Evaluation of the Use of Microalgae as a Novel Process for the Valorisation of Nutrient Rich Digestate in a Context of Circular Economy

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DECLARATIONS AND STATEMENTS

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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Date **24/03/2023**.....

This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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SUMMARY

Nutrient rich digestate resulting from the anaerobic digestion of waste from different feedstocks (kitchen waste, manure, agricultural waste, etc.) is mainly used in Northwest Europe as a fertiliser, but its heavy usage has led to soil eutrophication and its spreading onto arable land is now restricted under the Nutrient Vulnerable Zones policy. Consequently, digestate has become an underused resource and novel technologies to remediate this waste are now needed. In this thesis, microalgae were evaluated for their potential to bioremediate digestate, within the context of a circular economy. Chapter 1 presents a summary of digestate characteristics and its current use and discusses the circular economy concept focusing on microalgae as a bioremediation tool.

Digestate is characterised by its dark colour and high dry matter content, which can be bottlenecks to light penetration and consequently microalgal growth, hence treatment is essential to allow for microalgal growth on this waste. Chapter 2 investigated mechanical and biological processing of a digestate from kitchen waste and aimed to assess its potential to support microalgal growth. Digestate was shown to be a suitable nutrient source for *Chlorella vulgaris*, following membrane filtration, which was highly efficient at separating liquid and solid fractions of digestate. 2.5% of digestate was the optimal concentration used in this work, as higher levels could lead to ammonium toxicity, therefore, this chapter also highlighted the bottlenecks of digestate utilisation for microalgal cultivation.

To improve digestate uptake by microalgae, chapter 3 tailored different digestate sources to *Chlorella vulgaris* and *Scenedesmus obliquus* while maintaining a low pH to increase ammonium availability by reducing evaporation. Digestate from pig manure provided the best growth results in both strains, and while ammonium availability was increased, acclimation of strains to high ammonium levels was still necessary, limiting the use of high concentrations of digestate in cultures. Composition analysis of the microalgal biomass showed that nitrogen starvation, caused by higher pHs and reduced ammonium lead to lipid increase. This chapter demonstrated the importance of digestate and strain pairing to improve remediation and enhance scalability of valorising digestate within a circular economy.

Growth of microalgae on digestate was demonstrated in chapters 2 and 3, to pursue the circular economy approach and use the produced biomass, chapter 4 subsequently aimed to assess the feasibility of using a microalgal hydrolysate derived from digestate as a feed ingredient in the feed of Nile tilapia. Enzymatic hydrolysis was used to alleviate palatability issues encountered when whole cells are incorporated into feed. An incorporation of 10% of the waste-derived hydrolysate did not convey advantages in terms of the fish growth but increased fatty acid content demonstrating a commercial advantage of the ingredient in terms of flesh quality.

Increasing digestate uptake by microalgae is essential to increase viability of the circular economy concept presented in chapter 2, 3 & 4. Consequently, chapter 5 investigated the potential of microalgae-bacteria consortia for this purpose, by studying the bacterial community associated to *Chlorella vulgaris* and the influence of nutrient availability on this dynamic association. Sequencing analysis revealed that there was a likelihood of growth-promoting and other advantages conveyed by bacteria growing concomitantly with microalgae and the use of bacteria to increase digestate remediation by microalgae was discussed.

The different lines of research investigated in this thesis demonstrated the feasibility of valorising nutrient rich digestate using microalgae by developing a circular economy approach. My thesis has also highlighted some of the bottlenecks to the establishment of a sustainable and commercially viable microalgal industry in the UK and globally, and additional research contributing to bridging this gap were discussed in Chapter 6, giving an outlook on future research prospects in the field of microalgae biorefineries.

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ABBREVIATIONS

ω-3: Omega 3
ω-6: Omega 6
AD: Anaerobic Digestion
ANOVA: Analysis of Variance
ASV: Amplicon Sequence Variant
DHA: Docosahexaenoic acid
DOC: Dissolved Organic Carbon
DW: Dry Weight
EPA: Eicosapentaenoic Acid
FAs: Fatty Acids
FAME: Fatty acid methyl ester
FTIR: Fourier-Transformed Infra-Red
GCMS: Gas Chromatography Mass Spectrophotometry
GLM: Generalised Linear Model
IAA: Indole Acetic Acid
IBA: Indole-3-butyric acid
IPA: Indole-3-propionic acid
MUFAs: Monounsaturated Fatty Acids
MWCO: Molecular Weight Cut-Off
N: Nitrogen
NRD: Nutrient Rich Digestate
NVZ: Nitrate Vulnerable Zones
NEW: Northwest Europe
OD: Optical Density
ONT: Oxford Nanopore Technology
OTU: Operational Taxonomic Unit
P: Phosphorus
PUFAs: Poly Unsaturated Fatty Acids
SFAs: Saturated Fatty Acids
VFAs: Volatile Fatty Acids
XRF: X-Ray Fluorescence

CHAPTER 1. INTRODUCTION

1.1. THE CIRCULAR ECONOMY CONCEPT

1.1.1. Definition of the circular economy

A circular economy is defined as "an economic system designed with the intention that maximum use is extracted from resources and minimum waste is generated for disposal" (Deutz, 2020), hence moving away from the traditional linear economy where manufacturing of an end-product can generate by-products, with little or no value to the remaining supply chain or for new applications. There is no evidence for a single origin of the circular economy concept at a precise time in history, however in just a few years, the concept has become a key principle for industrial development in the European Union (Völker et al., 2020), the United States (ReMade Institute, 2021), Africa (World Economic Forum (WEF), 2020) and China (Winans et al., 2017; Zhu et al., 2019). The circular economy also aligns with the UK government's Net zero objectives by potentially limiting the generation of carbon waste from several industries of economic importance for the UK. A circular economy, or closing the loop within an industrial process, can be implemented in several ways. In this thesis, focus is made on a circular economy for which waste from an industry can be used to generate novel products, themselves re-introduced within the supply chain to generate additional value.

1.1.2. Examples of circular economy

To illustrate the concept of circular economy, a few examples from previous research are presented below, and for which, emphasis was made on the utilisation of waste within an industry to generate added value.

Recovery of fish waste is crucial in a growing aquaculture sector. To illustrate, Laso et al. (2018) re-used heads and spines from the processing of anchovies to produce fish meal which can then be used in aquafeed for various species of interest to the aquaculture sector. Another study by de la Caba et al. (2019) successfully transformed fish by-products into biodegradable packaging. A further application linked to animal farming includes a study from Berbel & Posadillo (2018), who showed that using waste from the olive oil manufacturing chain for cattle feed could produce milk with a lower saturated fat content,

resulting in improved milk quality for a reduced feed cost. The circular economy can be applied in other sectors, for example pineapple residue has been used in wound healing, drug delivery applications and medical implants, due to the biocompatibility, low cytotoxicity, and biodegradability of nanocellulose fibres found in the leaves (Banerjee et al., 2018). Vermicomposting is also an example of circular economy, for which waste are transformed into organic fertiliser, additionally, produced earthworm have potential to be used in animal feed thanks to high protein content (Rorat and Vandenbulcke, 2019; Tedesco et al., 2019).

Anaerobic digestion also constitutes an application of the circular economy concept, by converting agricultural by-products into biogas and electricity. For example, Hoehn et al. (2019) describes landfills used for food waste as biogas and energy generators via anaerobic digestion, however anaerobic digestion itself can generate by-products which will be discussed in the following sections.

1.1.3. Limitations and bottlenecks of the circular economy

The circular economy concept has been widely endorsed by industries, governments, and academia in many fields of research and for a vast array of applications. Therefore, it appears to be a logical approach towards sustainability in industry and aligns with objectives aiming to tackle pressures caused by climate change. Despite this, current policies remain vague and lack precise descriptions of how a circular economy can fully replace a linear one (Gregson et al., 2015), therefore, the trade-offs and potential problems of leaving a linear economy are often overlooked (Völker et al., 2020; Lazarevic & Valve, 2017).

Additionally, the word “circular” is misleading as a circular economy is far from being a strictly closed loop in which materials and products are recycled indefinitely. Indeed, the energy required for a circular economy can itself result in significant dissipation into the environment (Korhonen et al., 2018); contamination (Baxter et al., 2017) and wearing down of materials (Parrique et al., 2019) which also limit the application of a closed loop. This is particularly true for the example mentioned above of anaerobic digestion of waste providing a novel source of energy, but also a novel source of waste in the form of digestate.

Scalability of processes used in a circular economy can also constitute a significant challenge, indeed many technologies used in the remediation of waste are extremely costly

(*e.g.,* nanofiltration, desalinisation), and upfront investment costs can discourage industries from implementing circularity. Finally, and especially when considering waste, regulations linked to animal feed and food production systems can be justifiably strict, but also not up to date with the latest development and resulting novel products. Efforts should be made to develop circularity in accordance with policy makers to allow for progress and address this major bottleneck.

The circular economy concept is not a perfect loop for which no waste is produced in any manner, however this concept has been generating significant drive for stakeholders to consider more seriously their waste generation and is a promising approach to tackle current environmental issues, with the caveat of scalability and regulation to support the overall process.

1.2. ANAEROBIC DIGESTION OF WASTE: ENVIRONMENTAL IMPACT AND CURRENT SOLUTIONS FOR TREATMENT

1.2.1. The anaerobic digestion process and resulting digestate

The anaerobic digestion process

With the increase of global industrialisation, a considerable amount of waste is being generated. Consequently, it is necessary to find solutions to treat this waste to limit anthropogenic impact on natural ecosystems and environments. Waste can be biologically treated to produce biogas that is reused for energy purposes: this is the anaerobic digestion process that is a technology widely used for the treatment of carbon-rich organic waste. Anaerobic digestion (AD) is a biological process during which organic matter (*e.g*., food, animal, or agricultural waste) is converted by bacterial and archaeal communities cooperating to form a stable and self-regulating fermentation process that transforms the organic matter into biogases, primarily composed of methane (CH_4) and carbon dioxide (CO_2) (Doble and Kumar, 2005). This process occurs in a tank called a digester and can be conducted mesophilically at a temperature ranging from 38°C to 41°C or thermophilically at 52°C. The volume of biogas produced is dependent on the type of waste and its composition, as well as the quantity of waste treated and the operating temperature of the digester. Anaerobic digestion can be divided into six stages (Jeyaseelan, 1997): hydrolysis of

complex organic biopolymers into monomers; fermentation of amino acids and sugars; anaerobic oxidation of volatile fatty acids and alcohols; anaerobic oxidation of intermediary products such as volatile fatty acids; conversion of hydrogen to methane; conversion of acetate to methane. Commonly the AD process is divided into hydrolysis, acidogenosis, acetogenosis and methanogenosis stages. The resulting biogas is converted into electricity by a co-generator or directly used by injection after purification. Another by-product of the AD process is a nutrient rich liquid digestate with a high dry matter content (**Figure 1.1**).

Composition of digestate

The composition of digestate can be strongly impacted by the waste from which it is derived but also by geographical location and seasonality (Malhotra et al., 2022). Additionally, nutrient rich digestate can have an extremely varied composition, with many elements not characterised yet, such as humic compounds (Fernandes et al., 2020). In this work, the interest of digestate lies in its nutrient composition, mainly nitrogen and phosphorus. Nitrogen content of digestate can be highly variable, which is again related to the diversity of feedstock used in the anaerobic digestion process. Additionally, nitrogen is essentially found in the form of ammonium in digestate (Malhotra et al., 2022). Phosphorus, on the other hand is found in lower concentrations, ranging from 5.1 mg/L (Labbé et al., 2017, digestate source: cattle farm waste) to 716 mg/L (Massa et al., 2017, digestate source: buffalo farming wastewater enriched with whey). Additionally, phosphorus is mainly bound to the solid fraction of digestate (Gerardo et al., 2015), emphasising the importance of feedstock and its influence on digestate composition. Heavy metals are also commonly present in digestate, with elements such as copper, zinc or iron found in trace amounts (Kupper et al., 2014), which can be utilised as oligo-elements for fertilising purposes. Macro-elements such as potassium and calcium can be found in high concentrations in digestate and convey excellent fertilising properties to the product (Levine et al., 2011), with, for example concentrations of potassium ranging from 102 and 3389 $mg.L^{-1}$ in various digestates . Finally, volatile fatty acids (VFAs) are also found in significant amount in digestate (Huang et al., 2016; Weiland et al., 2010), and can range from 697.1 $mg.L^{-1}$ to 13.9 $g.L^{-1}$ (Markou, 2015; Olguín et al., 2015).

Physical properties of digestate

Digestate resulting from the anaerobic digestion of waste is often described as a thick dark sludge. Indeed, dry matter content of digestate can reach 6% (Fernandes et al., 2020). Particle size is also extremely varied, with some digestates being fully liquid and others with non-digested waste, such as grass, silage and other solid compounds. Additionally, digestate is characterised by an extremely dark colour and high turbidity (Wang et al., 2021). Whilst turbidity is linked to particles such as finely dispersed inorganic and organic compounds, colour of digestate is often caused by soluble organic compounds (Tawfik et al., 2022), and the resulting colour of digestate is mainly linked to feedstocks and the AD process itself; it is also believed that the combination of different humic compounds (which have not been fully characterised) could be responsible for the digestate colour. Digestate is also characterised by a high pH of 8 or 9 which is mainly linked to the degradation of VFAs which results in the production of ammonia (Vasnika & Badalikova, 2019).

Composition and properties of digestate described above demonstrate that treatment of digestate is essential when considering its incorporation within a circular economy, as its intrinsic properties can be incompatible with many applications that will be discussed further is the following section.

1.2.2. Current use and treatment of digestate

Current use of digestate

Digestate is currently used as soil fertiliser, and its spreading on arable land is a widely adopted method for closing the nutrient cycle, but also to substitute the use of fossil fertilisers, which are the main two drivers for this common practice (Logan & Visvanathan, 2019). The solid fraction of digestate is usually composted and used as a multifunctional soil improver in agriculture and horticulture or for topsoil production. The liquid fraction of digestate is applied on land, using trailing or injection methods to limit the time of exposure of digestate to air, which can result in evaporation and significant air pollution as well as loss of valuable nutrients (Logan & Visvanathan, 2019). Application of the “whole digestate” onto land remains the preferred method but only digestate of high quality can be used for this purpose, which results in the need for pre-treatment or extensive storage when seasonal restrictions are implemented depending on the type of crops fertilised (Logan &

Visvanathan, 2019). Generally, crops for which digestate is used for fertilising purposes are located close to the digester, to avoid transportation cost. When transportation is needed, solid and liquid fractions of digestate are separated and further concentrated to facilitate transportation. Finally, digestate has also been used in vermiculture, producing high-quality earthworm compost, and excess worms can be used as feed additives in pig, chicken, fish and shrimp production. This practice, however, is only applied in China at present as European legislations and public acceptance do not allow for an animal waste to be re-introduced into an animal feed (Bell, 2014; Gómez-Brandón et al., 2016). Digestate has also been investigated as a substrate for the production of pyrochar which can then be used as soil amenders (Monlau et al., 2015). Pyrochar is a charcoal-like residue obtained after pyrolysis which has seen growing interest as it plays a significant role in atmospheric carbon sequestration (M. Zhang & Ok, 2014), but its utilisation mainly relies on its porous-like structure which allows for chemical absorption and increased surface for compound exchange within soils (Ahmad et al., 2014). For example, digestates resulting from the digestion of dairy waste and whole sugar beet have been used for this purpose and showed a higher accessible surface in comparison to pyrochar from raw feedstock (Inyang et al., 2012).

Digestate has also been used in energy production, firstly by being recirculated into digesters to increase biogas production and allow for maximum utilisation of feedstock (Muha et al., 2015; Sambusiti et al., 2015; Seppälä et al., 2013). This option is widely spread as storage of digestate or spreading on land, both in the open air, can generate significant amounts of methane which then contributes to greenhouse gases emissions (Gioelli et al., 2011; Sambusiti et al., 2013). Digestate can also be used in bioethanol production, via biological fermentation, as digestate has a high content in cellulosic fibres, however additional treatments are required as some of these fibres are not easily degradable (MacLellan et al., 2013; Monlau et al., 2015; K. Wang et al., 2014). Finally, thermal conversion of digestate for fuel production has also been studied, with processes such as combustion, hydrothermal carbonisation or pyrolysis being investigated, but few studies have reported the environmental impact of these processes (Monlau et al., 2015).

Treatment methods for digestate

Treatment of digestate have been alluded to in the previous section as many of the applications described require a treatment step and raw digestate is rarely usable for direct applications. In this section, the described treatments of digestate are mainly aimed towards its utilisation as a nutrient substrate for microalgae cultivation, however most of the described treatments are also common in other utilisations of digestate.

The importance of separating liquid and solid fractions of digestate has been mentioned previously, and this treatment of digestate is required to either use both fractions for different processes or to remove the solid fraction of digestate which contains many contaminants and, when considering microalgal cultivation, constitute the main obstacle for light penetration in cultures, and hence photosynthesis (Wang et al., 2010). Settlement of digestate has been one of the first method implemented to separate liquid and solid fractions, as it requires little effort and is highly cost-effective. For example, Godos et al. (2009) showed that a digestate sedimentation at a residence time of 5 days reduced the total of suspended solid by 70%, however when considering processes for which a high quality liquid fraction of digestate is required, settlement alone is not sufficient. Dilution has been combined with settlement to reduce amount of solids present in digestate, and as a way to solubilise some of the mineral precipitates present in digestate, to release compounds of interest for diverse applications (Wahal & Viamajala, 2016). Water utilisation for this process should however be carefully considered to avoid unnecessary wastage and recycling processes would be highly valuable to implement.

Mechanical separation of digestate fractions is more costly but provides higher efficiency, centrifugation is one of these processes and has been shown to result in a clear liquid fraction with solids collected in a pellet. Rotation speed, temperature and centrifuging time are important factors to implement while using this technique and several studies have used centrifuging to treat their feedstock for algal cultivation (Marcilhac et al., 2015; Markou, 2015; Singh et al., 2011; Tao et al., 2017; Y. Wang et al., 2015). The centrifugation process is often associated with further processing steps such as flocculation or decantation to facilitate the separation of liquid and solid fractions in digestate. Combining several methods for the treatment of digestate has been reported in many studies (Cicci & Bravi, 2014; Ledda et al., 2016a; J. Xu et al., 2015; Zhou et al., 2012) as this provides the best

results in terms of solids removal and in some cases can provide higher contents of nutrients and other compounds of value (Wahal & Viamajala, 2016).

Membrane filtration is also highly advantageous for the treatment of digestate, as it allows for the removal of most particles and large microorganisms (*e.g.,* protozoa) potentially present in the digestate. This technique consists in the filtration of a product through a fine material (or membrane) using a combination of high pressure and small pore size in order to retain any particles but also bacteria, providing a mechanically sterilised permeate (*i.e.,* liquid obtained after membrane filtration). Membrane filtration has also been used for nutrient recovery from digestate (Gerardo et al., 2015). Filtration has been widely used for pre-treating digestate used for algal cultivation (Ledda, et al., 2016b; Massa et al., 2017; J. Park et al., 2010; L. Wang et al., 2010; Wen et al., 2017) but it remains a costly process which limits its scalability potential.

Many other pre-treatments of digestate can be used, such as autoclaving, acidification, alkalisation, precipitation, or flocculation. Autoclaving has been used in some studies in order to sterilise digestate (Dickinson et al., 2015; Hollinshead et al., 2014; Koreivienė et al., 2014; Kumar et al., 2010), and while this technique is very efficient for small volumes of digestate, it is unrealistic for high volumes. Another process used to treat digestate is flocculation, which combined with filtration can facilitate the elimination of dry matter contained in the liquid digestate (Veronesiv et al., 2017). However, the flocculation process involves the use of chemicals such as iron sulphate, which if used in high volume could be incompatible with large-scale applications. Alkalinisation and acidification of digestate are also methods used to treat digestate and more specifically to induce release of nutrients in the liquid fraction as well as for increasing flow rate when filtering digestate on membranes. For example, Gerardo et al. (2015) showed that a pH of 11 significantly increased the permeate flow rate during ultra-filtration and that a basic pH facilitated the extraction of nitrogen and phosphorus during the filtration process.

There are many treatments available to process digestate for further applications, however many of the treatments discussed here can be costly and require additional pre or post treatment to obtain high quality digestate. Consequently, scalability of these processes is extremely challenging and demonstrates the need for novel, low-cost treatment of digestate to incorporate this waste source into a circular economy.

1.2.3. Environmental impact of digestate utilisation

As described above, digestate is mainly used as a soil fertiliser, and is either spread on land as a whole or liquid and solid fractions are segregated and used separately. This practice is widespread and aligns with a circular economy approach by returning vital nutrients to the supply chain. In addition nitrogen and phosphorus, the main nutrients found in digestate, can also reduce soil erosion (Slepetiene et al., 2020). This technique also increases economic viability of the biogas industry and reduces reliance on chemical fertilisers (Möller & Müller, 2012).

