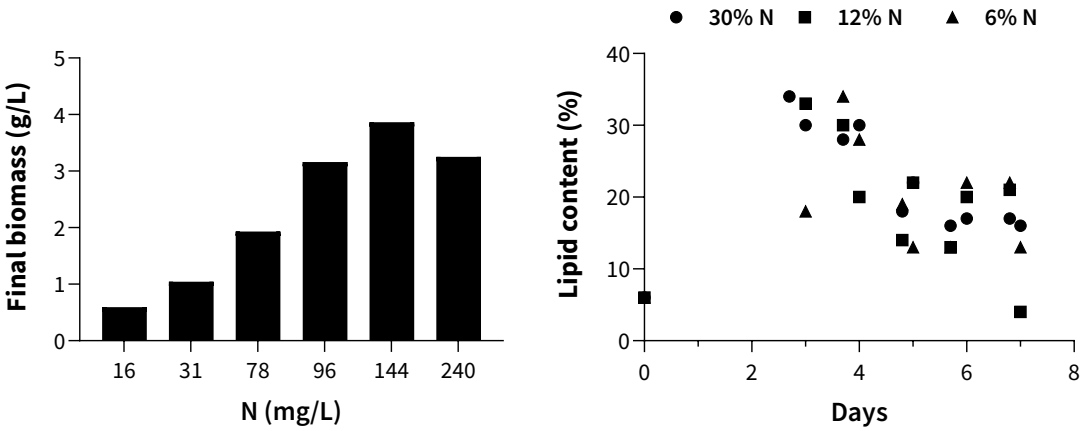


Figure 2. 9. Final biomass production and lipid evolution over time of *C. sorokiniana* NIES 2173 under different levels of N supply**.**

2. 5. Experimental designs

This section describes common experimental designs implemented along this thesis, and finishes with the specific details of each paper.

2. 5.1. Common experimental designs

Balanced nutrient limitation for lipid accumulation

The preliminary tests demonstrated that a limited amount of N resulted in higher biomass and lipid accumulation. However, the supply of the rest of the nutrients was not limited, resulting in an incomplete uptake of phosphates and sulfates, for example. Considering a cautious and efficient use of nutrients, in further experiments all nutrients were limited by diluting the original FWM recipe. Modified versions of the FWM (i.e., 1/4, 1/2) were prepared to induce nutrient limitation and consequently lipid accumulation in a given time and light intensity. This approach supported a one-stage starvation, where nutrient deficiency was naturally reached as the nutrients were depleted from the medium (Table 2. 4). Once the nutrients were completely consumed by the microalgae, the cultures were left growing under nutrient depletion to determine their compound accumulation capacities.

Table 2. 4. Nutrient composition of FWM.

FWM	mg/L
TN	267
NO_3^-	1064
NH_4^+	34
PO_4^{2-}	163
SO_4^{2-}	50

The FWM dilution was chosen accordingly to the objective and duration of each experiment:

In **Paper I** and **Paper II**, the nutrient content of the media was reduced by 50% (FWM 1/2) according to the preliminary tests. A longer period of cultivation was chosen to have a longer period of nutrient deficiency. This, with the objective of better comparing the potential of the six strains.

In **Paper III**, a dilution of FWM 1/4 was chosen to reduce the cultivation period with a faster trigger of lipid accumulation. These experiments were performed only on *C. vulgaris* NIES 227, after strain selection from **Paper I** and **Paper II**.

Similarly, AP-based media in **Paper II** and **Paper III** were designed following this strategy.

AP as a substrate for microalgae cultivation

These APs were characterized for their nutrient content (see *Nutrient and Media characterization*) (Table 2. 5). Two individual APs, corresponding to HTL reactions 14 and 57, were used in **Paper II** and **Paper III**.

Table 2. 5. Nutrient composition of APs used in **Paper II** and **Paper III**.

Content (g/L)	AP_{14}	AP_{57}	AP_{mix}
TOC	32.36	10.99	22.30
TC	33.44	11.03	23.60
TN	13.26	3.75	8.49
TON	10.34	2.94	5.67
NO_3^-	n.d.	n.d.	0.06
$NH4^+$	3.75	1.03	3.55
$PO4^{3-}$	2.85	2.04	3.13
$SO4^{2-}$	0.78	0.48	0.48

AP_{14}: used in **Paper II** and **Paper III**.
AP_{57}: used in **Paper III**.
AP_{mix}: used **Paper III**.
n.d: non-detected.

In order to formulate the AP-based media, two important factors were considered according to the nature of the HTL process and to previous studies: the dilution and nutrient content. First, according to the HTL process and considering a biomass concentration of 1 g/L in the culture and an HTL biomass load of 20 wt.%, a mass balance calculation would imply a dilution of 1/200 if all the AP was recycled in the culture. Secondly, previous studies have shown the importance of diluting the AP to reduce its inhibitory effects on microalgal growth (Biller et al., 2012). On the other hand, Garcia Alba et al. have also highlighted the importance of replenishing the missing nutrients after AP dilution to avoid nutrient deficiencies caused by high dilutions (Garcia Alba et al., 2013).

With that in mind, the macronutrient content of the AP (N, P and S), at a given dilution (1/50-1/1000), was considered in the preparation of the growth media. All AP-based media were normalized to have an equal concentration of macronutrients as FWM or modified versions (i.e., 1/4, 1/2) (Table 2. 4). Only the AP inorganic fractions (ammonium, nitrate, phosphate, and sulfates) were considered when replenishing the AP-based medium (Figure 2. 10). The final AP media were adjusted to pH 7.5 by addition of NaOH 2.5 M and sterilized with a 0.22-µm PES filter (Steritop 45 mm-Millipore Express Plus, Millipore, USA).

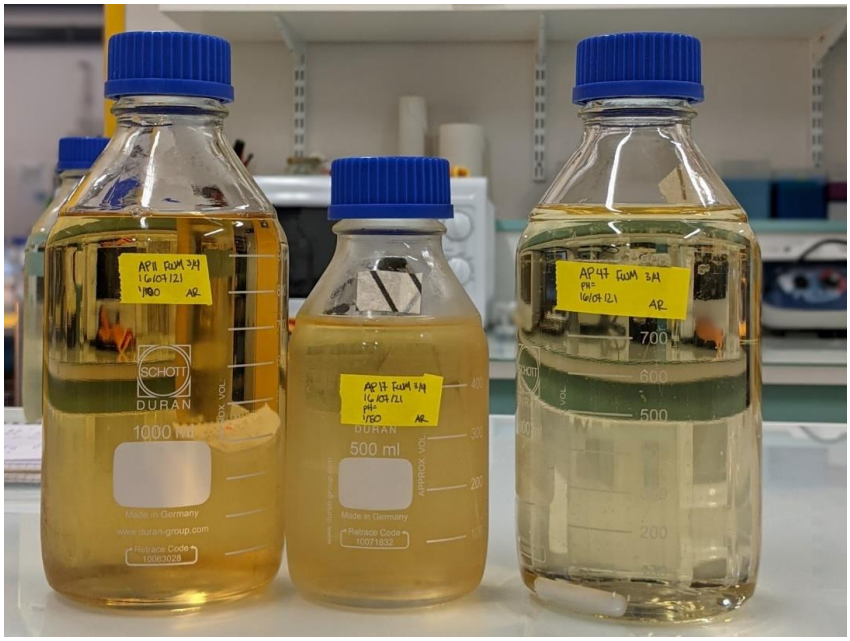


Figure 2. 10. AP-based media after dilution 1/200.

2. 5.2. Specific experimental designs

Paper I – Cultivation of high-lipid content microalgae

The six *Chlorellaceae* strains were cultivated for 17 days, in three biological replicates, to assess their biomass qualities as biofuel feedstocks. A balanced nutrient limitation (FWM 1/2) was used to trigger lipid accumulation while maintaining biomass accumulation. The relation between the biomass components and biofuel implications was explored. Erlenmeyer flasks (1 L) containing 500 mL of FWM 1/2 were inoculated with to an initial OD of 0.2 (880 nm). The light intensity was fixed to 230 µmol/m^2/s (20 h light and 4 h darkness). The rest of the environmental conditions were kept the same as for the stock cultures (T: 25°C, agitation: 130 rpm, CO_2: 2% v/v).

Biodiesel properties

In **Paper I**, the microalgae's fatty acid profiles were studied and their qualities for its utilization as biodiesel were estimated according to Arguelles and Martinez-Goss (2021).

Paper II – AP-grown microalgae biomass

In order to compare the qualities of the strains in AP-free FWM, the cultivation conditions were kept identical to **Paper I**. The study was divided into two phases to first evaluate the performance of the strains when cultivated in HTL-AP, and second to assess the effect of the AP under a longer period of cultivation aiming to accumulate lipids (as in **Paper I**). Phase I: the six strains were acclimated to an AP-based media (AP14, 1/200) with a nutrient content equivalent to FWM 1/2. The cultures were transferred to fresh media every 7–10 days. Phase II: after the fourth cycle, the microalgae were cultivated in duplicates for 18 days to study the effects of AP exposure on lipid and biomass accumulation. Finally, the resulting biomass was studied for its properties as HTL feedstocks in comparison to AP-free media (control conditions in **Paper I**).

Phase I: Acclimation phase

The first phase consisted of five successive cycles of acclimation to an AP-containing medium (1/200) with a nutrient content equivalent to the reference medium FWM 1/2. The cultures were rejuvenated to the initial inoculation cell density with fresh media every seven to ten days. The light intensity was set at 230 mol/m^2/s, with a photoperiod of 20 hours of light and 4 hours of darkness. Agitation was kept at 130 rpm and the air was enriched with 2% CO_2 in an automated incubator (ZWYC-290A, Labwit Scientific, Australia). NIES 269 was not included in the next experimental phase as it did not survive inhibition from the AP.

Phase II: Biomass and lipid production phase

After four successive cycles of dynamic growth under nutrient-replete conditions, the microalgae were cultivated for a prolonged fifth cycle of 18 days in order to assess the impact of the AP on lipid and biomass accumulation under nutrient-limited conditions. This duration was equivalent to **Paper I** in order to provide insights into the quality of AP-grown biomass compared to AP-free biomass. An additional biomass measurement was performed after 25 days without further growth monitoring to study the evolution of the biomass qualities.

Paper III - Microalgae adaptation and tolerance development to the AP

Based on the results from **Paper I** and **Paper II**, NIES 227 was selected for further studies under AP cultivation. The wild-type NIES 227 (WT) was submitted to a gradual acclimation to the AP, aiming for an adaptive laboratory evolution (ALE). This technique is a strategy toward strain improvement, where a specific environmental stress is applied mimicking natural evolution, commonly referred to as Darwinian selection (LaPanse et al., 2020). Two different ALE approaches were followed: a) batch cultivation and b) continuous adaptation in turbidostat mode. The resulting strains are further referred to as BA (from batch-adaptation) and TA (from turbidostat-adaptation).

Batch-adaptation to the AP

The WT *C. vulgaris* NIES 227 was subjected to two different ALE approaches aimed at increasing AP tolerance. First, conventional stock transfers 1:30 (v:v) were conducted periodically every 15 days over three months. Cultures were kept at constant conditions as the stock cultures, and particularly at a constant AP dilution (1/200) in control medium (FWM 1/2). For this first adaptation, the AP-based media were prepared using only one of the APs without mixture. (AP_{14} in **Table 2. 5**). The culture was followed under the microscope to visually check the fitness of the cultures. The obtained strain is further referred to as batch-adapted strain or BA.

Continuous adaptation in turbidostat mode

A second method was used to get a closer insight into the adaptation dynamics of the microalga to the AP. Starting from FWM 1/4, the WT *C. vulgaris* NIES 227 was grown in a 1-L panel-type photobioreactor (FMT-150 PSI, Czech Republic) (**Figure 2. 11**). The photobioreactor (PBR) was aerated at 1.5 vvm (2 vvm every 5 min to avoid microalgae deposition), and with a controlled temperature of 25°C and a controlled pH around 7.5. Continuous light was set at 550 µmol/m^2/s in the reactor, measured with a ULM-500 PAR-meter and its MQS-B surface probe (Walz, Germany). This light intensity corresponded to a non-null light intensity measured at the non-illuminated panel of the PBR throughout the experiment. This allows avoiding dark zones across the culture.

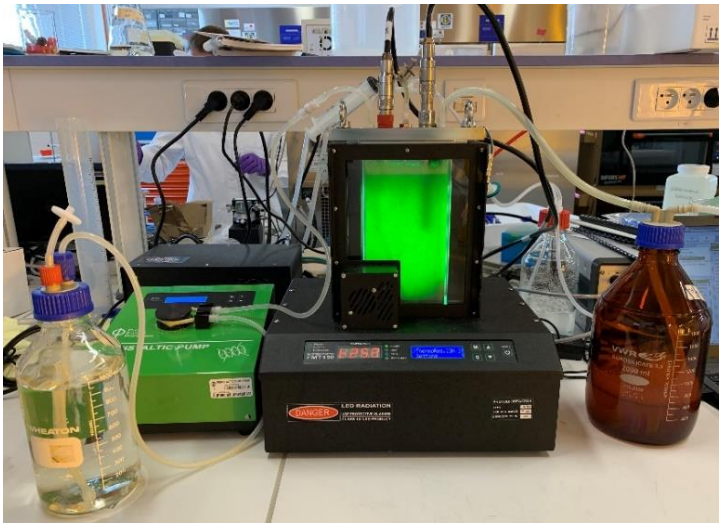


Figure 2. 11. Photobioreactor used for turbidostat cultivation in **Paper III**.

Once the culture reached an OD of 1.0 at 720 nm, the process was switched to turbidostat mode to keep the culture at this OD by adding a new AP-containing medium. The culture was exposed to an increasing concentration of AP_{mix}, starting from a dilution of 1/600 (0.17%) with a target of reaching a final AP dilution of 1/100 (1%) without significant negative effects. The adaptation in the photobioreactor was maintained for five phases with AP dilutions of 1/600, 1/500, 1/400, 1/300 and 1/250. This corresponded to acclimation cycles ranging from 10 to 15 days. After 95 days, due to a technical problem with the PBR, the culture was transferred to 1-L flasks and maintained under constant conditions in an incubator (light intensity: 200 µmol/m^2/s, T: 25°C, agitation: 130 rpm, CO_2: 2% v/v). The adaptation was continued with this set-up with dilutions of 1/250, 1/200 and 1/150 with manual dilution on each working day at an OD of 0.5 at 720 nm. The obtained strain is further referred to as a turbidostat-adapted strain or TA.

In contrast to batch adaptation, the culture was closely monitored in this strategy and several biomass parameters were measured: biomass concentration and productivity, OD, cell number and size, and photosynthetic activity or quantum yield. The biomass productivity was calculated as the product of the daily harvested volume (in L/d) and the biomass concentration (in g/L) divided by the PBR volume (1 L).

Strain purification

The pre-cultures for both adaptations were prepared under axenic conditions, but long-term manipulation of the cultures promoted the presence of bacteria. At the end of the adaptation, unlike the strain BA strain, the TA strain showed an important level of bacterial contamination. For both strains, the strains were recovered and/or purified after numerous transfers in petri dishes using FWM 1/4 with 1.5% agar. The efficacy of the purification was confirmed with scanning electron microscope observations of samples isolated during exponential growth (Figure 2. 12).

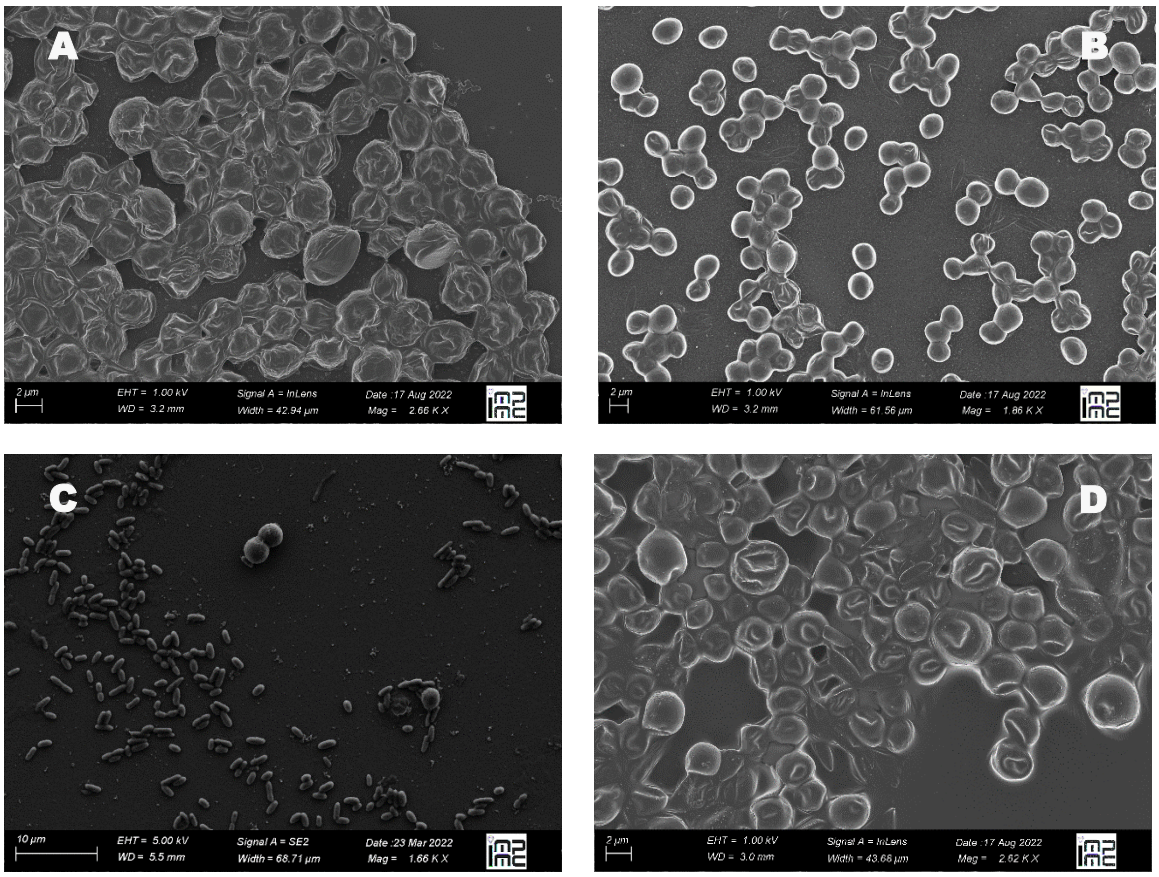


Figure 2. 12. SEM images of ALE *C. vulgaris* NIES 227 strains. From top to bottom, NIES wild-type NIES 227 (A), batch-adapted (B), turbidostat-adapted strain before strain purification (C), and after strain purification (D), respectively.

AP tolerance validation and persistence

To validate the effects of the adaptation strategies, several comparative tests were performed between the two adapted strains (BA and TA), and the WT of *C. vulgaris* NIES 227. Adapted strains were incubated in AP-free control medium (AP-) for over three months to ensure that tolerance persisted after the AP-stress stimulus was removed. The cultures were diluted periodically (every 10–15 days) and kept under the same conditions as the original stock cultures. After this period, a comparative culture test was performed in AP 1/200, between the two ALE strains precultivated in AP (AP+) and AP-free control medium (AP-). The WT without prior exposure to the AP was used as a reference and tested in the same AP and control medium.

This first experiment was performed using AP_{mix} with a dilution of 1/200 and nutrient replenishment equalizing the FWM 1/4 recipe. Flasks of 150 mL with 30 mL of AP_{mix} medium were inoculated with an initial OD of 0.2 (880 nm). Cultures were monitored daily for cell count and OD. Initial and final dry weights were measured. These experiments were performed with three biological replicates under non-axenic conditions, under the indicated environmental conditions, and with a continuous light intensity of 100 µmol/m^2/s.

To further investigate the performance of the adapted strains against the WT, a final experiment was performed after strain purification. A higher AP concentration (1/100) was also studied using a third AP (AP_{57}) in which none of the adapted strains were originally developed. The medium containing AP_{57} was prepared by diluting the AP_{57} 100- and 200-fold with FWM 1/4. Triplicate 250-mL cultures were inoculated into 500-mL flasks and maintained under constant conditions with a photoperiod of 16:8h (200 µmol/m^2/s).

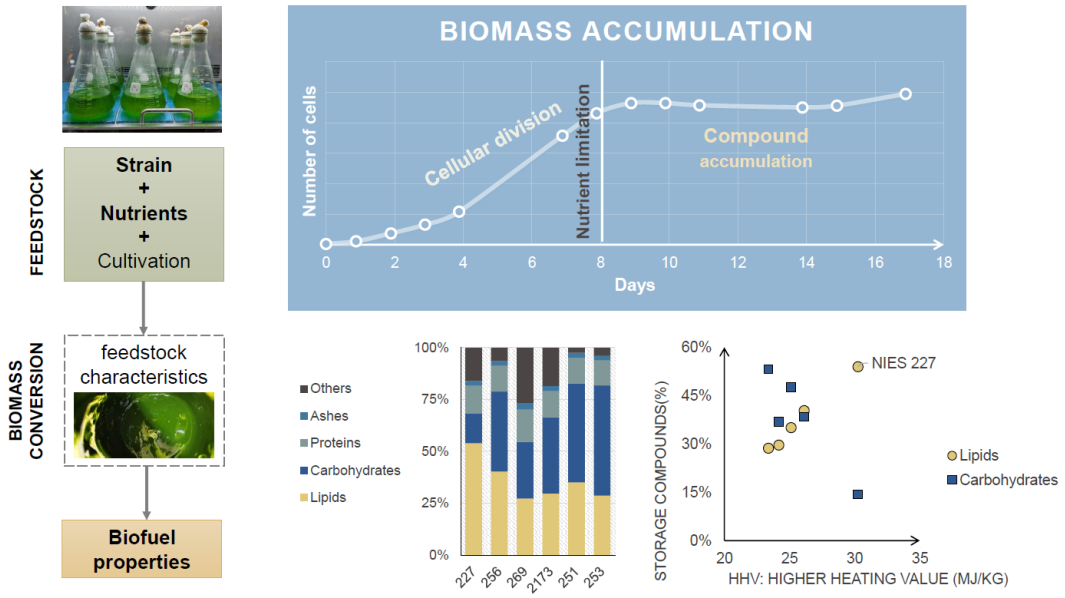
3. Results

This section contains the main findings of the experiments previously described. The first section presents the selection among six *Chlorellaceae* strains targeting lipid-based biofuels (**Paper I**). A second paper follows, concerning the impact of reusing AP as a substrate to grow new microalgae and its impact on the biomass as HTL feedstock (**Paper II**). Finally, the selected strain was subjected to adaptive laboratory evolution in order to improve its tolerance to the AP without compromising the quality of the biomass produced (**Paper III**).

Paper I - *Chlorellaceae* HTL feedstock selection under balanced nutrient limitation

This Paper has been published in **Ramírez-Romero et al. (2022)** "*Chlorellaceae* Feedstock Selection under Balanced Nutrient Limitation" Fermentation 8, 554.
https://doi.org/10.3390/fermentation8100554

Graphical abstract



Abstract

Microalgae are an attractive source of biomass for fossil fuel alternatives and renewable energy sources. Regardless of their potential, the development of microalgal biofuels has been limited due to the associated economic and environmental costs. For this, a strain selection was performed considering the biomass properties of six *Chlorellaceae* strains with a specific interest in lipid-based biofuels. The strains were cultivated under balanced nutrient limitations inducing a gradual limitation of nutrients that triggered reserve accumulation. The final biomass of each strain was characterized by its elemental and biochemical composition. Due to its high lipid content and overall composition, *Chlorella vulgaris* NIES 227 was identified as an ideal feedstock for biofuels with the best energy-content biomass. Its fatty acid profile also showed superior qualities for biodiesel production. Balanced nutrient limitation promoted not only the accumulation of storage compounds in all strains but also resulted in a low content of heteroatom precursors and ashes for biofuel applications.

I. 1. Motivation and research objective

To work toward environmentally friendly energy sources, it is critical to select strains while taking into account not only the yield and productivity of the compound of interest, but also the implications of the feedstock throughout the process (see Box 2). Therefore, this study aimed to identify a suitable strain for lipid-based biofuels among six potential strains. The possibility of AP reincorporation into microalgal cultivation was considered but not pursued yet in this paper. This effluent contains a rich and diverse load of inhibitory organic compounds. For this reason, it was essential to focus on strains that would be able to thrive in harsh conditions. *Chlorellaceae*, divided into two sister clades, *Chlorella* and *Parachlorella*, cover a wide variety of attributes that are attractive for the production of biomass for biofuel purposes (Krienitz et al., 2004). This microalgae family is well known for its fast growth rate and resistance to adverse growing conditions (Ummalyma et al., 2019). This family has also been shown to produce biomass with a high content of carbohydrates or lipids when grown under stress conditions (Přibyl et al., 2012; Li et al., 2013; Shen et al., 2015).

One-stage cultivation with balanced nutrient limitation was implemented, where nutrients were provided in concentrations that induce biomass accumulation while recovering all macronutrients from the medium. Once the microalgae consumed the nutrients from the media, the six strains were studied under nutrient-depleted conditions to potentially produce biomass with a high lipid content (Ramírez-Romero et al., 2022). The synergistic effect between microalgal metabolism and conscious use of nutrients resulted in various biomass compositions. The various biomass components and the subsequent consequences during the biomass conversion process were discussed. See experimental details in *Paper I – Cultivation of high-lipid content microalgae* (Materials & Methods 5. Experimental *designs*).

I. 2. Results and Discussion

I. 2.1. Growth and Biomass Accumulation under Nutrient Deficiency

During cultivation, all strains maintained a constant biomass accumulation, regardless of the reduced nutrient content in the medium (Figure I. 1A). Nitrogen was depleted from the mediums as they reached a biomass concentration of 2 g/L. This corresponded to the medium composition (FWM 1/2), designed to produce this amount of biomass from a *Chlorophyceae*-type strain without nutrient deficiencies. *C. sorokiniana* NIES 2173 had the highest final biomass concentration and overall biomass productivity, corresponding to 5.63 g/L and 0.33 g/L/d, followed by *C. vulgaris* CCALA 256 and *C. vulgaris* NIES 227. Moreover, *C. vulgaris* CCALA 269 had the lowest overall performance, with a final biomass concentration of 3.91 g/L and overall biomass productivity of 0.23 g/L/d (Table I. 1). In contrast to the produced biomass, nutrient limitations strongly affected the cell cycle of the microalgae. Cell division ceased soon after all macronutrients were depleted from the medium on day 9 (Figure I. 1B). Once cell division stopped, the cells continued to increase in volume, suggesting the accumulation of storage compounds, which was reflected by the continuous increase in biomass.