Very few long-term environmental and human health assessments have been performed in relation to the spreading of digestate on arable land, but is has been established that longer air exposure due to improper spreading techniques, or lack of uptake by crops result in the loss of nutrients by evaporation, but also by nutrient-run-off into deeper soil layers and into groundwater (Formowitz and Fritz, 2010). Additionally, disposal of digestate is extremely problematic and highly regulated, as it can contain compounds toxic to the environment (Jomova & Valko, 2011; Silkina et al., 2017), such as heavy metals or persistent organic pollutants but also medicines and antibiotics for which the bioaccumulation within ecosystems is still unknown (Gerardo et al., 2013). Other challenges for the sustainable management of digestate include the non-biodegradability of some of the organic matter found in digestate, the presence of organic pollutants and pathogenic bacteria (Shargil et al., 2015). Some of these challenges can be addressed by long storage period before utilisation of digestate, but due to a significant liquid fraction, storage can be very expensive and difficult to implement for the volumes of digestate created by large scale digesters.

Finally, the significant soil and water eutrophication resulting from the use of digestate on land (Guilayn et al., 2019), has led to the designation of Nitrate Vulnerable Zones (NVZs) under the European Nitrate Directive 91/676/CEE (European Community, 1991). This directive limits the annual load of nitrogen applied onto farmland and NVZs have been on the rise across Northwest Europe, resulting in the accumulation of approximately 10 million tons of excess digestate (Fuchs & Drosg, 2013), which again represents a significant cost to store or burry in slurry pits following the appropriate regulations. Consequently, there is a significant need to develop novel management systems for digestate, with the objective to integrate this underused resource into a circular economy.

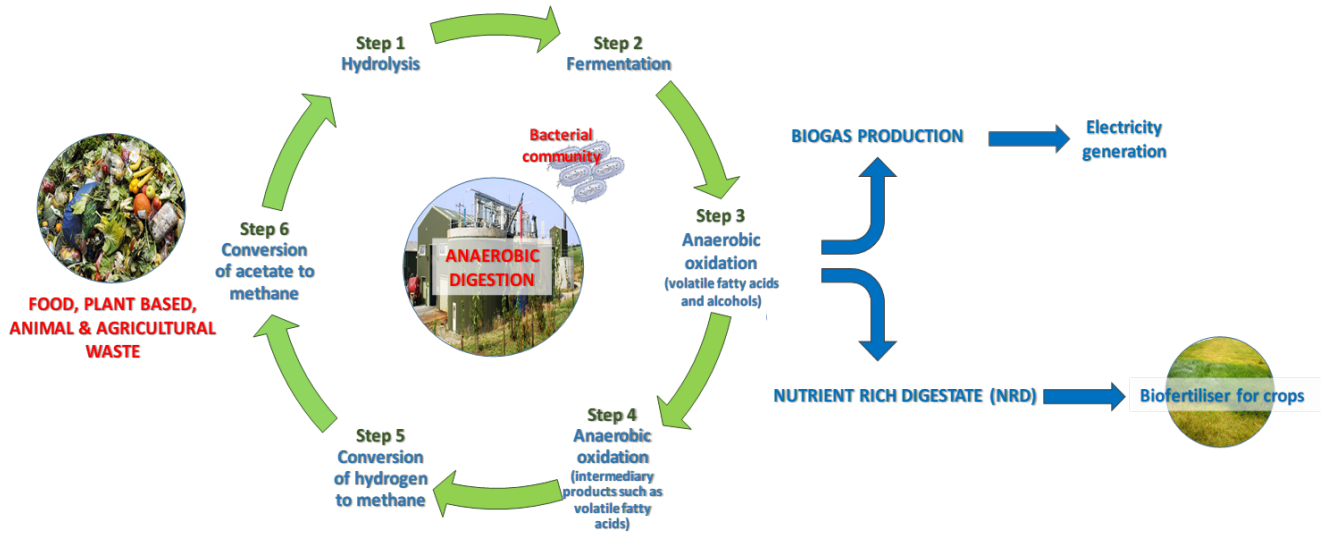

Figure 1.1: Summary of the anaerobic digestion process and current utilisations of digestate for the biogas industry and land fertilisation.

1.3. MICROALGAE FOR WASTE REMEDIATION

1.3.1. Background

Microalgae are photosynthetic microorganisms, found in a range of freshwater and saline environments and responsible for more than 70% of the global oxygen production (Vale et al., 2020). Their capacity to capture carbon for photosynthesis has made them very popular as a novel process to meet decarbonisation objectives in the current climate. Microalgae are also able to recover many types of waste, thanks to their ability to uptake nutrients such as nitrogen and phosphorus, which are found in many waste sources (Judd et al., 2015), but also for their capacity to accumulate heavy metals and other toxic compounds (Torres et al., 2017). Additionally, microalgae are of significant value for the food and feed sectors, thanks to their richness in bioactive compounds and their high protein content (Gifuni et al., 2019; Ledda et al., 2016a). Cultivation of microalgae at commercial scale has been heavily implemented in the past decades, mainly in warm countries with high solar irradiances. Several cultivation systems are used worldwide, but open ponds and closed photobioreactors are the two principal cultivation systems used (Uggetti et al., 2018). One of the major bottlenecks to high scale production and commercialisation of microalgae worldwide is the high investment costs required to set-up microalgal cultivation facilities,

consequently, using nutrients from waste sources, in addition to representing an eco-friendly process, also has potential to alleviate some of these production costs.

1.3.2. Microalgae for wastewater bioremediation

Microalgae are of particular interest for the bioremediation of wastewater, because they can remove toxic substances from their surroundings via their various ways of nutrition: heterotrophy, mixotrophy and photoautotrophy (Goswami et al., 2021; Jaiswal et al., 2020; Lima et al., 2020; Mohd Udaiyappan et al., 2017), allowing them to accumulate, absorb and metabolise these substances. Additionally, they can thrive on wastewaters by using nutrients such as phosphorus and nitrogen, which are present in abundance (Xia & Murphy, 2016). Indeed, microalgae have been found to be an efficient process for the remediation of textile wastewaters, effluents of the mining and pharmaceuticals industries, and effluents from tanneries. For example, de Cassia Campos Pena et al. (2019), showed that microalgae were able to recover 99.9% of $N\text{-}NH_3$ and 91.75% of $P\text{-}PO_4^-$ from tannery wastewaters, while reaching a biomass production of 1.7 $g.L^{-1}$.

Microalgae have also been investigated for their role in agricultural waste remediation, for example the microalgae *Nannochloropsis oculata* was able to remediate fully the pesticide lindane from agricultural run-off (Pérez-Legaspi et al., 2016). Dairy waste, which is very rich in nitrogen, in the form of proteins, nitrates, and phosphorus, is costly to process and thus microalgae are a promising process for their remediation. Kiani et al. (2023) showed that *Nannochloropsis oceanica, Chlorella vulgaris* and *Tetradesmus obliquus* were able to remediate 88% of proteins by breaking them down into smaller compounds for nutritional uptake, and that after 21 days of cultivation, all nitrates present in the dairy wastewater were fully remediated. Additionally, the microalgal biomass obtained after remediation can be of added-value, Pandey et al. (2019) showed that the microalgae *Scenedesmus sp.* grown in dairy effluents removed 100% of nitrate and 98.63% of phosphorus, but was also able to increase its lipid productivity up to 31.16 $mg.L^{-1}.d^{-1}$. A review by Vieira Costa et al. (2021) discussed that the use of dairy effluents to support microalgal growth could reduce the cost of production by 50%, which is an additional advantage of bioremediation as the scalability of microalgal products and their commercialisation is currently limited by the need for heavy upfront investments.

Remediation of aquaculture wastewater has also been investigated, as increasing eutrophication of natural waterbodies has been a significant issue in the recent years. Algal pond systems are more and more commonly implemented in shrimp aquaculture (Iber & Kasan, 2021), with the expectation that the produced biomass could generate additional revenue for the aquaculture sector. Kishi et al., (2018) showed that such systems were able to remediate 100% of ammonium, nitrate and phosphate from the wastewater, as well as up to 80% of organic matter. Another study by Tossavainen et al. (2019) showed that *Euglena gracilis* and *Selenastrum* sp. grown on pike perch wastewater achieved almost 100% of nitrogen and phosphorus removal, in addition the resulting microalgal biomass content in EPA and DHA were suitable for their incorporation into aquafeed, demonstrating potential as alternative for fish oil in feed.

1.3.3. Microalgae for digestate remediation

The literature shows that the main liquid digestates used for algal cultivation comes from Dairy, pig and poultry manure and litter as well as from food waste (Xia & Murphy, 2016). As described in the previous section, microalgae are also valuable in digestate treatment for their ability to incorporate nitrogen and phosphorus, found in high concentrations in digestate. Physical properties of digestate, namely its dark colour, high dry matter content and high ammonium concentration toxic to microalgae represent a bottleneck to the remediation of this waste. Pre-treatment methods are essential to alleviate inhibitory factors (Al-Mallahi & Ishii, 2022) and currently used techniques have been described in section *1.2.2*., but filtration, dilution and centrifugation remain the preferred treatments to allow for digestate to be used as a growth medium for microalgal cultivation. Pathogens can also present an issue for the remediation of digestate by microalgae (Xia & Murphy, 2016), but the treatments mentioned above also allow for sterilisation of digestate, via mechanical separation or high temperature treatment.

Following treatment, digestate has been found to provide a suitable growth medium for microalgae, Rude et al. (2022) demonstrated that several species of green microalgae could be cultivated on digestate resulting from the processing of food waste, with more than 95% recovery of nitrogen and phosphorus. *Dunaliella tertiolecta* and *Cyanobacterium aponinum* successfully removed 80–98.99% of total nitrogen and 65% of total phosphorus from food waste digestate, and had productivities of 0.88 and 0.34 g/L/day, respectively (Wu et al.,

2020). Other sources of digestate have been studied, for example, Hu et al. (2021) showed that green microalgae could recover nitrogen and phosphorus from liquid digestate from pig manure. These studies demonstrated the feasibility of using microalgae for digestate remediation and they discuss the need for pre-treatment of digestate to do so, however not much is found in the literature regarding the need to investigate digestate composition and tailor digestate sources to microalgal strains to improve uptake of digestate. Indeed, in most of the studies presented, while recovery of nutrients was almost total, this was only true for small amounts of digestate used in small scale cultures, but excess digestate is an increasing issue, with a vast amount of digestate currently stored and underused across Northwest Europe. Therefore, solutions must be developed to optimise the uptake of digestate by microalgae to increase significance and stability of this remediation process.

1.3.4. Use of waste-derived microalgal biomass – status and challenges

Within a context of circular economy, microalgal biomass produced from waste streams, and in the rest of this section on digestate, should be re-integrated into the process loop to bring added value to novel or existing applications.

Microalgal biomass grown on digestate can be used for bioenergy production (Bauer et al., 2021). Indeed, the lipid content of microalgae make them a good candidate for biofuel production, and while production cost has been a major bottleneck for this application, the use of waste streams alleviates some of the economic pressures. Bioethanol production is also a route for the use of digestate-derived biomass, as many strains have high carbohydrate content (Koutra et al., 2018; Martín-Juárez et al., 2017), and no lignin, which provides a significant advantage in comparison to other feedstocks. Additionally, biomass produced from digestate can simply go back into digesters, as microalgae can provide a good substrate for anaerobic digestion and associated bacterial population, producing satisfactory biogas yields (Gruber-Brunhumer et al., 2016), however, seasonality can impact efficiency of microalgal biomass as an anaerobic digestion feedstock (Bauer et al., 2021). Additionally, while waste is not discharged into the environment or stored for extensive periods of time in this kind of application, it could be argued that waste is only re-circulated and potentially not generating as much value as for other applications, for which novel products are obtained from the resulting biomass.

A biorefinery approach appears more suited to bring added value to digestate, and several models have been put forward. The cyanobacteria *Synechocystis sp*. has been investigated for its ability to produce the biodegradable biopolymer PHB (polyhydroxybutyrate) which could replace commercial plastics (Samantaray et al., 2014). The strain was investigated for its capacity to grow on digestate and PHB production was achieved (Kovalcik et al., 2017), demonstrating a pipeline for the generation of high-value compounds from digestate-derived biomass. Microalgal pigments also fall in this category, such as phycocyanin or carotenoids which are in high demand in the cosmetic, feed additive and food industries (Acien et al., 2017). *Chlorella vulgaris* and *Scenedesmus sp* were shown to produce more carotenoids when grown on diluted digestate (Koutra et al., 2018), however, the use of biomass grown from digestate for human health is questionable and current regulation needs to address this clearly.

Microalgal biomass grown on digestate also has potential to be used in animal feed, thanks to its high content in proteins, carbohydrates, and lipids, but also thanks to its anti-inflammatory and antioxidant properties of relevant interest in animal health (Hayes et al., 2017). For example, there is tremendous scope to use microalgae in aquafeed, for rotifers and artemia, but also directly for fish feed (Bauer et al., 2021). However, and similarly to food applications, there are requirements that novel products have to meet in terms of safety, hygiene and public health (Koutra et al., 2018). Levels of contaminants must consequently be tested before introducing a microalgal ingredient into a feed (Regulation (EC) No 258/97) and the use of digestate as a nutrient source remains a grey area in the legislation as it is unlikely that digestates from animal waste sources would be authorised within the supply chain and re-introduced under the form of a microalgal ingredient. However, other feedstock of digestate, such as plant waste, could be considered for such applications.

Microalgal biomass produced from waste digestate can be used in diverse applications, by bringing high-value products to the market conveying many benefits to the health, food, and feed sectors, however there are still challenges, primarily regulatory aspects which present a bottleneck to the implementation of circularity.

1.3.5. Hydrolysis for incorporation of microalgal ingredients into animal feed

At present, microalgal ingredients are incorporated as whole cells or specific extracts into animal feed (Madeira et al., 2017). Whole cells are a straightforward incorporation process but can cause issues related to palatability of feed and contaminants might still be present in the biomass, which is especially true when considering digestate as a nutrient source for microalgal cultivation. On the other hand, pure extracts are expensive to produce and constitute an additional challenge to the scalability of using microalgae for animal feed (Rizwan et al., 2018). Hydrolysis of microalgal biomass constitutes a promising third approach, as it allows for fractionation of proteins into smaller compounds and commercial enzymes are widely available at low cost (Sarker et al., 2020).

Chemical hydrolysis has been implemented on microalgal biomass for third-generation biofuel production, to release sugar molecules from the biomass and transform complex carbohydrates into more accessible and usable compounds (Debnath et al., 2021). Resulting sugars have been successfully used in bioethanol production, for example Seon et al. (2020) used acid hydrolysis of *Chlorella sp* for this purpose, however, chemical hydrolysis, which is performed at high temperature and pH can lack sensitivity and specificity, thus destroying some essential amino acids (Sharma et al., 2020). Enzymatic hydrolysis is performed at lower temperatures and controlled pH, and it usually uses protease mixtures; it has a higher specificity, and results in higher yields and purity of targeted compounds (Lucakova et al., 2022). Enzymatic hydrolysis on microalgal biomass results in functional peptides which have mainly been studied for their anti-inflammatory (Vo et al., 2013) and anti-oxidant (Ko et al., 2012) properties for health applications, but also for cosmetic.

The incorporation of hydrolysate into animal feed has been under-studied and the literature only discusses the potential of these ingredients, in terms of protein uptake and relevance of functional peptides (Nagarajan et al., 2021), however no literature was found on animal feed trials using hydrolysates from microalgal biomass, and let alone hydrolysates derived from a waste source. Consequently, there is significant scope to develop this line of research to provide additional value to microalgal biomass grown on waste. Additionally, it can be hypothesised that enzymatic hydrolysis could help to alleviate some of the hurdles linked to using digestate to produce animal feed ingredients, namely the presence of contaminants

that could be degraded by the hydrolysis process and encourage regulatory bodies to consider this approach within a circular economy.

1.4. Microalgae-bacteria consortium and their potential in waste bioremediation

1.4.1. Interaction between Microalgae and bacterial populations

It has been widely recognised that bacterial communities develop concomitantly with microalgae in natural environments (Amin et al., 2015; Natrah et al., 2014) and in culture (Subashchandrabose et al., 2011). The development of these bacteria is often characterised as contamination and can lead to culture decline, especially when algae are grown commercially for specific high value compounds, however, obtaining bacterial-free (*i.e.,* axenic) cultures is a challenge in culture systems and is a costly process, especially when upscaling to commercial production volumes (L. Xu et al., 2009). Consequently, there has been a growing body of literature looking more closely at the dynamic between microalgae and bacteria in culture, firstly to understand this complex relationship, but also to assess its potential for optimisation in culture processes.

Ramanan et al. (2016) suggested that the Phycosphere was one of the most ignored and distinctive habitats for bacteria, however this microenvironment is where most of the interactions between algae and bacteria take place. These interactions can be facultative or obligate and are categorised into nutrient exchange, signal transduction and gene transfer (Kouzuma & Watanabe, 2015).

Positive interaction between microalgae and bacteria

Bacteria can influence microalgal behaviours, including growth stimulation, morphogenesis and colonisation behaviours (Kouzuma & Watanabe, 2015). For example, microalgal bio-flocculation is facilitated by bacterial production of proteins and exopolysaccharides (Wang et al., 2016), benefiting the harvesting process in cultivation systems, showing a commercial interest of the microalgae-bacterial symbiosis. Substrate exchange is one of the main drivers for the existing interactions between microalgae and bacteria. Knowing that microalgae do not possess a nitrogen-fixing mechanism, associated bacteria facilitate the nitrogen integration by microalgae by reducing NO_2 and NO_3 into more assimilable nitrogen forms

such as ammonia. Furthermore, bacteria such as *Escherichia coli, Pseudomonas sp.* and *Bacillus sp.* were proven to provide inorganic phosphate to microalgae (Jiang et al., 2007).

Other than macronutrients exchange, bacteria also provide micronutrients essential to microalgal growth, such as vitamin B12 (Croft et al., 2005; Kazamia et al., 2012). Vitamins play an important role in the algae-bacteria interaction, as many microalgae are incapable of producing important vitamins for their growth, including cobalamin (Vitamin B12), thiamine and biotin (Natrah et al., 2014). Bacteria can induce an increase in chlorophyll content, a higher biomass production and they can result in more stable microalgal culture with delayed death phase (Bruckner et al., 2011; Y. Park et al., 2008; Thi Vu et al., 2010).

On the other hand, microalgae provide oxygen and DOC (Dissolved Organic Carbon) through photosynthesis, which are used by bacteria. The bacteria can also take advantage of microalgal dead cells and use them as a source of organic carbon. Bacteria can also play a detoxifying role by removing products such as hydrogen peroxide, which are produced during photosynthesis by the microalgae. Hünken et al. (2008) demonstrated this activity in several genus of *Proteobacteria*. It was also showed that aerobic bacteria such as *Pseudomonas* could reduce photosynthetic oxygen tension within the phycosphere (Mouget et al., 1995).

Negative interactions between microalgae and bacteria

The nature of the interaction between algae and bacteria can also be negative. For example, Desbois et al. (2008) characterised the antibacterial metabolites produced by the diatom *Phaeodactylum tricornutum* and found that different types of fatty acids, palmitoleic acid and hexadecatrienoic acid were responsible for the growth inhibition of various Gram-positive bacteria, however, the authors did not know if this activity was limited to specific bacteria or if a broad spectrum of bacterial strains was targeted (Natrah *et al.*, 2014). It has also been shown that bacteria can demonstrate algicidal activity that could be caused either by the release of algicidal compounds in the environment or by lysis of the microalgae cells (Mayali & Azam, 2004; B. Zhang et al., 2020). As for the antibacterial activity in microalgae, it was not clear whether the algicidal activity of bacteria was limited to certain microalgal species or whether it can also affect other species (Mayali & Azam, 2004).

Influence of abiotic parameters

There are a variety of relationships between microalgae and bacteria, mutualism being the most investigated, however commensalism also occurs and there is a thin line between both relationships (Ramanan et al., 2016). Indeed, many studies hypothesised that the change from mutualism to commensalism and vice-versa and further to competition between kingdoms was strongly related to changes in environmental factors (Bruckner et al., 2011; Sher et al., 2011). For example, Guo & Tong (2014) demonstrated that the relationship between *Chlorella vulgaris* and *Pseudomonas sp.* was changed from mutualism to competition when the cultivation conditions were shifted from photoautotrophic to photoheterotrophic. Grover (2000) demonstrated that a decrease in the DOC: P ratio could as well provoke a shift from mutualism to competition in a *Scenedesmus-Pseudomonas consortium*. Another example has been demonstrated by Grossart & Simon (2007) who showed that different bacteria affected the growth of *Thalassiosira rotula* very differently, and that the strain-specific effects were dependent on environmental conditions such as concentrations of nutrients, vitamins and trace metals. They also showed that the succession of communities that can occur in nature could also be influenced by environmental parameters.