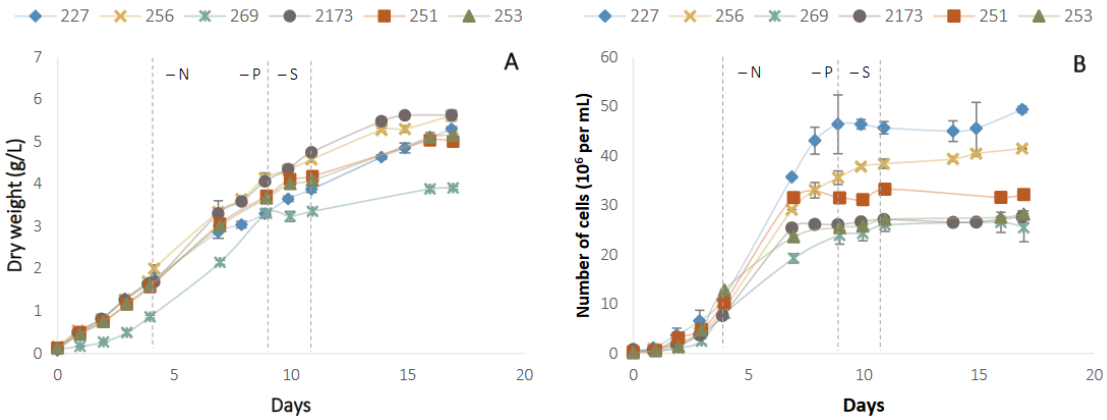


Figure I. 1. Growth comparison based on (A) Dry weight and (B) Number of cells of six *Chlorellaceae* strains: *C. vulgaris* NIES 227 and CCALA 256 & 269, *C. sorokiniana* NIES 2173, *P. kessleri* (CCALA 251 & 253). The dashed line denotes the onset of N (-N), P ,(-P).

Table I. 1. Microalgae growth, biomass, and lipid values comparison of six *Chlorellaceae* spp. strains. *C. vulgaris* NIES 227 and CCALA 256 & 269, *C. sorokiniana* NIES 2173, *P. kessleri* (CCALA 251 & 253).

Microalgae Strain	Growth Rate (μ/d)	Biomass			Lipids		Carbohydrates	
		(g/L)	Overall P (g/L/d)	Max P (g/L/d)	(%)	Max P (mg/L/d)	(%)	Max P (mg/L/d)
C. vulgaris NIES 227	1.67 ± 0.1	5.31 ± 0.1	0.31 ± 0.00	0.48 ± 0.01	54% ± 2	212 ± 3	14% ± 1	92 ± 04
C. vulgaris CCALA 256	1.55 ± 0.2	5.62 ± 0.1	0.32 ± 0.01	0.58 ± 0.08	40% ± 1	156 ± 4	38% ± 5	220 ± 24
C. vulgaris CCALA 269	1.20 ± 0.1	3.91 ± 0.0	0.23 ± 0.00	0.58 ± 0.03	27% ± 1	91 ± 3	27% ± 3	131 ± 16
C. sorokiniana NIES 2173	0.90 ± 0.1	5.63 ± 0.1	0.33 ± 0.01	0.70 ± 0.01	30% ± 4	149 ± 1	37% ± 6	244 ± 33
P. kessleri CCALA 251	1.60 ± 0.1	5.00 ± 0.0	0.29 ± 0.00	0.50 ± 0.00	35% ± 1	124 ± 3	48% ± 1	236 ± 19
P. kessleri CCALA 253	1.25 ± 0.1	5.16 ± 0.1	0.30 ± 0.00	0.47 ± 0.02	29% ± 1	108 ± 2	53% ± 1	199 ± 30

Interestingly, even if N was depleted from the medium on day 4 for most of the strains (except for CCALA 269, for which N depletion occurred on day 7), cell division did not stop immediately and a stationary phase was observed only after day 9 for all microalgae. After day 10, all strains were lacking, not only N, but also P and S (Figure I. 2). It is unknown how the lack of P and S could have affected each of the tested strains regarding their specific growth and compound accumulation. However, the reasons why N-starvation is the main trigger for the accumulation of storage compounds, like lipids and carbohydrates, have been well-studied (Zhu et al., 2018). In some strains, like in *C. vulgaris* CCALA 924, sulfur deprivation resulted in a higher starch accumulation than in N-deprived conditions (Brányiková et al., 2011). Sakarika et al. also observed higher intracellular lipid content under S-limitation and a higher maximum biomass and lipid productivity under P-limitation (Sakarika and Kornaros, 2017).

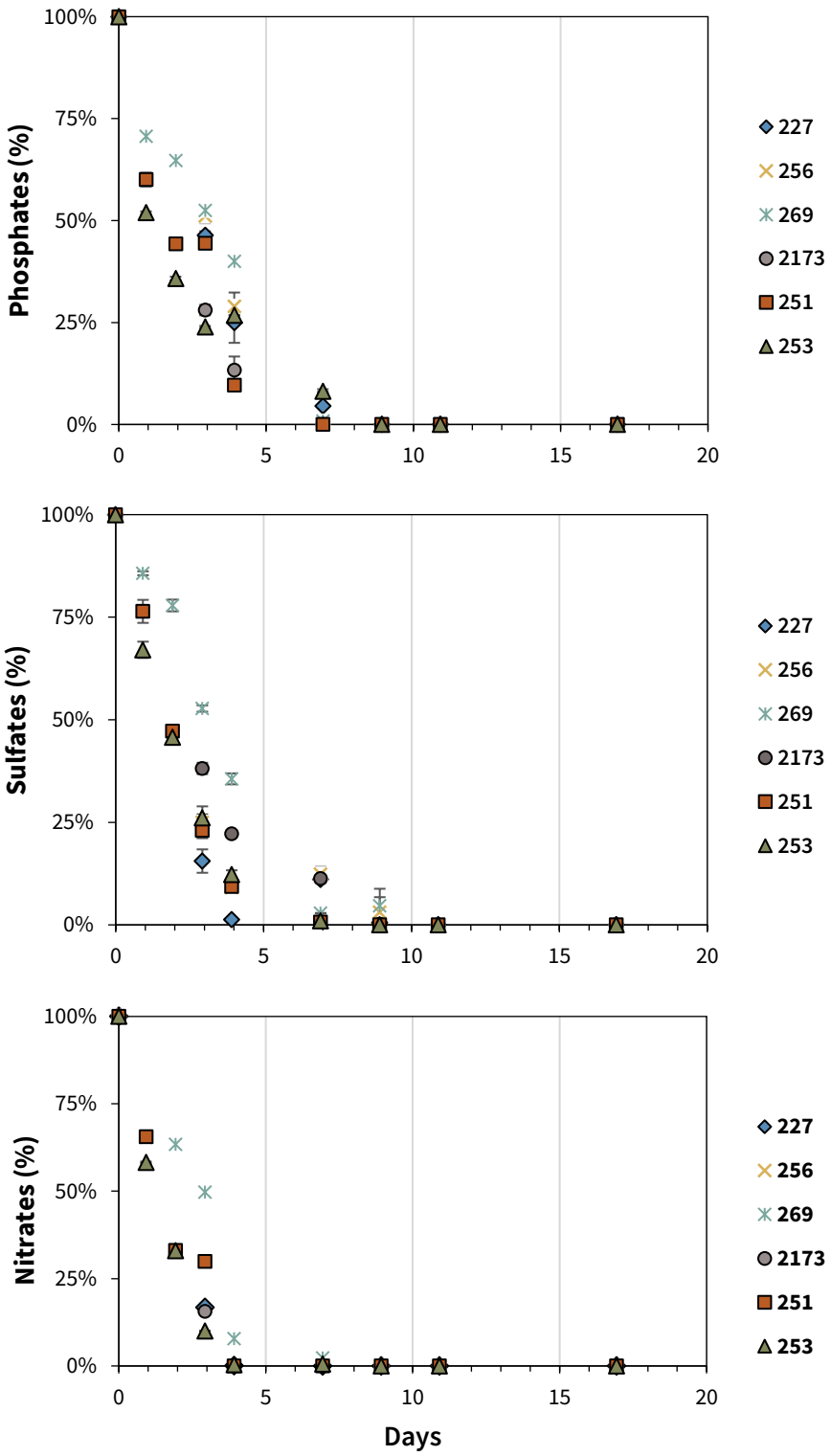


Figure I. 2. Nutrient availability (%) in culture media in relation to the initial content of: Phosphorous, Sulfur, and Nitrogen. *C. vulgaris* NIES 227, CCALA 256 & 269, *C. sorokiniana* NIES 2173, and *P. kessleri* CCALA 251 & 253.

Nitrogen is a vital nutrient for the biosynthesis of proteins necessary for cell growth and metabolic requirements (Markou et al., 2014). However, N-depletion from the media did not reflect the real metabolic status of the cells, since more impactful stress was produced as the cells continued to consume their internal N-reserves. Remarkably, when comparing the intracellular N-quotas between the onset of extracellular N-depletion (day 4) and after almost two weeks of intracellular N-starvation (day 17), a reduction of almost four times was observed among the strains (**Table I. 2**), indicating the consumption of intracellular N after N was completely depleted from the media. It was observed that microalgal cells continue to divide, by using their own N reserves, even after extracellular N-depletion. Cell division continued until reaching a limiting intracellular N content during the second week of cultivation. Based on the intracellular N quota on day four, protein content was drastically reduced in all strains, from an initial protein content of 40–45% to reach values as low as 12–16% on day 17 (**Figure I. 3**). Microalgae can assimilate high amounts of N for the buildup of proteins and nucleic acids, consisting of up to 40–60% of their dry weight (Templeton and Laurens, 2015). Under N-limiting conditions, microalgae degrade non-essential proteins to support basic cellular requirements (Di Caprio, 2021).

Table I. 2. Intracellular N content dynamics per (A) number of cells and (B) per biomass of six *Chlorellaceae* spp. strains: *C. vulgaris* NIES 227 and CCALA 256 & 269, *C. sorokiniana* NIES 2173, *P. kessleri* (CCALA 251 & 253).

		Intracellular N Quota					
	Day	**NIES 227**	**CCALA 256**	**CCALA 269**	**NIES 2173**	**CCALA 251**	**CCALA 253**
(A) pg of N per cell	4	1.29 ± 0.05	1.62 ± 0.03	1.88 ± 0.22	2.01 ± 0.18	1.41 ± 0.03	1.15 ± 0.01
	17	0.28 ± 0.02	0.33 ± 0.01	0.62 ± 0.08	0.51 ± 0.01	0.45 ± 0.01	0.52 ± 0.01
(B) mg of N per g of biomass	4	85.4 ± 1.9	88.1 ± 3.7	183.8 ± 8.9	89.9 ± 2.8	98.6 ± 2.9	98.7 ± 1.7
	17	26.0 ± 0.3	24.9 ± 0.6	40.0 ± 0.6	24.7 ± 0.4	29.2 ± 0.1	29.1 ± 0.3

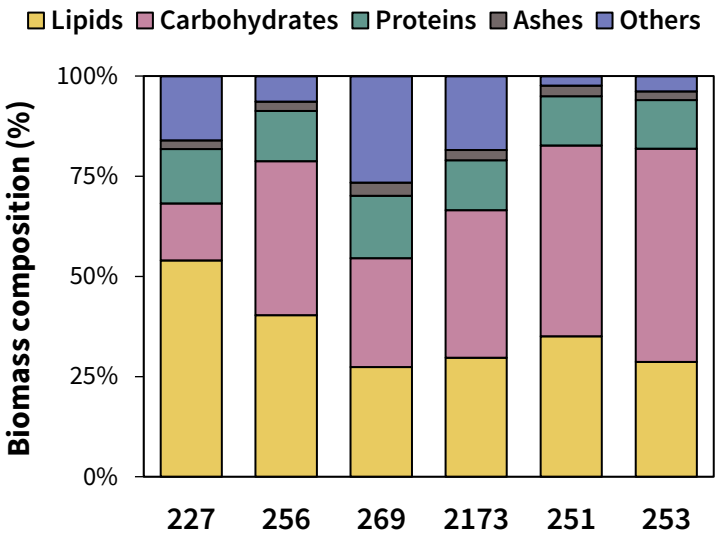


Figure I. 3. Final biomass (day 17) composition of six *Chlorellaceae* spp.: *C. vulgaris* NIES 227, CCALA 256 & 269, *C. sorokiniana* NIES 2173 and *P. kessleri* CCALA 251 & 253.

I. 2.2. Accumulation of Storage Compounds under Nutrient-Deficiency

After almost two weeks of N-starvation, the prolonged metabolic stress triggered the accumulation of storage compounds, such as lipids and carbohydrates, at diverse levels depending on the strain (**Figure I. 3**). Each strain presented a different biochemical and lipid profile, even among the same species of *Chlorellaceae*, showing potential and diversity. In general, all strains had a higher lipid content compared to the 10–20% found in nutrient-sufficient conditions (data not shown). Notably, different lipid accumulation patterns could be observed among the microalgae (**Figure I. 4**). NIES 227 had the earliest trigger for lipid accumulation and a continuous lipid increase since the onset of N-starvation. In contrast, for strains CCALA 251 and 253, lipid biosynthesis was triggered after almost two weeks of N-starvation. The highest lipid contents were observed in NIES 227 and CCALA 256, with 54% and 40%, respectively (**Table I. 1**). Although NIES 227 had only moderate biomass productivity, this strain significantly surpassed the lipid productivities of all the tested strains with a value of 212 mg/L/day, compared to CCALA 256 and NIES 273 with 156 mg/L/day and 149 mg/L/day, respectively.

In terms of carbohydrate content, NIES 227 had the lowest carbohydrate content at 14% in accordance with its high lipid content. On the contrary, the reduced lipid content observed in some strains coincided with a preference for carbohydrate storage. Four of the strains had a carbohydrate content of at least 35%. The highest carbohydrate content was found in both *P. kessleri* strains, CCALA 251 and 253, with 48% and 53%, respectively. Total carbohydrate content varies among strains and values between 19 to 69% have been reported under nutrient limitation (de Carvalho Silvello et al., 2022)

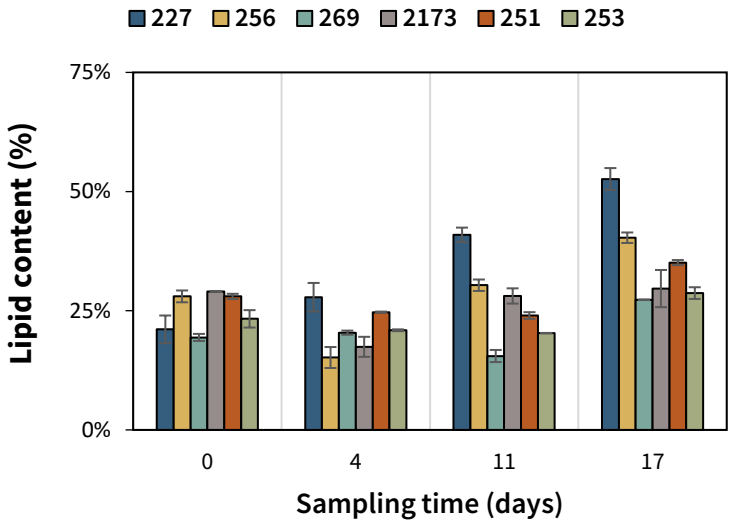


Figure I. 4. Lipid accumulation (as biomass %) under nutrient limitation over time: days 0, 4, 11 and 17 for six *Chlorellaceae* spp. strains: *C. vulgaris* NIES 227 and CCALA 256 & 269, *C. sorokiniana* NIES 2173, *P. kessleri* (CCALA 251 & 253).

The observed differences between the strains could be explained by the different rates of carbohydrate synthesis and degradation inherent to each strain. Nitrogen stress has been studied for its effect on lipid and starch accumulation, as well as the degree and extent to which this stress may cause different responses in microalgae, and remarkably within the same species (Procházková et al., 2014). The specific preference of each strain toward starch or lipid storage under nitrogen stress depends on each microalgae's metabolic capacities and energy requirements for the biosynthesis of these molecules (Li et al., 2015). Nordin et al. identified, by proteomic studies, that *Chlorella vulgaris* (UPSI-JRM01) responded to the absence of N by producing both storage compounds for different purposes (Nordin et al., 2020); where starch storage corresponded to an immediate

response to N deficiency, and subsequent carbohydrate degradation and lipid biosynthesis occurred for long-term energy storage. The transition from starch storage to lipid biosynthesis might differ from one strain to the other, explaining the differences in lipid and carbohydrate contents observed in this study.

Furthermore, a different growth rate was observed among the strains, resulting in a dissimilar number of cells for a similar amount of biomass. Cellular division in NIES 227 resulted in twice the number of cells compared to NIES 2173 for the same amount of nutrients. When comparing the intracellular N content or the protein content of the biomass, minor differences were observed. However, when comparing the intracellular N quotas, it was observed that each strain had different intracellular N reserves. Interestingly, the strains with the lower intracellular N-quota (< 0.5 pg-N/cell), such as NIES 227 (0.28 pg-N/cell) and CCALA 256 (0.33 pg-N/cell), had the highest lipid content. Markou et al. compared various levels of N-limitation in *C. vulgaris* (SAG 211-11b) cultures to induce diverse N-content in the biomass. It was found that a lower N content (29 mg-N/g) promoted a higher lipid content in the biomass (48%) compared to a higher N-content (41 mg-N/g) with 28% of lipids (Markou et al., 2017). In our study, it could be observed that the biomass N content could diverge from the intracellular N quotas, depending on the cellular division of the strains. A similar N content in the strains CCALA 256 (24.9 mg-N/g) and NIES 2173 (24.7 mg-N/g) resulted in different intracellular quotas of 0.33 pg-N/cell and 0.51 pg-N/cell respectively, and distinctive biomass compositions.

I. 2.3. Impact of the Biomass Composition as a Biofuel Feedstock

Elemental analysis is essential for predicting biomass carbon and energy content prior to HTL and identifying heteroatoms that could cause further problems (Hossain et al., 2019). For our study, the nutrient deficient conditions resulted in final biomasses with high C content and decreased N content. Typically, C content in microalgae varies between 45% and 55% of dry biomass (Markou et al., 2014). Values within 52% to 62% were found among the studied strains. The high-lipid biomass of NIES 227 presented the highest C and H (62.2% and 9.3%) content and the lowest O content (27%). In contrast, the high-carbohydrate biomass of CCALA 253 presented the lowest C and H contents (52% and 7.6%, respectively) and a high O content of 39.3% (Table I. 3).

Table I. 3. Microalgae CHONS elemental composition and calculated higher heating value (HHV) six *Chlorellaceae* spp. strains: *C. vulgaris* NIES 227 and CCALA 256 & 269, *C. sorokiniana* NIES 2173, *P. kessleri* (CCALA 251 & 253).

Microalgae Strain	C (wt.%)	H (wt.%)	O (wt.%)	N (wt.%)	S (wt.%)	HHV (MJ/kg)
***C. vulgaris* NIES 227**	62.2 ± 0.1	9.3 ± 0.1	27.0 ± 2.2	2.6 ± 0.0	0.101 ± 0.01	29.8
***C. vulgaris* CCALA 256**	56.3 ± 2.6	8.2 ± 0.3	35.1 ± 0.6	2.4 ± 0.1	0.097 ± 0.03	25.7
***C. vulgaris* CCALA 269**	55.7 ± 2.1	8.1 ± 0.5	33.4 ± 0.3	3.0 ± 0.1	0.099 ± 0.01	25.4
***C. sorokiniana* NIES 2173**	53.5 ± 0.3	7.9 ± 0.1	40.6 ± 0.8	2.4 ± 0.0	0.102 ± 0.01	23.7
***P. kessleri* CCALA 251**	54.7 ± 0.4	7.9 ± 0.1	35.3 ± 1.2	2.4 ± 0.1	0.104 ± 0.01	24.7
***P. kessleri* CCALA 253**	52.0 ± 0.2	7.6 ± 0.1	39.3 ± 1.5	2.4 ± 0.0	0.100 ± 0.01	22.9

The results showed the influence of the C content present among the different macromolecules. For instance, carbon in the biomass decreased when carbohydrates were the predominant storage compound, since the carbohydrate backbone has a lower C content (44%), compared to lipids and proteins, at 76% and 53%, respectively (Di Caprio, 2021). A positive correlation (R^2 = 0.80, p-value: < 0.05) was found between the lipid content of the strains and HHV. The opposite trend was observed with the sugar content (R^2 = 0.77, p-value: < 0.05). The highest HHV was found in NIES 227 (29.8 MJ/kg), in comparison to CCALA 253 (22.9 MJ/kg), which had the highest sugar content. Correspondingly, C and O have a contrasting effect on the potential HHV of the biomass: C having a positive correlation and O having the opposite effect (Hossain et al., 2019).

Microalgae are considered high-N content biomass, mostly varying within 5–9% and reaching values as high as 12%, in comparison to the 1–5% found in other biofuel feedstocks, and 1% or even less, in lignocellulosic biomass (Zhan et al., 2018; Leng et al., 2020b). In this study, 2–3% of N was measured in the final biomass of all strains, which could potentially decrease the N content of the biofuel and consequent emissions. Sulfur, a less-mentioned biomass component, varies from 0.15% to 1.6% in microalgal biomass (Grobbelaar, 2004). This element can also have repercussions during the biomass conversion and further SOx emissions during the biofuel combustion. Remarkably, this element was also on the lower end in all the strains used in the current study, with a commonly shared value of 0.10 ± 0.01%.

Mineral and trace element levels also vary among microalgae (2–20%) depending on the specific metabolic requirements and the availability in the medium (Grobbelaar, 2004; Lababpour, 2018). This ash fraction of the feedstock could later interfere with biomass conversion to biocrude and its further upgrading, and it could cause damage to the combustion system (Huang et al., 2016). Remarkably, a low ash content of 2.2–3.3% was found in the final biomass of the strains (Figure I. 3). A comparison of low-ash and high-ash microalgal biomass conversion revealed a preference for lower ash content biomass, as high-ash biomass reduced conversion efficiency (Liu et al., 2020b).

I. 2.4. Fatty Acid Profile for Biodiesel Applications

An alternative route for biofuel production is the extraction and transesterification of lipids to produce biodiesel (see Box 1). In parallel to lipid productivity, it is also important to assess the fatty acid (FA) profile of the microalgae. In this study, and in the final biomass, 65–80% of the total FA profile corresponded to C18 chains in all strains, followed by 18% up to 33% of C16 chains (Figure I. 5). Major differences were observed in the saturation levels of the different FA profiles.

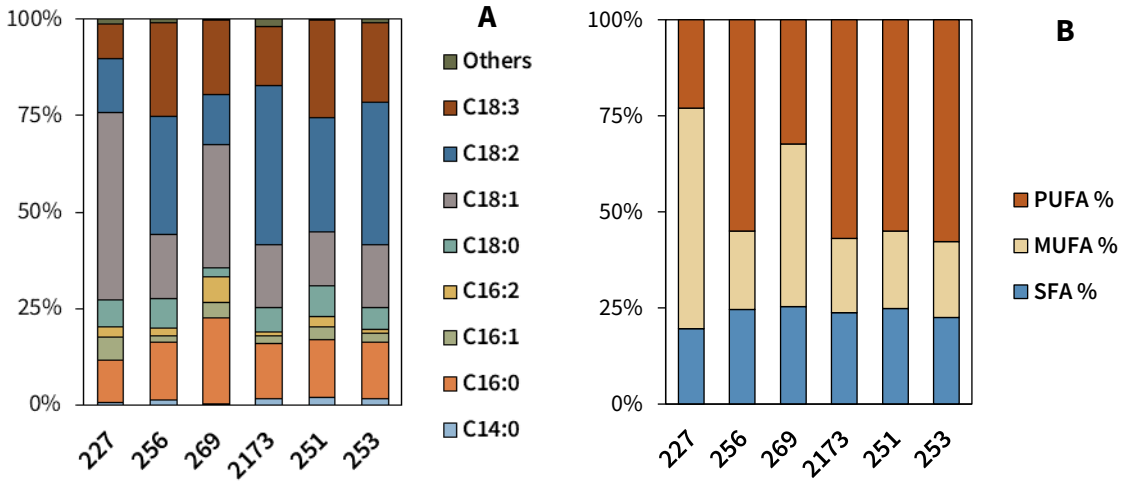


Figure I. 5. Lipid composition of six *Chlorellaceae* strains. (A) FAME profile and (B) Saturation distribution: Saturated fatty acids (SFA), Mono-unsaturated fatty acids (MUFA), Poly-unsaturated fatty acids (PUFA). *C. vulgaris* NIES 227, CCALA 256 & 269, *C. sorokiniana* NIES 2173 and *P. kessleri* CCALA 251 & 253

NIES 227 and CCALA 269 presented 55% and 36% of the total FAs as mono-unsaturated FAs (MUFAs), predominantly in the form of oleic acid (C18:1), with 49% and 32%, respectively. For the other strains, about 60% of the FAs were in the form of PUFAs, which are not ideal for biodiesel but are of great nutraceutical interest (Patel et al., 2020). Notably, the other four strains presented a high content of linoleic acid (C18:2), known for its health properties, with values ranging from 30% to 41%.

To obtain a biodiesel with balanced properties, some generalizations can be made, but it is the entire composition that dictates the qualities of the biodiesel. For this, the overall quality of the FA profiles were assessed for their utilization as biodiesel, and their potential properties were estimated according to Arguelles et al. (Arguelles and Martinez-Goss, 2021). Based on these properties, NIES 227 presented not only an outstanding lipid content but also a high-quality FA profile for its direct use as biodiesel (**Table I. 4**). Interestingly, this strain was the only one that met the requirements set by the European standard for biodiesel (European Committee for Standardization, 2009), such as having a linoleic acid (C18:3) content lower than 12% (9%), a cetane number of at least 51 (51), and an iodine value of less than 120 (99.9).

Certain fatty acids (FA) may have opposing effects on various biodiesel properties. Poly-unsaturated FAs (PUFA), for example, have better properties at low temperatures, but they decrease the oxidation stability of the biodiesel (Stansell et al., 2012). Stansell et al. reported that a feedstock ideally should have a high content of MUFA and highlighted the importance of the composition of saturated FA (SFA) (Stansell et al., 2012). In general, a balance between a low unsaturation level of the FA and a higher proportion of longer FA chains (C16–C18) is desirable (Arguelles and Martinez-Goss, 2021).

Table I. 4. Biomass properties for biodiesel applications.