Signalling and communication processes – compound exchange

Quorum sensing is a well-documented communication system between bacterial species and it relies on the biosynthesis and excretion of small molecules (auto inducers) controlling cell density and gene expression (Amin et al., 2012). Microalgae on the other hand were shown to produce signalling molecules such as pheromones to influence different aspect of their life cycle. Most of these pheromones are lipid-based hydrophobic molecules able to cross cell membranes without the need of cell-wall receptors. Vardi *et al.* (2006) also hypothesized that nitric oxide functioned as an info chemical in diatoms and represented a signalling mechanism to nearby cells to immunise them against stress conditions. Bacterial and algal signals are present and play a role in different biological mechanisms; furthermore, these signals seem to be mainly lipid-based molecules that does not require a high amount of energy to go across cell membranes (Amin *et al.*, 2012).

A variety of phytohormones have been found in algae (Tate et al., 2013). The main ones belong to the auxin and cytokinin. These phytohormones when supplemented to cultures have the potential to enhance performances of algae. Indeed, while phytohormones are present in green algae, their level is extremely low (Stirk et al., 2002). A few research groups have studied the effect of auxin compounds addition on algae. For example, Jusoh et al (2015) demonstrated that when added to algal culture, the auxins indole-3-acetic acid (IAA), indole-3-n-propionic acid (IPA), and indole-3-n-butyric acid (IBA) significantly enhanced the growth performances of *Chlorella vulgaris* by increasing the cell count. Amin et al. (2015) showed that a *Sulfitobacter* was promoting diatom cell division by secreting the hormone indole-3-acetic acid (IAA). The production of IAA by the bacteria was based on the transformation of tryptophan secreted by the diatom *Pseudonitzschia multiseries*. In this case, tryptophan and IAA acted as signalling molecules. Indeed, Tryptophan secretion by the diatom could attract bacteria but only bacteria able to convert it to IAA could create a positive feedback loop between algae and bacteria and demonstrate a mutualistic relationship.

Microalgae exude organic matter, which is the result of photosynthetic activity. These polysaccharides enrich the phycosphere and attract heterotrophic bacteria (Mühlenbruch et al., 2018). Indeed, it was reported that 50 % of the dissolved organic carbon released by phytoplankton in marine environment was used by bacteria (Azam et al., 1983). Furthermore, other authors observed that polysaccharides are an important factor influencing the community of heterotrophic bacteria associated with phytoplankton (Grossart et al., 2005). Conversely, it has been widely studied that the production of polysaccharides by microalgae could be dramatically changed by a modification of environmental factors such as temperature, light and nutrient availability (Wetz & Wheeler, 2007). This implies that the bacterial community attracted to these polysaccharides could also be modified seasonally in nature or by altering abiotic conditions in laboratory experiments.

Furthermore, it has been hypothesised that microalgae able to modulate their polysaccharide excretion would be able to attract selectively heterotrophic bacteria. Indeed, Amin *et al*. (2012) suggested that phytoplankton could ‘cultivate’ heterotrophic bacteria by

offering specific signalling molecules (Seymour et al., 2017), which are in return metabolised only by bacteria able to detect them.

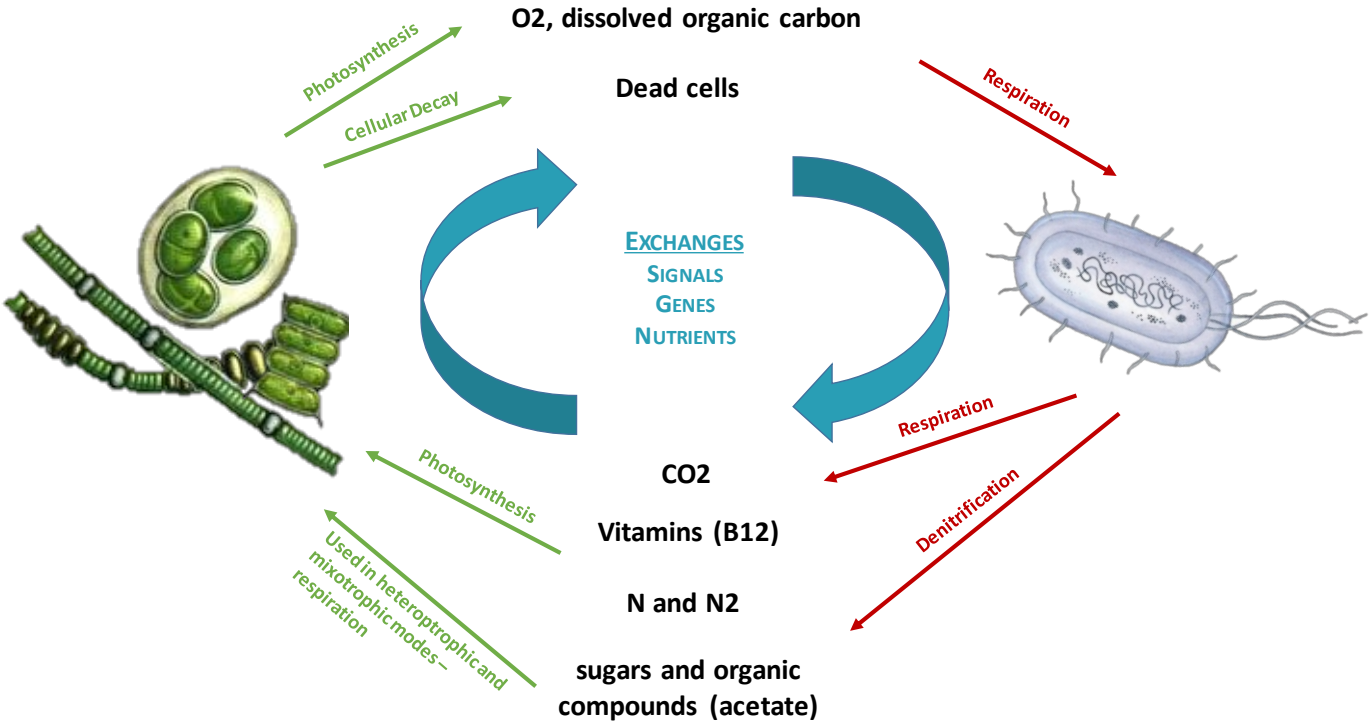

Figure 1.2: Schematic representation of exchanges between Algae and Bacteria within the phycosphere- Case of mutualistic relationship.

The literature has demonstrated that microalgae and bacteria develop in the same environment and that they interact (on different levels) with each other (Geng et al., 2016; Ramanan et al., 2015, 2016). This interaction is highly dependent on compound exchange (**Figure 1.2**) and can be influenced by environmental parameters, making the relationship between microalgae and bacteria and very dynamic and complex one, however, there is scope for engineering of consortia for different biotechnology application, including waste remediation.

1.4.2. Use of algae-bacteria consortium for the remediation of digestate

Examples of microalgae-bacteria interaction for waste remediation

The relationship between microalgae and bacteria has been exploited in the remediation of different waste streams (Ji et al., 2018), mainly because bacteria can increase growth of microalgae which in turn can uptake more nutrients from waste (Z. Hu et al., 2018),

however, microalgae-bacteria consortium can also be more robust to environmental changes and bacteria can also contribute to waste remediation. For example, the growth-promoting bacteria *Rhizobium sp.* facilitated nitrogen assimilation from wastewater by *Chlorella vulgaris* (Kim et al., 2014). Maza-márquez et al. (2014) also demonstrated that a microalgae-microbe consortium could efficiently degrade toxic contaminants such as phenolic compounds.

Additionally, some recent studies have focused on algae bacteria community assembly. For example, (Ji et al., 2019) investigated the initial incubation ratio between microalgae and bacteria and studied the removal efficiency according to this ratio. The authors found that a ratio of 1:3 of *Chlorella vulgaris-Bacillus licheniformis* provided the best results in terms of remediation of total dissolved phosphorus and nitrogen. Hu et al., 2018 suggested that artificial microalgae-bacteria consortia should be constructed by species with functional diversity when considering remediation systems.

Consortium engineering for waste remediation

A few studies investigated the growth performances of microalgal strains exposed to bacteria not belonging to the algae natural phycosphere (or not found in cultures), hence creating an artificial consortium. *Azospirillum sp*. has been the bacteria the most studied for this purpose and its association to microalgae resulted in increased dry weight, cell number, size, and a general enhancement of growth performances. Furthermore, chlorophyll content, lipid productivity, intra-cellular phosphate and carbohydrate and starch content were also improved by the bacteria (Amavizca et al., 2017; Choix et al., 2012; De-Bashan et al., 2002). These enhanced performances have been mainly attributed to the growth hormone Indole-3-Acetic Acid (IAA) produced by the bacteria *Azospirillum sp*. (De-Bashan et al., 2008; Meza et al., 2015). Improved growth in microalgae were also shown to convey higher nutrient removal efficiency from waste. Indeed, De-Bashan et al. (2008) showed that a *Chlorella vulgaris- Azosprillum brasilense* system was able to remove NH_3-N, nitrate-nitrogen and phosphorus at an efficiency rate of respectively 100%, 15% and 36% (the algae alone removed respectively 75%, 6% and 9%).

This demonstrates that the performances of an artificial consortium can be comparable or even greater than the ones of a natural algae-bacteria consortium. Indeed, Cho et al. (2015)

showed that an artificial consortium containing *Chlorella vulgaris* and bacteria belonging to the Bacteroidetes and α-Proteobacteria groups increased algal growth but also helped inducing flocculation and increasing lipid productivity, which are two valuable characteristics for microalgae grown commercially. The literature has studied the use of these microbial consortium for wastewater remediation (Fallahi et al., 2021b, 2021a; Tait et al., 2019) but not specifically for digestate remediation. Remediation of digestate by algae-bacteria consortium appears as a promising approach to increase the uptake of digestate by microalgae and improve scalability of the process, however it can also be argued that introducing additional bacterial communities to microalgal cultures could impact the implementation of a circular economy, especially when focus is made on animal feed applications.

1.5. Aims and objectives of the thesis

Waste generation is an increasing issue in our society, the circular economy concept attempts to alleviate this growing pressure by recycling and re-introducing waste within the supply chain, which is not without its challenges. Here, I have introduced the concept of circular economy and discussed the challenges presented by the increasing amount of digestate which is not valorised in Northwest Europe. I have discussed the potential of microalgae to remediate this source of waste and introduced a few of the applications for which microalgal biomass produced from digestate can be utilised, providing added value to the anaerobic digestion sector. While microalgae convey a clear advantage to many applications, especially animal feed, digestate at the base of their production remains a concern from a regulatory perspective, but also from a public acceptance viewpoint. Consequently, very few studies have attempted to close this loop and have tested the feasibility of using a digestate-based microalgal ingredient into animal feed.

The overall aim of this thesis was to evaluate the use of microalgae for the valorisation of nutrient rich digestate in a context of circular economy.

To answer this question, Chapter 2 firstly investigated routes for the valorisation of excess digestate, focusing on mechanical and biological processing of digestate. Additionally, this chapter aimed to assess the potential of digestate to support microalgal growth at laboratory scale and pilot scale, in order to develop the initial step of a circular economy

by utilising an underused source of waste. To do so, a series of digestate treatments were investigated, namely dilution combined with settlement, and membrane filtration using a range of pore sizes. The resulting processed NRD was used as a nutrient source for the cultivation of *Chlorella vulgaris,* a species widely recognised in algal wastewater remediation (Judd et al., 2015). *C. vulgaris* was cultivated from laboratory up to pilot-scale (800L), with the objective to explore the microalgae potential for converting excess nutrients from NRD into biomass.

Following up on findings from Chapter 2, Chapter 3 aimed to improve uptake of digestate by microalgae, to increase the bioremediation potential of digestate and improve the scalability of valorising this resource using microalgae within a circular economy. Therefore, tailoring of digestates from different feedstocks to microalgal strains, while manipulating pH to limit ammonium losses was studied, to improve remediation using a dual approach based on abiotic and biological factors. Additionally, the composition of the generated biomass was analysed to determine if different digestate would influence microalgal composition, bringing additional information on the potential of microalgae grown on digestate as a high-value product for further applications and their re-incorporation into the supply chain.

After achieving growth of microalgae on digestates in Chapters 2 and 3, Chapter 4 aimed to utilise the resulting microalgal biomass into aquaculture applications. Indeed, this chapter aimed to assess the feasibility of using a microalgal hydrolysate derived from digestate as a feed ingredient in aquaculture, thus implementing circularity. In this work, a hydrolysate from the green microalgae *Scenedesmus obliquus* grown on nutrient rich digestate was used as a feed ingredient in the diet of Nile Tilapia (*Oreochromis niloticus*). Hydrolysis was chosen as a novel biomass processing technique to alleviate palatability and smell related issues for feed uptake. Focus was made on the potential of the hydrolysed microalgal ingredient to provide additional benefits to the aquaculture sector in terms of flesh quality and gut health, without compromising growth of Nile Tilapia.

Whilst Chapters 2,3 and 4 evaluated the feasibility of developing a circular economy by producing an aquafeed using a digestate-derived microalgal ingredient, chapter 5 looked into a novel direction, by investigating the bacterial community developing in cultures of *Chlorella vulgaris* under different nutrient regimes and the potential they can represent

within the context of this thesis, namely digestate bioremediation by microalgae. To do so, the growth performances of *C. vulgaris* under 3 nutrient regimes (low, medium and high nutrient supply) was studied, along with the biochemical composition of the microalgae. Findings were linked to the abundance and composition of the bacterial community found in cultures of *C. vulgaris* to identify any changes in the bacterial community under different nutrient regimes and determine if these changes could be related to mutualism effects between *C. vulgaris* and the identified bacteria. Finally, these interactions were assessed to determine if they could influence the basic biochemical composition of *C. vulgaris*. Discussion was made regarding the potential of the algae-bacteria relationship to be utilised for improving digestate remediation by microalgae, and contribute to the circular economy concept established in this thesis.

Finally, chapter 6 summarises and discusses the thesis main findings within a context of circular economy and addresses some of the bottlenecks identified as part of this work. Future research prospects are also discussed, as well as their contribution to bridging the gaps identified in this work.

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CHAPTER 2. VALORISING NUTRIENT-RICH DIGESTATE: DILUTION, SETTLEMENT AND MEMBRANE FILTRATION PROCESSING FOR OPTIMISATION AS A WASTE-BASED MEDIA FOR MICROALGAL CULTIVATION

2.1. ABSTRACT

Digestate produced from the anaerobic digestion of food and farm waste is primarily returned to land as a biofertiliser for crops, with its potential to generate value through alternative processing methods at present under explored. In this work, valorisation of a digestate resulting from the treatment of kitchen and food waste was investigated, using dilution, settlement, and membrane processing technology. Processed digestate was subsequently tested as a nutrient source for the cultivation of *Chlorella vulgaris,* up to pilot-scale (800L). Dilution of digestate down to 2.5% increased settlement rate and induced release of valuable compounds for fertiliser usage such as nitrogen and phosphorus. Settlement, as a partial processing of digestate offered a physical separation of liquid and solid fractions at a low cost. Membrane filtration demonstrated efficient segregation of nutrients, with micro-filtration recovering 92.38% of phosphorus and the combination of micro-filtration, ultra-filtration, and nano-filtration recovering a total of 94.35% of nitrogen from digestate. Nano-filtered and micro-filtered digestates at low concentrations were suitable substrates to support growth of *Chlorella vulgaris.* At pilot-scale, the microalgae grew successfully for 28 days with a maximum growth rate of 0.62 day^{-1} and dry weight of 0.86 $g.L^{-1}$. Decline in culture growth beyond 28 days was presumably linked to ammonium and heavy metal accumulation in the cultivation medium. Processed digestate provided a suitable nutrient source for successful microalgal cultivation at pilot-scale, evidencing potential to convert excess nutrients into biomass, generating value from excess digestate and providing additional markets to the anaerobic digestion sector.

Keywords: digestate; membrane filtration; settlement and dilution; microalgae; Chlorella vulgaris; pilot-scale

2.2. INTRODUCTION

Anaerobic digestion (AD) is commonly used in Europe for the treatment of food and farm waste. The AD process is a biological mechanism during which bacterial and archaeal

communities convert carbon-rich organic waste into biogases, primarily methane and carbon dioxide (Doble and Kumar, 2005). Another by-product of the AD process is a nutrient-rich digestate (NRD). NRD is rich in carbon, nitrogen (N), phosphorus (P) and other macro and micronutrients (Papadimitriou et al, 2008; Tambone et al, 2017). NRD is primarily used as organic fertiliser and is directly applied onto farmland (Fuchs and Drosg, 2013). However, the use of digestate as a soil fertiliser increases the risk of nutrient runoff and penetration of groundwater resources, leading to soil and water eutrophication (Guilayn et al., 2019). Consequently, Nitrate Vulnerable Zones (NVZs) have been designated under the European Nitrate Directive 91/676/CEE (European Community, 1991) that limits the annual load of nitrogen applied onto farmland. NVZs are on the increase across Northwest Europe, resulting in the accumulation of approximately 10 million tons of excess digestate (Fuchs and Drosg, 2013).

Alternatives to farmland spreading have been investigated, such as using solid digestate for energy production or conversion into added-value products (char or activated carbon) (Monlau et al., 2015), however valorisation of digestate has been underexplored and solutions have yet to be firmly established to create value from this excess NRD. The present study focused on mechanical and biological treatments of digestate to increase its value and marketability. The partial processing of digestate was investigated first, by establishing methods for the separation of liquid and solid fractions of digestate using simple low-cost techniques. This approach is known to reduce the volume of digestate, hence minimising the processing cost, and facilitating the transportation of digestate to other locations less impacted by soil eutrophication (Guilayn et al., 2019). On the other hand, complete processing of digestate results in a clear liquor, directly dischargeable into the environment, however this process involves more complex and costly separation techniques such as centrifugation. Ultimately, both partial and complete processing of digestate result in nutrient segregation (*e.g.* P in the solid fraction and N in the liquid fraction (Tambone et al., 2017), which allows for the exploitation of digestate, for example in fertiliser formulation, due to the targeting of specific compounds.

Membrane filtration as a technology for the complete processing of digestate has generated a lot of interest in recent years. This technique allows for the removal of particles and microorganisms (*e.g.* protozoa, bacteria) potentially present in digestate (Ledda et al., 2016;

Massa et al., 2017; Mayhead et al., 2018; Park et al., 2010; Wang et al., 2010; Wen et al., 2017). Membrane technology is also used as a means to recover and concentrate nutrients from digestate (Abou-Shanab et al., 2013; Gerardo et al., 2015; Khan and Nordberg, 2018; Olguín et al., 2015; Silkina et al., 2017; Zacharof et al., 2019). During the process, particulates remain in the retentate (*i.e.* fraction of liquid not going through the membrane and resulting in a thick sludge following concentration); while the liquid nutrient-rich fraction permeates the membrane.

The complete processing of digestate using membrane filtration can have significant upfront costs, especially for large-scale applications. There is subsequently scope to utilise excess digestate and consequent nutrients, for example by conversion into added-value biomass for new markets. One of these novel methods includes the use of micro and macro nutrients present in NRD for microalgal cultivation (Fathi et al., 2013; Judd et al., 2015; Luo et al., 2017; Silkina et al., 2019). The majority of the compounds of interest found in NRD and useful for microalgal production are bound to the solid fraction (*e.g.* phosphorus), which limits their bioavailability and requires digestate processing for use as a waste-based medium for microalgal cultivation. Coupling the partial and complete processing of digestate to its biological treatment via microalgal production could be a promising technology, increasing digestate value and recouping the cost of heavy processing methods currently used. Furthermore, microalgae production is a promising technology, allowing for large scale applications (Stiles et al., 2018).

The aim of this work was to investigate routes for the valorisation of excess digestate. To do so, a series of digestate treatments were investigated, namely dilution combined with settlement, and membrane filtration using a range of pore sizes. The resulting processed NRD was used as a nutrient source for the cultivation of *Chlorella vulgaris,* a species widely recognised in algal wastewater remediation (Judd et al., 2015). *C. vulgaris* was cultivated up to pilot-scale (800L), with the objective to explore the microalgae potential for converting excess nutrients from NRD into biomass.

2.3. MATERIAL AND METHODS

2.3.1. Nutrient-rich digestate

Raw NRD (*i.e.,* sampled directly from the digester tank and not modified) from the industrial anaerobic digestion of kitchen food waste was sourced from the Langage-AD facility located in Plymouth (Devon, UK). The composition of the collected NRD was measured throughout the year by an external laboratory (data not shown) and was stable throughout the year, demonstrating a robust anaerobic digestion process. The NRD was transported to Swansea University and stored at 4°C, to avoid bacterial development. A 500 mL aliquot of NRD was taken to measure initial pH, dry weight, ammonium (NH_4-N), P and heavy metal composition (**Table 2.2**).

NRD was first treated using dilution and settlement and the unsettled layer of NRD was then processed using membrane filtration at a different range of molecular weight cut-offs (MWCO), namely micro-filtration (MWCO: 0.2 µm), ultra-filtration (MWCO: 10 kDa) and nano-filtration (MWCO: 500Da). The permeates from nano-filtration and micro-filtration were used for the cultivation of *C. vulgaris*.

2.3.2. Dilution and settlement – partial processing of digestate

Experimental design and sample collection

Raw NRD was diluted in triplicate to 2.5%, 5%, 10% and 20% with deionised water in 500 mL glass cylinders. Lower percentages were implemented to anticipate microalgal needs in terms of nitrogen concentration as high levels of dilution are required to reduce toxicity linked to ammonium for microalgal growth. Additionally, initial experiments (data not shown) did not show significant differences between higher digestate concentrations (i.e. >20%) and a 100% control. Sedimentation rate was measured for 24 hours. Conditions were compared against a raw NRD (*i.e.,* undiluted) control tested in quadruplicate. The sedimentation rate of NRD was calculated by measuring the height (in cm) of the first visible separation layer of NRD in the glass cylinders. Measurements were taken every hour for the first five hours of the experiment and for the last two hours of the experiment. After 24 hours, samples were collected in layers of 100 mL using a sterile syringe connected to a sterile tube, and samples were stored at 4°C before analysis. Five layers were collected for

each cylinder, defined as Layer 1 (from the top; L1), Layer 2 (L2), Layer 3 (L3), Layer 4 (L4) and Layer 5 (at the bottom of the cylinder; L5).

Dry weight, turbidity, and particles distribution analysis

Dry weight (in $g.L^{-1}$) was measured by filtering 5 to 60 mL samples (the volume was dependent on the dilution factor) using pre-dried and pre-weighed filters (Whatman 47 mm GF/C glass microfiber filters, pore size: 1.2µm, method based on Silkina et al. (2019)). Samples were oven dried for 24 hours at 80°C and dry weight was calculated as the weight difference between the dried-filtered sample and the pre-weighed-filter in relation to the volume of sample filtered as shown below:

$$dw\ (g.L^{-1}) = ((fs-f)/Vs) * 1000$$

Where dw is the dry weight in $g.L^{-1}$, fs is the weight of the filtered and dried sample (g), f is the weight of the pre-dried filter (g) and Vs is the volume of sample filtered (mL).

Sample turbidity was determined using absorbance measured at a wavelength of 750nm using a spectrophotometer (SPECTROstart^{Nano}, BMG Labtech). Particle distribution analysis was performed using a coulter-counter (Beckman) according to the method described in Mayers et al. (2013). Particle numbers in the range of 1-20 µm were assessed.

Nutrients and elemental composition

For each sampled layer, the NH_4-N concentration was measured using an ammonium reagent kit (Spectroquant®), based on the colorimetric quantification of NH_4-N (method analogous to EPA 350.1, APHA 4500-NH3 F, ISO 7150-1, and DIN 38406-5). The absorbance of treated samples was measured at 690 nm according to supplier instructions and measured against a calibration curve to determine NH_4-N concentration. P was measured using a reagent test kits (Spectroquant®), also based on colorimetric reactions (method analogous to EPA 365.2+3, APHA 4500-P E, and DIN EN ISO 6878). Absorbance was recorded at 410 nm and P concentration was assessed as for ammonium.

Elemental analysis of collected samples was completed using an X-ray fluorescence equipment (XRF, Rigaku Nex CG). XRF is a technique which allows distinction between atoms, based on their X-ray fluorescence spectra: electrons from a sample are excited by the X-ray and emit a fluorescence radiation characteristic to a particular material

(Shapovalov et al., 2007). 200 µL of each sample was deposited on ultra-carry discs and left to dry for 12 hours. Samples were then processed for elemental analysis by XRF.

2.3.3. Membrane Filtration – complete processing of digestate

Experimental design and step-filtration process

Micro-filtration, ultra-filtration and nano-filtration were used to process the unsettled layer of raw NRD. Between each filtration step, the N, P and heavy metal composition of the NRD permeates were assessed.

Micro-filtration of NRD was implemented using a ceramic membrane at a pore size of 0.2 µm and a trans-membrane pressure ranging from 1.5 to 2.5 bars was applied (Koch membrane systems Inc.). The entire set-up comprised a 60L capacity tank connected to a pump drawing the NRD into the membrane for filtration. The permeate was collected at one end of the membrane and the remaining sludge (*i.e.,* retentate) was pumped back into the tank and mixed with the remaining digestate for further filtration. Micro-filtration of NRD continued until the volume of retentate left in the tank reached 10 L. The obtained retentate was a thick and concentrated sludge.

The micro-filtered NRD permeate was then ultra-filtered at a pore size of 10 kDa (membrane: hollow fibre cartridge, GE Healthcare, UFP-10-E-6A). The ultra-filtration system functioned similarly to the one described for the micro-filtration step. A smaller retentate tank was used (20L) and ultra-filtration was stopped when the level of retentate collected reached 5L.

Micro-filtered NRD was also filtered using a nano-filtration membrane at a pore size of 500 Da. Nano-filtration was performed using frontal filtration in a high-pressure bench scale unit (HP4750, Sterlitech, Kent, WA, USA, NF270). The unit was continuously pressurised at 30 bar using nitrogen gas and a stirring speed of 300 rpm. The system had a maximum operation volume of 200 mL. A new membrane was installed between every trial to eliminate the risk of fouling and to avoid compromising permeate composition.

Nutrient and elemental composition analysis

The NH_4-N, P and elemental composition of permeates resulting from the different filtration steps detailed above were analysed as described in section *2.3.2.*

2.3.4. Chlorella vulgaris cultivation - Biological processing of digestate

Processing of nano-filtered digestate at laboratory scale

The permeate resulting from the nano-filtration of NRD was used at different concentrations for the cultivation of *C. vulgaris.* Concentrations of 2.5% ([N] = 62.45 $mg.L^{-1}$; [P] = 0.07 $mg.L^{-1}$), 5% ([N] = 124.9 $mg.L^{-1}$; [P] = 0.13 $mg.L^{-1}$), 10% ([N] = 249.8 $mg.L^{-1}$; [P] = 0.27 $mg.L^{-1}$), 15% ([N] = 374.7 $mg.L^{-1}$; [P] = 0.39 $mg.L^{-1}$) and 20% ([N] = 499.6 $mg.L^{-1}$; [P] = 0.53 $mg.L^{-1}$) of nano-filtered NRD mixed with deionised water were tested. These conditions were compared against a 2.5% concentration ([N] = 101.3 $mg.L^{-1}$; [P] = 1.27 $mg.L^{-1}$) of micro-filtered NRD selected as a control.

Cultures of *C. vulgaris* were grown in triplicates for each digestate concentration, and the control, using 250 mL flasks, with a cultivation volume of 150 mL. An inoculum of 15 mL (10% of the total cultivation volume, corresponding to an OD 750nm of 0.13) provided by the Centre for Sustainable Aquatic Research (Swansea University, UK) was used. Culture turbulence was provided by stirring and cultures were grown at a temperature of 25°C under an illumination of 100 $\mu mol.m^{-2}.s^{-1}$ and a 12 Light:12 Dark photoperiod. Nano-filtered digestate was added at the beginning of the experiment, in the concentrations mentioned above, and cultivation occurred for eight continuous days without the addition of any other nutrient.

Processing of micro-filtered digestate at pilot-scale

The unsettled layer of undiluted digestate was chosen in this pilot-scale trial due to the simplicity of processing (settlement took place in a 60L tank for 24 hours). Following settlement, micro-filtration was used to treat the unsettled layer of digestate prior to microalgal cultivation. Micro-filtration was selected due to practicality of the system, allowing for the processing of a large volume of NRD, necessary to this pilot-scale application. The micro-filtered permeate of digestate was diluted to 2.5% of the total photobioreactor volume (800L). This dilution corresponded to N and P concentrations of 101.3 $mg.L^{-1}$ and 1.27 $mg.L^{-1}$, respectively.

An inoculum of 80 L (10% of the PBR total volume, corresponding to an OD 750nm of 0.13 and a DW of 0.168 $g.L^{-1}$) of C. *vulgaris* was used to start cultivation into a horizontal tubular

photobioreactor, located in a greenhouse at Swansea University. The greenhouse temperature was maintained at 25°C, natural light and photoperiod were used with an average light intensity throughout the experiment of 848.42±64.92 $\mu mol.m^{-2}.s^{-1}$. The pH was maintained at 7.5 using automated CO_2 injection. *C. vulgaris* was cultivated for 51 days in semi-continuous mode. Harvesting occurred every 6 to 7 days to enable maximum N and P uptake from NRD while *C. vulgaris* was still in a state of exponential growth. Approximately 30% of the PBR total volume was harvested, followed by water renewal and digestate addition, maintaining a 2.5% concentration of micro-filtered NRD in the cultivation system.

Growth measurements

Growth rate of *C. vulgaris* was assessed through daily measurements of the absorbance at 750 nm for the laboratory scale experiment and every other day for the pilot-scale trial. Absorbance at 750nm was utilised to measure biomass as this specific wavelength avoids light absorption by pigments, and so can be treated as a light scattering measurement (Chioccioli et al., 2014). Growth rate was calculated during the exponential phase of growth using the following equation:

$$\mu = \ln (OD_2/OD_1) / (t_2-t_1)$$

Where μ is the specific growth rate (day^{-1}), OD_1 and OD_2 are the optical density measured at 750 nm at time 1 (t_1) and time 2 (t_2).

Dry weight (in $g.L^{-1}$) of produced biomass was assessed in the pilot-scale trial, where the volume of culture was sufficient for regular sampling of *C. vulgaris*. Three replicates of 20 mL each were sampled every other day and dry weight was assessed as described in section *2.3.2.*

Nitrogen and phosphorus were measured in the culture supernatant every other day in the pilot-scale trial and assessed according to the method detailed in section *2.3.2.*

2.3.5. Statistical Analysis

Statistical analysis was carried out on settlement rate, OD750 nm, dry weight, particle count, NH_4-N and P concentrations, and elemental analysis using the R project software. The OD 750nm and dry weight were also analysed for the microalgae cultivation trials. Crossed factors ANOVAS were carried out on normally distributed data and normality was determined using Shapiro tests. When statistical significance was found, post hoc Tukey tests were implemented. Results were deemed significant when p-value was below 0.05.

2.4. RESULTS AND DISCUSSION

2.4.1. Dilution and Settlement – Partial processing of digestate

Settlement Rate of diluted NRD

As shown in **Figure 2.1**, a faster settlement rate was observed in highly diluted digestate in comparison to lower levels of dilution tested. Specifically, digestate settled at an average rate of 0.314 (± 0.005) $cm.h^{-1}$ at a 2.5% concentration while the settlement was only at a rate of 0.04 (± 0.002) and 0.057 (± 0.003) $cm.h^{-1}$, respectively for a 20% concentration and 100% concentration (*i.e.,* control). Both the 20% concentration of digestate and the control presented a low and constant settlement rate during the 24 hours of the experiment (**Figure 2.1**) and showed very little sedimentation of the NRD beyond four hours. On the other hand, for a 2.5% concentration of digestate, a high settlement rate of 1.553 (± 0.05) $cm.h^{-1}$ was recorded at the beginning of the experiment, only to reach a plateau after four hours (significantly different from the control, p-value < 0.05); similar results were found for a 5% concentration (**Figure 2.1**). These results demonstrated that dilution significantly accelerated the settlement rate of NRD, increasing the potential for separation of solid and liquid fractions. A direct correlation can be made between these results and a reduced heavy particles content in the diluted digestate, responsible for an enhancement of settlement properties. Indeed, a reduced amount of particles in the digestate column was likely to reduce the level of interaction between these particles (or Brownian motion), resulting in the faster settlement rate observed as particles were more free to simply settle due to gravity. Furthermore, results indicated that sedimentation occurred during the first few hours of settlement, showing that settlement techniques would represent an efficient and low-cost process for higher amounts of digestate within a shorter processing time.

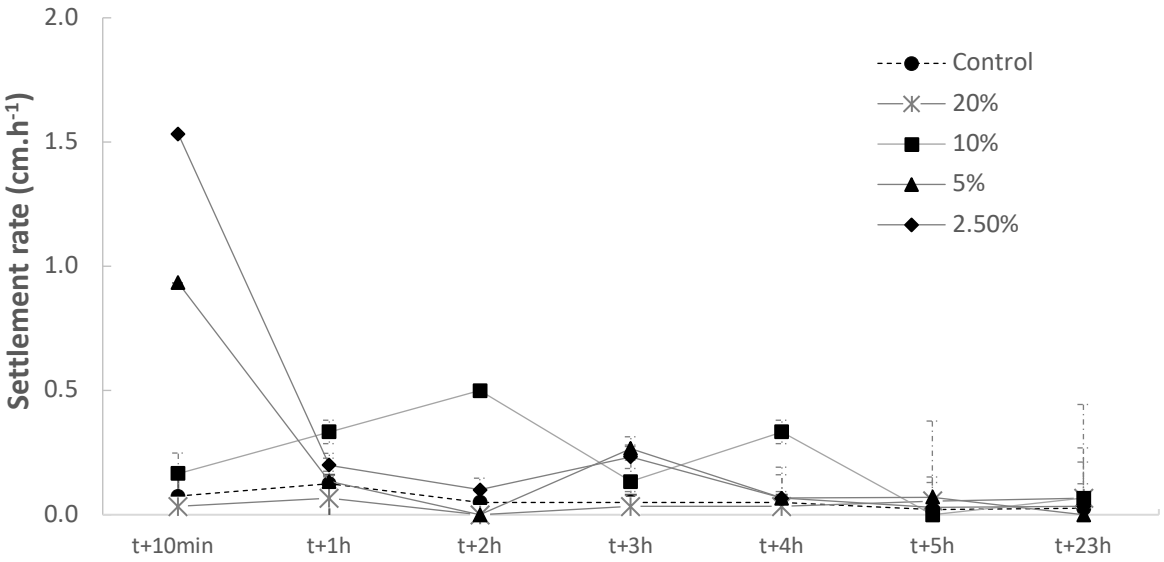

Figure 2.1: Settlement rate of diluted digestate at concentrations of 2.5%, 5%, 10% and 20% compared to a raw undiluted digestate (control). Settlement rate measured from t+10 min to t+23 hours. Error bars represent standard deviation of data on three replicates for diluted digestate and four replicates for control.

Dry weight, turbidity, and particle distribution of settled layers

Dry weight, turbidity and particle distribution were significantly higher in L5 for all tested conditions (p-value <0.01, Tukey test: L1=L2=L3=L4<L5). Similarly, DW was three-fold higher in L5 (12.18 $g.L^{-1}$; **Figure 2.2**) compared to the other four layers (ranging from 3.66 to 4.01 $g.L^{-1}$; **Figure 2.2**). Similar results were observed for optical density and particle count, where OD is an indicator of turbidity and particle count and DW are a direct assessment of the particulate matter present in a sample. Therefore, these results showed that the vast majority of particles present in the column were found in L5 at the end of the experiment, confirming that settlement and decantation occurred at a significant degree during the time of experiment. Findings from Godos et al. (2009) reported that when a swine manure digestate had been settled at a residence time of five days, the total of suspended solids was reduced by 70% in the digestate column. In the present study, sedimentation of the Langage AD digestate (originating from the anaerobic digestion of kitchen food waste) was faster, exhibiting that sedimentation time can depend on the nature of the settled digestate.

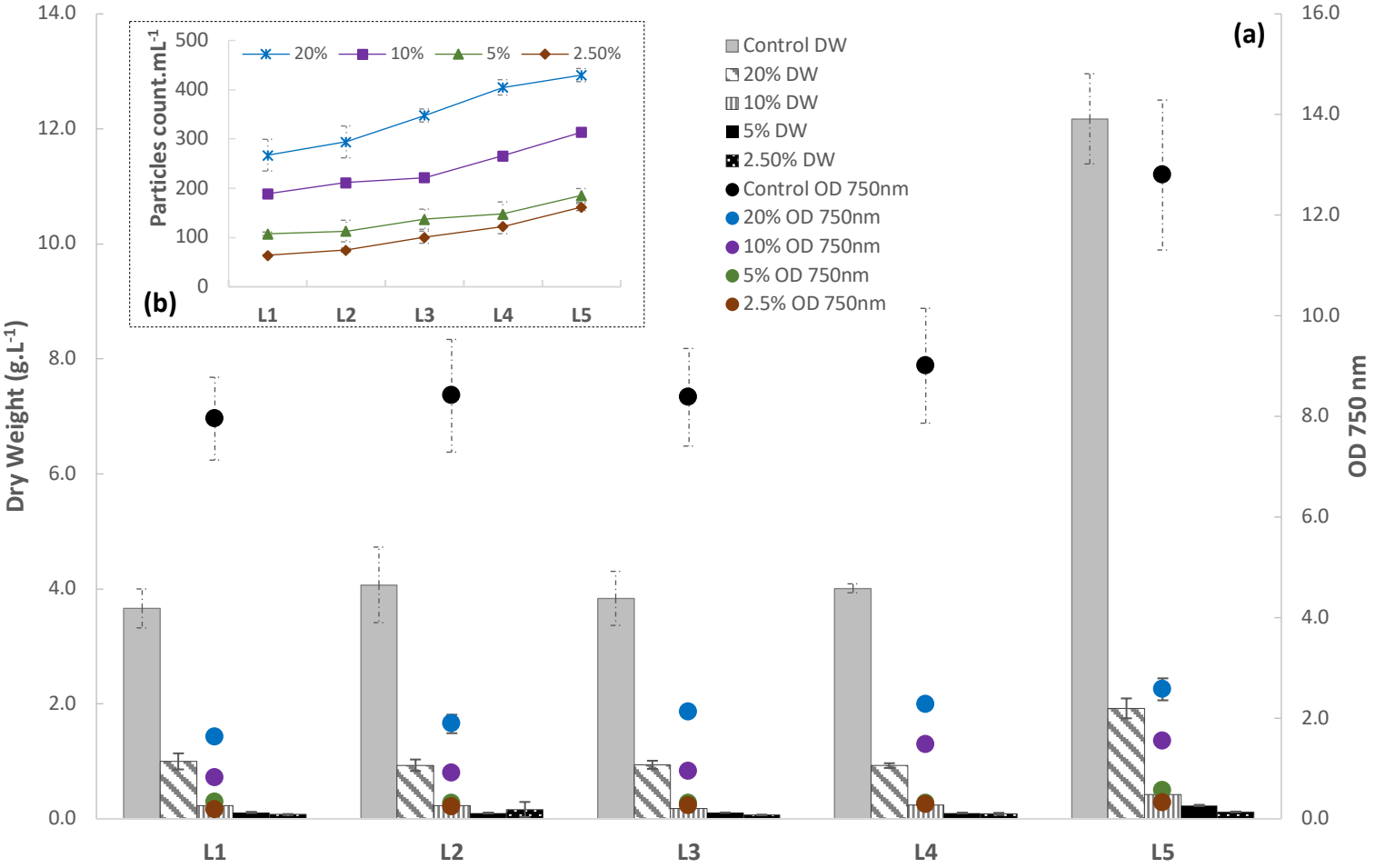

Figure 2.2: (a) Dry weight (left axis, bar plot) and OD 750 nm (right axis, coloured dots) and (b) particle count data of diluted digestate at concentrations of 2.5%, 5%, 10% and 20% for 5 layers collected after 24 hours of experiment. Dry weight and OD 750 are compared to a raw undiluted digestate (control). Error bars represent standard deviation of data on three replicates for diluted digestate and four replicates for control.

Nutrient Analysis of settled layers

Results of nutrient analysis showed a maximum NH_4-N concentration of 6928 $mg.L^{-1}$ in L4, in the control (*i.e.,* undiluted digestate, **Figure 2.3a**). P content was similar in all collected layers for digestate concentrations of 2.5%, 5% and 10% (p-value < 0.05). Recalculated values of NH_4-N and P content from treated digestate did not show an increase of either nutrient, except for the NH_4-N content for a 2.5% concentration, where the highest concentration of NH_4-N measured in L2 was 422 $mg.L^{-1}$. This result corresponds to an equivalent concentration of 16.88 $g.L^{-1}$ for a 100% concentration of digestate, which is 2.4 times higher than the highest measured concentration in the control. This suggests that a high level of water dilution could have caused a release of NH_4-N. However, it is unlikely that dilution alone was responsible for such an increase in the NH_4-N content, and it could

be argued that digestate was still active to some extent, resulting in protein degradation, producing NH_4-N ions measured in the study (Jokela and Rintala, 2003). The increase in P was consistent with findings from the literature, where water dilution has been shown to dissolve some of the solid particles binding P, and releasing the compound in the liquid fraction of digestate (Gerardo et al., 2015). Furthermore, digestate dilution was found to solubilise mineral precipitates and release other compounds of interest (Wahal and Viamajala, 2016). Dilution has been widely used as a digestate treatment, especially when NRD was used as a waste based medium for microalgal cultivation (Abu Hajar et al., 2017; Franchino et al., 2016; Lu et al., 2015). In these studies, dilution was a means to reduce the load of some potentially toxic elements, such as NH_4-N; this work demonstrated that dilution could also release important nutrients for microalgal cultivation and allowed for some manipulation of these nutrient concentrations.