Microalgae Strain	CN (min)	HHV (MJ/Kg)	C18:3 (wt.%)	IV (g I_2/ 100 g of fat)	SV (mg/ KOH g)	OS (h)	CFPP (°C)
C. vulgaris **NIES 227**	51.6 ± 0.2	46.5 ± 0.1	9%	99.9 ± 0.6	196.7 ± 0.2	7.7 ± 0.1	−1.5 ± 0.1
C. vulgaris **CCALA *256***	42.5 ± 0.0	45.3 ± 0.0	24%	138.9 ± 0.0	198.7 ± 0.1	4.7 ± 0.1	0.9 ± 0.0
C. vulgaris **CCALA 269**	50.4 ± 0.1	46.3 ± 0.2	19%	108.8 ± 0.4	191.1 ± 0.2	6.2 ± 0.1	−5.5 ± 0.7
C. sorokiniana **NIES 2173**	43.7 ± 0.7	45.4 ± 0.4	15%	133.8 ± 1.57	198.8 ± 2.8	4.7 ± 0.1	−2.3 ± 1.7
P. kessleri **CCALA 251**	42.6 ± 0.2	45.4 ± 0.1	25%	138.9 ± 0.6	198.4 ± 0.2	4.7 ± 0.1	0.5 ± 0.1
P. kessleri **CCALA 253**	41.8 ± 0.1	45.2 ± 0.0	21%	140.6 ± 0.3	201.1 ± 0.3	4.6 ± 0.1	3.1 ± 0.0
European Standard EN 14214 (2009)	≥51	-	< 12%	≤ 120	-	≥ 8	≤ 5/≤ −20

Cetane number (CN), iodine value (IV), saponification value (SV), higher heating value (HHV), oxidation stability (OS), degree of saturation (DU), cold filter plugging point (CFPP).

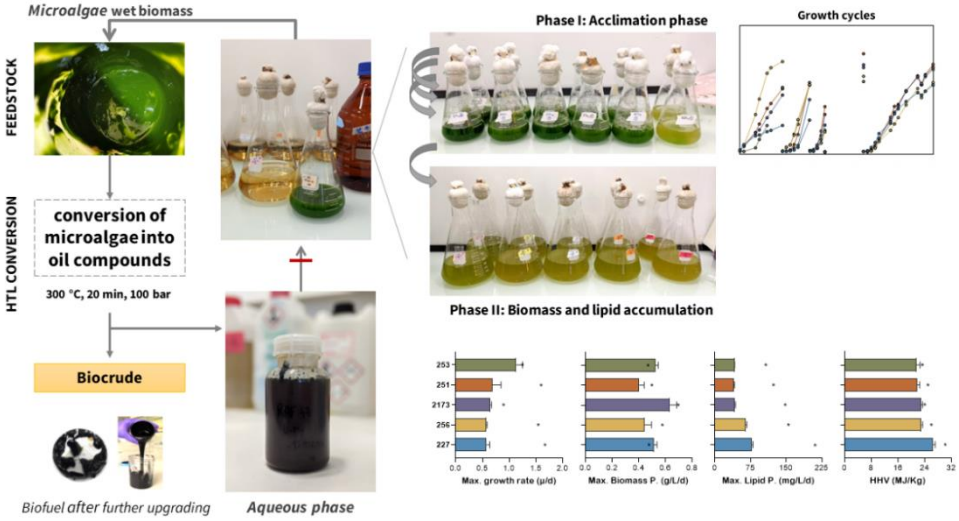
I. 3. Conclusion

The synergy of a balanced medium and strain-specific metabolism resulted in different biochemical and lipid profiles. The observed metabolic diversity, even among the same *Chlorella* species, showed the potential of this microalgal family for different applications. *C. vulgaris* NIES 227 was identified as an ideal lipid-based feedstock due to its superior lipid productivity and reduced content of heteroatom precursors. In parallel, *Parachlorella* strains presented the potential for sugar-based applications. A balanced nutrient limitation is proposed to produce high-lipid or high-sugar biomass. This one-stage strategy supported biomass accumulation with advantageous properties as biofuel feedstocks while fully recovering the macronutrients from the media. These results demonstrated the potential of individual strains as feedstocks for biofuels, but the question of the impact of reusing AP as a substrate to produce microalgal biomass remains open.

Paper II - Impact of reusing HTL aqueous phase on microalgal biomass quality

This Paper is under preparation for submission to Biomass and Energy (March, 2023).

Graphical abstract



Abstract

The potential success of microalgal biofuels greatly depends on the sustainability of the chosen pathway to produce them. Hydrothermal liquefaction (HTL) is a promising route to convert wet algal biomass into biocrude. Recycling the resulting HTL aqueous phase (AP) aims not only to recover nutrients from this effluent but also to use it as a substrate to close the photosynthetic loop and produce algal biomass again and process this biomass again into new biocrude. With that purpose, the response to AP recycling of five *Chlorellaceae* strains was monitored over five cultivation cycles. After four successive cycles of dynamic growth under nutrient-replete conditions, the microalgae were cultivated for a prolonged fifth cycle of 18 days in order to assess the impact of the AP on lipid and biomass accumulation under nutrient-limited conditions. Using AP as a substrate reduced the demand for external sources of N, S, and P while producing a significant amount of biomass (2.95–4.27 g/L) among the strains, with a lipid content ranging from 16% to 36%. However, the presence of the AP resulted in a biomass with suboptimal properties, as it slowed down the accumulation of lipids and thus reduced the overall energy content of the biomass in all strains. Although *Chlorella vulgaris* NIES 227 did not have the best growth on AP, it did maintain the best lipid productivity of all the tested strains. Understanding the impact of AP on microalgal cultivation is essential for further optimizing biofuel production via the HTL process.

II. 1. Motivation and Research Objective

Hydrothermal liquefaction (HTL) is a promising route for converting wet algal biomass into biocrude. During HTL, biocrude is obtained along with an aqueous phase (AP) that contains a high amount of nutrients essential for microalgal growth. However, HTL also produces hundreds of different compounds, some of which can be inhibitory to microalgae. Recycling the AP is proposed to not only to recover nutrients from this effluent but also to use it as a substrate to close the process loop and produce new microalgal biomass, and process this biomass again into new biocrude. When recycling AP to produce new microalgal feedstock, it is critical to understand how the AP affects not only microalgal growth but also the biomass yield and composition. Despite the fact that several previous studies have been conducted to study AP inhibition, less attention has been targeted to study the impact of recycling of AP on the biomass quality for biofuel production via HTL.

With this in mind, this study compared five *Chlorellaceae*-type strains. This set of five *Chlorellaceae* was previously investigated for their strain-specific potential as biomass sources for lipid-based biofuels (**Paper I**). In order to identify a robust strain capable of adjusting to the effects of the AP while maintaining a high-quality feedstock, the response and acclimation of these strains to the AP were monitored over five cycles of cultivation (Phase I). Finally, after four successive cycles of dynamic growth, the fifth cycle was prolonged in order to trigger compound-accumulating conditions and assess the impact of the AP on lipid and biomass accumulation (Phase II). See experimental details in *Paper II – AP-grown microalgae biomass* (Materials & Methods 5. Experimental *designs*).

II. 2. Results and Discussion

Phase I

II. 2.1. Microalgae Acclimation to the AP

The growth rate during the exponential phase and tolerance of each strain to the AP (1/200) were monitored during five batch cycles (**Figure II. 1**). The growth rate under control medium from **Paper I** was used as a baseline to measure the impact of integrating the AP into microalgal cultivation. The results showed a net inhibitory effect was all AP-exposed strains when comparing them to the growth rate under AP-free control media (see **Figure I. 1**). During the initial cycle of exposure to the AP, the growth rate observed among the strains ranged from 0.38 to 0.44 /d (**Table II. 1**). This growth rate corresponded to a significantly lower growth rate than in the absence of AP (0.74–1.08 /d). After the initial exposure to the AP, CCALA 256 and CCALA 251 were the least impacted, with an initial reduction in the growth rate of about 33% for both of them compared to their corresponding growth rate in control medium. Among the tested strains, NIES 227 was the most affected, with a growth rate reduction of 65%.

Table II. 1. Growth rate of the microalgae during several cycles of acclimation compared to the control medium.

Microalgae strain	$Control_1$	Cycle 1	Cycle 2	Cycle 3	Cycle 4*	Cycle 5
C. vulgaris NIES 227	1.08	0.38	0.36	0.71	n.a.	0.59
C. vulgaris CCALA 256	0.79	0.52	0.53	0.64	n.a.	0.54
C. sorokiniana NIES 2173	0.86	0.38	0.24	0.69	n.a.	0.58
P. kessleri CCALA 251	0.75	0.50	0.47	0.67	n.a.	0.45
P. kessleri CCALA 253	0.74	0.44	0.59	0.81	n.a.	0.48

Average growth rate during exponential phase from **Paper I** used as a reference.
Growth rates calculated during the exponential phase (day 1-3) according to the OD at 880 nm.
*n.a., cycle not monitored.

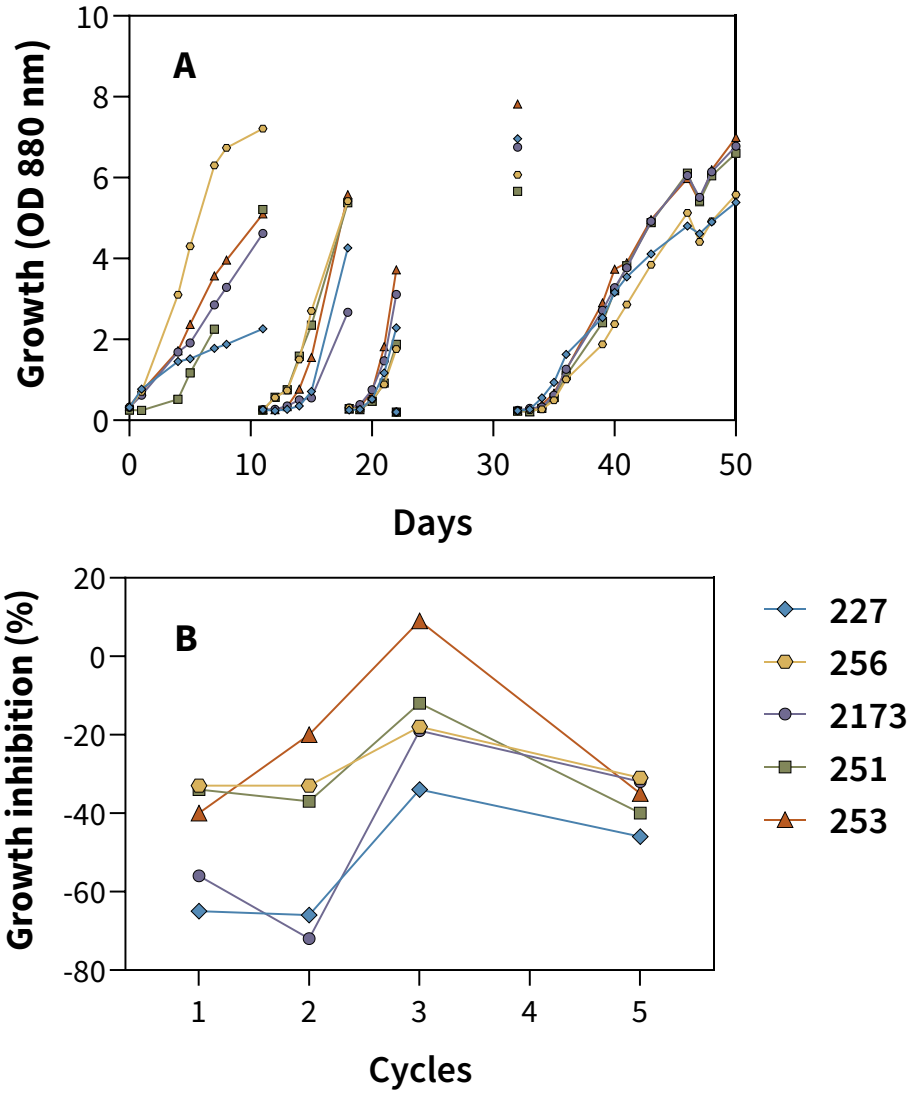


Figure II. 1. Growth upon acclimation to the AP over five cycles of cultivation (A), and growth rate inhibition compared to control conditions (B). For Cycle 4, only the OD at start and at the end of the cycle were measured. Growth inhibition was not calculated for this cycle.

Interestingly, after two cycles of acclimation, all strains presented decreased inhibition and an optimized growth rate (0.64–0.81 /d) on Cycle 3. In the last cycle, however, the growth rate decreased, probably as a result of the older inoculum with microalgal cells beyond the exponential phase. Unlike previous cycles, Cycle 5 started after eleven days of Cycle 4, rather than 5–7 days. Renewing microalgae cultures during the exponential growth phase is important for acclimating microalgae to harsh conditions (LaPanse et al., 2020). This can allow microalgae to have optimal conditions for a longer period of time, in terms of light availability and nutrient supply and help select robust cells that are better adapted to the inhibitory conditions.

Nonetheless, when compared to the initial exposure to the AP, the last cycle resulted in an overall improved growth rate (0.45–0.59 /d) among most of the strains, except for CCALA 251 (Cycle 1: 0.45 vs Cycle 5: 0.50). NIES 227 and NIES 2173 demonstrated the greatest capacity to acclimate to the AP among the tested strains, with the greatest improvement over repeated cycles of exposure to the AP. NIES 227 nearly doubled its initial growth rate in AP from 0.38 to 0.71 /d on Cycle 3, and it reached the highest growth rate in the final cycle (Cycle 5: 0.59 /d). Similarly, NIES 2173 was able to double its initial growth rate during the third cycle. Interestingly, CCALA 253 presented a net improvement in its growth rate, increasing from 0.44 (Cycle 1) to 0.81 /d (Cycle 3), in contrast to the 0.74 /d observed in the control medium.

Phase II

II. 2.2. Biomass Accumulation under AP Cultivation

After acclimation, the fifth cycle was left to grow for a longer period to study the impact of the AP on production and quality of microalgal biomass (**Figure II.3**). When comparing to AP-free cultivation on **Paper I** —and despite the improved growth rate after acclimation— the results on AP show that inhibition had an overall negative effect on the final number of cells and thus the final biomass concentration (**Figure II. 2**). In terms of final biomass, the AP-acclimated strains were able to reach significant biomass concentrations ranging from 2.95 to 4.27 g/L. However, due to their compromised growth rates, these strains did not yield optimal biomass values (from a 15% decrease in CCALA 251 up to a 44% decrease in NIES 227), compared to what was found in the absence of AP in **Paper I** (5–5.6 g/L). Nevertheless, these values seem promising compared to other studies where biomass concentrations of less than 2 g/L were typically found in AP-containing

cultures (Table II. 2). Overall, an extended initial lag phase was observed with the presence of AP, followed by a shorter exponential phase (Figure II. 2).

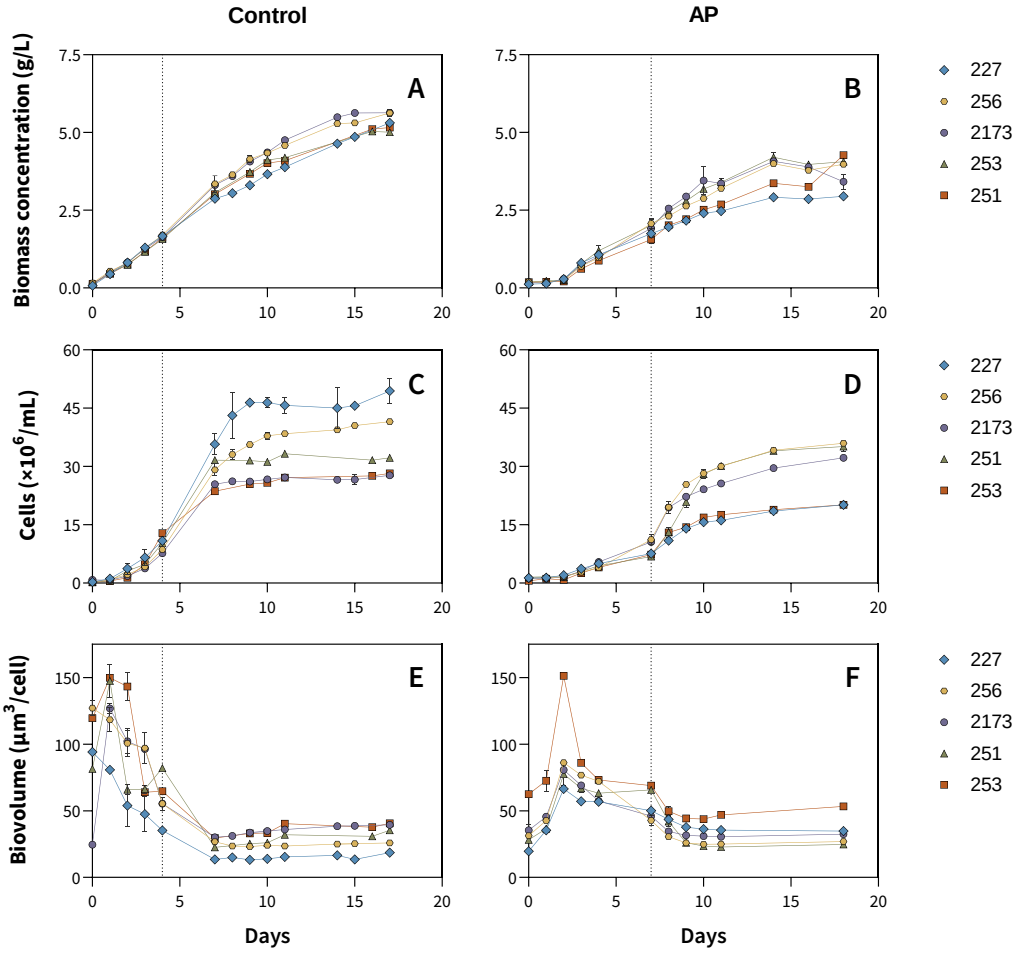


Figure II. 2. Comparison of microalgae cultivation in control medium from **Paper I**, and on AP-based media after five cycles of acclimation, left and right, respectively. A-B) Biomass accumulation, C-D) Number of cells, and E-F) Cellular volumes. Dotted line represents the onset of N-deficiency in the media caused by nutrient consumption.

Table II. 2. Studies of the use of the AP for microalgal biomass production.

HTL Conditions	AP dilution	Cultivation conditions	Nutrient replenishment	Microalgae cultivated in AP	Dry weight (g/L)	Biomass *P.* (g/L/d)	Growth rate (µ/d)	Lipid content (%)	Reference
Dunaliella salina 10 wt.%, 300°C, 45 min	1/400	14 days, 28°C, 1.5% CO_2, 100 µmol/m^2/s, 12 h on/12 h	Yes/ +Seawater (TN: 65 mg/L, NH_4: 11.38 mg/L, NO_3: 7.9 mg/L) *	*Dunaliella salina* KAU19	1.97	<0.20	n.a.	21%	(Almutairi, 2022)
SEQHTL *Chlorella sorokiniana* 10 wt.%, 160°C, 20 min	1/1–1/30	10 days, 26°C, 0.5% CO_2, 12 h on/12 h	Yes/ +1.5 g/L $NaNO_3$ (N-NO_3: 208 mg/L) *	*Chlorella vulgaris*	2.11	0.14	n.a.	19%	(L. Chen et al., 2017)
				Chlorella sorokiniana	1.4	0.21	n.a.	19%	
Chlorella vulgaris 22 wt.%, 250–380°C, 3–5 min	1/200	15 days, 25°C, 50 µmol/m^2/s, 24 h	Yes/ mBBM (10mM N) **	*Chlorella vulgaris* UTEX 395	n.a.	n.a.	0.21	11%	(Chen et al., 2020)
Teraselmis sp. 15 wt.%, 325-350°C, 30–60 min	1/300	Semi-continuous cultures, 14 days, 700 µmol/m^2/s, 12 h on/12 h	Yes/ Guillard f/2 (TN: 28 mg/L) **	*Teraselmis* sp.	0.53–0.66	n.a.	n.a.	20–25%	(Das et al., 2020)
Batch HTL *Spirulina platensis* 20 wt.%, 350°C, 60 min	1/500	12 days, 25°C, 5% CO_2, 80–110 µmol/m^2/s	No	*Chlorella minutissima*	0.52	0.04	0.08	8–10%	(Jena et al., 2011)
	1/100			*Chlorella minutissima*	0.41	0.03	0.09	8–10%	
Catalyst TiO_2 HTL Green macroalgal blooms 10:1 ratio, 270°C, 45 min	1/400	Yeast co-cultivation (5%), 14 days, 0.5% CO_2, 200 µmol/m^2/s, 16 h on/18 h	Yes/ +1% BBM*	*Scenedesmus abundans*	0.96	n.a.	n.a.	32%	(Kumar et al., 2019)
				Chlorella minutissima	1.0	n.a.	n.a.	28%	
Batch HTL Wastewater sludge 10:1 ratio, 255°C, 45 min	1/200	14 days, 0.5% CO_2, 200 µmol/m^2/s, 16 h on/18 h	Yes/ +5% BBM*	*Scenedesmus abundans*	0.64	n.a.	n.a.	28%	(Kumar et al., 2022)
				Chlorella minutissima	0.77	n.a.	n.a.	25%	
Batch HTL *Chlorella sorokiniana* 10 wt.%, 270°C, 20 min	1/200	18 days, 2% CO_2, 200 µmol/m^2/s, 20 h on/4 h	Yes/ FWM 1/2 (N-NO_3: 134 mg/L) **	*Chlorella vulgaris* NIES 227	2.95	0.52	0.59	36%	This study***
				Chlorella vulgaris CCALA 256	3.98	0.45	0.54	21%	
				Chlorella sorokiniana NIES 2173	3.41	0.43	0.58	17%	

[1]: Media used as main nutrient source and N content as a reference

*AP composition not considered: nutrient replenishment in addition to the AP nutrient content.

** AP composition considered for the final media formulation and nutrient content.

***Values found on Cycle 5 after 18 days of cultivation

n.a.: data not available

Following a brief acclimatization period, a transitory positive effect was observed in all strains during the exponential phase, which could be attributed to nutrient sources found in the AP, such as organic acids and NH_4^+, which is metabolized faster than NO_3^- by microalgae (Watson et al., 2020). However, as presented, this growth rate was not maintained constant, probably due to an asynchronous action of the multiple toxic compounds present in the AP.

Despite having the highest growth rate under AP-free medium, NIES 227 was overall the most negatively affected strain by the AP, with a strong decrease in the final number of cells equivalent to 2.95 g/L . Interestingly, the final number of cells in NIES 2173 and CCALA 251 was 16% and 9% higher than in the control medium, respectively. However, the cells of these strains were smaller in size. Inhibitory conditions can reduce cellular biovolume due to toxic effects on cell growth and metabolism. Kim et al. (2016) reported a decrease in biovolume, compared to the control, in *Chlorella* sp. at high dosages of inhibitory silver particles (Kim et al., 2016). In contrast, non-inhibitory mixotrophic growth can promote higher accumulation of storage compounds resulting in increased biovolumes (Marchello et al., 2018). On AP-supported cultivation, Stablein et al. similarly observed a decrease in cellular density but increased biovolumes caused by mixotrophic growth, as seen on NIES 227, and suggested that the cells were metabolically active but unable to divide (Stablein et al., 2021).

II. 2.3. Production of Lipid-Rich Biomass under AP Cultivation

The presence of the AP resulted in lower lipid content in all strains, compared to values of at least 29% found in **Paper I** for a similar period of time. After 18 days of AP cultivation during the fifth cycle, the highest lipid content was observed in NIES 227 at 36%, higher than values previously reported in other AP studies, typically falling under 30% (**Table II. 2**). This strain remarkably reached up to 43% lipids on day 25 (**Table II. 3**). These values, however, are still lower than those previously reported in NIES 227 studies without AP incorporation. Shen et al. reported a lipid content of 62.6% under mixotrophic conditions with N-free media (Shen et al., 2019). Similarly, this strain was able to reach up to 56% in control conditions with the reference medium FWM 1/2 after 17 days and 28% only after four days of cultivation (see **Figure I. 4**); in contrast, 29% after 18 days with the presence of AP.

Table II. 3. Lipid content (%) in the microalgal biomass.

Time (days)	NIES 227	CCALA 256	NIES 2173	CCALA 251	CCALA 253
4	11.3 ± 0.1	9.5 ± 0.1	9.5 ± 0.2	9.6 ± 0.3	9.1 ± 0.5
11	20.9 ± 0.3	11.4 ± 0.2	11.6 ± 0.2	9.6 ± 0.2	11.1 ± 0.1
18	36.2 ± 0.2	20.5 ± 0.1	16.5 ± 2.1	12.6 ± 0.2	16.0 ± 0.1
25	43.3 ± 0.1	25.7 ± 0.8	28.2 ± 0.8	23.6 ± 2.0	15.9 ± 1.5

Other than NIES 227, no other strain was able to accumulate a substantial amount of lipids in the presence of AP (**Figure II. 3**). The rest of the microalgae had low lipid content, ranging from 21% in CCALA 256 down to as low as 13% in CCALA 251. As a comparison, CCALA 256 produced up to 40% in the AP-free reference medium. Similarly, Přibyl et al. reported this former strain as the highest lipid producer compared to nine other strains, with a lipid productivity of 0.604 g/L/d and a lipid content of 57% (Přibyl et al., 2012). On the contrary, *P. kessleri* strains (CCALA 251 and CCALA 253) presented a strong evolution toward carbohydrate-rich biomass when exposed to the AP, at 34% and 47%, respectively. A similar pattern was observed under control conditions, with a carbohydrate content of 48% and 53%, respectively.

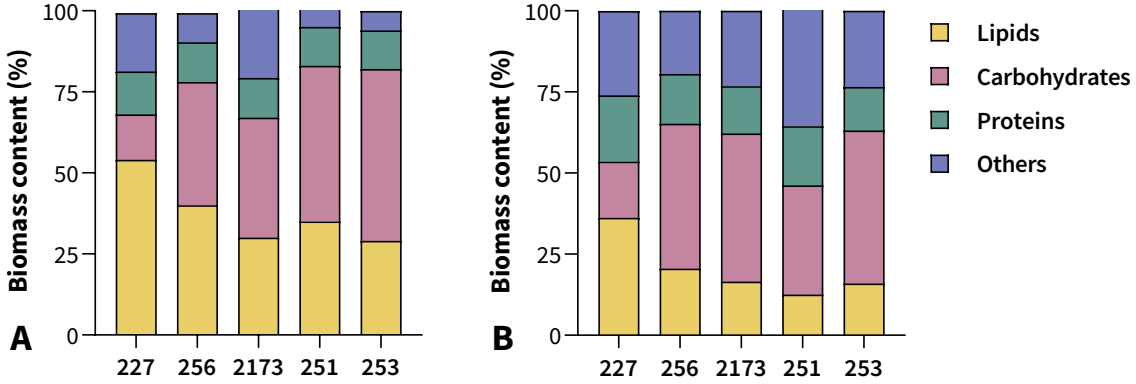


Figure II. 3. Biomass composition of control biomass (A) in comparison to AP-grown microalgae after 17 and 18 days of cultivation, respectively.

In this study, the accumulation of lipids was targeted by limiting the initial N content in the media. In **Paper I**, the gradual nutrient consumption caused N-depletion both extra- and intracellularly in the microalgae (see **Figure I. 2** and **Table I. 2**). When grown in AP for 11 days, no nutrient, or a very small fraction (< 0.9%) was still detected in the culture media, and N in particular was depleted 100% from the media since day 7 (see Appendix **Table B. 1**). However, the impaired growth of microalgae had an impact on their nutrient consumption rate and their final protein and N content. In accordance, a 13% (CCALA 253) up to 53% (NIES 227) increase in N content was observed on AP compared to control conditions (**Table II. 4**).

Table II. 4. Elemental composition of control and AP-grown microalgal biomass.

	C (wt.%)		H (wt.%)		N (wt.%)		O (wt.%)		S (wt.%)	
Strain	Control	AP	Control	AP	Control	AP	Control	AP	Control	AP
227	62.2 ± 0.1	56.7 ± 1.2	9.3 ± 0.1	7.9 ± 0.2	2.6 ± 0.1	4.1 ± 0.1	27.0 ± 2.2	28.2 ± 0.7	0.10 ± 0.01	0.11 ± 0.08
256	56.3 ± 2.6	51.3 ± 0.5	8.2 ± 0.3	7.4 ± 0.2	2.4 ± 0.1	3.1 ± 0.1	35.1 ± 0.6	38.0 ± 1.4	0.10 ± 0.03	0.13 ± 0.02
2173	53.5 ± 0.3	50.8 ± 0.5	7.9 ± 0.6	7.4 ± 0.2	2.4 ± 0.2	2.9 ± 0.1	40.6 ± 0.8	38.8 ± 0.2	0.10 ± 0.01	0.12 ± 0.02
251	54.7 ± 0.4	49.3 ± 0.8	7.8 ± 0.1	7.1 ± 0.3	2.4 ± 0.1	3.6 ± 0.1	35.3 ± 1.2	40.3 ± 1.0	0.10 ± 0.01	0.15 ± 0.03
253	52.0 ± 0.2	48.7 ± 1.4	7.6 ± 0.1	7.0 ± 0.6	2.4 ± 0.1	2.7 ± 0.4	39.3 ± 1.5	40.1 ± 0.4	0.10 ± 0.01	0.08 ± 0.01

As cellular division was significantly hampered, the stress caused by nutrient deficiency was not experienced neither to the same extent nor for the same length of time as under AP-free conditions. Where the onset of N depletion in control medium began at day four, in contrast to AP where it began until day seven. Furthermore, additional N-sources found in AP might also be consumed by microalgae, thereby preventing the same level of N-deficient conditions encountered in control media.

Moreover, the compound-specific effect was not explored during this work, but it remains important to study in order to determine the specific action of the multiple compounds present in the AP on microalgae biosynthesis at a metabolic level. Deciphering the beneficial and inhibitory effect of AP compounds on biomass productivity as on lipid biosynthesis would certainly allow to gain better insight on the reuse of AP for microalgae cultivation.

II. 2.4. Impact of the AP on the Biomass Elemental Composition

During HTL, the whole biomass is processed to produce biocrude. Lipids are the main source of the hydrocarbon molecules recovered in biocrude (Biller and Ross, 2011). The overall biochemical composition of microalgae results in a specific molecular and elemental composition of the biomass, which actually influences its distribution into the different HTL products (Cheng et al., 2017). In general, feedstocks with higher C and H contents are more suitable for the production of biofuels, as they can potentially yield more energy per unit of feedstock (Channiwala and Parikh, 2002). **Figure II. 4** illustrates the elemental ratios of the AP-grown microalgae at two timepoints (days 18 and 25) in comparison to values found on control media as a reference for AP-free biomass (see **Table I. 3**). The intracellular N content resulted in a N/C ratio ranging from 0.047 to 0.063 at day 18, which was significantly higher than in AP-free medium (0.038 ± 0.001).

Longer cultivation periods were found to improve the elemental distribution of the biomass, with a decrease in N/C (18% ± 6%) and O/C (9% ± 4%), as all cultures evolved for another week (**Figure II. 4**). In contrast to C and H content, low N and O contents are generally desirable, as these elements can cause the biofuel to have a lower energy content, which can reduce the fuel's efficiency and performance (Ratha et al., 2022). In addition, high N and O contents can lead to excessive NOx emissions during combustion (Zhan et al., 2018).

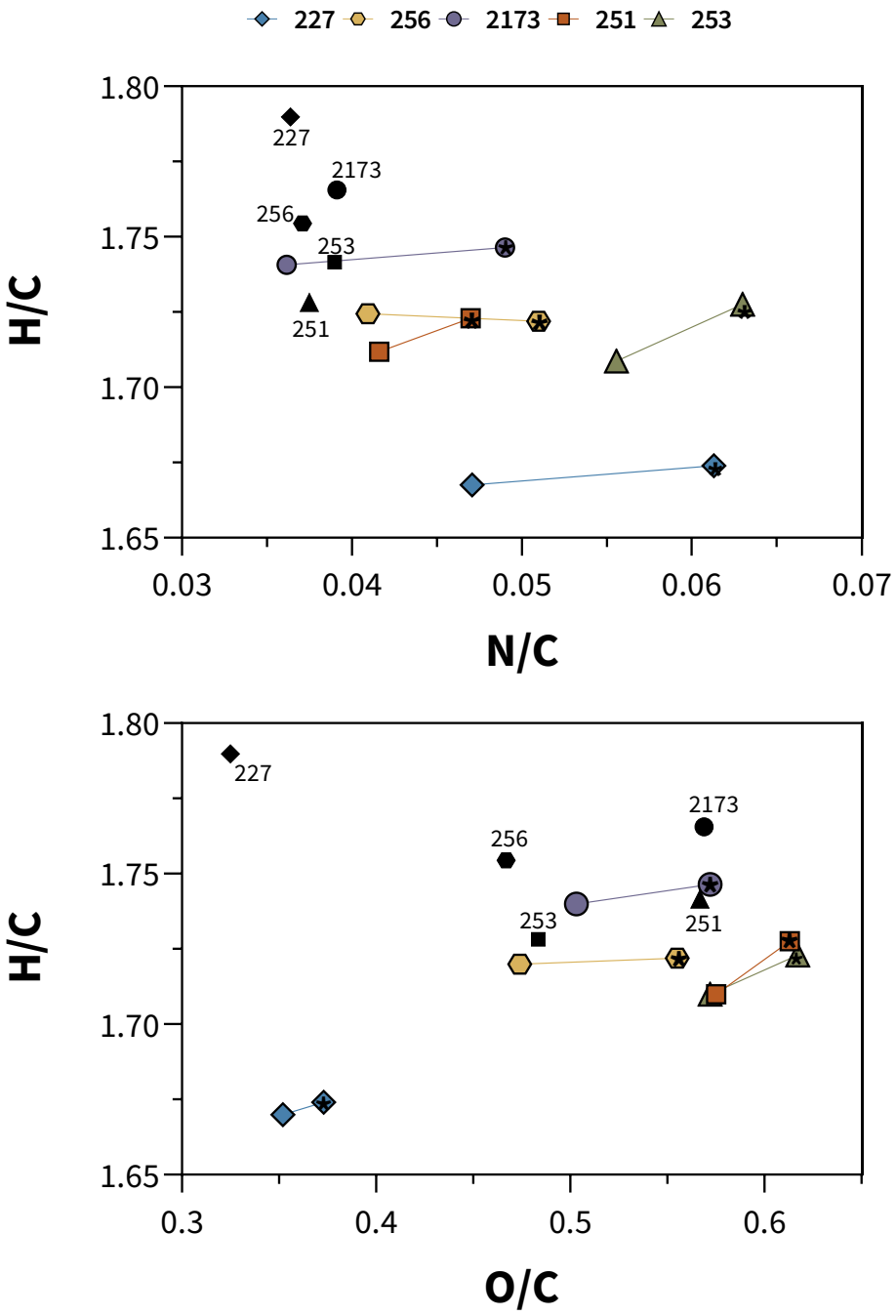


Figure II. 4. Van Krevelen's diagram of microalgal biomass grown in AP 1/200 on day 18 (*), and day 25. Black figures correspond to values reported in **Paper I** in the absence of AP, respectively to each strain.

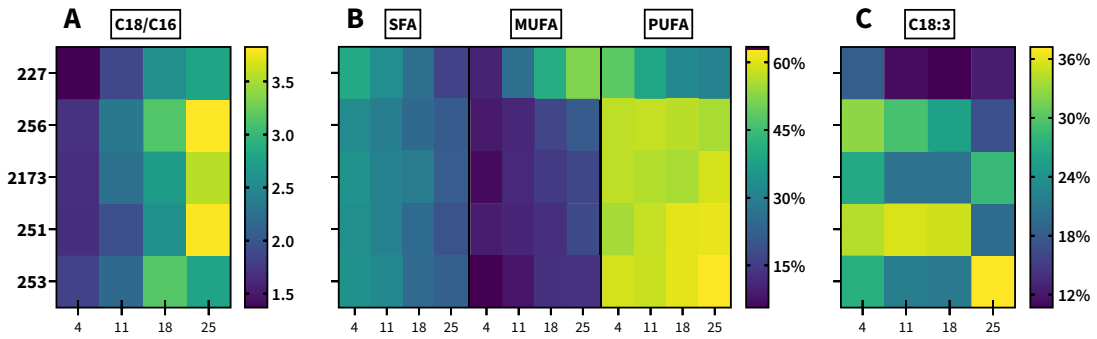


Figure II. 5. Dynamics of lipid profile at different sampling points. Heatmap A illustrates the C18/C16 ratio found in the microalgae. Heatmap B illustrates SFA, MUFA and PUFA content (%). Heatmap C shows the C18:3 content in the biomass (%). X-axis denotes the cultivation time in days: t4, t11, t18 and t25.

Along the cultivation period, the content of saturated fatty acids (SFA) decreased overall with time and presented the least variation among the strains, ranging from 17% to 23% on the final day of cultivation. The content of mono-unsaturated fatty acids (MUFA) increased over time for NIES 227 but decreased for the rest of the strains ($p < 0.001$), while the inverse was observed for poly-unsaturated fatty acids (PUFA; $p < 0.001$). The final composition of NIES 227 stood out favorably as it presented a strong preference for MUFA (52%), as opposed to the rest of the strains (14%–22%) (see Appendix Table B. 3). The other four strains, on the other hand, produced mostly PUFA (56%–64%) that could be of nutraceutical interest but are disadvantageous for biofuels. Interestingly, the overall FA distribution remained comparable to AP-free biomass for a similar period of time (see Figure I. 5).

During biocrude upgrading, the different fatty acids undergo reactions of dehydration and decarbonization. However, PUFA can result in molecules that are harder to process, affecting the quality of the resulting bio-oil and its economic cost (Bezergianni et al., 2018). Notably, SFA and MUFA (C14–18) have shown good recovery in HTL biocrude (>83%) compared to PUFA (<2.5%) (Leow et al., 2015). The presence of multiple double bonds in unrefined fatty acids may lead to the formation of free radicals that promote charring and NOx emissions upon burning (Omidvarborna et al., 2015). Excessive unsaturation levels also increase the biofuel's risk of undergoing oxidative degradation and negatively affect its cetane number (Moser et al., 2009). Furthermore, C18:3 is regulated in biodiesel to values under 12% (European Committee for Standardization, 2009). Remarkably, under AP-cultivation, and as observed in **Paper I** (see Table I. 4), this value was only attained by NIES 227

that maintained a stable 11% ± 1% throughout the cultivation since day 11; as opposed to the rest of the strains, which ranged from 23% ± 4% in NIES 2173 up to 32% ± 7% in CCALA 251.

II. 2.6. Overall AP Impact on Microalgal Biomass Production

In comparison to the first exposure to the AP, acclimation improved overall the growth rate of the microalgae under AP cultivation. However, compared to **Paper I**, AP integration still resulted in reduced microalgae growth and, therefore, decreased biomass and lipid productivity. This impaired growth resulted in a biomass with a 10% (± 3%) lower energy yield (**Figure II. 6**). For instance, *C. vulgaris* NIES 227 reached an energy content of 26.62 MJ/kg under AP cultivation, in contrast to 29.8 on AP-free media (see **Table I. 3**). Nevertheless, an important maximum biomass productivity (0.41–0.64 g/L/d) was found during the exponential phase, indicating that—to some extent—the AP can support microalgal growth before the cumulative damage of the multiple toxic compounds.

This effluent has both positive and negative effects on microalgae. On one side it is a potential source of macronutrients and extra nutrients that are not always found in control media and assimilable by microalgae, including short-chained fatty acids, glucose and urea. At the same time, this complex mix also contains a wide array of molecules that are inhibitory to microalgae and that combined have a synergistic inhibitory effect. For instance, the presence of a high diversity of heterocyclic N-containing organic compounds has been found to have a higher toxicity effect when combined than when individually tested (Alimoradi et al., 2020).

Interestingly, regardless of the inhibitory effects, the obtained biomass productivity, and in particular the high-quality biomass of NIES 227, remains important compared to previous studies on AP (**Table II. 2**). Chen et al. reported biomass productivity of 0.14 and 0.21 g/L/d in *C. vulgaris* and *C. sorokiniana* when grown in an AP obtained after sequential HTL and diluted to 1/25 (L. Chen et al., 2017). Kumar et al. studied *Scenedesmus abundans* and *Chlorella minutissima* in co-cultivation with yeast, which have a strong detoxification capability by fermenting organic compounds (Kumar et al., 2019). The authors reported a significant lipid content in the strains at 32% and 28%, respectively, and a final biomass concentration of nearly 1 g/L for both strains, lower than in the current study.

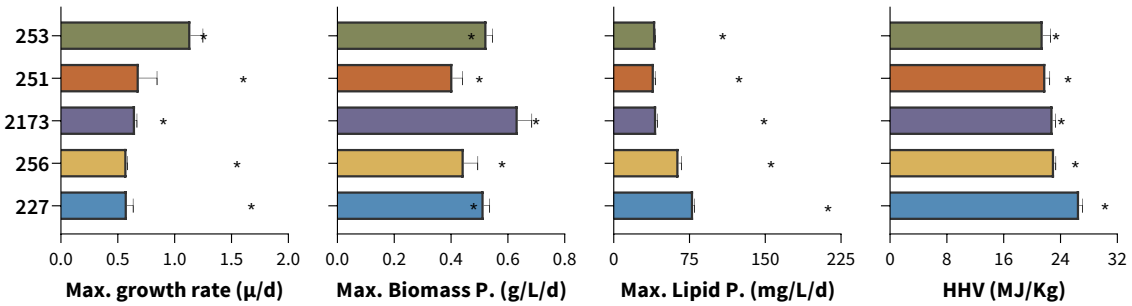


Figure II. 6. Microalgae characteristics grown in AP for 18 days. Values reported **Paper I** of AP-free cultures are illustrated by asterisks (*).

It was observed that lipid content during culture evolution mainly depends on strain-specific metabolism and intracellular nutrient levels. The five strains followed similar patterns under both control conditions and with the addition of AP. For instance, *C. vulgaris* NIES 227 showed a superior lipid content in both control and AP conditions, resulting in AP-grown biomass with an energy content of 26.62 (MJ/kg). In contrast, *P. kessleri* strains produced carbohydrate-rich biomass in both cases. Secondly, it can be assumed that the lower lipid content could be due to the lower growth rate and thus the slower onset of N-deficiency. Longer AP cultivation times would be necessary to produce energy-rich biomass in oleaginous microalgae. Different strategies to improve lipid accumulation when using AP could be adopted.

It is important to emphasize that each AP may have unique inhibitory effects on microalgae, as AP composition varies depending on the feedstock composition and the HTL reacting conditions. Microalgae-based HTL can result in AP with a TN going from 4 to 15 g/L, and a TOC ranging from 10 to 50 g/L. At the same time phenol and derived compounds exhibit varying levels of toxicity in microalgae, with EC_{50} values ranging from 2.10 to 200 mg/L (Aruoja et al., 2011). Similarly, N-organics EC_{50} can range from as low as 0.05 mg/L to as high as 140 mg/L (Pham et al., 2013). Therefore, the inhibitory effects of different APs can vary, leading to different results, depending on the specific inhibitory compounds present in the AP and their concentrations. To mitigate this inhibition, coupling AP-supported microalgae cultivation with other strategies can be useful and should be further studied. For instance, acclimation, co-cultivation with yeast or bacteria, or prior recovery of inhibitory compounds can alleviate the inhibition to some degree.

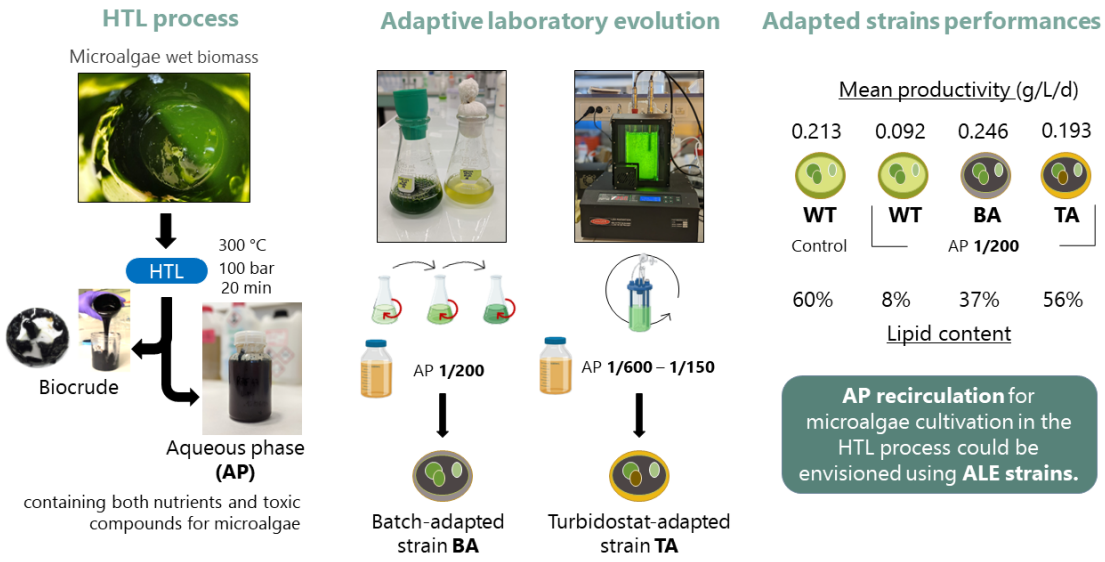
II. 3. Conclusion

The impact of reusing HTL-AP for the production of new microalgal biomass was investigated in this study. It was demonstrated that a few cycles of acclimation to the AP are effective in optimizing microalgal growth rate, as this HTL effluent is inhibitory to microalgae. The five tested strains presented an optimized growth rate after their acclimation to the AP compared to their initial exposure to the effluent. However, when cultivated in AP, and although acclimated, the strains could not reach optimal lipid and biomass productivities. The addition of AP resulted in a biomass of lower quality for biofuel production via HTL. Furthermore, an important strain-specific influence was observed on biomass quality. *C. vulgaris* NIES 227 despite having a moderate biomass production of 2.95 g/L, it showed the highest lipid content of 36% that resulted in the highest energy content at 26.62 MJ/kg. The use of AP as a substrate for microalgae cultivation was found to be feasible, but further optimization is necessary to produce high-quality HTL feedstocks. Furthermore, a punctual positive effect of the AP was observed on microalgae growth.

Paper III - Microalgae adaptation as a strategy to recycle the aqueous phase from hydrothermal liquefaction

The results of this Paper have been published in Ramírez-Romero et al., (2023) "Microalgae adaptation as a strategy to recycle the aqueous phase from hydrothermal liquefaction" in Bioresource Technology 128631. https://doi.org/10.1016/j.biortech.2023.128631

Graphical Abstract



Abstract

Hydrothermal liquefaction (HTL) produces biocrude oil from wet algae along with an aqueous phase (AP). This effluent contains minerals that can be reused for cultivating new microalgae but whose utility remains limited due to the presence of inhibitors. Reduced photosynthetic performance, growth, and null lipid accumulation were observed in wild-type C. *vulgaris* NIES 227 cultivated in AP (1/200). Batch transfers and turbidostat mode were used to study adaptive laboratory evolution. Both methods effectively counterbalanced AP toxicity and restored the fitness of the microalgae. After adaptation, a higher AP addition was achieved, from 1/600 to 1/200, without inhibition. As compared with the wild type in control medium (0.261 g/L/d), both adapted-strains maintained competitive productivity (0.310 and 0.258 g/L/d) of lipid-rich biomass (37%–56%). The improved tolerance of the adapted strains persisted after the removal of AP and under axenic conditions. Adaptive laboratory evolution is suggested for AP reutilization in the algae production process.

III. 1. Motivation and Research Objective

Although AP has been shown to support microalgal growth, optimal productivities are generally not achieved as in control media (Watson et al., 2020). The results from **Paper II** demonstrated the potential of using AP as a substrate for microalgae cultivation where consecutive growth cycles diminished the AP inhibitory effect, but optimal values were not observed as in **Paper I** under AP-free media. Therefore, additional steps to reduce this inhibition are necessary when aiming for high-quality microalgal biomass and biocrude.

Several studies have reported significant improvement after short-term acclimation of microalgae to toxic compounds or effluents with inhibitory effects (Lee et al., 2021) (see **Table 1. 2**) Specifically, in studies focused on cultivation in AP, higher tolerances in microalgae were achieved after acclimation, but inhibition persisted depending on the AP composition and dilution (Hognon et al., 2015). Furthermore, most AP studies have focused on microalgal growth but failed to investigate its impact on the final biomass composition. For biofuel production, the initial feedstock composition plays a key role in the biofuel quality and yield of the process (Ratha et al., 2022).

The aim of this work was to study whether a prolonged period of adaptation to the AP could offset the negative effects of this effluent on microalgae growth observed in **Paper II**. **Paper III** focuses on *C. vulgaris* NIES 227, which was already identified as an ideal feedstock for lipid-based biofuels in **Paper I**. Furthermore, despite its compromised growth rate when grown in AP in **Paper II**, this strain was able to maintain higher lipid productivity compared to the other strains. The adaptation of this strain aimed to: i) offset the negative effect of the AP on the growth rate and biomass productivity; ii) maintain the capacity of this strain to accumulate a high amount of lipids; and iii) increase the AP concentration in the medium and, with this, its contribution to the nutrient content of the medium. The wild-type strain (WT) was adapted through adaptive laboratory evolution (ALE) (Ramírez-Romero et al., 2023). This technique is a strategy toward strain improvement where a specific environmental stress is applied mimicking natural evolution, commonly referred to as Darwinian selection (LaPanse et al., 2020). Here, this approach could increase the tolerance of the strain to the multiple compounds present in the AP. See experimental details in *Paper III - Microalgae adaptation and tolerance development to the AP* (Materials & Methods 5. Experimental designs, *page* 49).

III. 2. Results and Discussion

III. 2.1. Dual Effect of the AP as a Substrate for Microalgae Cultivation

C. vulgaris NIES 227 was cultivated under different dilutions of AP to investigate the concentration-dependent effect on the WT. All AP dilutions had a negative impact on the initial growth rate (< 48 h) of the strain compared to the control medium. An extended lag phase was observed, possibly caused by a gradual physiological adjustment of the microalgae to the AP (Figure III. 1). Remarkably, higher AP-dilutions (1/600) had a positive effect on microalgal final concentration, yielding a higher final OD at 720 nm (6.2) than the control medium (4.8). An expected negative effect was observed when the AP concentration increased at dilutions below 1/400. Dilution 1/50 had a dramatic adverse effect on the microalgae growth, where a lower OD than the initial (0.30) was obtained at the end of the cultivation (0.14).

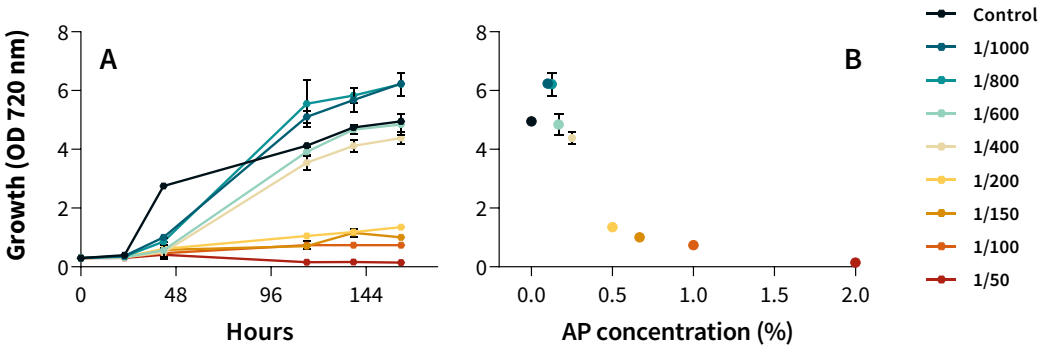


Figure III. 1. AP concentration-dependent effect on *C. vulgaris* (WT) NIES 227. A) Growth of WT under different AP dilutions in comparison to the control medium (FWM 1/4); B) Final culture optical density (OD 720 nm) in relation to the AP concentration (%).

The results showed a dual effect of AP in *C. vulgaris* NIES 227. At higher dilutions (1/800 and 1/1000), an increase in microalgae growth was observed. On one hand, it could be suspected that the inhibitors are diluted enough for toxicity to disappear, and on the other hand, assimilable nutrients absent in the control medium (e.g., short-chain organic acids) are boosting microalgal growth (Watson et al., 2020).

The potential of using AP as a nutrient source lies in finding a balance between its nutrient-rich nature and its inhibitory effect. Accordingly, previous studies have emphasized the importance of diluting the AP to counterbalance its toxicity. Biller et al. observed microalgal growth comparable to the control medium (BBM) at a dilution of 1/400, where the amount of inhibitory compounds (TOC, phenols, and Ni) was not as high as at 1/50 (Biller et al., 2012). Similarly, Jena et al. observed a significantly higher amount of biomass produced with a 1/500 AP-based medium compared to lower AP dilutions (Jena et al., 2011). In contrast, microalgal monocultures were reported to be killed or severely inhibited at lower AP dilutions (1/100 and 1/50) (Godwin et al., 2017).