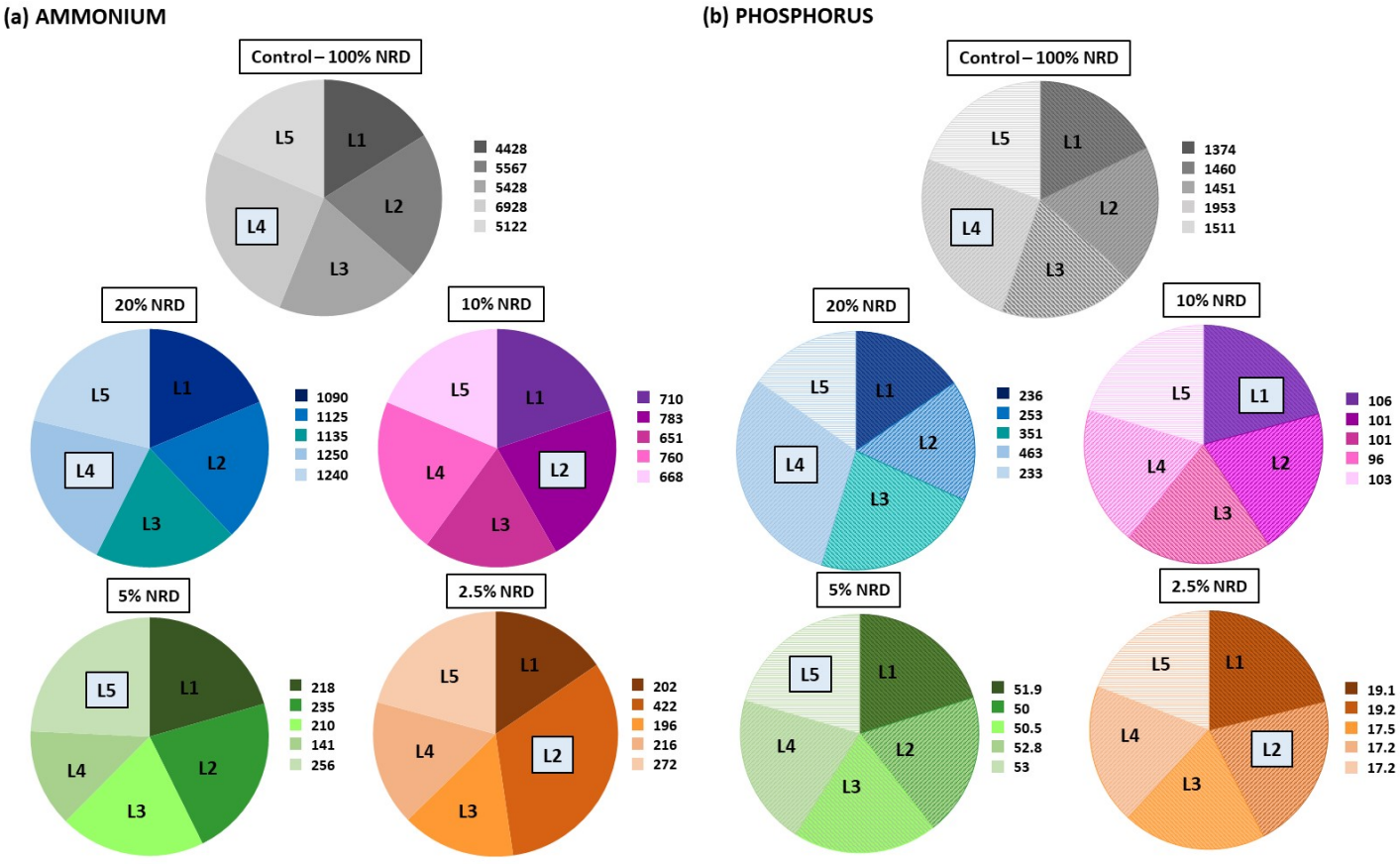

Figure 2.3: Ammonium (a) and Phosphorus (b) (in $mg.L^{-1}$) content of diluted digestate at concentrations of 2.5%, 5%, 10% and 20% compared to a raw undiluted digestate for five layers collected after 24 hours of experiment. Layers presenting the higher concentration of nitrogen or phosphorus are outlined in a blue frame. Standard deviations were not significant and are not shown.

Elemental Analysis of settled layers

Results from the elemental analysis on each of the collected layers of diluted and undiluted NRD at the end of the 24 hours experiment are compiled in **Table 2.1.** Each element concentration was significantly higher in L5 (p-value < 0.01, Tukey test: L1=L2=L3=L4<L5) compared to the remaining layers (L1 to L4) that were not significantly different from each other. For example, in the control, calcium and iron were both significantly higher (p-value <0.01) with respective concentrations of 590.7 $mg.Kg^{-1}$ and 128.7 $mg.Kg^{-1}$ in L5, while the concentrations in the remaining layers ranged from 176.3 to 204.3 $mg.Kg^{-1}$ and 68.6 to 80 $mg.Kg^{-1}$ for calcium and iron, respectively (**Table 2.1**). Similar results were found across the range of tested dilutions.

Results of elemental analysis demonstrated that dilution contributed to the release of some elements from the solid fraction to the liquid fraction. To illustrate, zinc had a concentration of 1.5 $mg.Kg^{-1}$ in L5 for a digestate diluted down to 2.5%, corresponding to a recalculated concentration of 60 $mg.Kg^{-1}$ in a 100% NRD. This is 5 times higher than the amount of zinc measured in L5 for the control (Zn = 12.9 $mg.Kg^{-1}$, **Table 2.1**). The same observation was made for sodium, aluminium, sulphur, chlorine, potassium, calcium, iron and copper. However, some elements, such as magnesium and manganese, were not detected in NRD diluted down to 2.5%, 5% and 10% (**Table 2.1**). Heavy metals, especially zinc and copper, are toxic to photosynthetic organisms at high concentration (Papadimitriou et al., 2008) but are essential oligo-elements when supplemented at the right concentration, including in microalgal cultivation systems (Kropat et al., 2015).

Table 2.1: Elemental Analysis (in $mg.Kg^{-1}$) of diluted digestate at concentrations of 2.5%, 5%, 10% and 20% compared to a raw undiluted digestate for five layers collected after 24 hours of experiment. Highest concentrations for each element are highlighted in bold. Measured potassium (K), Copper (Cu) and Zinc (Zn) were all under the safety limit for fertiliser standards (respectively <19.5 $Kg.t^{-1}$; <300 and <600 $g.t^{-1}$).

Treatment	Layers	Na	Mg	Al	S	Cl	K	Ca	Mn	Fe	Cu	Zn
Control	L1	1213.3±82.2	15.9±0.5	36.6±2.5	62.4±5.2	898±61.7	951±73.8	176.3±11.9	0.84±0.0	68.6±5.8	0.8±0.0	9.1±0.8
	L2	1286.7±4.7	15.9±2	39.3±1.3	70.1±1.7	951.3±11.4	1007.3±24.2	201.7±9.8	0.94±0.1	79.1±4.5	0.92±0.1	10.5±0.4
	L3	**1333.3±47.8**	17.5±0.7	38.4±1.3	66.2±2.6	940±32.8	987.7±23.2	188.3±8.7	1.04±0.1	73.7±3.0	0.81±0.1	9.7±0.3
	L4	1330±69.8	19.7±1.5	39.2±1.1	69.3±2.4	955.3±18.8	1016.7±17.0	204.3±2.1	1.03±0.1	80±2.3.0	0.91±0.1	10.2±0.3
	L5	1326.7±60.2	**30.9±8.3**	**52.8±6.9**	**96.5±14.1**	**1073.3±57.9**	**1083.3±45.0**	**590.7±239.1**	**1.25±0.2**	**128.7±26.0**	**0.98±0.2**	**12.9±1.6**
20%	L1	**745.5±1.5**	12.5±1.0	16.8±0.2	19.7±0.2	**584±9.0**	**466.5±0.5**	49.2±0.9	0.22±0.0	16.6±0.4	0.71±0.1	2.9±0.0
	L2	601.5±32.5	9.8±0.6	15.2±0.4	16.5±0.0	561±18.0	443±8.0	47.3±0.0	0.29±0.0	16.4±0.2	0.52±0.1	2.8±0.1
	L3	561±30.0	9.2±1.4	15.4±0.3	16.1±0.6	547.5±13.5	452±12.0	49.8±2.5	0.23±0.0	16.5±0.8	**0.81±0.0**	2.9±0.2
	L4	629.5±10.5	10.1±1.0	16.4±1.0	17.9±0.5	579±18.0	462.5±6.5	49.4±2.1	*ND*	16.4±0.5	0.67±0.0	**3±0.3**
	L5	623.5±5.5	**41.1±6.0**	**25.7±1.4**	**30.2±2.3**	527±33.0	437±16.0	**177.5±47.5**	**0.64±0.0**	**37±2.4.0**	0.64±0.1	2.9±0.4
10%	L1	254.5±23.5	6.5±0.5	8.5±0.1	14.3±0.8	276±11.0	223.5±10.5	41±1.9	*ND*	9.2±0.3	0.74±0.0	1.9±0.1
	L2	239±22.0	6.4±0.2	8.2±0.4	13.3±0.2	268.5±9.5	220±9.0	40.5±1.5	*ND*	8.8±0.1	0.75±0.1	1.9±0.0
	L3	250.5±15.5	6.9±0.7	7.9±0.4	13.4±0.4	266.5±9.5	219.5±9.5	40.3±1.3	*ND*	8.8±0.3	0.71±0.0	2.1±0.0
	L4	240±10.0	5.5±0.1	8±0.0	13±0.1	268.5±0.5	215.5±6.5	39.2±0.4	*ND*	9±1.0	**0.87±0.0**	2.2±0.1
	L5	**284.5±10.5**	**17.7±7.1**	**11.3±1.9**	**17.2±1.3**	**280±11.0**	**228±7.0**	**69.4±26.4**	*ND*	**12.9±2.9**	0.75±0.2	**2.4±0.1**
5%	L1	96.4±12.6	*ND*	4.4±0.3	4.9±0.8	131.5±12.5	112±11.0	16.4±1.6	0.2±0.0	5.7±0.3	**0.68±0.1**	1.5±0.1
	L2	99.4±4.6	*ND*	4.2±0.0	4.6±0.0	125±1.0	105.5±1.5	15.7±0.0	**0.3±0.0**	5.3±0.1	0.62±0.0	1.5±0.1
	L3	**112±0.0**	*ND*	4.4±0.0	4.9±0.0	**134±0.0**	**112±0.0**	17±0.0	*ND*	5.3±0.0	0.6±0.0	1.3±0.0
	L4	102.5±0.5	1.4±0.0	4.4±0.1	4.8±0.0	125±1.0	105±1.0	16.5±0.2	0.2±0.0	5.3±0.0	0.67±0.0	1.6±0.0
	L5	99.5±4.5	**5.3±0.9**	**6.2±0.2**	**6.5±0.0**	128.5±0.5	110.5±0.5	**32.7±1.1**	*ND*	**7.3±0.3**	0.66±0.1	**1.8±0.1**
2.5%	L1	**66.1±15.2**	*ND*	2.7±0.3	2.7±0.4	**84.5±15.5**	**67.9±12.3**	8.5±0.3	0.2±0.0	3.5±0.1	0.57±0.1	1.3±0.0
	L2	53.2±15.4	*ND*	3.1±0.4	2.5±0.4	69.3±2.7	56±2.1	8.9±1.4	*ND*	3.5±0.5	0.57±0.1	1.5±0.2
	L3	64.2±5.1	*ND*	2.6±0.2	2.5±0.0	73.2±0.7	55.8±0.3	7.7±0.0	*ND*	3.5±0.1	0.58±0.0	1.1±0.1
	L4	54.2±2.5	*ND*	2.8±0.1	2.4±0.1	71.6±0.4	56.6±0.6	8±0.1.0	*ND*	3.6±0.1	**0.69±0.1**	1.2±0.1
	L5	55.6±5.8	**4.4±1.5**	**4.3±0.7**	**3.9±0.7**	72.3±2.7	59.4±2.0	**15.9±0.8**	**0.2±0.0**	**5.2±0.5**	0.58±0.0	**1.5±0.1**

Analytical results indicate that the combination of dilution and settlement as a partial processing of digestate was a promising technique to separate liquid and solid fractions of digestate. Additionally, NRD sedimentation occurred in a few hours, resulting in a settled layer of NRD (or solid fraction), transportable at a lower cost once dried, and with the potential to be used in fertiliser formulation (Alburquerque et al., 2012). In the unsettled layer of digestate, results indicated that macronutrients (N and P) and micronutrients (zinc, calcium, iron) were all released, and these compounds are all essential to microalgal growth. Hence, there is potential to use this partial processing of NRD as part of the upstream process to produce a waste-based medium for microalgae cultivation. However, it is important to highlight that only high levels of dilutions proved to be beneficial for this purpose, inducing consequent water usage when scaled-up for mass cultivation of microalgae. If dilution is to be considered in large-scale cultivation systems, water from culture dewatering (*i.e.,* harvesting, downstream process) could be recycled and used for the dilution of digestate for several utilisation cycles, reducing the cost linked to water usage and avoiding water penalties. Additionally, despite evidencing benefits for microalgal growth in terms of nutrient release, a significant number of particulates were remaining in the unsettled layers of diluted and raw digestates, particulates that provide a substrate for the development of contamination, potentially harmful to microalgal growth (Xia and Murphy, 2016). To illustrate, when testing the growth of *Chlorella sp*. on raw digestate from a municipal waste treatment facility, Cho et al. (2011) found that bacterial load and suspended solids were responsible for a lack of growth in the microalgae. Light penetrability was also very limited in a raw digestate, compromising photosynthetic performances by microalgal cultures (Marazzi et al., 2017; Rusten and Sahu, 2011). Here, while dilution improved transparency of the digestate and reduced the particulates load, these were nevertheless sub-optimal for microalgal growth, bringing a risk to compromise cultures, especially when considering large-scale systems and commercial viability of biomass production. Hence, there is an argument for exploring additional treatments of digestates following settlement and dilution, in an effort to maximise microalgal production. Filtration of the unsettled layer of digestate can be explored to remove remaining particulates and associated contaminations and increase light penetrability for optimal microalgal growth.

2.4.2. Membrane Filtration – complete processing of digestate

The N, NH_4-N and P content of NRD following each filtration step (micro-filtration, ultra-filtration and nano-filtration) is presented in **Figure 2.4**. Elemental analysis is summarised in **Table 2.2**. **Figure 2.4** shows that most of the P was retained at the micro-filtration step, with a concentration of only 50.7 $mg.L^{-1}$ measured in the permeate. Concentrations of 25.76 $mg.L^{-1}$ and 2.66 $mg.L^{-1}$ were found in the ultra-filtration and nano-filtration permeates respectively, showing a significant decrease in comparison to the raw digestate, which had a P concentration of 665 $mg.L^{-1}$. Results demonstrated that most of the P was bound to the solid fraction of NRD, as most of the solid particles were removed during micro-filtration. Findings from Tambone et al. (2017) confirm this result, as they showed that dry matter and nutrients were concentrated in the solid fraction of processed digestate. A concentration of 4052 $mg.L^{-1}$ of N was measured in the permeate after micro-filtration and concentrations of 1846 $mg.L^{-1}$ and 2496 $mg.L^{-1}$ were found in the ultra-filtration and nano-filtration permeates, respectively. Thus, some of the N was lost during the different filtration steps, but concentration stayed relatively high, even following nano-filtration. The obtained results were consistent with results from Adam et al. (2018), who recovered 53% of NH_4-N in nano-filtered digestate. The significant reduction in P after each filtration step, resulted in a N to P ratio of 80, 71 and 939 in the micro-filtered, ultra-filtered and nano-filtered permeates, respectively.

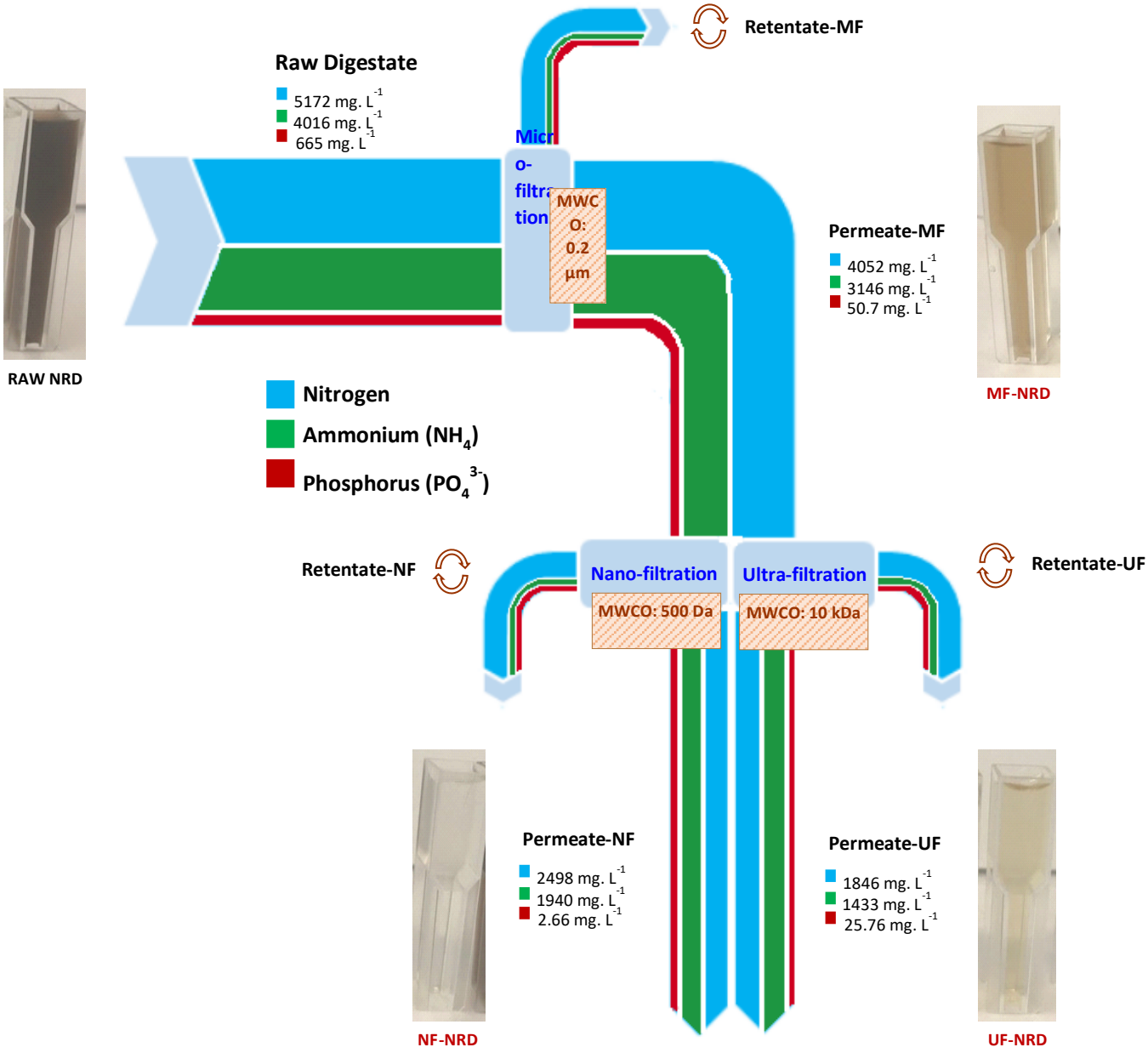

Figure 2.4: Concentration flow diagram of the membrane filtration process for nutrient-rich digestate for total nitrogen (blue), ammonium (green) and phosphorus (red). Arrow thickness represents relative concentrations between the three nutrients. MF: micro-filtration; UF: ultra-filtration; NF: nano-filtration. Permeate composition is shown after every filtration step, circular arrows indicate the recirculation of retentate during the filtration process.

Table 2.2: Composition of raw NRD (initial composition) and permeates following membrane filtration: micro-filtration, ultra-filtration and nano-filtration. The highest concentration of each element is highlighted in bold.