III. 2.2. Evolution of the adaptation in turbidostat mode

Dilution 1/600 was chosen as the initial conditions for the gradual adaptation by turbidostat mode, since only minor differences were observed in the final OD compared to the control. This ALE approach was monitored for over four months while the concentration of AP was gradually increased. During this period, the WT was able to grow from a starting AP dilution of 1/600 to 1/150 without any negative effects being observed (**Figure III. 2**). Looking at the productivity of the PBR culture, an improved average productivity was found, from 0.63 ± 0.47 g/L/d in the first AP dilution (1/600) to 2.23 ± 0.86 g/L/d in the last AP dilution (1/250). In parallel, the maximum photosynthetic activity (QY as Fv/Fm) of the strain remained stable despite the increasing AP concentration. The QY was not measured right at the beginning of the culture, and thus it cannot be determined whether the lower QY at dilution 1/600 was due to the initial addition of AP or to the adjustment of the microalgae to the PBR. Nevertheless, the QY remained constant at 0.69 ± 0.04 from dilution 1/500 to the last dilution (1/150), which is a typical value for non-stressed microalgae cells (Schagerl et al., 2022).

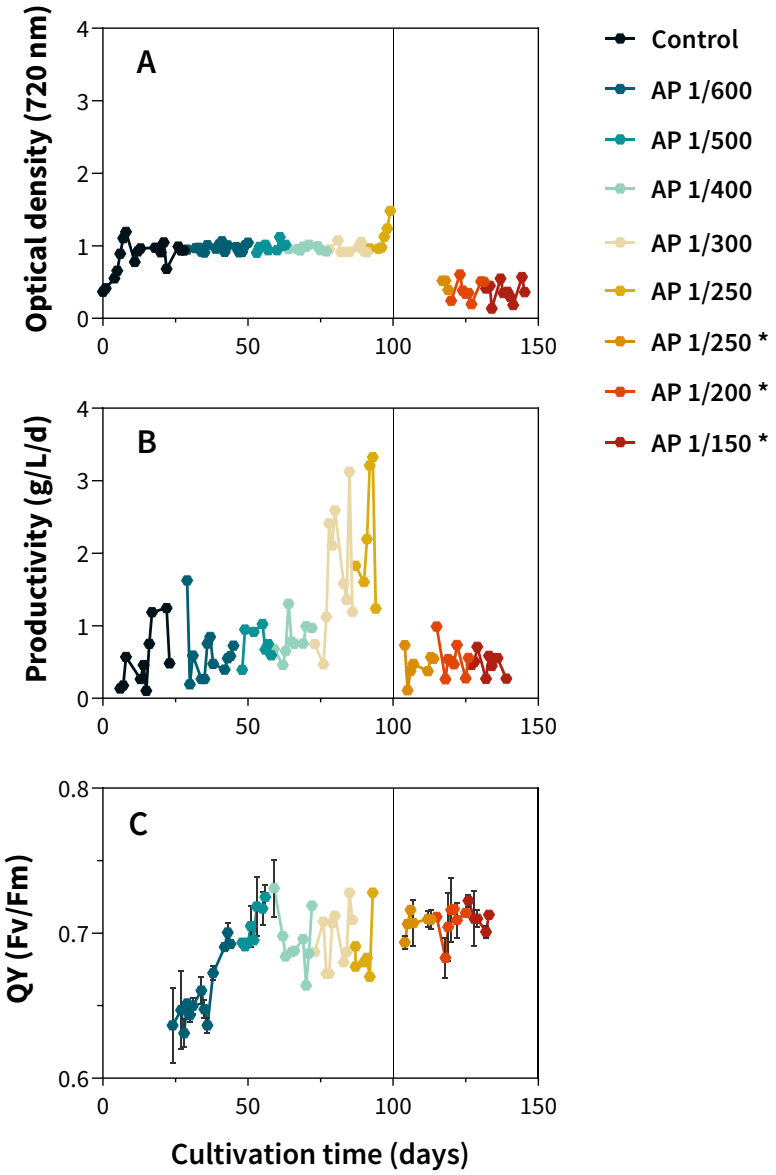


Figure III. 2. Parameters followed during the adaptation in turbidostat mode (TA strain). The optical density of the culture was kept constant at approximately 1.0. The maximum photosynthetic activity (QY) was followed since the addition of AP (1/600). Line at day 100 and asterisks in the legend denote the transfer of the culture to flasks and manual ALE dilution due to PBR failure.

III. 2.3. Potential of adapted strains to thrive in AP-based media

After completing two ALE strategies, batch transfers and turbidostat mode, the obtained BA and TA strains were compared with the WT. The first comparison was performed with the aim of evaluating the acquired ability to tolerate the AP_{mix} at a dilution of 1/200 (with nutrient replenishment equivalent to FWM 1/4). In addition, the persistence of the adapted phenotype was examined after the AP stress stimulus was removed for several months (de-adaptation). The results showed that both ALE strategies offset the negative effect of the AP observed in the WT (**Figure III. 3**). A comparable final OD to the WT (7.03 ± 0.07) was observed in the ALE strains (7.05–7.79), independent of the pre-culture media. Remarkably, tolerance was maintained in both adapted strains after a period in the absence of AP (AP-). Compared to the prolonged exposure of AP (AP+), no significant differences were observed in the final growth of each ALE strain (*p*-values of 0.70 and 0.94, respectively, for TA and BA).

As previously observed in **Paper II**, and reported by numerous authors (Biller et al., 2012; Jena et al., 2011; Stablein et al., 2021), the results of this third study showed that the WT on AP has a strong growth inhibition (-57%) with respect to OD (3.03 ± 0.12) compared to the control WT. Cell division in WT was affected during AP cultivation, resulting in smaller cells (2.7 µm vs. 3.1 µm). Nevertheless, after 96 h of exposure to the AP, the WT almost caught up with the WT in the control medium, with 35 and 38 × 10^6 cells/mL, respectively (**Figure III. 3B**). In contrast, the BA strain presented the lowest final number of cells (27 × 10^6 cells/mL), but with a much larger cell diameter (3.8 µm) and volume (9.0 × 10^8 μm^3/mL). The divergent growth rates resulted in distinctive cellular volumes and compositions of the final biomass. Overall, the AP increased biomass accumulation in both adapted strains even after the de-adaptation phase (**Figure III. 4**). The BA cultures produced 30%–33% more biomass (5 ± 0.09 g/L) than the control WT (3.8 ± 0.02 g/L), followed by the TA cultures, which had an 11%–15% higher final biomass (4.3 ± 0.12 g/L). Similarly, an increase in cell density and biomass has been reported in several microalgae under mixotrophic growth with organic carbon (Meng et al., 2020; Shen et al., 2019). This is consistent with the final DW observed in ALE cultures without the observed negative effects of the AP.

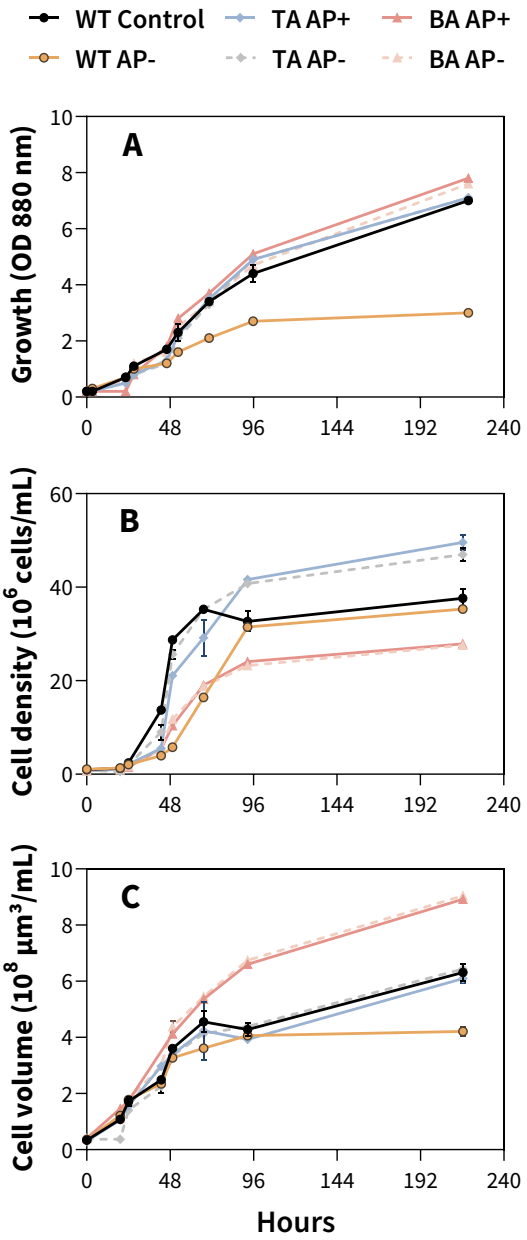


Figure III. 3. Growth of *C. vulgaris* NIES 227 wildtype (WT) in reference conditions (control) and on AP 1/200 without previous acclimation (AP-), in comparison to the turbidostat-adapted strain (TA) and the batch-adapted strain (BA).

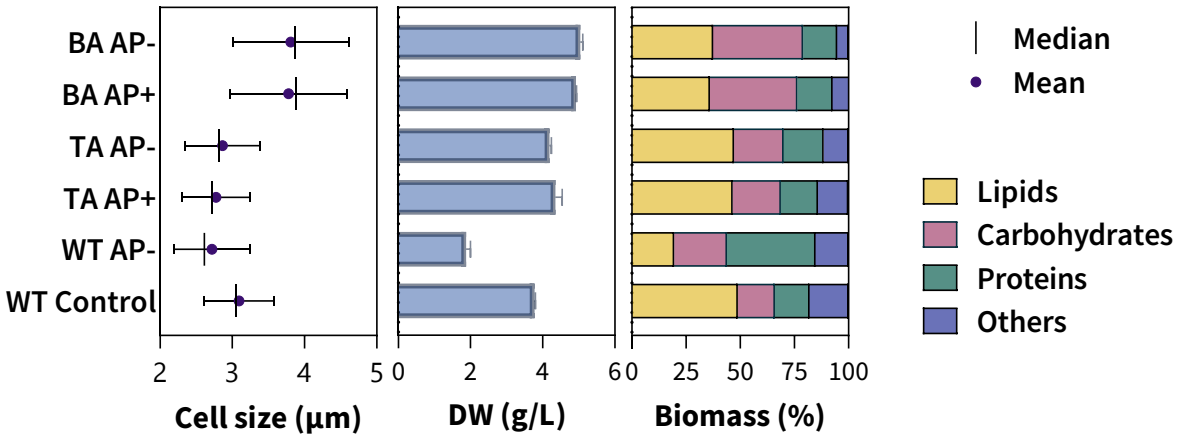


Figure III. 4. Final microalgae characteristics of *C. vulgaris* NIES 227 wildtype (WT) and adapted strains: batch-adapted (BA), and turbidostat-adapted (TA); after an extended exposure to the AP after adaptation (AP+), or after a period of AP removal and/or without acclimation for the WT (AP-).

The closest biomass composition to the control WT was found in TA, 49% and 47% of lipids, respectively (Figure III. 4). BA accumulated not only lipids (36%), but also a higher amount of carbohydrates (40.9%) compared to the 17% found in the control WT. In contrast, the AP highly affected the WT in various aspects: reduced biomass production (-50%, 1.9 g/L), reduced lipid content (-60%, 19.6%), and increased protein content (+56%, 40.9%). Microalgal biomass targeted for HTL biocrude production should ideally contain a high-lipid content and a low amount of heteroatom precursors like proteins and carbohydrates (see Box 2). Protein and carbohydrates contribute to the biocrude yield and C recovery, however, they also promote the presence of heteroatoms in the biocrude, and carbohydrates favor biochar formation. The protein content poses another challenge, as recalcitrant N-containing compounds require further upgrading than carbohydrate derivatives, thereby decreasing the quality of the biofuel (Haider et al., 2020).

III. 2.4. Cultivation of adapted strains under high content of AP

During the prolonged TA cultivation, the sterility of the culture was significantly compromised. This strain was heavily contaminated by bacteria at the end of the ALE cultivation. Although axenic conditions are difficult to keep over long operating time, and not particularly adapted when recycling effluents (Muñoz and Guieysse, 2006), it was still important to determine whether the positive results were due to the adaptation of the microalgae itself or to the presence of bacteria. For this, a concluding experiment was performed after complete strain purification and under axenic conditions. Figure III. 5 shows that the performance of both ALE strains outperformed the WT in AP, even at a lower dilution of AP (1/100). Particularly, the BA strain had a higher tolerance to the AP compared to the TA. The higher content of AP in the media resulted in a higher final biomass (3.8 ± 0.1 g/L) compared to AP 1/200 (3.5 ± 0.1 g/L) and the WT in control medium (3.3 ± 0.1 g/L). In contrast, the TA strain remained tolerant to AP 1/200 (3.0 ± 0.1 g/L) and the closest to the WT high-lipid content (56% and 60%, respectively). However, TA's biomass accumulation and lipid content were more affected at dilution 1/100 (2.1 ± 0.1 g/L, 39%). Although the lipid content of TA in 1/100 and both BA cultures (37% each) was not as high as the WT, it remained significantly important compared to the WT in AP 1/200 (8%).

Furthermore, maximum photosynthetic activity (QY) was measured to determine the effect of the AP on the microalgae's health. It can be observed that the ALE strains had a comparable evolution of the QY during cultivation compared to the WT in control medium (**Figure III. 5C**). Distinct phases can be identified during the cultivation: an adjustment to the cultivation conditions within the first three days, followed by a 22%–35% decrease in all cultures. The declining QY coincides with the ongoing stress induced by N-depletion and consequent lipid accumulation. In contrast, the impact of the AP in the WT was observed immediately after 48 h of exposure under nutrient-sufficient conditions. Interestingly, the WT seemed to adjust to the AP presence, and an improved QY of 0.75 was observed after six days. In the ALE strains, it can be assumed that the main stress is coming from the N-stress, as detected in the control WT, as opposed to the WT in AP. The weaker growth of the WT in AP resulted in a slower consumption of N during cultivation, where no complete consumption of N or P was achieved, 93% and 86%, respectively (Figure III. 6).

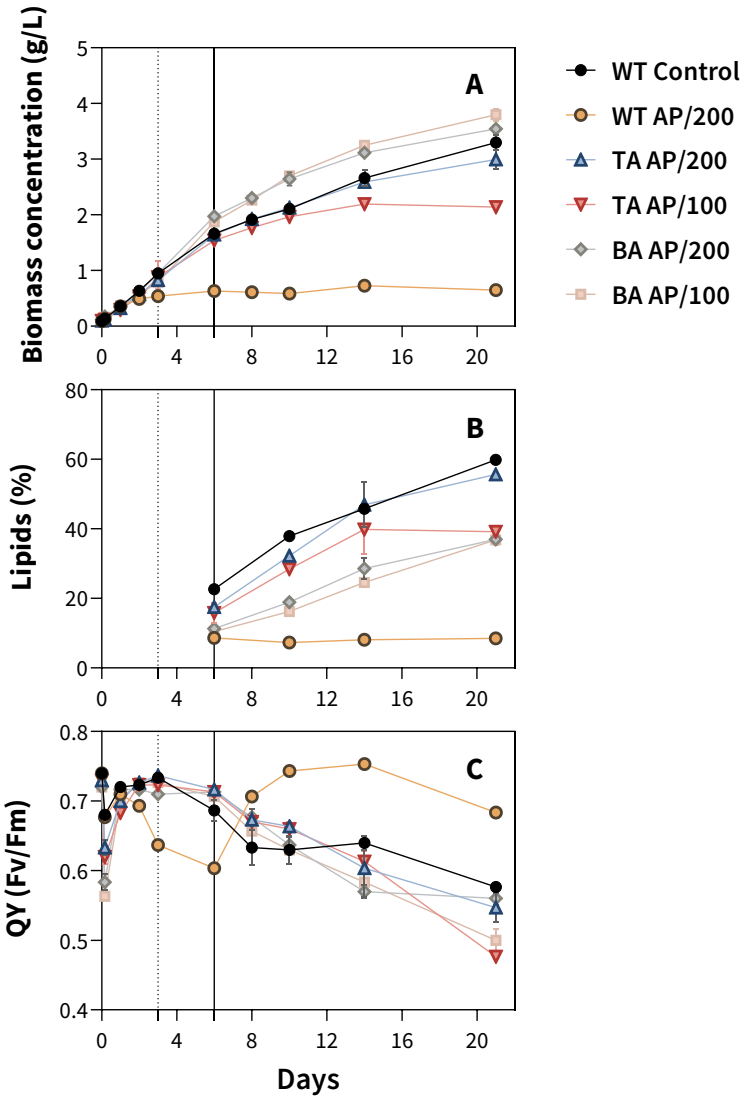


Figure III. 5. Biomass and lipid accumulation of adapted strains (TA and BA) in comparison to WT in control medium and in the presence of AP (1/200). Adapted strains were grown at a dilution of 1/200 and 1/100. Dashed lines illustrate the onset of N-depletion for all. Dashed lines illustrate the onset of N-depletion for all cultures (except TA AP/100 and WT AP/200). Continuous lines illustrate the onset of N-depletion for TA AP/100. N-depletion in WT AP/200 was not reached.

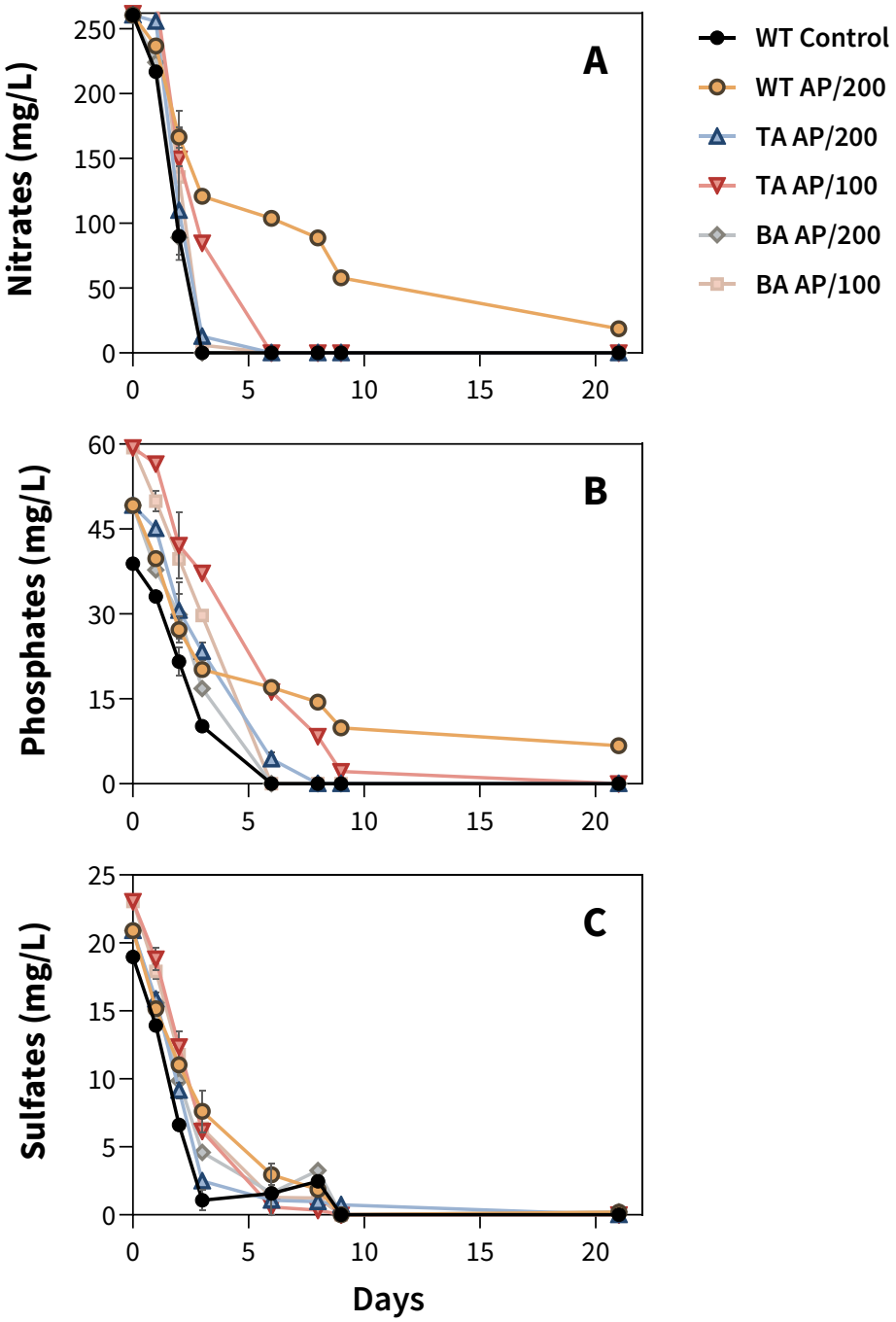


Figure III. 6. Nutrient consumption of ALE strains in AP diluted 1/200 and 1/100 when compared to wildtype in control medium and AP diluted 1/200.

Microalgae cultivated under N-limitation often show a decrease in QY compared to N-replete cells (Negi et al., 2016). The initial QY change in the WT in AP, however, could more likely be due to diminished autotrophic growth in AP. Similarly, Stablein et al. reported diminished microalgal growth in AP, species- and concentration-dependent, in parallel to an inhibited photosynthetic performance (Stablein et al., 2021). A decrease in QY, however, could also be due to a change from autotrophic to mixotrophic growth supported by C sources present in the AP (Rohit and Venkata Mohan, 2018).

III. 2.5 Productivity of ALE strains

As important as the final lipid content, the productivity of each strain during cultivation was also studied. Biomass productivity was calculated on a daily basis (Figure III. 7), and interestingly, a maximum biomass productivity of 0.27 ± 0.02 g/L/d was identified for the WT in AP, right next to the control WT (0.27 ± 0.01 g/L/d). However, this productivity was observed on day one, right after a large productivity drop that coincided with its declining photosynthetic performance after exposure to the AP.

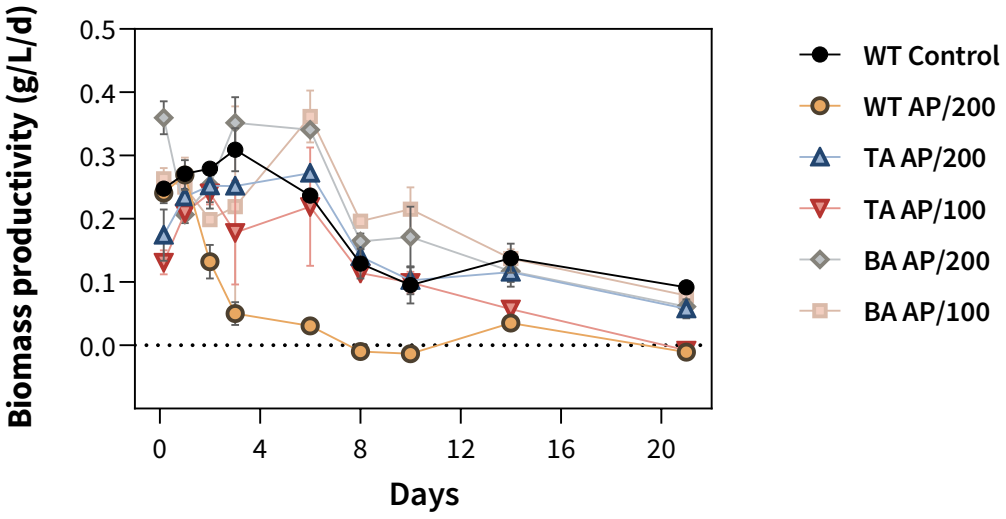


Figure III. 7. Biomass productivity dynamics.

Figure III. 8 illustrates the variation in productivity during cultivation. As no substantial sign of biomass and lipid accumulation was observed in the WT during the AP cultivation, the biomass and lipid productivities were significantly compromised. Consequently, this strain reached a much lower average productivity of 0.08 ± 0.01 g/L/d when grown in AP for over twenty days. In contrast, even at higher concentrations of AP, both ALE strains showed superior performance throughout the cultivation. Cultivated in AP 1/200, the adapted strains presented an average biomass productivity of 0.18 ± 0.02 g/L/d (TA) and 0.23 ± 0.02 g/L/d (BA), compared to 0.20 ± 0.02 g/L/d found in WT cultivated on AP-free media. In terms of lipid accumulation, once again, the TA strain reached a competitive lipid productivity close to the control WT (TA: 0.10 g/L/d against WT: 0.11 g/L/d). Remarkably, the lipid productivities of both ALE strains were almost 100-times higher than the WT in AP (WT: 0.001 g/L/d).

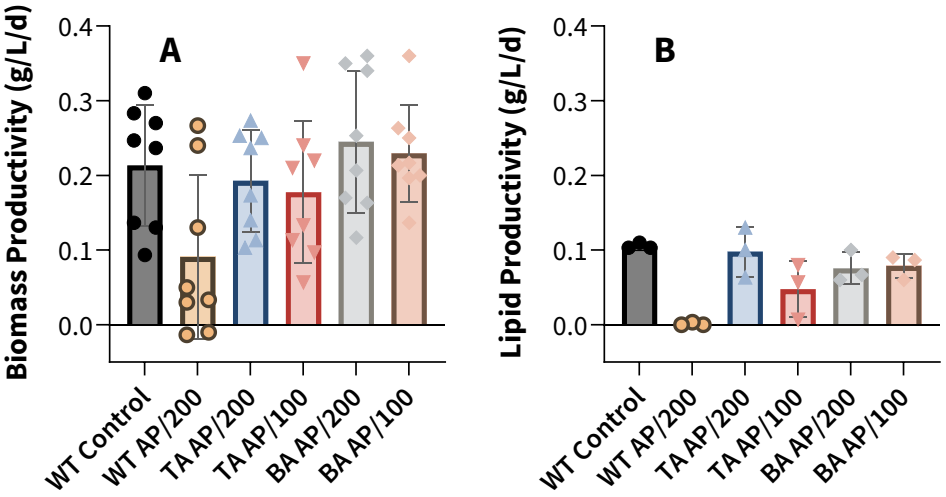


Figure III. 8. Biomass and lipid productivity of adapted strains (TA and BA) in comparison to the WT in control medium, and in the presence of AP (1/200). Adapted strains were grown under an AP dilution of 1/200 and 1/100. Datapoints represent different timepoints during the cultivation.

In **Paper I** NIES 227 was able to produce high amounts of lipids under N-depleted conditions. However, this metabolic trigger was not met for the WT in AP, as N-NO_3 was still present in the medium at the end of the cultivation (**Figure III. 9**). In contrast, the ALE strains consumed all of the inorganic N in three days (except for TA AP/100, which consumed it all until day six).