	Raw NRD (Initial composition)	Micro-filtered NRD permeate	Ultra-filtered NRD permeate	Nano-filtered NRD permeate
pH	8.0	-	-	-
DW	5.94%	-	-	-
NH_4^+-N *mg.L^{-1}*	**4016**	3146	1433	1940
P *mg.Kg^{-1}*	**665**	50.7	25.8	2.7
Ca *mg.Kg^{-1}*	**6756**	37 ±0.6	33.7 ±1.5	19.6 ±0.3
K *mg.Kg^{-1}*	**2015**	1203 ±12.5	815.2 ± 16.6	849.6 ±22.7
Mg *mg.Kg^{-1}*	**113.7 ±7**	6.0 ±0.5	3.6 ±0.5	ND
Na *mg.Kg^{-1}*	1150 ±104.4	2146 ±77.6	1300 ±40.8	**2230 ±98.9**
Al *mg.Kg^{-1}*	**109.3 ±2.1**	20.9 ±0.8	13.7 ±0.9	20.9 ±0.8
Cu *mg.Kg^{-1}*	1.6	0.6 ±0.0	0.78 ±0.1	**2.3 ±0.1**
Fe *mg.Kg^{-1}*	6.2	**8.2 ±0.2**	5.2 ±0.21	2.3 ±0.1
Zn *mg.Kg^{-1}*	**32.9**	1.1 ±0.1	1.2 ±0.2	1.3 ±0.2

In this study, while filtration was very efficient in cleaning the NRD by removing particles and maintaining suitable levels of N for microalgal cultivation, this method significantly reduced the amount of bioavailable P. However, studies showed that using membrane filtration in association with chemical pH adjustment could increase the amount of P released in the permeate. Gerardo et al. (2015) demonstrated that P could be recovered from dairy manure digestate through a series of diafiltration (*i.e.,* addition of equal amounts of water for effective dialysis of solutes) and that pHs of 3 and 7 led to recovery of 96.4% and 97.2% of P respectively. hence, there are methods available to increase the amount of P recovered after NRD treatment, and despite incurring cost, these methods can be considered to improve the N to P ratio and tailor it to microalgal needs.

While P appeared to decrease consistently with each filtration step, other compounds were found in higher concentrations in the nano-filtered permeate, in comparison to permeates resulting from ultra-filtration and micro-filtration. As an example, N was 1.35 times higher in the nano-filtered product compared to the ultra-filtration permeate. Elemental analysis

(**Table 2.2**) also showed that sodium, copper and zinc were found in higher concentrations in the nano-filtered permeate when compared to both ultra-filtered and micro-filtered permeates. The nano-filtration process has the characteristic to concentrate metals due to the small pore size of the membrane, and the divalent charge of the species interacting with the charged active layer on the membrane surface. This specificity could explain why the aforementioned elements were found in higher concentrations following nano-filtration (Al-rashdi et al., 2013; Gherasim and Mikulá, 2014).

Analysis revealed that nano-filtration was the only process resulting in a colourless permeate (**Figure 2.4**), and magnesium was the only compound not detected in the nano-filtered digestate in comparison to micro and ultra-filtered NRDs. There is no evidence suggesting that magnesium is solely responsible for digestate colouration. Hence, it is likely that the digestate colour resulted from the interaction of several compounds, such as humic substances, which are large organic compounds and were not assessed in this research. Furthermore, it is challenging to assess the exact compound (or mixture of), responsible for digestate colouring, as digestate colour can also vary between AD facilities due to the nature of the waste input (Marcilhac et al., 2014).

Membrane filtration was an efficient treatment of digestate and allowed for its complete processing by producing a clear liquor after nano-filtration, potentially dischargeable to receiving waters (Fuchs and Drosg, 2013). Additionally, the nutrient segregation following each filtration step enables the tailoring of nutrients for specific fertiliser utilisation, contributing to the valorisation of raw digestate; however, this multi-step technology is costly and not readily scalable when considering the current need for processing vast amounts of excess NRD produced across Northwest Europe. Using treated NRD as a medium for microalgae cultivation has potential to provide a solution to alleviate NRD processing cost and add value to excess NRD, currently underused and under valorised by the AD sector.

2.4.3. Chlorella vulgaris growth trial

Results of laboratory scale cultivation

Nano-filtered digestate was used as nano-filtration was the only process successful at removing the NRD colour, allowing for the testing of a greater range of digestate

concentrations. The OD at 750 nm as a measurement of *C. vulgaris* growth showed significant differences between some of the concentrations tested (p-value < 0.01, Tukey test = NF 2.5> NF5 = MF2.5>NF10>NF15). *C. vulgaris* grew best on a 2.5% concentration of nano-filtered NRD with a maximum OD of 0.93 reached after 8 days of experiment and a growth rate of 0.46 day^{-1} (**Figure 2.5**). A 5% concentration of nano-filtered NRD showed the second-best results with a final OD of 0.75 (growth rate of 0.19 day^{-1}). Similar performances were observed for the micro-filtered NRD control (growth rate of 0.17 day^{-1}). Neither 10% nor 15% concentrations of nano-filtered digestate yielded significant growth of *C. vulgaris* (**Figure 2.5**).

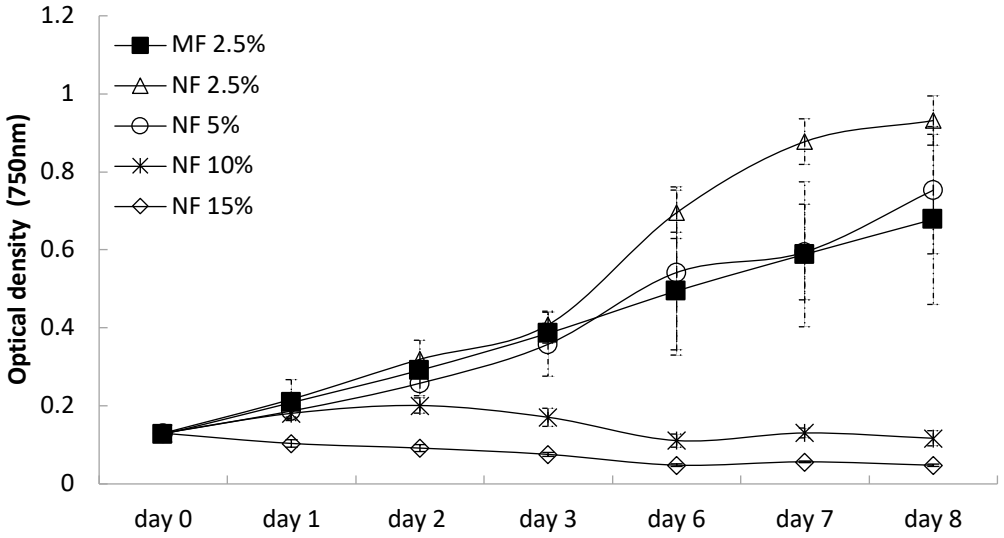

Figure 2.5: OD 750nm measurements in *C. vulgaris* cultures grown on different concentrations of nano-filtered digestate and a micro-filtered digestate control. Error bars represent standard deviation of data on three replicates.

The N:P ratio in both micro and nano-filtered digestates was different to the recommended N:P ratio for efficient growth (Redfield ratio of 16:1-N:P; Geider and Roche, 2002; Rhee and Gotham, 1980). The significant reduction of P resulting from the membrane filtration process was responsible for the N:P ratio values. Some studies reported supplementing treated digestate with artificial medium to improve microalgal growth. For example, Hollinshead et al. (2014) supplemented digestate from municipal waste sludge with BG11 medium (at a ratio of 1:4-digestate:BG11) and obtained a growth rate of 0.14 day^{-1} in *Synechocystis sp*. Additionally, unpublished data from ALG-AD project partners reported

that an addition of monosodium phosphate (NaH_2PO_4) to digestate sourced from pig manure (initial P concentration of 25 $mg.kg^{-1}$) resulted in a cell count of 1.5x108 $cells.mL^{-1}$ in cultures of *Auriantiochitrium mangrovei.* However, P supplementation results in additional production costs, especially at higher cultivation scales.

Magnesium was not detected in the nano-filtered NRD, however, magnesium is required for microalgal growth (Becker, 1994), as it acts as a chelator in the chlorophyll complex, which was found to be a limiting factor for microalgae development (Park et al., 2010). Another study supported these findings and identified magnesium as a limiting factor for the growth of microalgae on digestate sourced from pig slurry (Bjornsson et al., 2013).

In this work, low concentrations of NRD were found to be more suitable for the cultivation of *C. vulgaris*, however more work is needed to tailor specific nutrients and oligo-elements necessary to facilitate microalgal growth. Indeed, results indicated that micro and macro nutrients such as P and magnesium could be supplemented to improve growth further, but this work confirmed that *C. vulgaris* could grow on processed digestate and evidenced potential for larger scale applications and the consequent valorisation of excess NRD into microalgal biomass. Furthermore, while comparing nano-filtered digestate which was colourless (**Figure 2.4**) and micro-filtered digestate that had retained some colour (**Figure 2.4**), our results showed that growth was similar for a 2.5% diluted micro-filtered digestate and a 5% diluted nano-filtered digestate. Based on this result, it could be assumed that while using a highly diluted and filtered digestate, colour was not impacting *C. vulgaris* growth negatively.

Some studies however, demonstrated that digestate colour could have a detrimental impact on microalgal growth and associated removal of N due to limitation of light availability for photosynthesis (Marcilhac et al., 2014). Hence, a high level of dilution (implemented to reduce the load of NH_4-N in the NRD), can also be beneficial to microalgal growth by attenuating NRD colour and avoiding compromising photosynthetic performances.

Results of pilot-scale cultivation

C. vulgaris was cultivated for 51 days in an 800 L photobioreactor in semi-continuous mode under a concentration of 2.5% micro-filtered digestate. Harvesting and water renewal occurred every 6 to 7 days. Temperature, pH, and light intensity were stable throughout the time of cultivation. Results of OD at 750 nm and dry weight showed that growth was continuous for 28 days with an average growth rate of 0.143 day^{-1} (a maximum growth rate was recorded at day 3: 0.620 day^{-1}, and maximum OD 750 nm: 5.24). Productivity averaged at 47.57 $mg.L^{-1}.day^{-1}$ (with a maximum productivity recorded at day 17: 93.33 $mg.L^{-1}.day^{-1}$, and maximum dry weight: 0.86 $g.L^{-1}$) (**Figure 2.6a**). Decrease in growth was observed after 28 days of cultivation, reaching a final OD 750 nm of 1.79 and a final dry weight of 0.267 $g.L^{-1}$. N and P concentrations dropped significantly during the two first days of cultivation and a slight drop in both concentrations was observed following each addition of microfiltered digestate, showing that both N and P were used by *C. vulgaris* (**Figure 2.6b**). However, data from the full duration of the experiment revealed a slow increase in both N and P, showing that consumption of nutrients from the micro-filtered NRD by *C. vulgaris* was only partial, resulting in an accumulation of both compounds in the cultivation system.

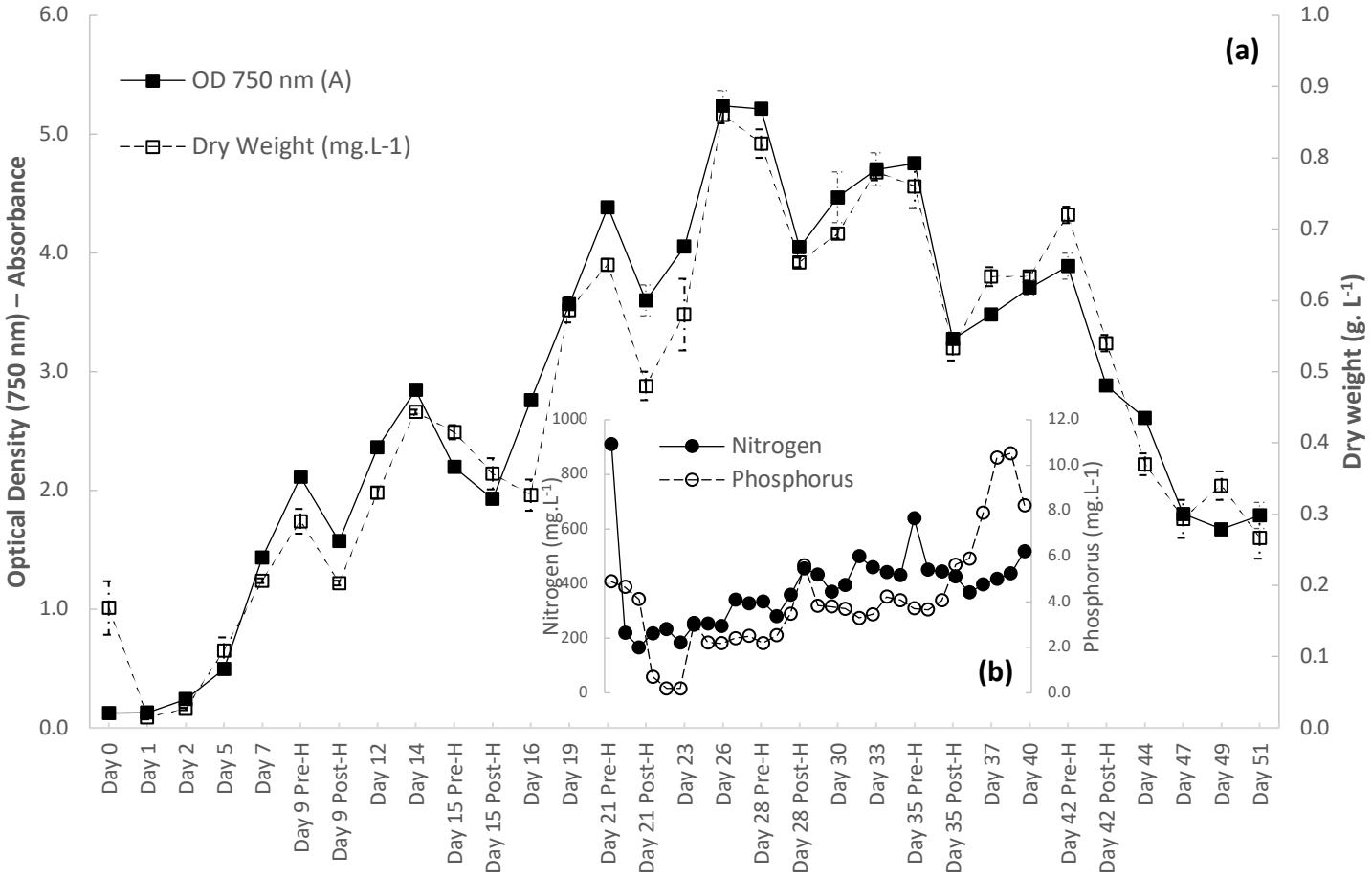

Figure 2.6: (a) OD 750 nm measurements (left axis) and dry weight measurements (in $g.L^{-1}$, right axis) in *C. vulgaris* grown in a 800 L photobioreactor. (b) Nitrogen (left axis) and phosphorus (right axis) in $mg.L^{-1}$ measured in the culture supernatant. Horizontal axis is valid for both sub figures (a) and (b): Pre-H: pre-harvest; Post-H: post-harvest. Error bars represent standard deviation of data on three replicates.

The decline of *C. vulgaris* in culture was concomitant with the increase in N and P in the cultivation medium. NH_4-N has been found to be toxic to microalgae in high concentrations (maximum tolerance of total ammoniacal nitrogen of 500 $mg.L^{-1}$ reported for *C. vulgaris* (Uggetti et al., 2014; Xia and Murphy, 2016). N had a final concentration of 518.4 $mg.L^{-1}$ (**Figure 2.6b**), corresponding to a NH_4-N concentration of 402.5 $mg.L^{-1}$. The high concentration and accumulation of NH_4-N in the system could be an element of response for the decline of *C. vulgaris* after 28 days of cultivation. Adjusting the addition of NRD in the culture could counteract the NH_4-N accumulation, for example in Marazzi et al. (2017), authors were only adding digestate (from piggery manure) when NH_4-N was fully depleted in the cultivation system, and the concentration of N was maintained below a maximum of 160 $mg.L^{-1}$, thus avoiding toxicity linked to an accumulation of NH_4-N.

Heavy metals such as aluminium, copper and zinc are present in significant amounts in digestate, and microalgae have been widely studied for their capacity to absorb heavy metals, contributing to their popularity in bioremediation technologies (Kropat et al., 2015; Papadimitriou et al., 2008). However, in the case of digestate, microalgae must cope with a mixture of different metals, and other organic compounds such as humic substances. Consequently, toxicity could have been induced by the accumulation and interference between such compounds that are absorbed by microalgae and potentially interfere with growth and cell development (Al-rub and Ashour, 2006; Mehta and Gaur, 2008; Zhou et al., 2012).

In addition, bacterial contaminations are likely to occur in large-scale systems (Subashchandrabose et al., 2011) and could have compromised the growth of *C. vulgaris* by competing with the microalgae for nutrients, but also by producing algicidal compounds responsible for the lysis of microalgal cells (Mayali and Azam, 2004). While aiming to bioremediate digestate using microalgae, an artificial consortium of several species could be considered to improve yields and use contaminations as an advantage, as they are highly likely to occur in large-scale systems. Further down the line, microalgae-bacteria consortia could be explored, as bacteria can contribute to NH_4-N remineralisation and improve remediation performance by microalgae (Hu et al., 2018; Luo et al., 2017; Sniffen et al., 2016).

Despite a growth decline after 28 days of cultivation, *C. vulgaris* presented regular growth with a high dry weight, demonstrating that cultivation of *C. vulgaris* at a large scale using digestate as a feedstock is a promising technology with the potential to convert excess nutrients from digestate into valuable biomass. Knowing that the decline in growth was likely linked to an accumulation of NH_4-N, and heavy metals in the cultivation system, solutions such as a complete harvest of the biomass and renewal of the medium could be implemented. An adjustment of the digestate addition to maintain an NH_4-N concentration below toxicity level, could also contribute to an improvement of growth beyond the 28 days of cultivation achieved in this study.

2.5. CONCLUSIONS

In this work, it was evidenced that dilution combined with settlement provided a low-cost method for processing digestate, allowing for transportation of smaller volumes of NRD to areas outside of the NVZs legislation. Release of compounds for fertiliser usage was also induced by dilution, including valuable compounds for microalgal cultivation. Membrane filtration was efficient at separating liquid and solid fractions of NRD and at recovering nearly 95% of both N and P, while significantly reducing heavy metals in the NRD permeates. Nano-filtration produced a clear liquor potentially dischargeable into the environment, showing that nano-filtration could clean-up NRD. This process remains costly and is not readily scalable for industrial uses, reinforcing the need for NRD valorisation by finding new markets for excess nutrients. Cultivation of *C. vulgaris* at pilot scale (800L) on micro-filtered digestate was successful for 28 continuous days, demonstrating the potential for biological processing of NRD to enable the conversion of excess nutrients into valuable biomass. Additional research would benefit the studied processes, for example by incorporating a pre-membrane filtration dilution step for large-scale applications, improving further microalgal growth conditions and yields by enhancing nutrient balance. The studied technology is scalable and can lead to the production of high amounts of microalgal biomass. *C. vulgaris* is a GRAS specie (Generally Regarded as Safe) for the food and feed sector, hence the production of microalgae using excess nutrients from digestate has the potential to open new markets for the AD sector and add value to increasing volumes of produced digestate.

2.6. STATEMENT OF WORK

This chapter is presented in "**Valorising nutrient-rich digestate: Dilution, settlement and membrane filtration processing for optimisation as a waste-based media for microalgal cultivation"** by Fleuriane Fernandes*, Alla Silkina, Claudio Fuentes-Grünewald, Eleanor E. Wood, Vanessa L.S. Ndovela, Darren L. Oatley-Radcliffe, Robert W. Lovitt and Carole A. Llewellyn; published on the 3rd of September 2020 in Waste Management (Volume 118, December 2020, pages 197-208). All the work was carried out by the author of this thesis apart from the following: Nanofiltration of microfiltered digestate which was performed by Dr Alla Silkina and Dr Darren L. Oatley-Radcliffe. Eleanor E. Wood assisted the author of this

thesis with sample collection for Dilution and settlement – partial processing of digestate and Vanessa L.S. Ndovela assisted the author of this thesis with sample collection for *Chlorella vulgaris* cultivation - Biological processing of digestate: Processing of micro-filtered digestate at pilot-scale. Other author contributions were of an advisory, supervisory, or proof-reading nature.

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CHAPTER 3. MICROALGAE CULTIVATION ON NUTRIENT RICH DIGESTATE: THE IMPORTANCE OF STRAIN AND DIGESTATE TAILORING UNDER pH CONTROL

3.1. ABSTRACT

The bioremediation of digestate using microalgae presents a solution to the current eutrophication issue in Northwest Europe, where the use of digestate as soil fertiliser is limited, thus resulting in an excess of digestate. Ammonium is the main nutrient of interest in digestate for microalgal cultivation and improving its availability and consequent uptake is crucial for optimal bioremediation. This work aimed to determine the influence of pH on ammonium availability in cultures of two green microalgae, additionally screened for their growth performances on three digestates produced from different feedstocks, demonstrating the importance of tailoring a microalgal strain and digestate for bioremediation purposes. Results showed that an acidic pH of 6–6.5 resulted in a better ammonium availability in the digestate media, translated into better growth yields for both *S. obliquus* (GR: 0.099 ± 0.001 day^{-1}; DW: 0.23 ± 0.02 g L^{-1}) and *C. vulgaris* (GR: 0.09 ± 0.001 day^{-1}; DW: 0.49 ± 0.012 g L^{-1}). This result was especially true when considering larger-scale applications where ammonium loss via evaporation should be avoided. The results also demonstrated that digestates from different feedstocks resulted in different growth yields and biomass composition, especially fatty acids, for which, a digestate produced from pig manure resulted in fatty acid contents of 6.94 ± 0.033% DW and 4.91 ± 0.3% DW in *S. obliquus* and *C. vulgaris*, respectively. Finally, this work demonstrated that the acclimation of microalgae to novel nutrient sources should be carefully considered, as it could convey significant advantages in terms of biomass composition, especially fatty acids and carbohydrate, for which, this study also demonstrated the importance of harvesting time.

Keywords: digestate; microalgae; pH control; ammonium; bioremediation

3.2. INTRODUCTION

Nutrient rich digestate is a by-product resulting from the anaerobic digestion (AD) of food and farm waste and is currently used as liquid fertiliser across North West Europe (Fuchs & Drosg, 2013); however, in the last decade, nutrient run-off into ground water and natural environments has been an increasing issue (Guilayn et al., 2019), leading to the

implementation of the Nitrate Vulnerable Zones (NVZs) policy, designated under the European Nitrate Directive 91/676/CEE, that limits the annual load of nitrogen applied onto arable land. Consequently, the majority of AD plants are under pressure to find alternative solutions for their excess digestate, which is currently stored or buried. Microalgae have been widely studied to remediate wastewater from water treatment processes, aquaculture facilities, and other industries (Chai et al., 2021; Silkina et al., 2019; Wollmann et al., 2019) and there has been a growing interest in utilising their bioremediation properties to tackle the growing digestate issue in Northwest Europe (Chong et al., 2022; Silkina et al., 2020; Stiles et al., 2018). Indeed, digestate composition shows a vast potential to support microalgal growth, especially in terms of macronutrients: phosphorus and nitrogen (Papadimitriou et al., 2008; Tambone et al., 2017), and also other microelements, which at the right concentrations, can provide essential nutrition to microalgae (Bauer et al., 2021). While some studies have shown the capacity of microalgae to grow on digestate, only a few have looked at optimising the process, and more precisely increasing the digestate uptake by microalgae. Indeed, digestate is characterised by high turbidity and pH (Dębowski et al., 2017; Fernandes et al., 2020), as well as a high particle content which can decrease light availability and consequently disturb the photosynthesis process and thus microalgal growth (Marcilhac et al., 2014). Additionally, the wide range of feedstocks used in the AD industry, result in a variety of digestates with very different compositions (Häfner et al., 2022; Lamolinara et al., 2022). Therefore, there is a need to tailor specific nutrients to suit microalgal growth requirements. Different strains of microalgae can also have different abilities to uptake digestate, demonstrating the need to screen specific strains for different types of digestates, allowing for the optimisation of the bioremediation process.

The value of digestate for microalgae cultivation lies in its high nitrogen content, more specifically ammonium which is the preferred nitrogen source for microalgae, indeed the metabolic cost to process ammonium into organic matter is lower than for other forms of nitrogen (Mandal et al., 2018; Salbitani & Carfagna, 2021). However, ammonium is also known for its tendency to evaporate, reducing availability for microalgae (Shen et al., 2020). Indeed, volatilisation of ammonium is a common phenomenon in cultures of microalgae grown on digestate, due to the high pH environment caused by both digestate, which has a naturally elevated pH, and microalgal growth which increases pH in cultures during

respiration. This combined effect results in a shift of ammonium from liquid to gaseous form (Chuka-ogwude, Ogbonna, Borowitzka, et al., 2020). Consequently, pH should also be considered when using microalgae to remediate digestate as this factor could play an essential role in improving ammonium uptake by microalgae, and therefore digestate remediation.

Beyond remediation purposes, growing microalgae on digestate also has significant potential to provide high value products for an array of industries, such as the animal feed or biofertiliser and biostimulant sectors (Chanda et al., 2019; Chuka-ogwude, Ogbonna, & Moheimani, 2020b; Kapoore et al., 2021). Indeed, within the context of a circular economy, it is crucial to generate value from the biomass grown using waste nutrients from digestate, contributing to reducing the production cost of microalgal systems. However, strain screening for specific digestates while looking closely at the biomass composition has been investigated only superficially, despite the tremendous potential to optimise microalgal composition by tailoring digestates.

In the presented work, two green microalgae (*Chlorella vulgaris* and *Scenedesmus obliquus*), were selected for their ability to grow on waste products (Chuka-ogwude, Ogbonna, & Moheimani, 2020b; Judd et al., 2015) and cultivated on three digestates produced from the anaerobic digestion of three different feedstocks, aiming to identify which strain would perform better on the different digestates tested. Furthermore, to improve ammonium uptake by the microalgae (and hence remediation potential), pH was also manipulated to avoid ammonium evaporation during culture, improving its availability for microalgal growth. Combining pH manipulation with strain and digestate screening and tailoring presents novelty to the field of digestate bioremediation using microalgae, by aiming to improve remediation potential using a dual approach based on abiotic and biological factors. Additionally, the composition of the generated biomass was analysed to determine if different digestate would influence differently on the microalgal composition, bringing additional information on the potential of microalgae grown on digestate as a high-value product for further applications.

3.3. MATERIALS AND METHODS

3.3.1. Digestates

Digestates resulting from the anaerobic digestion of different waste streams were used in the experiment. The digestate provided by the Langage AD facility (UK, described as SU hereafter) was produced from kitchen and food waste. Digestate from COOPERL (France) resulted from the processing of pig manure and agricultural waste; finally, digestate supplied by INNOLAB (Belgium) was plant-based with a feedstock dominated by potato peel. Resulting digestates had all a dark-brown colour and a high turbidity (see data from Fernandes et al., 2020). Consequently, all three digestates were treated according to the technique developed in Fernandes et al.(Fernandes et al., 2020) to allow microalgal cultivation. To summarise, digestates were filtered using membrane filtration at a pore size of 0.22 µm and stored at 4°C prior to the experiment.

The ammonium (NH_4^+) and phosphorus (PO_4^{3-}) compositions of each digestate were assessed using test kits, determining the level of dilution (using di-ionised water) required to bring the ammonium concentration below 100 $mg.L^{-1}$ (corresponding to 2% of the total culture volume of 800 mL) for the growth experiment and providing an insight on the N:P ratio. Results are summarised in **Table 3.1**.

Table 3.1: Ammonium and phosphorus composition of the three digestates used in the experiment.

	NH_4^+	PO_4^{3-}	N:P	Dilution factor required
SU	4815 $mg.L^{-1}$	202 $mg.L^{-1}$	23.8	48
COOPERL	3102 $mg.L^{-1}$	440 $mg.L^{-1}$	7.1	31
INNOLAB	5295 $mg.L^{-1}$	395 $mg.L^{-1}$	13.4	53

3.3.2. Experimental design

The microalgae *Chlorella vulgaris (C. vulgaris)* and *Scenedesmus obliquus (S. obliquus)* were used in the present study for their known ability to grow on waste (Judd et al., 2015). Both microalgae are freshwater species belonging to the Chlorophyta division. *C. vulgaris* and *S. obliquus* were grown in triplicates separately on the three digestates and on an F/2 control

medium to assess repeatability of the work. The composition of the F/2 control was based on the CCAP formulation, however, the main source of nitrogen in the medium, which is sodium nitrate ($NaNO_3$), was replaced by ammonium chloride (NH_4Cl, Sigma-Aldrich, Dorset, UK) in this experiment. Ammonium chloride was selected, allowing a comparison of the nitrogen consumption between the F/2 control and the digestate conditions, where ammonium was the main form of nitrogen available. Ammonium chloride was added to the F/2 medium to obtain a concentration of 100 $mg.L^{-1}$. Furthermore, magnesium in the form of $MgSO_4.7H_2O$ (Sigma-Aldrich, Dorset, UK) was added to the medium at a concentration of 0.2 $g.L^{-1}$ to reflect microalgal needs in micronutrients.

Culture conditions and inoculation

Cultures were grown in 1L Erlenmeyer flasks, at a total working volume of 800 mL, which allowed for a sufficient volume of culture for sampling throughout the experiment. Cultures were maintained at a temperature of 25°C, under a light intensity of 70 $\mu mol.m^{-2}.s^{-1}$ and a photoperiod of 12L:12D. Flasks were supplied with filtered (0.22 µm) air for constant aeration and culture agitation was provided by a shaking platform at a speed of 90 rpm, twelve hours a day. Inoculum volume was calculated to obtain an initial optical density at 750 nm of 0.5.

Nutrient addition in the form of F/2 medium and the three digestates occurred at Day 0 and no further nutrients were added for the rest of the experiment which was continued for 10 days.

pH manipulation

The pH was measured daily and adjusted using solutions of 1M HCl or 1N NaOH (Sigma-Aldrich, Dorset, UK), to maintain a pH between 6 and 6.5. The use of an acidic pH aimed to reduce losses of ammonium due to evaporation from the culture media and increase its availability for microalgae. The pH manipulation condition (pHM^+) was compared against no manipulation of the pH (pHM^-).

3.3.3. Growth measurements

1 mL of culture was sampled daily by pipetting, to assess growth rates through measurements of the absorbance at 750 nm. This specific wavelength was used as it avoids

light absorption by pigments and can be treated as a light scattering measurement (Chioccioli et al., 2014). The growth rate was calculated using the following equation:

$$\mu = ln (OD_2/OD_1) / (t_2-t_1)$$

Where μ is the specific growth rate (day^{-1}), OD_1 and OD_2 are the optical density measured at 750 nm at time 1 (t_1) and time 2 (t_2).

Flowcytometry was also performed daily on the same sample to assess the cell counts in the cultures.

Dry weight (in $g.L^{-1}$) was measured at Day 0 and Day 10 (last day of the experiment) by filtering 20 mL of a sample using pre-dried and pre-weighed filters (Whatman 47 mm GF/C glass microfiber filters, pore size: 1.2µm). Samples were oven dried for 24 hours at 80°C and dry weight was calculated as the weight difference between the dried-filtered sample and the pre-weighed-filter in relation to the volume of sample filtered as follow:

$$dw (g.L^{-1}) = ((fs-f)/Vs) * 1000$$

Where dw is the dry weight in $g.L^{-1}$, fs is the weight of the filtered and dried sample (g), f is the weight of the pre-dried filter (g) and Vs is the volume of sample filtered (mL).

3.3.4. Nutrient Analysis

Every other day, namely at Day 0, 2, 4, 6, 8 and 10, 7 mL of cultures were sampled to perform ammonium and phosphorus analysis. The NH_4^+ concentration was measured using an ammonium reagent kit (Spectroquant®), based on the colorimetric quantification of NH_4^+ (method analogous to EPA 350.1, APHA 4500-NH3 F, ISO 7150-1, and DIN 38406-5). The absorbance of treated samples was measured at 690 nm according to supplier instructions and measured against a calibration curve to determine NH_4^+ concentration. PO_4^{3-} was measured using a reagent test kit (Spectroquant®), also based on colorimetric reactions (method analogous to EPA 365.2+3, APHA 4500-P E, and DIN EN ISO 6878). Absorbance was recorded at 410 nm and P concentration was assessed as for ammonium.

3.3.5. Determination of total proteins and carbohydrates

Pellets of biomass for biochemical analysis were sampled every other day. 15 mL of culture were collected and centrifuged for 30 minutes at 4500 rpm. The supernatant was discarded,

and the pellets were washed with 2mL of DI water. Samples were further centrifuged at 10 000 rpm for 25 minutes. Supernatants were discarded and pellets were stored at -80°C for further analysis.

For the determination of the biochemical composition, a multi-assay procedure was modified for the quantification of total protein and carbohydrate(Chen & Vaidyanathan, 2013). Previously collected pellets were freeze-dried and weighed (1 – 1.5 mg) in 2 mL Safe-Lock microcentrifuge tubes. The dry pellets were resuspended in 24.3 µL of phosphate buffer (pH 7.4) and 1.8 mL of 25% (v/v) methanol in 1 N of NaOH along with an equal volume of glass beads (425–600 µm i.e., acid washed). Cells were treated using a cell disruptor (Scientific Industries Inc., NY, USA) for 3 cycles (ten-minute bead beating and two-minutes stand). For carbohydrate analysis, two aliquots of 0.2 mL extract were transferred to 2 mL PTFE capped glass vials: for the control by adding 1.2 mL 75% H_2SO_4; for the experimental samples by adding 0.4 mL 75% H_2SO_4 and 0.8 mL anthrone reagent. Samples were incubated at 100°C for 15 min followed by measurement in polystyrene cuvettes (absorbance at 578 nm). The remaining extract after cell disruption was stored at -80°C in 4 mL PTFE capped glass vials and later saponified by incubating at 100°C for 30 min. Saponified extracts of 25 µL were first placed directly into 96-well assay plates with the following additions: controls, 0.2 mL BCA reagent alone (Thermo Scientific); experimental, 0.2 mL BCA/Cu mix (Thermo Scientific) and incubated at 37°C for 30 min, measuring (absorbance at 562 nm) (Kapoore et al., 2019). The carbohydrate and protein assay standard curves were generated separately and are presented in **Figure 3.1**.

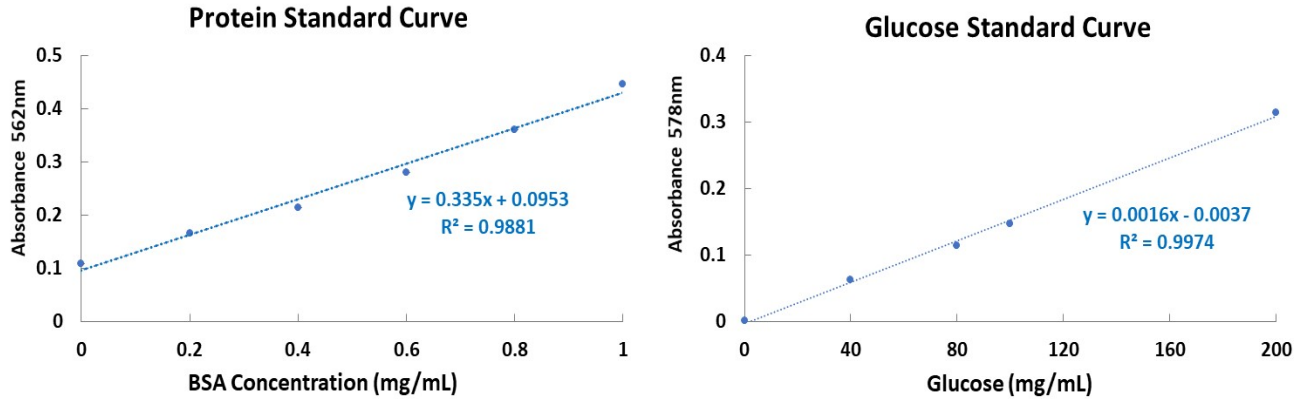

Figure 3.1: Protein and glucose standard curves for total protein and carbohydrate analyses

3.3.6. Determination of fatty acid methyl esters (FAMEs)

All chemicals and analytical reagents were of high-performance liquid chromatography grade unless stated otherwise (Sigma–Aldrich, Dorset, UK). On the day of analysis, freeze-fried algal biomass was weighed (~1 to 2 mg) followed by direct transesterification as described elsewhere (Hamed et al., 2020; Kapoore, 2014; Roccuzzo et al., 2020). 290 µL of toluene, 300 µL of 0.5M sodium methoxide and 10 µL of hexane containing C13:0 as an internal standard were added to the weighed samples, followed by incubation at 80°C for 20 min. After cooling at room temperature, 300 µL of 10% boron tri-fluoride in methanol were added and the mixture was incubated at 80°C for 20 min. After cooling to room temperature, 300 µL of water and 600 µL of hexane were added. The mixture was vortexed for 1 min and centrifuged at 18,000 g at 4°C for 10 min. The organic phase was recovered, measured and evaporated to dryness under inert nitrogen gas using a six-port mini-vap evaporator (Sigma-Aldrich, Dorset, UK).

The dried fatty acid methyl esters (FAMEs) were reconstituted in 300 µL hexane prior to identification and quantification on a GC-ToF-MS (Waters Corporation, Massachusetts, USA) using a TR-FAME capillary column (25 m × 0.32 mm × 0.25 µm). The sample volume of 1 µL was injected in split injection mode at 250˚C. The split ratio was 1:20, split flow 30 mL min^{-1} and helium carrier gas flow 1.5 mL min^{-1}. GC oven temperature was held at an initial temperature of 150˚C for 1 minute, then increased by 10˚C min-1 up to 250˚C and held at 250˚C for 1 minute; total run time was 17mins. The GC-ToF-MS was operated in EI mode at 70 eV, scanning m/z 50-700 with an initial 2-minute solvent delay. EI source temperature was held at 200˚C and GC re-entrant temperature at 250˚C. Fatty acids were identified by comparing the obtained retention times with that of known standards (37 component FAME mix, Supelco™). A Representative GC-ToF-MS chromatogram of 37 FAME Mix (C8-C24) standard is available in the supplementary material. Data acquisition and post-acquisition processing for peak identification was performed using MassLynx (version 4.1) software (Waters Corporation, Massachusetts, USA). A six-point calibration curve was generated using one internal (C13:0) and two external standards (C17:0 and C19:0). Quantitation of FAMEs in the biomass extract was then determined by comparing experimentally derived component peak areas with the calibration curve generated by the reference internal and external standards. In total, n=3 replicates (biological) were run, among which FAMEs

identified only in 2 or more replicates were considered as true hits. The data were later normalised to the dry weight of the biomass and FAMEs were reported on a percentage dry weight basis.

3.3.7. Statistical analysis

Statistical analysis was carried out on the studied factors using the R project software. Crossed factors ANOVAS were carried out on normally distributed data and normality was determined using Shapiro tests. When statistical significance was found, post hoc Tukey tests were implemented. Results were deemed significant when the p-value (p) was below 0.05.

3.4. RESULTS

3.4.1. Growth performances: Absorbance at 750 nm and Dry Weight

Chlorella vulgaris grew better on all three digestates in comparison to the F/2 control medium, both under pHM^+ and pHM^- conditions ($p<0.001$, **Figure 3.2**). Growth under pHM^- was consistent over the 10 days of experiment, and the highest growth rate was recorded for the SU digestate (0.09 day^{-1}) (**Figure 3.2 & 3.3**). In pHM^+, the growth of *C. vulgaris* was less linear, and cell clumping was observed in the cultures, this was reflected in the cell count data obtained by flowcytometry, for which no clear trends were observed (**Figure 3.3**). The highest absorbance at 750 nm was recorded for the Innolab digestate, reaching 1.26 ± 0.15 after 6 days of the experiment, this was however followed by a decrease until the end of the experiment (**Figure 3.2)**. The Cooperl digestate showed second best performance with an absorbance of 1.08 ± 0.05, followed by the SU digestate (0.93 ± 0.05) at the end of the experiment.