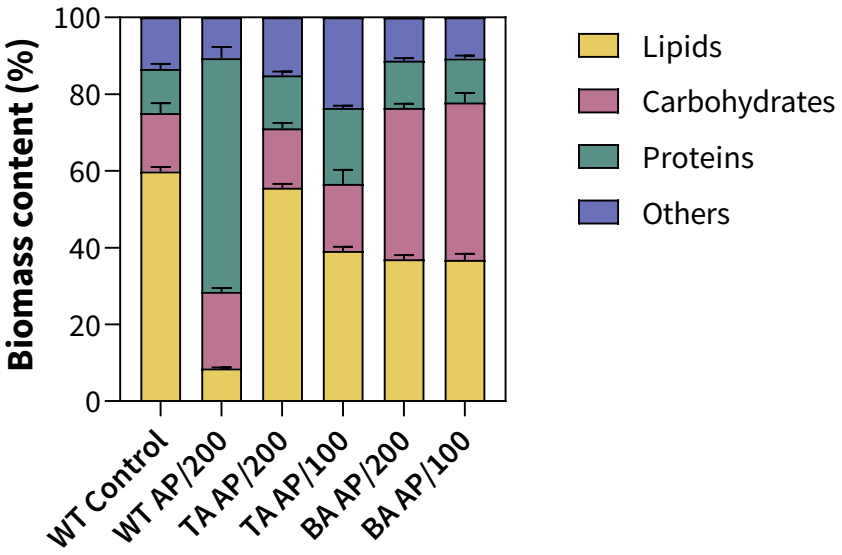


Figure III. 9. Final biomass composition of ALE strains in AP diluted 1/200 and 1/100 against wildtype in control medium and AP diluted 1/200.

Additionally, the low cellular density in WT in AP resulted in higher intracellular N-quotas and, therefore, constrained lipid biosynthesis (**Table III. 1**). Markou et al. compared several levels of N-limitation in *C. vulgaris* (SAG 211-11b), and a relationship was observed between the N-supply and the intracellular N-content. A concentration of 7.5 mg-N/L in the medium promoted higher accumulation of lipids (48%) compared to a supply of 150 mg-N/L that resulted in a higher N-quota (76 vs. 29 mg-N/g) and consequently a lower lipid content (15%) (Markou et al., 2017).

Table III. 1. Intracellular N-quotas per cell and g of biomass.

	WT FWM	WT AP/200	TA AP/200	TA AP/100	BA AP/200	BA AP/100
A) pg of N per cell	0.215 ± 0.016	1.646 ± 0.020	0.277 ± 0.032	0.366 ± 0.024	0.262 ± 0.002	0.274 ± 0.023
B) mg of N per g of biomass	21.3 ± 1.5	91.3 ± 2.6	24.9 ± 0.4	32.7 ± 2.7	21.7 ± 0.9	22.1 ± 0.4

III. 2.6 Enhancing Microalgal Tolerance to Toxic Compounds via ALE

Several strategies have been implemented to adapt microalgae to toxic compounds or effluents containing important levels of inhibitors (Table 1. 2). Wang et al. reported the adaptation of a *Chlorella* sp. to high concentrations of phenol (700 mg/L) (L. Wang et al., 2016). The resulting strain doubled its maximal biomass concentration, in contrast to the wild type, while removing up to 500 mg/L of phenol in seven days. Two more studies were conducted particularly for microalgae cultivation in AP, with reported information on acclimation to AP. Hognon et al. reported improved growth on *C. reinhardtii* after six cycles of acclimation to AP with a final DW higher than in control medium (Hognon et al., 2015). Similarly, *Picochlorum* sp., acclimated to 1/1000 AP, was able to maintain photosynthesis and respiration even at an AP diluted 1/50, compared to the control cells that were highly inhibited at a dilution of 1/100 (S. Wang et al., 2016). To the authors' knowledge, this work is the first application of ALE for the reuse of AP as a source of nutrients for microalgae cultivation.

In this study, ALE was proposed for strain improvement of *C. vulgaris* NIES 227 targeting HTL-AP integration for microalgal feedstock production. It was important to explore the plasticity of *C. vulgaris* NIES 227 to adapt to the toxic effects of the AP while preserving its oleaginous capacity. No important negative effect was observed as the ALE in turbidostat-mode evolved. As observed in Figure III. 3, the WT in AP showed cell division recovery when exposed to the AP after four days, and after six days, a restored photosynthetic rate was observed (Figure III. 5).

These results indicate that the WT was capable of acclimating to the AP within less than one week, compared to the imposed cycles of 10 to 15 days. The transition to a lower dilution was performed automatically by the turbidostat-mode corresponding to a dilution rate of 1/d. Furthermore, it can also be observed that a negative effect in terms of productivity and QY is observed only after 24 h of exposure.

The two ALE methods presented in this study were proved to be effective strategies to offset the inhibitory effects of the AP. Although two distinct strains were obtained, they both demonstrated the ability to produce high-lipid biomass in the presence of AP. From one side, batch adaptation resulted in a practical method to perform ALE with minor monitoring. At the end, the BA strain presented superior tolerance to the AP compared to the TA, while surpassing the WT biomass productivity. However, due to its divergent growth, the BA strain composition was different compared to the WT. In contrast, a semiautomatic adaptation was performed in a PBR under turbidostat-mode where the culture was closely monitored to avoid extensive stress to the microalgae's fitness. This latter strategy produced a strain (TA) that could maintain the distinctive lipid-producing capacity of *C. vulgaris* NIES 227. However, its tolerance to the AP was not as robust as that of the BA strain at lower AP dilutions. Further strain-specific optimization would be necessary to increase the growth rate and lipid content of each ALE strain, respectively.

Notably, the strains demonstrated important levels of tolerance once they were recovered after ALE and were kept in control medium prior to the presented experiments, three months and seven months, respectively. Although genomic analysis would be required to confirm genetic changes beyond the observed phenotypes, it is highly suspected that the strains not only adjusted physiologically to the AP (acclimation), but that these cellular responses elicited genetic changes in the strains (adaptation) (Borowitzka, 2018). After ALE and the stress AP removal, both strains did not show major damages as seen in the WT in AP: compromised growth, declining photosynthetic capacity under nutrient sufficiency, and null lipid productivity that resulted in a low-quality biomass for HTL. In contrast, both ALE strains maintained competitive productivities in AP against the control WT in reference media.

III. 3 Conclusion

ALE was proven to be an effective strategy to offset AP inhibition in *C. vulgaris* NIES 227. Despite the presence of AP, the two resulting strains were able to maintain competitive biomass and lipid productivity against the WT, characteristics that are essential for HTL purposes. Remarkably, after removing the stress stimulus, the acquired tolerance remained under axenic conditions. Strain selection and optimization are essential for any microalgal application, yet they remain strategic for the production of sustainable energy sources. Without prior detoxification, ALE may allow AP reintegration into the system for new microalgae biomass production. Additional research is needed to investigate the multiple recycling cycles of AP for microalgal cultivation.

4. General discussion and perspectives

The main objective of this thesis was to explore the potential and limitations of recycling the aqueous phase (AP) obtained from hydrothermal liquefaction (HTL) for the production of new microalgal biomass, with the ultimate goal of developing circular and sustainable processes. To achieve this objective, significant efforts were dedicated to identifying a suitable strain for HTL and high-quality feedstocks while also recycling AP. The ability of the strains to maintain robustness without compromising biomass quality was a crucial criterion in the selection process. Although microalgae were able to grow using recirculated AP, further measures were necessary to mitigate its inhibitory effects. Several cycles of acclimation under dynamic growth on AP resulted in optimized growth but impacted the biomass quality. This study concluded with the improvement of an oleaginous strain through prolonged exposure to the AP using adaptive laboratory evolution (ALE). Two different methods were employed resulting in two distinct strains, batch-adapted (BA) and turbidostat-adapted (TA). The closing section of this manuscript discusses the implications, limitations, and future recommendations derived from this research.

4. 1. Chlorellaceae as a Source of Biomass for Biofuels

This work was focused on a small study group of six *Chlorellaceae* strains, aiming to find a suitable strain for lipid-based biofuels. The study group consisted of one *Chlorella sorokiniana* (NIES 2173), three *Chlorella vulgaris* (NIES 227, CCALA 256 and CCALA 269), and two *Parachlorella kessleri* (CCALA 251 and 253). This family is one of the most studied microalgae, and covers a wide variety of characteristics that are attractive for different applications. The ability of these strains to accumulate energy-rich molecules and their robustness make them valuable for producing HTL feedstocks, while their freshwater nature also prevents salt accumulation during HTL, resulting in a reduced ash content in the biocrude.

The rich potential of this taxonomic family and the distinctive profiles of the strains, even within the same species, were highlighted during **Paper I**. Among these strains, *C. sorokiniana* NIES 2173 presented a high biomass production, and was able to produce both lipids and carbohydrates at interesting levels (more than 30%). While *C. vulgaris* CCALA 256 did not have the highest lipid productivity in our study, Přibyl et al. (2012) described this strain as having the highest lipid productivity among other strains but at a much higher light intensity (500 μmol/m^2/s), than this study (230 μmol/m^2/s) (Přibyl et al., 2012). CCALA 269 (CCAP 211-11b) has been equally reported in numerous studies, to be a promising candidate for lipid-based applications (Sakarika and Kornaros, 2017). Despite having a good set of characteristics for biodiesel, under the studied conditions, an ideal lipid and biomass productivity were not achieved for this strain in the current study. This strain had overall limited growth, possibly indicating that favorable conditions were not met for this specific strain.

Moreover, the two *P. kessleri* strains, CCALA 251 and 253, remain promising candidates for sugar-based applications like the production of bioethanol, due to their high carbohydrate content and productivity. However, the high content of carbohydrates found in these latter strains restricts their use in lipid-based conversion pathways like HTL (Cheng et al., 2017). These compounds decrease the overall biocrude yield and quality, making the process economically inefficient and thus not feasible for industrialization.

4. 1.1. Oleaginous *Chlorella vulgaris*

All throughout this work, *C. vulgaris* NIES 227 stood out as a promising candidate for lipid-based biofuels, and exhibited remarkable qualities beyond its oleaginous nature. Previous research has demonstrated that this strain can reach lipid contents of up to 88%, with a biomass and lipid productivity of 108 mg/L/d and 129 mg/L/d, respectively (Shen et al., 2015). These values were found under heterotrophic conditions, adding 12 g/L of glucose in combination with N- and P-limitation. While this outstanding lipid content was not achieved in the current study, autotrophic conditions and a balanced nutrient limitation led to an important amount of lipids (up to 54%), and a much higher biomass and lipid productivity, 310 mg/L/d and 212 mg/L/d, respectively. Moreover, NIES 227 began to accumulate lipids the earliest, and showed a continuous lipid increase since the onset of N-starvation (see **Figure I. 4**). For instance, after only four days of cultivation in **Paper I**, NIES 227 produced up to 28% of lipids. In contrast, similar levels were found in NIES 2173 (29%) only after one more week of cultivation, and in CCALA 253 (29%) two weeks later. This metabolic ability would promote an optimized use of energy and resources to produce lipid-rich biomass from NIES 227. Furthermore, NIES 227 presented not only an outstanding lipid content but also a high-quality fatty acid profile (see **Table I. 4**). This strain was the only one that met critical requirements set by the European standard for biodiesel, where the other strains fell far behind with important levels of PUFAs (more than 50%) affecting their overall biodiesel properties.

4. 2. Importance of strain selection for bio-oil production

Two particularly noteworthy impacts of the current work were that the selection of *C. vulgaris* NIES 227 followed scalable production of lipid-rich biomass and an optimized HTL biomass conversion. As part of the RAFBIOALG project, the selection of this strain was transferred to the production at pilot scale of HTL feedstocks, with lipid contents of up to 56% (see **Table 2. 2**). This highlights the potential of the strain to produce lipid-rich biomass beyond laboratory conditions, which is essential for the commercialization of microalgal applications (published in **Paper IV**) (Chambonniere et al., 2022).

After HTL, this lipid-rich biomass also resulted in a bio-oil yield of 46 wt.%, compared to only 19.8 wt.% obtained from the initial RAFBIOALG strain *C. sorokiniana* NIES 2173 (to be published in Paper V) (da Costa Magalhães et al., 2022). The overall composition of *C. vulgaris* NIES 227 was found to be the most suitable between the two strains, as it contained not only a high amount of lipids, but also a lower carbohydrate and protein content (See Figure 2. 4). This resulted in bio-oil with lower heteroatom content and higher energy content after HTL and upgrading via hydrotreating (HDT). In contrast, *C. sorokiniana* NIES 2173 produced a bio-oil with a higher amount of N- and O-cyclic compounds.

These findings emphasize the importance of selecting microalgae strains with high lipid content for biofuel production, as it can significantly improve the efficiency and economics of the HTL process (Figure IV. 1). Moreover, the overall composition of the microalgae strain should also be considered aiming toward the production of sustainable biofuels. The latter is because during HTL, the biomass influences not only the biofuel yield and properties, but also its environmental impact. This is the case for heteroatom precursors (cyclic N-, O- and S- containing molecules) present in the biomass, such as proteins and carbohydrates. These molecules contribute to NOx and SOx emissions during the combustion of the biofuel (Obeid et al., 2019). Therefore, a low content of these molecules is highly desirable in any biofuel feedstock.

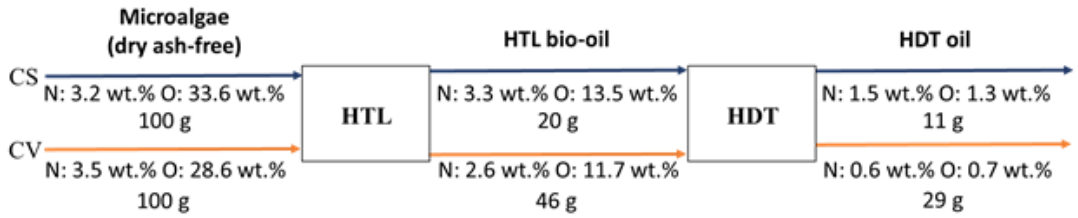


Figure IV. 1. Comparison of HTL process efficiency between *C. sorokiniana* NIES 2173 (CS) and *C. vulgaris* 227 (CV). RAFBIOALG results published in da Costa Magalhães et al., 2022.

This thesis demonstrated the potential of *C. vulgaris* NIES 227 as an ideal strain for lipid-based biofuels with scalable production of lipid-rich biomass and optimized HTL biomass conversion, contributing to the growing knowledge of microalgae as a high-quality biomass source. However, significant economic and resource-related challenges still hinder microalgal processes as a viable alternative to traditional fossil fuels, emphasizing the need for resource efficiency and eco-friendliness in microalgae cultivation for their success. While this work did not delve into these aspects, it urges the exploration of more sustainable processes to cultivate microalgae.

4. 3. Balancing cultivation media

Optimizing the nutrient supply while reducing excess mineral fertilizers and nutrient loss are key aspects of resource efficiency. Preliminary assays of this work showed that high nutrient supply was unnecessary. Conversely, reduced nutrient supply promoted biomass and compound accumulation in all strains, as observed throughout this work. Furthermore, this balanced nutrient limitation resulted in low heteroatom content in the biomass and advantageous properties as biofuel feedstocks. Limiting the N supply promoted the consumption of intracellular N reserves in the strains, and resulted in a N content of lower than 3% in all strains, in comparison to nutrient sufficient conditions with almost 10%. This in synergy with the oleaginous nature of NIES 227 also resulted in a limited amount of O, as lipids were preferred in contrast to carbohydrates. To produce sustainable energy sources, it is crucial to optimize nutrient use by finding a balance between nutrient supply and biomass production. Throughout this work, a one-stage cultivation was implemented to gradually induce natural nutrient deficiency, resulting in complete nutrient uptake and reducing nutrient losses. The results suggest the potential of using a tailored medium to steer microalgal metabolism, with advantages such as simplicity and total nutrient use which must be considered in AP-based medium, or any substrate for microalgae cultivaion with similar objectives as the ones presented.

4. 4. Recycling AP for microalgal biomass production

Recycling AP as a nutrient source for microalgae cultivation is presented in this work as an important pathway to promote circularity and reduce the reliance on synthetic fertilizers. AP contains a rich array of nutrients required for microalgae growth, and its use as a nutrient source can diminish its environmental impact by recovering nutrients that would otherwise be discharged to the environment. Several studies have assessed the AP inhibitory effect of microalgae but failed to focus on how the overall biomass quality would be affected as an HTL feedstock. To the author's knowledge, and at the time of the writing of this work, the majority of studies related to AP-supported cultivation, does report at best the lipid content of AP-grown biomass but mainly focus on the inhibitory effect of this effluent, and not on the opportunities that arise from its use. This research aims to bridge this gap by exploring the impact of AP on microalgal biomass for HTL conversion, which has been missing in the published literature, but is crucial for promoting sustainable and efficient microalgae cultivation.

Throughout this thesis, the impact of AP inhibition on biomass quality was thoroughly investigated. When AP was reused as a substrate, a significantly lower growth rate was observed compared to AP-free control media. This hindered the uptake of nutrients, delayed nutrient depletion from the media, and ultimately limited the accumulation of compounds in microalgae. However, longer cultivation periods resulted in improved biomass quality, where the overall biomass composition was determined by the metabolic nature of each strain in synergy with the limited nutrient supply. While compound accumulation was reduced in all strains, similar patterns were observed between the strains when comparing their growth in the presence and absence of AP (**Paper I** and **Paper II**, respectively). For instance, *C. vulgaris* NIES 227 exhibited a superior lipid content and the highest energy content among all strains in both control and AP conditions, while *P. kessleri* strains (CCALA 251 and 253) produced carbohydrate-rich biomass in both cases (see **Figure II. 3** and **Figure II. 6**).

4. 5. ALE for Enhancing Microalgal Tolerance to Toxic effluents

These findings indicate that selecting strains should be based firstly on their metabolic capabilities and secondly on their tolerance to AP. Yet, both aspects must be considered, since AP inhibition should still be mitigated, and the ability of any strain to adapt and resist the effects of this effluent is necessary for successful use of AP. To effectively recycle AP for the production of high-quality biomass and biofuels, a combination of strategies must be implemented. **Paper II** presented results demonstrating the potential to reduce the inhibitory effects of AP after several cycles of acclimation. In **Paper III**, two different approaches were tested to adapt NIES 227 to an extended and constant exposure to AP. To the author's knowledge, this work is the first application of ALE for the reuse of AP as a nutrient source for microalgae cultivation, where NIES 227 was adapted to the toxic effects of AP while preserving its oleaginous capacity.

The two ALE methods presented in this work (BA: batch-adaptation, and TA: turbidostat-adaptation) proved to be effective strategies to offset the inhibitory effects of AP. Although two distinct strains were obtained, they both demonstrated the ability to produce high-lipid biomass in the presence of AP. Batch adaptation resulted in a practical method to perform ALE with minor monitoring, resulting in a strain that presented superior AP tolerance compared to TA, while surpassing the WT biomass productivity. However, the BA strain composition had a lower lipid content compared to the WT. In contrast, a semi-automatic adaptation was performed in a PBR under turbidostat-mode, where the culture was closely monitored to avoid extensive stress to the microalgae's fitness. This latter strategy produced a strain (TA) that could maintain the distinctive lipid-producing capacity of NIES 227, but its tolerance to AP was not as robust as that of the BA strain. Further strain-specific optimization would be necessary to increase the growth rate and lipid content of each ALE strain, as well as further ALE cycles and optimized culture conditions.

Notably, both strains demonstrated important levels of tolerance once they were recovered after ALE and kept in control medium prior to the presented experiments, three months and seven months, respectively. Although genomic analysis would be required to confirm genetic changes beyond the observed phenotypes, it is highly suspected that the strains not only adjusted physiologically to the AP (acclimation) but also elicited genetic

changes in the strains (adaptation) (Borowitzka, 2018). After ALE and removal of AP stress, both strains did not show major damage as seen in the WT in AP, and compared to the results from **Paper II**: compromised growth, declining photosynthetic capacity under nutrient sufficiency, and null lipid productivity that resulted in low-quality biomass for HTL. In contrast, both ALE strains maintained competitive productivities in AP against the control WT in reference media.

Enhancing the AP tolerance of microalgae also plays a significant role in nutrient recovery, reducing the environmental impact of the whole process (Herrera et al., 2021). This effluent carries a rich array of minerals needed for microalgal growth (**Figure IV. 2**).

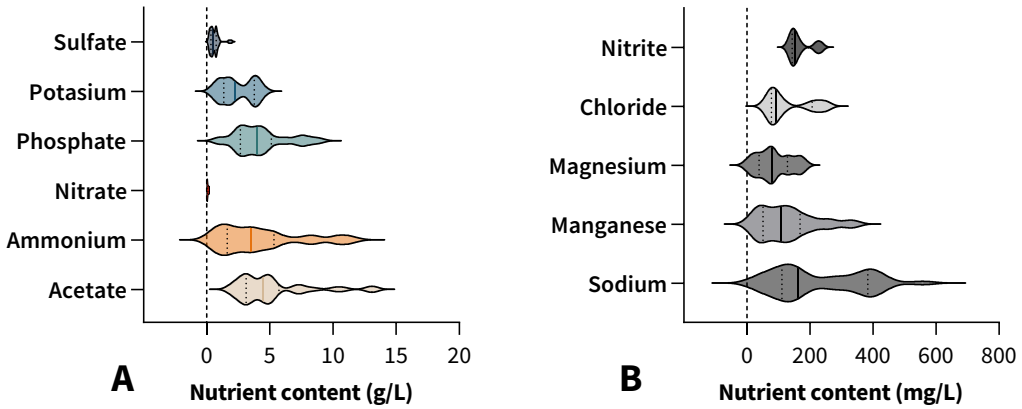


Figure IV. 2. Mineral nutrients found in AP. Violin plots illustrate the kernel density distribution of AP composition from 37 individual APs collected from different HTL reactions throughout the RAFBIOALG project. A) Macronutrients and carbon-sources like acetate, and b) other nutrients found at lower quantities in AP. Mean represented by solid lines, and quartiles represented by dotted lines.

However, the capacity of microalgae to recover nutrients is limited by its metabolic status and biomass productivity (Acién Fernández et al., 2018). In this work, a greater substitution of N, P, and S mineral salts in the growth media was possible as the AP concentration was increased while maintaining stable microalgal growth and nutrient uptake. When coupling AP for the cultivation of microalgae, the percentage and rate of nutrient uptake may depend on the performance of the microalgae, in addition to the initial concentrations present in the AP and the process dilution. For instance, at a dilution of 1/600, with a neutral AP effect on non-adapted microalgae, only

7% N, 13% P and 6% S are covered by AP_{mix} addition into FWM 1/4. In contrast, at a lower AP dilution (e.g., AP_{mix} 1/100), the nutrient contribution from the AP to the culture medium is more important at values of 41% N, 77% P, and 39% S.

Several approaches can be used to optimize microalgal processes besides ALE, while considering AP integration, including genetic modifications in order to increase lipid productivity, culture optimization to increase growth rate in AP, and co-cultivation with other microorganisms such as yeast, which have a strong detoxification potential metabolizing non-assimilable compounds by microalgae, and coupling AP cultivation with wastewater integration in order to reduce even more the addition of mineral fertilizers. All of which present different advantages and disadvantages that must be weighted against the overall cost, environmental impact, and complexity of the microalgae-to-biofuel processes.

Genetic modifications can result in fast and targeted optimization, but they require detailed information on the metabolic pathways and genomes of each strain. They also raise bio-security questions and their regulation may limit their use worldwide. On the other hand, ALE does not require detailed genome information, and may not raise bio-security or regulatory concerns. However, ALE is more time-consuming and the outcome might not result in the desired objective. For example, in **Paper III**, two different adapted strains were obtained, even though the adaptation started from the same wildtype. The latter result implies that although ALE can be successful in improving strain tolerance, the outcome may not be consistent and it is dependent on the chosen operational method.

When aiming for strain optimization to toxic effluents, where a complex mix of compounds is present, and from which the totality of the composition is unknown at a metabolic level, it is currently hard to pinpoint specific genetic modifications. Furthermore, at present, the focus should be on the ALE method to produce adapted microalgae strains to complex inhibitory effluents. The genomic information of these strains needs to be identified and used as a basis for gene editing in the future. Further studies of ALE strains adapted to toxic effluents like the AP can greatly aid to elucidate the desired genetic profile of strains with superior tolerance.

4. 5. Strategies for Successful AP-Recycling in Microalgae Production

The findings of this thesis have shed light on both the advantages and challenges of recycling AP as a substrate for microalgae cultivation. While AP was found to have negative effects on microalgae growth, it was also shown to be a rich source of nutrients that significantly increased biomass productivity in ALE strains. These results provide a better understanding of the efficient use of AP for microalgae cultivation, emphasizing the need to recycle AP not only to recover nutrients but also to use it as a substrate to produce new microalgal biomass and ultimately, more biocrude.

To integrate HTL-AP successfully in microalgae cultivation, several aspects must be considered, and further recommendations are proposed. Feedstock quality varies during the evolution of microalgae cultures and is highly influenced by strain-specific metabolism and robustness, as well as both extra- and intracellular nutrient levels. Before recycling this effluent it needs to be diluted, taking into account the mass balance of the HTL process and the tolerance of the strain of interest. Without any pre-treatment or without unrealistically high AP dilutions, AP inhibits microalgae growth and affects biomass quality.

The extent of this inhibition depends on strain-specific tolerance, AP dilution, and AP characteristics. These chemical and physical characteristics are influenced by the HTL reacting conditions and feedstock composition (see **Figure IV. 4B**) and have a significant impact on the toxicity of the effluent and nutrient content (Biller et al., 2012). It is important to note that these differences may result in different opportunities to reuse each AP. For example, AP with high levels of toxic compounds, such as N-containing heterocycles may cause stronger inhibition and be less suitable for microalgae cultivation (Zhang et al., 2016). The natural brown color of this effluent varies according to the reacting HTL temperature, where a darker color is found at lower temperatures (270°C), compared to higher temperatures (345°C). This coloration may affect the light capturing in microalgae, which can impact its photosynthetic activity (Alimoradi et al., 2020; de Mooij et al., 2016).

Furthermore, AP dilutions must be followed by nutrient replenishment to cover the needs of the microalgae biomass production stage. Nutrient replenishment must be cautiously tailored to the strain of interest, culture conditions, and each AP. Following the same strategy of balanced nutrient limitation, AP-based substrates should be designed ensuring a balance between biomass and lipid productivity without excessive mineral supply to avoid nutrient losses.

Producing high-quality biomass is a critical goal for microalgae cultivation, but this can be challenging when using AP. Therefore, it is crucial to prioritize strain selection based on biomass quality followed by their ability to tolerate AP. This can be achieved by conducting comprehensive screening to identify strains that exhibit both high growth rates and lipid content in the presence of AP. While longer cultivation times can potentially produce energy-rich biomass in non-adapted microalgae, it is important to note that optimal biomass quality must be considered throughout the cultivation process in an efficient manner.

In addition to strain selection, implementing adaptation techniques can be utilized to optimize growth rates and biomass quality under AP-supported cultivation. These adaptation strategies can include gradual exposure of microalgae to increasing concentrations of AP, thereby allowing them to adapt to the effluent's unique chemical composition. Overall, effective cultivation of microalgae using AP as a growth medium requires careful consideration of both strain selection and cultivation strategies. By prioritizing high-quality biomass production and implementing adaptation techniques, it may be possible to optimize growth rates and maximize the potential of microalgae cultivation in AP-supported systems.

4. 6. Limitations of this study and Future Recommendations

4. 6.1. Elucidating Nutrient Sources Found in AP

AP is a complex mixture that contains hundreds of different chemical species, as illustrated in Figure IV. 3, but only a small fraction of these compounds has been identified. A better understanding of the chemical composition of AP is essential for determining unidentified inhibitory compounds, assimilable compounds for microalgae, recalcitrant and non-biodegradable compounds, and remarkable compounds of interest. The complex mixture of AP organic compounds has numerous applications, including the manufacture of liquid fuels, high-value chemicals, and cosmetics (Watson et al., 2020). Nitrogen-organic compounds that are microalgae inhibitors can be recovered and used as intermediate chemicals in the production of pharmaceuticals, agrochemicals, and feed additives (Higashio and Shoji, 2004). Among these molecules are pyridines, methylpyridines, pyrazines, and acetamide.

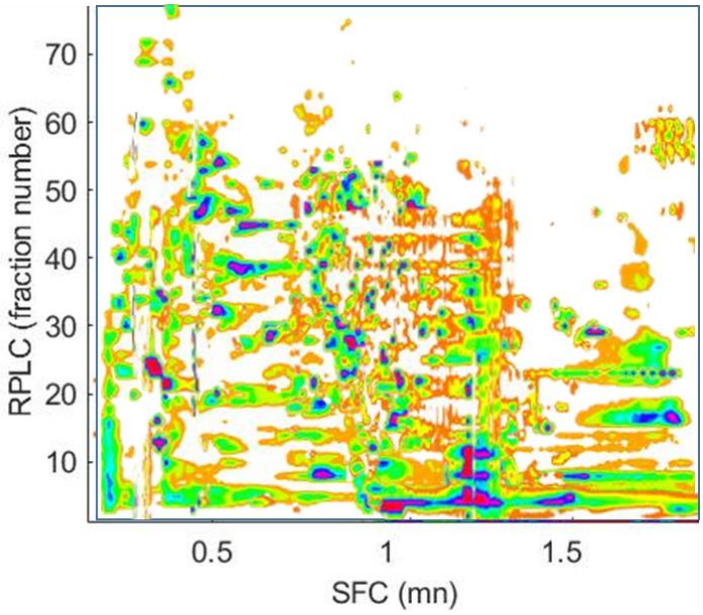


Figure IV. 3. AP compound qualitative characterization by two-dimensional analysis: RPLC (Reversed-Phase Liquid Chromatography) x SFC (Supercritical Fluid Chromatography). Each spot is a molecule responding in MS (mass spectrometry), after partitioning by hydrophobicity (RPLC) and polarity (SFC). AP sample from *C. sorokiniana* NIES 2173 (Internal RAFBIOALG results from Karine Faure).

While only certain macronutrients (N, P and S) were considered when designing the AP-based media, it is expected that other nutrient sources are also consumed by microalgae. Microalgae are known to assimilate several organic compounds present in AP, including acetate, glucose, glycerol, urea, amino acids, and even phenol (Pastore et al., 2020; Shen et al., 2016; L. Wang et al., 2016). These organic compounds promote mixotrophic growth in microalgae, leading to higher biomass productivity (as seen in the BA strain after ALE on **Paper III**). Further in-depth characterization of AP organic compounds would help to identify assimilable compounds for microalgae and their effects on lipid-biosynthesis.

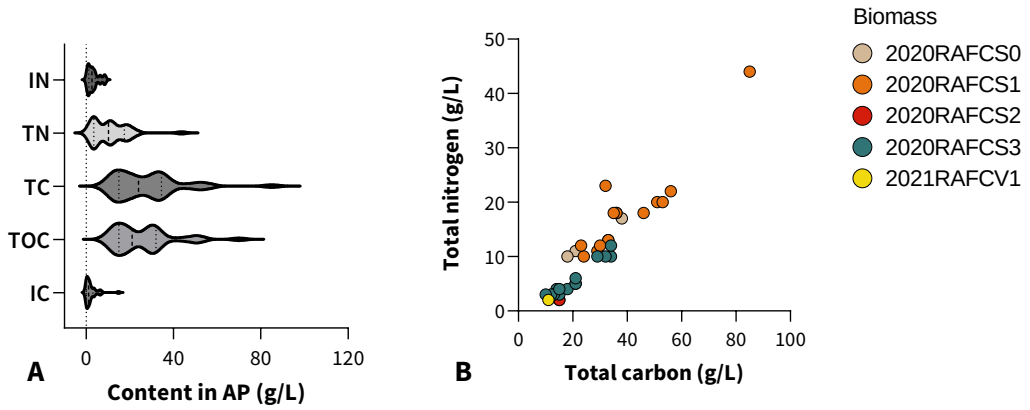


Figure IV. 4. Carbon and nitrogen content found in AP. A) Distribution of the different fractions of C and N found in numerous APs (n=37). B) Distribution of TC and TN according to each HTL reaction, and respective feedstock. Total carbon (TC), total organic carbon (TOC), inorganic carbon, total nitrogen (TN), and inorganic nitrogen (IN). IN corresponds to $N\text{-}NH_4$ and $N\text{-}NO_3$ found in AP.

Inorganic N is present in the AP in the form of ammonium nitrogen and nitrate nitrogen, which accounts for only 13% to 50%, depending on the AP, of the total AP nitrogen (**Figure IV. 4**). During the course of this study, it was found that microalgae completely consumed the mineral nutrients supplied by the AP, leading to a reduction in the demand for mineral fertilizers. Furthermore, other micronutrients such as potassium, magnesium, iron, and other trace minerals present in the AP were not considered for AP-based media formulation, which could have further reduced the demand for these minerals in microalgal substrates. Therefore, it is crucial to conduct more research to identify assimilable compounds and determine the optimal amount needed by microalgae for better AP-based substrate formulation.

4. 6.2. Biomass-to-substrate virtuous loop

Successful AP recirculation is a step toward more sustainable processes (Fernandez et al., 2018; Yang et al., 2018), and it must be as simple as possible in order to reduce its cost and environmental impact. ALE can promote the integration of AP back into the system without the need for additional detoxification steps with regards to biomass production. However, when aiming for circular processes, the accumulation of non-biodegradable compounds may limit the recirculation of the AP after several cycles (Leng et al., 2020a). Hence, it is necessary to identify potential recalcitrant compounds after multiple cycles of reusing AP for microalgae cultivation. This could aid in the selection of appropriate pre-treatment steps for removing recalcitrants that are not assimilable by microalgae and determining their fate after multiple HTL reactions. Some of the pretreatments studied in previous research to recover these compounds include membrane separation to recover carboxylic acids (Zhang et al., 2018), separation of phenolic compounds using resins activated charcoal adsorption (K. Chen et al., 2015), and solvent extractions to recover N-containing compounds (Zhang et al., 2016).

4. 6.3. Seeking Sustainable Microalgal-Processes

To achieve greater sustainability in microalgae processes, it is essential to consider potential environmental impacts at each stage of the process. Achieving this goal is a complex task that requires a multidisciplinary approach implementing various strategies targeting economic, technical, and environmental improvements. Critical steps in this direction include developing more efficient and cost-effective microalgae cultivation systems that use sustainable materials and reduce energy use. For example, harvesting by using floculants can replace energy-intensive practices such as centrifucation. Also, using microalgae strains that are resistant or adaptable to temperature changes can reduce the need for heating during winter or air conditioning during summer, thereby optimizing energy use.

Further research is also necessary to understand and model biomass decomposition during HTL to maximize biocrude yield and quality while considering reacting conditions such as heat supply and residence time. Moreover, integrating microalgae processes with wastewater, AP recirculation, and other process streams (e.g., harvesting water) can significantly reduce the environmental impact by reducing the use of mineral fertilizers and water needs while avoiding discharge to the environment. Finally, implementing a biorefinery framework can maximize

the use and valorization of all waste streams and byproducts from HTL, such as the use of biochar as a soil amendment, or the recycling of the HTL gas fraction to recover CO_2, and recovery of high-value compounds of AP, contributing to a more sustainable and environmentally friendly microalgae production system.

4. 7. Concluding Remarks

This thesis underscores the importance of considering every step of the microalgae cultivation process, from strain selection to the obtention of the final biofuel, to develop environmentally friendly and circular processes that produce both high-quality biomass and biofuels. The promising results of C. *vulgaris* NIES 227 for lipid-based biofuels further highlight the need for continuous research and development in this field. In particular, this study demonstrated the potential of recycling AP as a nutrient source for microalgae cultivation to produce high-quality biomass for HTL conversion. The findings of this work emphasize the importance of mitigating the inhibitory effects of AP to effectively produce high-quality biomass. Additionally, ALE emerges as a promising approach to improve the tolerance of microalgae to AP toxicity, which can be implemented to manage other toxic compounds or effluents containing growth inhibitors. These insights into the adaptation of microalgae strains to AP's toxic effects and the potential for strain-specific optimization can pave the way for the development of sustainable and efficient microalgae cultivation processes. To conclude, this thesis provides valuable insights into the development of sustainable and efficient microalgae cultivation processes, ultimately contributing to the transition toward sustainable energy sources.

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6. Appendix A

Extended Summary (French)
Culture de microalgues sur phase aqueuse issue de liquéfaction hydrothermale

INTRODUCTION

La course aux biocarburants à base de microalgues face aux carburants fossiles a été contrainte par les coûts économiques et environnementaux de leur production [1]. Afin de rivaliser avec les carburants fossiles, il est essentiel de considérer et d'optimiser chaque étape du processus, de la sélection de souche et de la production de biomasse à sa conversion et à son amélioration en biocarburant [2]. Le projet ANR RAFBIOALG a été conçu pour combler certaines des lacunes de connaissances impliquées le long de la chaîne algues-à-carburant par liquéfaction hydrothermique (HTL) et amélioration ultérieure de l'huile bio. Envisageant un processus circulaire, cette thèse se concentre sur le début de la chaîne de production avec la culture d'une microalgue sélectionnée, en fonction de sa productivité lipidique et de ses qualités de biomasse, et sa capacité à prospérer sur les effluents HTL. La sélection de la souche et la production de combustible sont des étapes clés de la chaîne de production [3]. La microalgue sélectionnée peut non seulement influencer la productivité de la biomasse lors de la culture, mais elle peut également représenter différents défis et/ou opportunités le long du processus [4]. Lors de la HTL, la biomasse est exposée à des températures allant de 250°C à 450°C et à une pression allant de 100 à 350 bars. Dans ces conditions subcritiques, la biomasse se décompose en composés plus simples [5]. En particulier, la conversion des lipides (composés de stockage d'énergie dans les microalgues) en biocarburant constituera une partie importante du rendement et des qualités du biocarburant [6]. Par contre, les protéines et les glucides, qui jouent un rôle secondaire sur le contenu énergétique du biocarburant, pourraient en fait introduire des composés contenant O et N dans le biocarburant [7].

Après HTL, un biocrude est obtenu avec d'autres sous-produits : une phase gazeuse, une phase aqueuse et une phase solide [8]. La phase aqueuse est particulièrement intéressante, car elle représente 20 % de l'énergie perdue, et en même temps, elle contient des nutriments nécessaires pour la croissance des microalgues [9]. Le recyclage de ce sous-produit pourrait remplacer jusqu'à 50 % des apports nutritifs [10]. L'utilisation de l'AP comme substrat pour les microalgues reste limitée, en raison de la présence de composés toxiques tels que : les phénols, les composés N-hétérocycliques, des composés non identifiés et, dans certains cas, des concentrations élevées de certains composés comme le NH_4/NH_3 [11,12]. Il est nécessaire de surmonter cette inhibition si l'on souhaite produire une biomasse couplée à l'AP. Plusieurs approches ont été explorées précédemment pour réduire cette inhibition, par exemple : des dilutions élevées de l'AP jusqu'à 500 fois, des étapes de désintoxication (charbon actif, résines et membranes), l'utilisation de polycultures et un apport en nutriments supplémentaires [13-15]. L'approche suivie au cours de cette thèse est basée sur la capacité des microalgues à s'acclimater et à s'adapter aux effets négatifs causés par l'AP. Lorsque l'inhibition est surmontée, l'AP peut en fait favoriser une meilleure croissance et une meilleure consommation des sources de C par la croissance mixotrophe des microalgues [16]. Un recyclage réussi de l'AP pour la production de biomasse HTL dépend de la sélection des souches, de l'utilisation des nutriments et des moyens de surmonter l'inhibition. Avec la bonne souche, il pourrait être possible de recycler l'AP jusqu'à un certain degré tout en maintenant une productivité en biomasse et en lipides efficace. Avec ces points à l'esprit, les objectifs spécifiques de cette thèse sont, tout d'abord, d'identifier une souche microalgale appropriée pour les applications de biocarburant couvrant certains critères : une productivité élevée en lipides et en biomasse, et une croissance robuste. A cette fin, six souches du genre *Chlorellaceae* ont été étudiées en conditions limitées en nutriments pour produire une biomasse à haute teneur en lipides. Deuxièmement, la réponse et l'acclimatation de ces souches à l'AP ont

été suivies sur plusieurs cycles pour identifier les effets de l'AP sur la productivité en lipides et en biomasse. De plus, avec l'objectif de mieux comprendre les effets de l'AP, plusieurs expériences ont été réalisées avec la souche sélectionnée *Chlorella vulgaris* NIES 227; sous une adaptation en lot et en continu à l'AP.

MATERIAUX ET METHODES

Liquéfaction hydrothermale et production d'AP

Dans le cadre du projet RAFBIOALG, de nombreuses conversions HTL ont été réalisées au CEA de Grenoble par Lucie Matricon et Anne Roubaud dans le but d'optimiser le rendement en biobrut de *Chlorella sorokiniana* NIES 2173 et *Chlorella vulgaris* NIES 227. Les APs collectés de chaque HTL ont été stockés à -20 °C jusqu'à leur utilisation ultérieure.

Souches de microalgues

Six souches de la famille des *Chlorellacea* ont été étudiées en tant que candidates potentielles pour les matières premières des biocarburants. Les souches ont été acquises auprès de l'Institut national d'études environnementales (NIES, Japon) et de la Culture Collection of Autotrophic Organisms (CCALA, République tchèque). Le groupe d'étude se compose d'une *Chlorella sorokiniana* (NIES 2173), de trois *Chlorella vulgaris* (NIES 227, CCALA 256 et CCALA 269) et de deux *Parachlorella kessleri* (CCALA 251 et 253). Ils sont désignés individuellement par leur numéro d'identification respectif (c'est-à-dire NIES 227).

Milieux de culture

Le milieu d'eau douce (FWM) a été utilisé comme milieu de référence pour toutes les expériences et les milieux à base d'AP. Ce milieu a été conçu pour couvrir les besoins en nutriments de 4 g/L de biomasse d'une microalgue de type chlorophycée [17]. Le FWM est composé de 1459 mg/L $NaNO_3$, 102 mg/L NH_4Cl, 233 mg/L. KH_2PO_4, 143 mg/L $MgSO_4$-$7H_2O$, 1,5 mg/L $CaCl_2$-$2H_2O$, et 1 mL/L de solution d'oligo-éléments de Hutner [18]. Des versions modifiées du FWM (c'est-à-dire 1/4, 1/2) ont été préparées pour induire une limitation des nutriments et par conséquent une accumulation de lipides dans un temps et une intensité lumineuse donnés. La dilution a été déterminée en fonction de l'objectif et de la durée de chaque expérience. Pour les expériences de culture AP, des nutriments inorganiques à une certaine dilution AP (1/600-1/50) ont été considérés pour une concentration finale de N, P et S équivalente au FWM (N : 267 mg/L, P : 53 mg/L et S : 20 mg/L), ou à la dilution respective. Le milieu a été ajusté à un pH de 7,0-7,5 et stérilisé à l'autoclave et/ou filtré (milieu à base d'AP) avec une membrane stérile de 0,2 µm.

Conditions de culture

Les cultures mères ont été maintenues dans un incubateur automatisé (ZWYC-290A, Labwit Scientific, Australie), avec une intensité lumineuse de 20 µmol/m2/s et dans des conditions environnementales constantes (T : 25°C, agitation : 130 rpm, CO_2 : 2% v/v). Les expériences suivantes ont été réalisées dans le même incubateur et dans les mêmes conditions environnementales, sauf indication contraire. Pour les cultures expérimentales, l'intensité lumineuse (100-230 µmol/m/s) et la photopériode ont été ajustées en fonction de l'objectif et de la durée de chaque expérience. Toutes les cultures ont été conservées dans des flacons Erlenmeyer stériles avec des milieux autoclavés et/ou filtrés.

Plan d'expérience

Culture de microalgues à haute teneur en lipides

Les six souches ont été cultivées pendant une période de 17 jours, en trois répétitions biologiques, afin d'évaluer leurs qualités de biomasse en tant que matières premières pour biocarburants. Une limitation équilibrée des nutriments a été utilisée pour déclencher l'accumulation de lipides tout en maintenant l'accumulation de biomasse. La teneur en nutriments du FWM a été réduite de 50 % afin d'induire un appauvrissement naturel en nutriments (en particulier N, S et P). Les qualités spécifiques des souches et leur biomasse finale ont été étudiées en profondeur afin d'identifier une souche idéale pour les matières premières des biocarburants.

Biomasse de microalgues cultivées en AP

Les souches ont ensuite été acclimatées à un milieu à base d'AP (AP14, 1/200) avec une teneur en nutriments équivalente à FWM 1/2. Les cultures ont été transférées dans un milieu frais tous les 7-10 jours. Après le

quatrième cycle, les microalgues ont été cultivées en double pendant 17 jours afin d'étudier les effets de l'exposition à l'AP sur l'accumulation de lipides et de biomasse. Enfin, la biomasse résultante a été étudiée pour ses propriétés en tant que matière première HTL. La NIES 269 n'a pas été incluse dans cette phase expérimentale, car elle n'a pas survécu à l'inhibition de l'AP. La NIES 227 a été sélectionnée pour d'autres études sur l'AP pour la culture des microalgues.

Adaptation et développement de la tolérance des microalgues à l'AP
Le type sauvage NIES 227 (WT) a été soumis à une acclimatation progressive à l'AP, en vue d'une évolution adaptative en laboratoire (ALE). Cette technique est une stratégie d'amélioration des souches, dans laquelle un stress environnemental spécifique est appliqué pour imiter l'évolution naturelle, communément appelée sélection darwinienne [19]. Deux approches différentes de l'ALE ont été suivies : a) la culture par lots et b) l'adaptation continue en mode turbidostat. Les souches obtenues sont appelées BA (pour batch-adapted) et TA (pour turbidostat-adapted). La souche BA a été obtenue après le transfert régulier de la WT dans un milieu frais avec 0,5 % d'AP. Pour la souche TA, une culture WT a été exposée à une concentration croissante d'AP à partir de 0,17% (1/600) avec pour objectif d'atteindre une concentration finale de 1% (1/100) sans effets négatifs significatifs. Enfin, les deux souches adaptées ont été comparées à la WT, pour leur capacité à se développer dans l'AP et les caractéristiques de la biomasse.

Mesures analytiques
Paramètres de croissance
La croissance des microalgues a été suivie par la détermination du nombre et de la taille des cellules, de la densité optique (OD) et du poids sec (DW). La densité de la culture a été mesurée à 880 nm par UV-Vis Epoch2 (BioTek Instruments, USA). Le poids sec a été déterminé avec 2-5 ml de culture. Les cellules ont été centrifugées à 4500 rpm pendant 10 minutes (Allegra X15R, Beckman Coulters, USA). Le surnageant a été recueilli pour la détermination des ions par chromatographie ionique et le culot a été lavé avec de l'eau distillée et centrifugé à nouveau. Le culot a été récupéré dans des plaques d'aluminium pré-pesées et séché à 105 °C pendant au moins 24 heures pour déterminer le poids sec (PS) de l'échantillon.

Caractérisation des nutriments de l'AP et consommation de nutriments
Les sources de nutriments (c'est-à-dire l'acétate, l'ammonium, le nitrate, le phosphate et le sulfate) présentes dans l'AP et le FWM ont été analysées par chromatographie ionique (IC 940, Metrohm, Suisse). Les échantillons d'AP et les surnageants de culture ont été dilués et filtrés avec un filtre Whattman 0,22 µm. Un analyseur TOC/TN (Shimadzu TOC- L, Japon) a déterminé la teneur en carbone organique total (COT) et en azote total (AT) de la phase aqueuse.

Caractérisation de la biomasse
Les aliquotes de culture ont été centrifugées, lavées avec de l'eau distillée et centrifugées à nouveau. Les culots congelés ont été lyophilisés et conservés à -20 °C en vue d'analyses ultérieures. La teneur en lipides a été quantifiée à l'aide d'une méthode gravimétrique adaptée d'Axelsson et Gentili [20]. Une deuxième méthode a été développée pour quantifier la teneur en lipides totaux et caractériser la composition en acides gras des microalgues. Des échantillons de 5 à 20 mg de biomasse lyophilisée ont été soumis à une transméthylation pour obtenir des esters méthyliques d'acides gras (EMAG). Les FAME totaux et le profil des acides gras (AG) ont été déterminés à l'aide d'un système de chromatographie en phase gazeuse avec détection par ionisation de flamme (GC-FID). Les différents profils d'AG ont également été étudiés et les qualités pour son utilisation en tant que biodiesel ont été estimées selon Deshmukh et al [21]. La teneur en hydrates de carbone a été mesurée selon un protocole Dubois modifié [22]. La teneur en protéines a été calculée sur la base d'un rapport de conversion N-protéines de 5,04 selon Templeton et Laurens [23]. La biomasse lyophilisée a été analysée via l'analyse élémentaire CHONS par Bruno Magalhaes à l'IRCELYON. Le pouvoir calorifique supérieur (PCS) a été estimé sur la base de la composition élémentaire finale selon Tian et al [24].

RÉSULTATS

Sélection de souches pour les applications de biocarburants

Six *Chlorellas* différentes ont été étudiées en tant que matière première potentielle pour les applications de biocarburants microalgaux. Toutes les souches ont maintenu une accumulation constante de biomasse malgré la teneur réduite en nutriments du milieu (Figure A.1). *Chlorella sorokiniana* NIES 2173 avait la concentration finale de biomasse et la productivité globale de biomasse les plus élevées, correspondant à 5,63 g/L et 0,33 g/L/d ; suivie par *C. vulgaris* CCALA 256 et *C. vulgaris* NIES 227. *C. vulgaris* CCALA 269 a eu la performance globale la plus faible, avec une concentration finale de biomasse de 3,91 g/L et une productivité globale de biomasse de 0,23 g/L/j. Contrairement à la biomasse produite, la division cellulaire a cessé lorsque les nutriments ont été épuisés dans le milieu (Figure A.1).

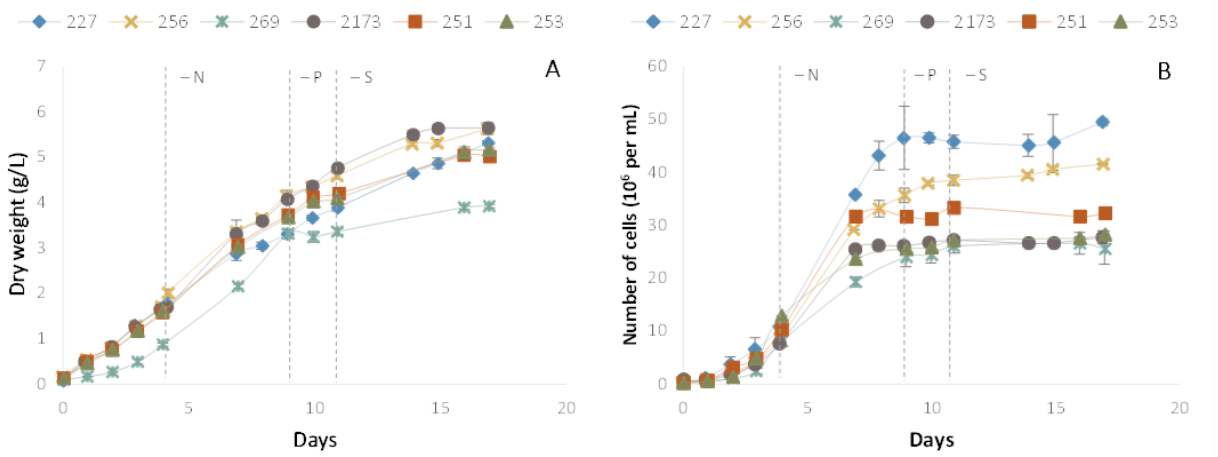


Figure A.1. Comparaison de la croissance basée sur A) le poids sec et B) le nombre de cellules de six souches de *Chlorellaceae* spp : *C. vulgaris* NIES 227 et CCALA 256 & 269, *C. sorokiniana* NIES 2173, *P. kessleri* (CCALA 251 & 253). La ligne pointillée indique le début de la privation de N (-N), de P (-P) et de S (-S) (sauf pour CCALA 269, pour laquelle l'appauvrissement en N s'est produit le 7e jour).

Le stress causé par l'épuisement des nutriments a déclenché l'accumulation de composés de stockage tels que les lipides et les hydrates de carbone à des niveaux différents en fonction de la souche (Figure A.2). En général, toutes les souches avaient une teneur en lipides élevée par rapport aux conditions suffisantes (~ 10 %). Les teneurs en lipides les plus élevées ont été observées chez NIES 227 et CCALA 256 avec 54% et 40%, respectivement. La teneur réduite en lipides observée chez certaines souches coïncide avec une préférence pour le stockage des glucides. Quatre des souches avaient une teneur en hydrates de carbone d'au moins 35 %. La teneur en hydrates de carbone la plus élevée a été observée dans les deux souches de *P. kessleri*, CCALA 251 et 253, avec 48 % et 53 %, respectivement. La souche NIES 227 avait la plus faible teneur en hydrates de carbone avec 14%. Alors que les protéines des microalgues peuvent varier de 7 % à 40 %, la biomasse finale présentait une faible teneur en protéines dans toutes les souches (12-16 %) [23]. Dans cette étude, les chaînes C18 correspondaient à 65-80% du profil total des AG dans toutes les souches (Figure A.4). Des différences majeures ont été observées dans le niveau de saturation des AG. NIES 227 et CCALA 269 présentaient 50 % et 40 % des AG totaux sous forme d'AGMI. Pour les autres souches, 60 % des AG étaient des AGPI, qui ne sont pas idéaux pour le biodiesel mais pourraient présenter un intérêt nutraceutique [33]. Sur la base de ces propriétés estimées, la souche NIES 227 présente non seulement une teneur exceptionnelle en lipides, mais aussi un profil d'AG qui pourrait être prometteur pour son utilisation directe en tant que biodiesel. Il est intéressant de noter que le profil FA de cette souche était le seul à respecter certaines réglementations (EN14214 [25]), comme une teneur en acide linoléique (C18:3) inférieure à 12 % (9 %) et un indice de cétane (50,24) proche de la spécification européenne d'au moins 51 (données non montrées).

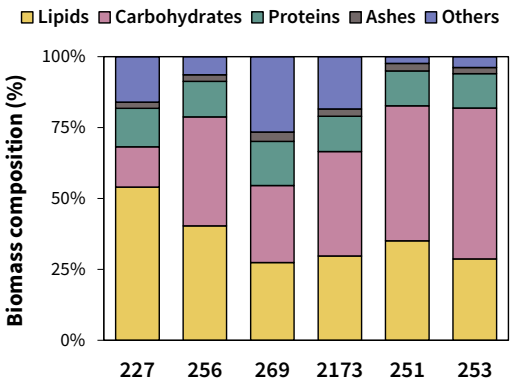


Figure A.2. Composition de la biomasse finale (jour 17) de six souches de *Chlorellaceae* : *C. vulgaris* NIES 227, CCALA 256 & 269, *C. sorokiniana* NIES 2173 et *P. kessleri* CCALA 251 & 253.

En fonction de la composition de la biomasse spécifique à l'espèce, le contenu énergétique de la biomasse finale peut être prédit sur la base de la composition élémentaire (Tableau A.1). La biomasse riche en lipides de NIES 227 présentait la teneur la plus élevée en C et H (62,2 % et 9,3 %) et la teneur la plus faible en O (27 %). En revanche, la biomasse riche en glucides de CCALA 253 présentait les teneurs en C et H les plus faibles (52 % et 7,6 % respectivement) et une teneur en O élevée (39,3 %). Le HHV le plus élevé a été trouvé dans la NIES 227 (29,8 MJ/kg), par rapport à la CCALA 253 (22,9 MJ/kg), qui avait la teneur en sucre la plus élevée. Une partie de la complexité de la production d'un biocarburant réside dans les composés N- et O-hétérocycliques qui peuvent affecter les qualités du biocarburant et qui, en même temps, doivent être réglementés. Les microalgues sont considérées comme une biomasse à forte teneur en azote, atteignant des valeurs de 10 % [26]. Dans cette étude, la teneur en N a été considérablement réduite pour toutes les microalgues, ce qui peut conduire à une teneur en N globalement plus faible dans le biocarburant et, par conséquent, à une réduction des émissions de NOx. Une teneur finale en N comprise entre 2 et 3 % a été mesurée dans la biomasse finale de toutes les souches.

Tableau A.1. Composition élémentaire des CHONS de microalgues et pouvoir calorifique supérieur (PCS) calculé de six souches de *Chlorellaceae* : *C. vulgaris* NIES 227 et CCALA 256 & 269, *C. sorokiniana* NIES 2173, *P. kessleri* (CCALA 251 & 253).

Souche de microalgues	**C (wt.%)**	**H (wt.%)**	**O (wt.%)**	**N (wt.%)**	**S (wt.%)**	**HHV (MJ/kg)**
C. vulgaris NIES 227	62,2 ± 0,1	9,3 ± 0,1	27,0 ± 2,2	2,6 ± 0,0	0,101 ± 0,01	29,8
C. vulgaris CCALA 256	56,3 ± 2,6	8,2 ± 0,3	35,1 ± 0,6	2,4 ± 0,1	0,097 ± 0,03	25,7
C. vulgaris CCALA 269	55,7 ± 2,1	8,1 ± 0,5	33,4 ± 0,3	3,0 ± 0,1	0,099 ± 0,01	25,4
C. sorokiniana NIES 2173	53,5 ± 0,3	7,9 ± 0,1	40,6 ± 0,8	2,4 ± 0,0	0,102 ± 0,01	23,7
P. kessleri CCALA 251	54,7 ± 0,4	7,9 ± 0,1	35,3 ± 1,2	2,4 ± 0,1	0,104 ± 0,01	24,7
P. kessleri CCALA 253	52,0 ± 0,2	7,6 ± 0,1	39,3 ± 1,5	2,4 ± 0,0	0,100 ± 0,01	22,9

Transformation des effluents du procédé HTL en biomasse : effets de la réutilisation de l'AP pour les matières premières des microalgues

Une fois que le potentiel des six chlorelles à produire des biocarburants a été déterminé, la croissance et la tolérance de chaque souche à l'AP ont été contrôlées pendant plusieurs cycles de traitement par lots. Un effet inhibiteur net a été observé dans toutes les souches par rapport au taux de croissance en l'absence de PA (Figure A.3). CCALA 256 était la souche la moins affectée avec une réduction initiale du taux de croissance de 33% et NIES 227 était la plus affectée avec une réduction de 65%. Après deux cycles d'acclimatation à l'AP, une diminution de l'effet inhibiteur et une optimisation du taux de croissance ont été observées chez toutes les souches. Après deux cycles d'acclimatation, NIES 2173 a pu doubler son taux de croissance dans l'AP, et CCALA 253 a présenté un effet positif net sur le taux de croissance par rapport aux conditions de contrôle. Au cours du quatrième cycle, les microalgues ont été cultivées pendant une période plus longue afin d'évaluer leur biomasse et leur accumulation de lipides. Toutes les souches ont eu une réduction de la biomasse finale allant de -44% pour NIES 227 à -15% pour CCALA 251. L'accumulation de lipides a également été affectée par la présence de l'AP. La division cellulaire étant fortement compromise, le quota final de nutriments intracellulaires était plus élevé puisqu'il n'y avait pas autant de cellules pour les mêmes concentrations de nutriments. Ainsi, le stress causé par la carence en nutriments n'a pas été atteint au même degré et dans le même laps de temps que dans les conditions de contrôle. Il n'est pas clair, et cela sort du cadre de cette thèse, s'il y a un effet ponctuel causé par l'AP sur la voie métabolique de la biosynthèse des lipides. Comme observé dans les conditions de contrôle, à l'exception de NIES 227, qui a atteint une teneur en lipides de 36%, aucune autre souche n'a été capable d'accumuler une quantité substantielle de lipides. Les autres microalgues avaient une teneur en lipides nettement plus faible, allant de 21 % pour CCALA 256 à 13 % pour CCALA 251.

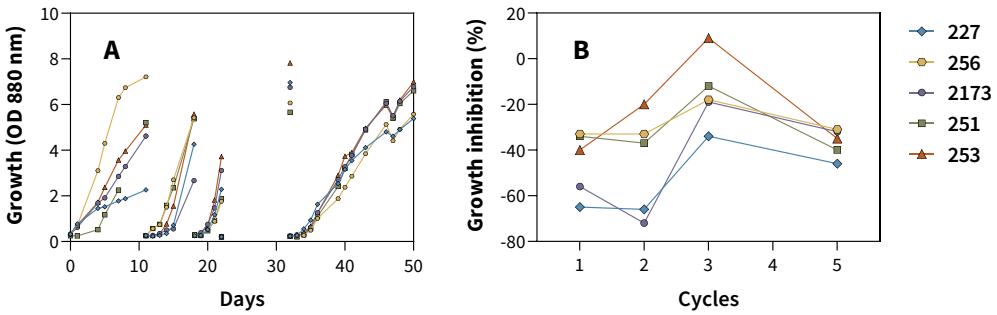


Figure A.3. Acclimatation à l'AP sur quatre cycles de culture (A) et inhibition du taux de croissance par rapport aux conditions de contrôle (B). Biomasse et accumulation de lipides après trois cycles d'acclimatation à l'AP (C et D). La ligne en pointillé représente le début de l'épuisement de l'azote.

Les microalgues cultivées dans l'AP ont été évaluées par rapport à leurs qualités dans des conditions de contrôle. Bien que la croissance et la biomasse aient été soutenues en présence d'AP, les valeurs optimales n'ont pas été observées. Une diminution globale de la productivité lipidique et du taux de croissance a été observée dans toutes les souches (Figure A.4). Bien qu'ayant le taux de croissance le plus élevé dans les conditions de contrôle, NIES 227 a été la souche la plus affectée par l'AP avec une forte diminution de la concentration finale de biomasse et du taux de croissance. Cependant, cette microalgue a été capable de compenser la réduction de la production de biomasse en ayant la teneur en lipides la plus élevée tout au long de l'expérience et donc la productivité lipidique et le contenu énergétique les plus élevés. Dans l'ensemble, NIES 227 présentait les meilleures qualités pour une matière première HTL et d'autres applications de biocarburants, et bien qu'elle n'ait pas eu la meilleure résistance initiale à l'AP, elle a maintenu l'accumulation de lipides la plus élevée et a montré la capacité de développer une tolérance au mélange inhibiteur. Sur la base de ces résultats, la NIES 227 a été sélectionnée pour une adaptation plus poussée à l'AP et à des études connexes.

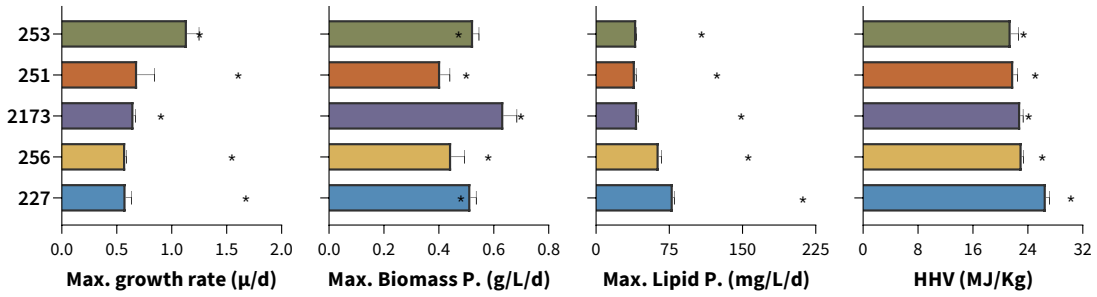


Figure A.4. Effet de l'AP sur plusieurs caractéristiques des microalgues par rapport aux conditions de contrôle. La barre de contrôle illustre les résultats moyens des souches de *Chlorella* dans le milieu de contrôle (FWM ½) et en l'absence d'AP.

L'adaptation des microalgues : une stratégie pour prospérer dans un effluent toxique
En raison des qualités de biocarburant observées chez NIES 227, notre objectif suivant était d'étudier si une période d'acclimatation plus longue augmenterait la tolérance des microalgues et réduirait les effets négatifs de l'AP. Après avoir réalisé deux stratégies d'ALE, en transfert de lots (BA) et en mode turbidostat (TA), les souches obtenues ont été comparées à la souche WT. Avant l'expérience, les souches adaptées ont été cultivées avec et sans AP pendant plusieurs mois afin d'évaluer la persistance du phénotype adapté. Les deux stratégies ALE ont compensé l'effet négatif de l'AP, alors que la WT semblait être fortement affectée par l'AP : production de biomasse réduite (-50%), teneur en lipides réduite (-60%) et teneur en protéines augmentée (+56) (Figure A.5). En revanche, la TA a présenté une composition de biomasse similaire à celle de la WT dans le milieu témoin (47% et 49% de lipides respectivement). La BA a accumulé non seulement des lipides (36%) mais aussi une grande quantité d'hydrates de carbone (40%). Il est intéressant de noter que l'AP a renforcé l'accumulation de biomasse chez les deux souches adaptées, même après la période de désadaptation. La souche BA a eu une augmentation de 30% de la biomasse par rapport à la WT, à côté de la TA qui a eu une augmentation de 15% par rapport à la WT dans le milieu de contrôle. Il est intéressant de noter que la tolérance est restée dans les deux souches adaptées, même après une période d'absence de PA après l'adaptation, sans différence importante avec les souches soumises à une exposition prolongée au PA (Figure A.6). La souche TA est restée similaire à la souche WT en termes de division cellulaire et de volume et taille cellulaires finaux. La souche BA a montré un nombre de cellules final beaucoup plus faible mais un volume cellulaire beaucoup plus important.

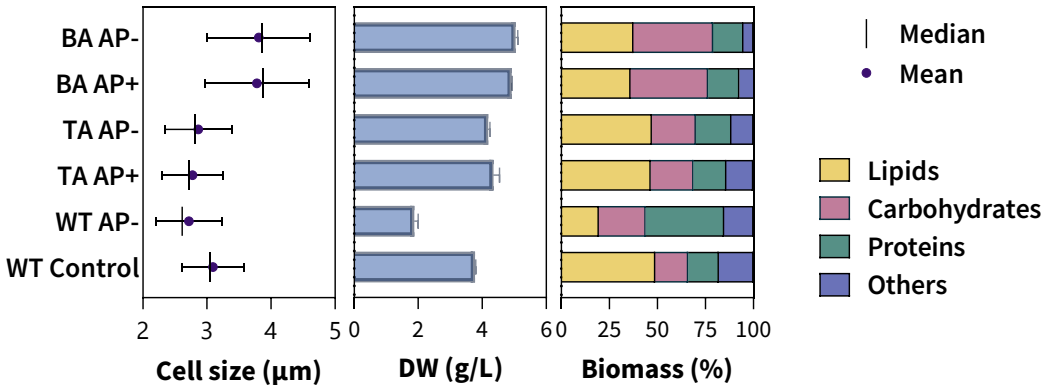


Figure A.5. Caractéristiques finales des microalgues de la souche sauvage (WT) NIES 227 et des souches adaptées : adaptées au traitement par lots (BA) et adaptées au turbidostat (TA) ; après une exposition prolongée à l'AP après adaptation (AP+), ou après une période de désadaptation et/ou sans acclimatation pour la souche WT (AP-).

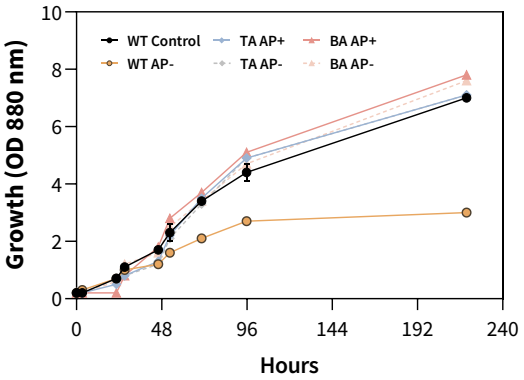


Figure A.6. Croissance de *Chlorella vulgaris* NIES 227 de type sauvage (WT) sur AP sans acclimatation préalable (AP-), en comparaison avec la souche adaptée au turbidostat (TA) et la souche adaptée au batch (BA). Les lignes pointillées illustrent les souches adaptées après une période de désadaptation de 3 mois, en comparaison avec une exposition prolongée à l'AP après l'adaptation (AP+).

Au cours de l'adaptation prolongée, la stérilité de la culture a été compromise, en particulier pour l'AT. Cette souche était fortement contaminée par des bactéries. Bien que les conditions axéniques soient difficiles à maintenir au-delà des conditions de laboratoire, et notamment lors du recyclage des effluents, il était important de déterminer si les résultats positifs obtenus étaient dus à la présence de bactéries ou à l'adaptation elle-même des microalgues. Pour cette raison, une deuxième expérience a été réalisée pour évaluer la tolérance des souches dans des conditions axéniques et en testant cette fois une concentration plus élevée d'AP (AP 1/200 et 1/100). Il est intéressant de noter que les performances des deux souches adaptées sont restées supérieures à celles de la WT en AP, même avec une concentration plus élevée d'AP (Figure A.7). La productivité de la biomasse de la WT a été compromise pendant la culture AP, avec un impact encore plus négatif en termes de productivité lipidique, car aucun signe d'accumulation de lipides n'a été observé. En revanche, les deux souches adaptées ont été capables d'avoir une performance supérieure même à des concentrations plus élevées d'AP. Pendant la culture avec AP 1/200, les souches adaptées ont présenté une productivité moyenne de biomasse de 0,193 (TA) et 0,245 g/L/d (BA), par rapport à la WT dans le milieu de contrôle 0,213 et 0,090 g/L/d dans AP. La souche TA était similaire à la WT dans le milieu témoin et a presque atteint une productivité lipidique similaire (TA : 0,098 g/L/d vs WT : 0,11 g/L/d). La productivité en lipides des deux dernières souches était presque 100 fois plus élevée que celle de la WT dans le milieu AP (WT : 0,001 g/L/d).

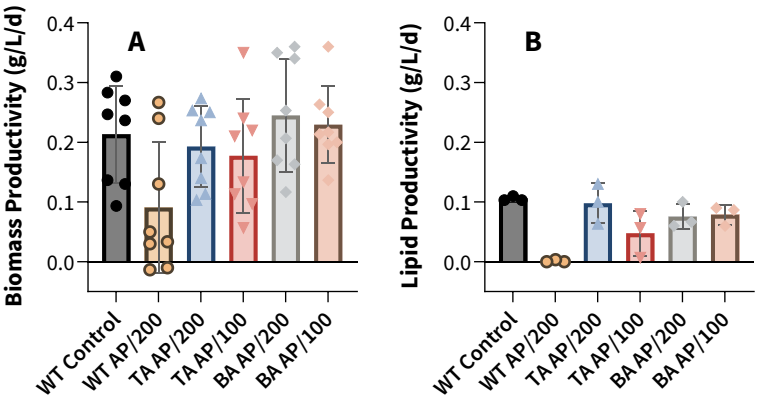


Figure A.7. Biomasse et productivité lipidique des souches adaptées (TA et BA) par rapport à la WT en milieu témoin et en présence d'AP (1/200). Les souches adaptées ont été cultivées sous une dilution de 1/200 et 1/100. Les points de données représentent différents moments de la culture.

CONCLUSION

L'interaction entre les conditions de culture et le métabolisme spécifique de six souches a résulté en différents profils biochimiques et lipidiques. La diversité métabolique observée, même parmi les mêmes espèces de *Chlorella vulgaris*, a démontré le potentiel de cette famille de microalgues pour diverses applications. *Chlorella vulgaris* NIES 227 a été identifiée comme une matière première riche en lipides idéale en raison de sa productivité lipidique supérieure et de sa faible teneur en précurseurs d'hétéroatomes. NIES 227 a également montré sa capacité à avoir une croissance robuste sous des conditions limitantes en nutriment et à tolérer les composés inhibiteurs de la phase aqueuse (AP). La combinaison de ces caractéristiques en fait une source prometteuse de biomasse pour les biocarburants. Lors de l'élaboration d'une boucle vertueuse de biomasse-substrat, plusieurs choses doivent être considérées et plus amplement expliquées. Une compréhension profonde des propriétés nutriment-biomasse a été établie, comme nous l'avons montré, une limitation nutritive équilibrée a provoqué l'accumulation de lipides, remarquablement chez NIES 227, avec une consommation complète de macronutriments.

La compréhension de ces interactions a permis de mettre en œuvre une stratégie similaire lors de l'inclusion de l'AP dans le milieu. Cela a permis la réduction de certains apports en nutriments. Un équilibre entre la division cellulaire et la famine cellulaire a été atteint dans les conditions de contrôle étudiées. Toutefois, la présence de l'AP a compromis le taux de croissance des microalgues et, par conséquent, le stress intracellulaire attendu pour déclencher l'accumulation des lipides a été compromis. Cela a entraîné une biomasse à faible contenu énergétique, en raison de la faible teneur en lipides et de la teneur plus élevée en précurseurs d'azote et d'oxygène. Cela indique qu'une accumulation lipidique et une croissance microalgale doivent être optimisées dans des substrats contenant de l'AP.

Malgré le fait que l'AP ait soutenu la croissance des microalgues, des productivités optimales n'ont pas été atteintes comme dans les milieux de contrôle. Après plusieurs cycles d'acclimatation, cette inhibition a été réduite mais n'a pas été complètement compensée. Des mesures supplémentaires sont nécessaires pour pré-traiter l'AP afin de diminuer l'inhibition de l'AP lors de sa mise en œuvre pour la culture de microalgues. Une adaptation prolongée s'est avérée être une stratégie efficace pour éliminer les effets inhibitifs de l'AP, tout en stimulant la croissance et l'accumulation lipidique des microalgues. La sélection et l'amélioration des souches sont essentielles pour produire toute application microalgale, mais restent stratégiques pour la production de sources d'énergie durables. En particulier, pour le HTL, où la souche sélectionnée devrait idéalement avoir une haute productivité lipidique tout en ayant une croissance robuste en présence de l'AP. L'augmentation de la tolérance à l'AP permettra aux microalgues de prospérer et de maintenir une productivité de biomasse stable tout en récupérant une plus grande quantité de nutriments.

Des recherches supplémentaires sont nécessaires pour comprendre la richesse et la complexité de l'AP. Cela facilitera la compréhension de ses effets sur la croissance et le métabolisme des microalgues. De plus, une meilleure valorisation de l'AP pourrait être envisagée. Les mécanismes de l'AP provoquant l'inhibition chez les microalgues sont une interaction complexe entre la composition globale de l'AP, la richesse et les concentrations des inhibiteurs, et la turbidité du l'effluent.

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7. Appendix B

Table B. 1.Nutrient consumption (nitrates, phosphates, sulfates) per strain, across time (days). The media preparation was done at day zero.

Nitrates (mg/L)	**Time (days)**	**NIES 227**	**CCALA 256**	**NIES 2173**	**CCALA 251**	**CCALA 253**
	0	521.1	521.1	521.1	521.1	521.1
	1	503.1	497.3	499.9	494.6	505.6
	2	389.6	409.7	191.2	435.7	370.2
	3	278.0	294.2	289.6	na	na
	4	263.4	251.5	240.4	289.5	220.3
	7	0.1	0.0	0.0	0.0	0.0
	9	0.0	0.3	0.1	0.0	1.2
	10	0.0	1.1	0.0	0.0	0.0
	11	0.3	0.2	0.0	0.0	0.0
Phosphates (mg/L)	**Time (days)**	**NIES 227**	**CCALA 256**	**NIES 2173**	**CCALA 251**	**CCALA 253**
	0	74.2	74.2	74.2	74.2	74.2
	1	66.5	61.0	58.1	63.1	47.0
	2	54.3	50.0	18.4	51.0	29.8
	3	48.7	45.3	33.3	na	na
	4	67.0	61.7	28.3	57.6	23.9
	7	21.7	12.4	0.0	14.4	0.0
	9	12.7	0.0	0.0	1.4	0.0
	10	4.3	0.0	0.0	0.0	0.0
	11	1.2	0.0	0.0	0.0	0.0
Sulfates (mg/L)	**Time (days)**	**NIES 227**	**CCALA 256**	**NIES 2173**	**CCALA 251**	**CCALA 253**
	0	33.5	33.5	33.5	33.5	33.5
	1	28.7	30.1	30.0	30.7	29.4
	2	22.0	24.4	12.7	26.2	23.9
	3	15.4	20.4	22.2	na	na
	4	15.0	22.1	24.8	22.3	21.1
	7	0.0	3.3	5.6	4.3	1.9
	9	0.0	0.0	2.7	1.3	0.0
	10	0.0	2.0	0.5	0.4	0.0
	11	0.3	0.0	0.0	0.0	0.0

Table B. 2. Results of the linear mixed effects models to test for the effects of cultivation time (day 4, 11, 18, 25), strain (NIES 227, CCALA 256, NIES 2173, CCALA 251, CCALA 253), and their interaction on SFA, MUFA, and PUFA. Significant results are in bold font.

	SFA				**MUFA**				**PUFA**			
	DFn	*DFd*	*F*	*P*	*DFn*	*DFd*	*F*	*P*	*DFn*	*DFd*	*F*	*P*
Time	1.7	12.5	169	**<.001**	1.22	8.93	299	**<.001**	1.29	9.44	23.6	**<.001**
Strain	4	10	2.56	0.104	4	10	348	**<.001**	4	10	1607	**<.001**
(T) x (S)	12	22	5.12	**<.001**	12	22	55.6	**<.001**	12	22	111	**<.001**

Table B. 3. Results of Tukey's multiple comparisons to test for the pairwise differences between strains (NIES 227, CCALA 256, NIES 2173, CCALA 251, CCALA 253) on the final cultivation day (day 25), for SFA, MUFA, and PUFA respectively. Significant results are summarized with an asterisk: * < 0.05 ; ** < 0.01 ; *** < 0.001.

	Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	P	Summary
SFA (Time = day 25)	227 vs. 256	-0.0531	-0.0760 to -0.0301	0.021	*
	227 vs. 2173	-0.0547	-0.0698 to -0.0396	0.003	**
	227 vs. 251	-0.0327	-0.0667 to 0.00123	0.052	ns
	227 vs. 253	-0.0608	-0.103 to -0.0188	0.03	*
	256 vs. 2173	-0.00163	-0.0354 to 0.0321	0.788	ns
	256 vs. 251	0.0203	-0.0345 to 0.0752	0.144	ns
	256 vs. 253	-0.00772	-0.0696 to 0.0542	0.404	ns
	2173 vs. 251	0.022	-0.00381 to 0.0478	0.065	ns
	2173 vs. 253	-0.00609	-0.0376 to 0.0254	0.483	ns
	251 vs. 253	-0.0281	-0.0547 to -0.00148	0.045	*
MUFA (Time = day 25)	227 vs. 256	0.304	0.290 to 0.318	0.017	*
	227 vs. 2173	0.344	0.337 to 0.352	<.001	***
	227 vs. 251	0.339	0.325 to 0.353	0.017	*
	227 vs. 253	0.386	0.354 to 0.418	0.009	**
	256 vs. 2173	0.0401	0.0240 to 0.0562	0.018	*
	256 vs. 251	0.0351	0.0340 to 0.0362	<.001	***
	256 vs. 253	0.0818	0.0380 to 0.126	0.021	*
	2173 vs. 251	-0.00498	-0.0212 to 0.0113	0.176	ns
	2173 vs. 253	0.0417	0.0127 to 0.0707	0.031	*
	251 vs. 253	0.0467	0.00285 to 0.0905	0.045	*
PUFA (Time = day 25)	227 vs. 256	-0.251	-0.256 to -0.246	0.005	**
	227 vs. 2173	-0.29	-0.299 to -0.280	0.003	**
	227 vs. 251	-0.307	-0.361 to -0.252	0.018	*
	227 vs. 253	-0.325	-0.336 to -0.315	0.004	**
	256 vs. 2173	-0.0385	-0.0532 to -0.0237	0.015	*
	256 vs. 251	-0.0555	-0.112 to 0.00127	0.052	ns
	256 vs. 253	-0.074	-0.0900 to -0.0581	0.015	*
	2173 vs. 251	-0.017	-0.0622 to 0.0282	0.152	ns
	2173 vs. 253	-0.0356	-0.0433 to -0.0278	0.001	**
	251 vs. 253	-0.0186	-0.0623 to 0.0251	0.134	ns