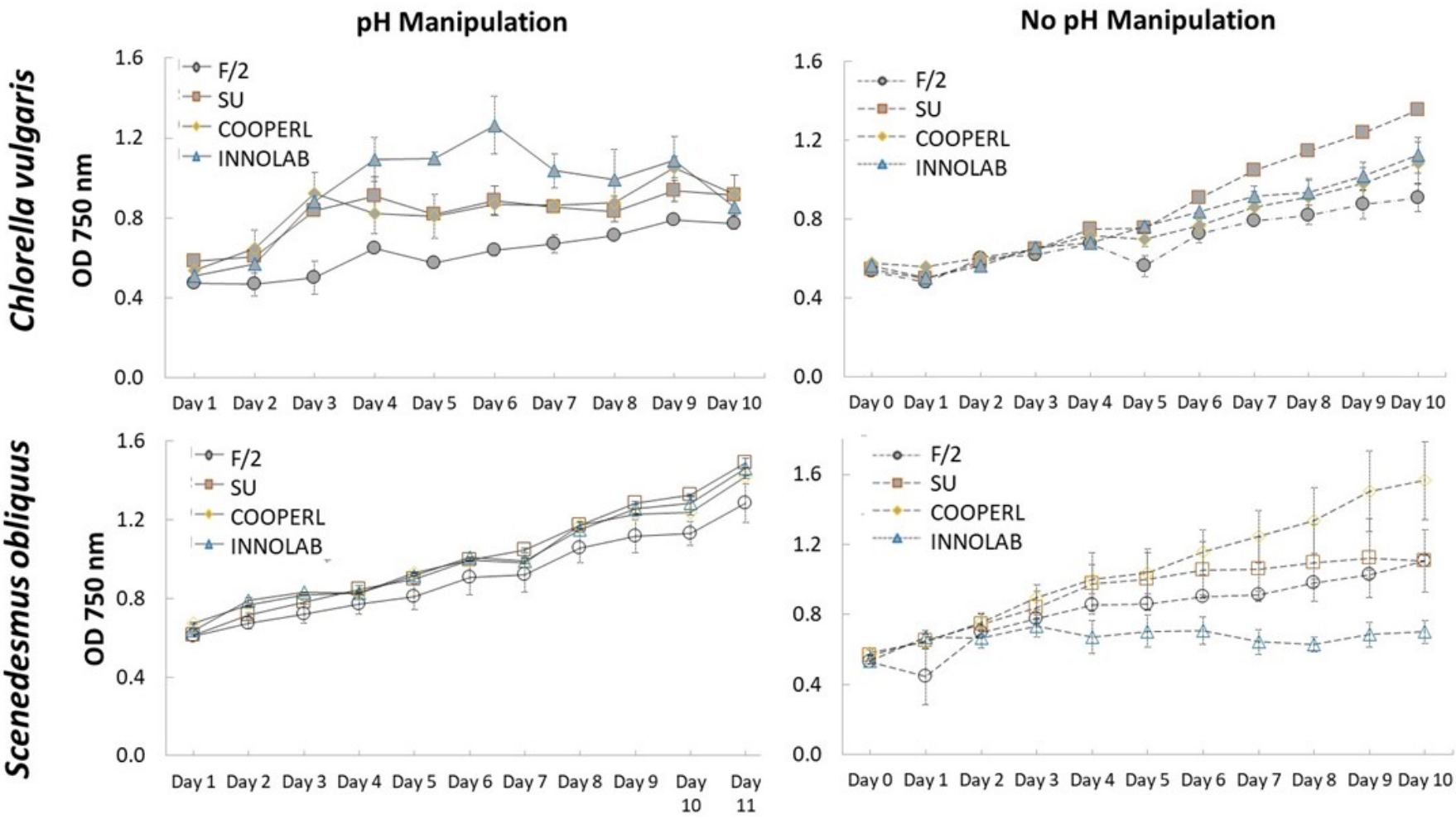

Figure 3.2: Absorbance at 750 nm for *C. vulgaris* and *S. obliquus* grown on three different digestates (F/2: orange square; SU: yellow diamond: COOPERL; blue triangle: INNOLAB) under pHM^{+}/ pHM^{-} for 10 days of experiment. Error bars represent the standard deviation on three replicates.

Under pHM^-, *S. obliquus* showed the best performances in the Cooperl digestate ($p<0.001$; GR: 0.099 day^{-1}), followed by the SU digestate (GR: 0.074 day^{-1}), the F/2 control (GR: 0.067 day^{-1}) and Innolab digestate (GR: 0.027 day^{-1}). These differences were also apparent in the cell count data (**Figure 3.3**). Under pHM^+, growth was more consistent between conditions and performances were very similar between digestates (GR: 0.081 day^{-1}; 0.067 day^{-1} and 0.078 day^{-1}, for SU, Cooperl and Innolab digestates, respectively). Growth was slightly lower for the F/2 control (GR: 0.059 day^{-1}), but no significant differences were found between all the tested conditions ($p>0.05$, **Figure 3.2**). Cell count also showed a similar increase over the time of the experiment for all digestate tested and a plateau was observed between day 9 and 11 of the experiment (**Figure 3.3**).

Additionally, there were no significant differences between the performances of the two strains when pH was not manipulated ($p<0.05$), however, *S. obliquus* showed significantly better growth under an acidic pH ($p<0.001$), reaching overall a higher absorbance at 750 nm at the end of the 10 days of experiment (**Figure 3.2**).

Dry weight analysis showed discrepancies at Day 0 between the different conditions tested, despite inoculum concentrations being the same for both cultures of *C. vulgaris* and *S. obliquus* (**Figure 3.5**). Indeed, despite the use of membrane filtration to treat the three different digestates, a significant number of particles can still be found in the filtration permeates, therefore contributing to the dry weights recorded at the start of the experiment.

Dry weight significantly increased during the time of experiment ($p<0.01$) and differences observed between conditions after 10 days were mainly related to microalgal growth. Results showed that dry weight in cultures of *C. vulgaris* was higher in the Innolab digestate and under pHM^- (0.28 ± 0.025 $g.L^{-1}$), while in pHM^+, dry weight was the highest in the Cooperl digestate (0.49 ± 0.012 $g.L^{-1}$). Overall, DW was significantly higher in cultures of *C. vulgaris* in pHM^+ for all digestates tested and the F/2 control ($p<0.001$), even doubling in some of the studied conditions (**Figure 3.4**).

In cultures of *S. obliquus*, the pH manipulation resulted in a higher dry weight in the Innolab digestate and the F/2 control ($p<0.001$), however, no significant changes were observed in the Cooperl and SU digestates, with a DW of around 0.23 ± 0.002 $g.L^{-1}$ in $pHM^{-/+}$ and in both

digestates ($p>0.05$). Finally, *C. vulgaris* yielded a higher dry weight at the end of the experiment under pHM^+, in comparison to cultures of *S. obliquus* under the same conditions of pH (**Figure 3.4**).

3.4.2. Nutrient consumption: ammonium and phosphorus

The concentration of NH_4^+ decreased rapidly after the start of the experiment in both cultures of *C. vulgaris* and *S. obliquus*, and for all three digestates tested when the pH was not manipulated. The decrease continued steadily and final NH_4^+ concentration in the different media was significantly lower than at the beginning of the experiment ($p<0.001$, **Figure 3.5**). No decrease in the NH_4^+ concentration was observed in the F/2 control which maintained a consistent content of ammonium throughout the experiment and for both strains. The observed decrease in ammonium however was not in accordance with the dry weight data measured previously (**Figure 3.4**), and it could be assumed that the recorded decrease was mainly linked to evaporation of the ammonium, most likely induced by the naturally high pH of digestate. Indeed, NH_4^+ did not decrease in the F/2 control where the pH remained neutral. However, the microalgae did not consume this source of nitrogen despite its availability.

Under pH manipulation, the concentration of NH_4^+ remained the same in cultures of *C. vulgaris,* for all three digestates and the control, with a concentration averaging 83.7 ± 1.83 $mg.L^{-1}$ ($p>0.05$). Therefore NH_4^+ was not consumed by *C. vulgaris* during the 10 days of experiment. The same result was observed in cultures of *S. obliquus*, up to day 8 of the experiment. However, NH_4^+ decreased significantly between day 8 and day 10 ($p<0.001$) and reached values below 20 ± 3.93 $mg.L^{-1}$ in the three tested digestates, showing a rapid consumption of ammonium by the microalgae (**Figure 3.5**). In this instance, it was demonstrated that the ammonium evaporation did not occur under an acidic pH, however, NH_4^+ was still not consumed by the microalgae, or only until a later stage of the experiment. Therefore, it could be hypothesised that both *C. vulgaris* and *S. obliquus* were acclimating to the novel source of nitrogen (ammonium from digestate or ammonium chloride in the F/2 control).

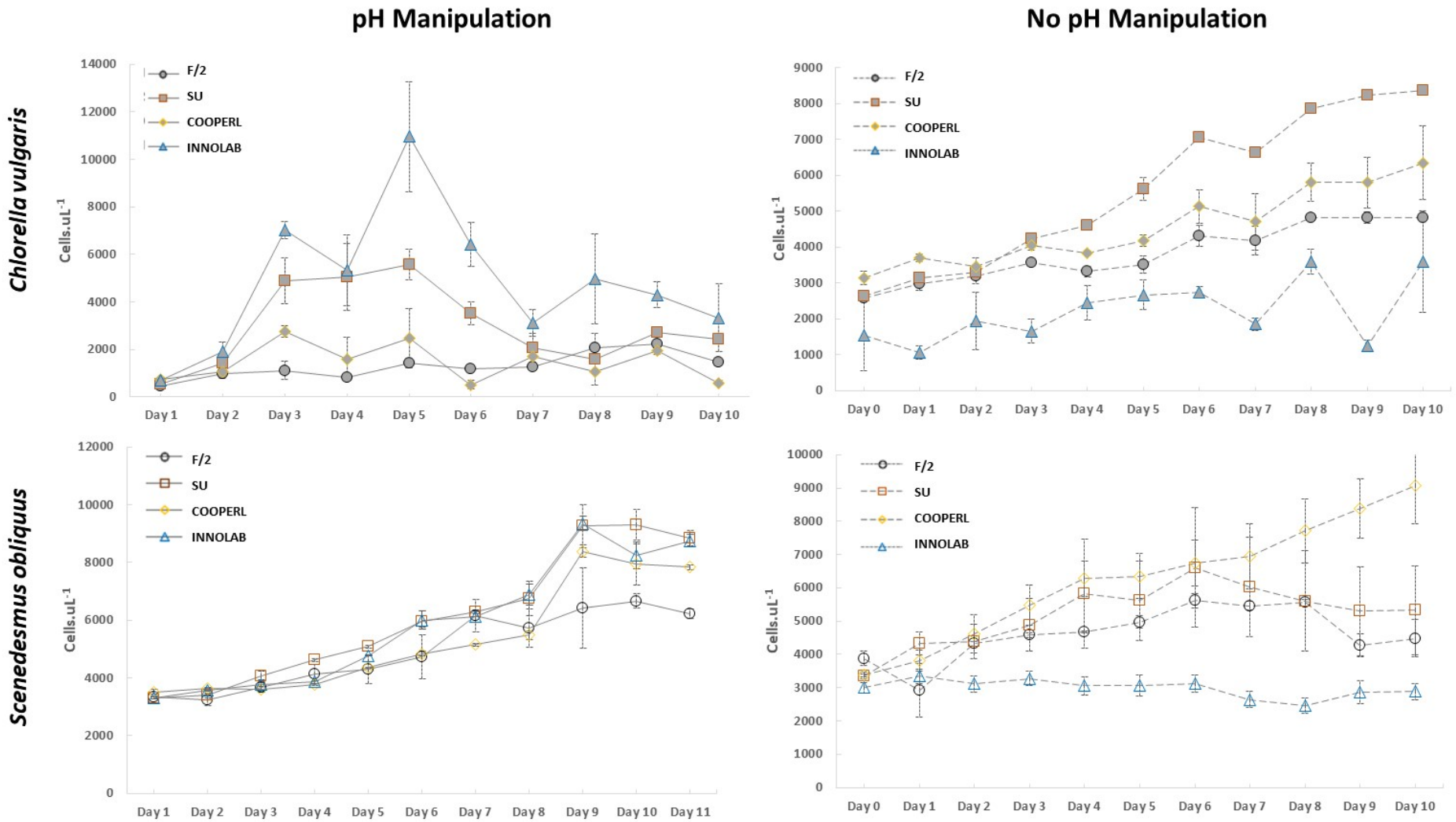

Figure 3.3: cell count per µL for *C. vulgaris* and *S. obliquus* grown on three different digestates (F/2: orange square; SU: yellow diamond: COOPERL; blue triangle: INNOLAB) under pHM^{+}/ pHM^{-} for 10 days of experiment. Error bars represent the standard deviation on three replicates.

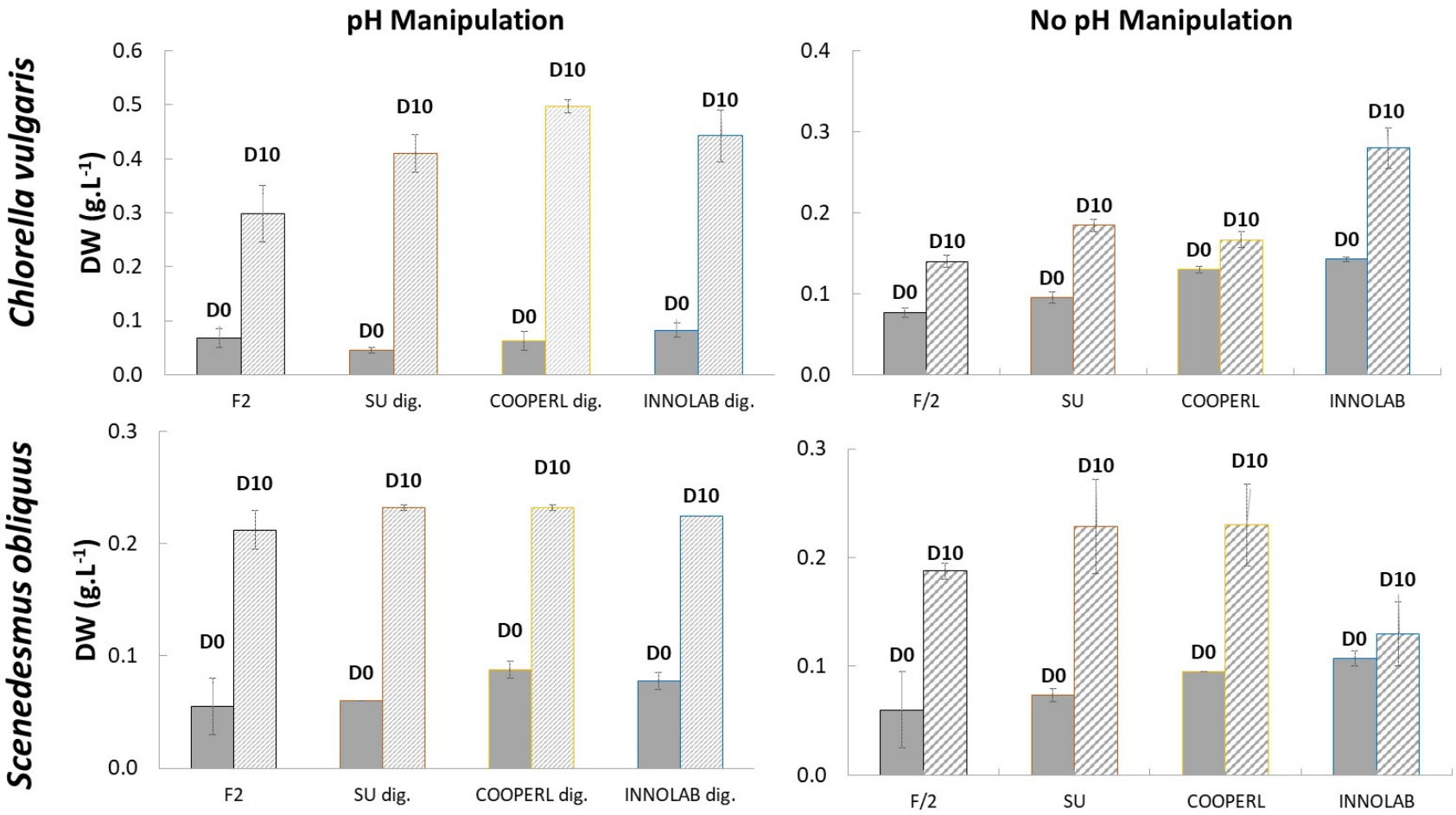

Figure 3.4: Dry weight of *C. vulgaris* and *S. obliquus* grown on three different digestates under pH manipulation and no pH manipulation on day 0 and 10 of the experiment. Error bars represent the standard deviation on three replicates.

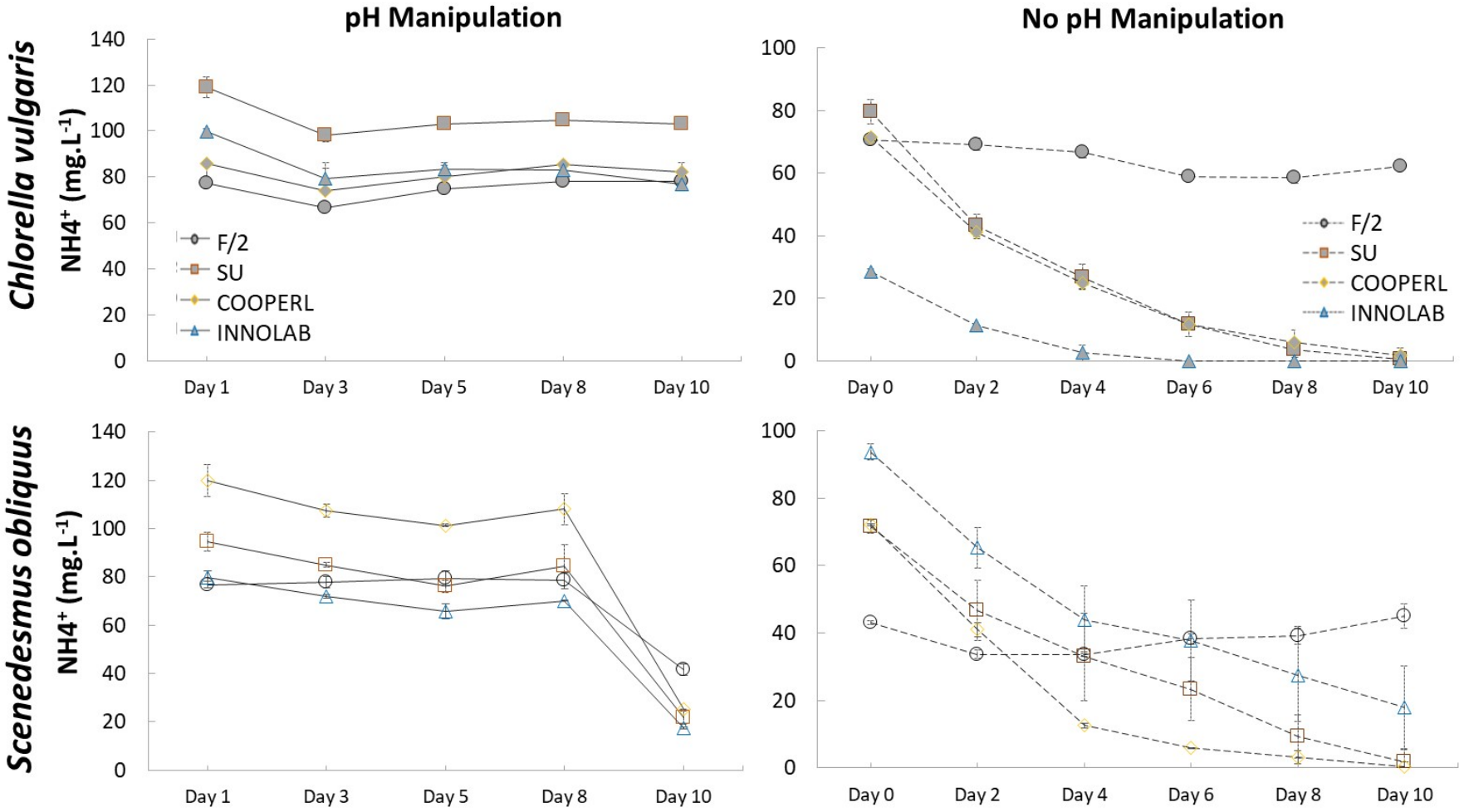

Figure 3.5: Concentration of ammonium NH_4^+ in cultures of *C. vulgaris* and *S. obliquus* grown on three different digestates (F/2: orange square; SU: yellow diamond: COOPERL; blue triangle: INNOLAB) under pHM^+/ pHM^- for 10 days of experiment. Error bars represent the standard deviation on three replicates.

Phosphorus decreased over time in cultures of *C. vulgaris* when pH was not manipulated, showing consumption of the nutrient, especially in the Cooperl and Innolab digestates. A decrease in the phosphorus concentration was also observed in cultures of *S. obliquus* in pHM^- for all the conditions tested, except for cultures grown in the Innolab digestate, where phosphorus increased after 4 days of cultivation reaching 8.86 ± 0.154 $mg.L^{-1}$ (**Figure 3.6**). Growth data presented previously showed that *S. obliquus* performed less well when grown on the Innolab digestate (**Figure 3.2,3.3 & 3.4**). Microalgal cells are known to release phosphorus when decaying, therefore the observed increase in phosphorus could be linked to cells of *S. obliquus* degrading in the Innolab digestate and releasing stored phosphorus.

Under an acidic pH, phosphorus increased during the time of experiment in cultures of *C. vulgaris* but decreased overall in cultures of *S. obliquus* (**Figure 3.6**). While regulating the pH using HCl, lower pHs were recorded in cultures of *C. vulgaris*, and a pH lower than 6 was measured several times across the time of experiment. Under an acidic pH, particles dissolve more easily, and in this study, it was possible that particles remaining in the different digestate would have released phosphorus when dissolving in the acidic environment, explaining the observed results. Additionally, growth data showed that *S. obliquus* performed better in pHM^+, which could also explain the decrease in phosphorus for this strain, using the nutrient for growth purposes.

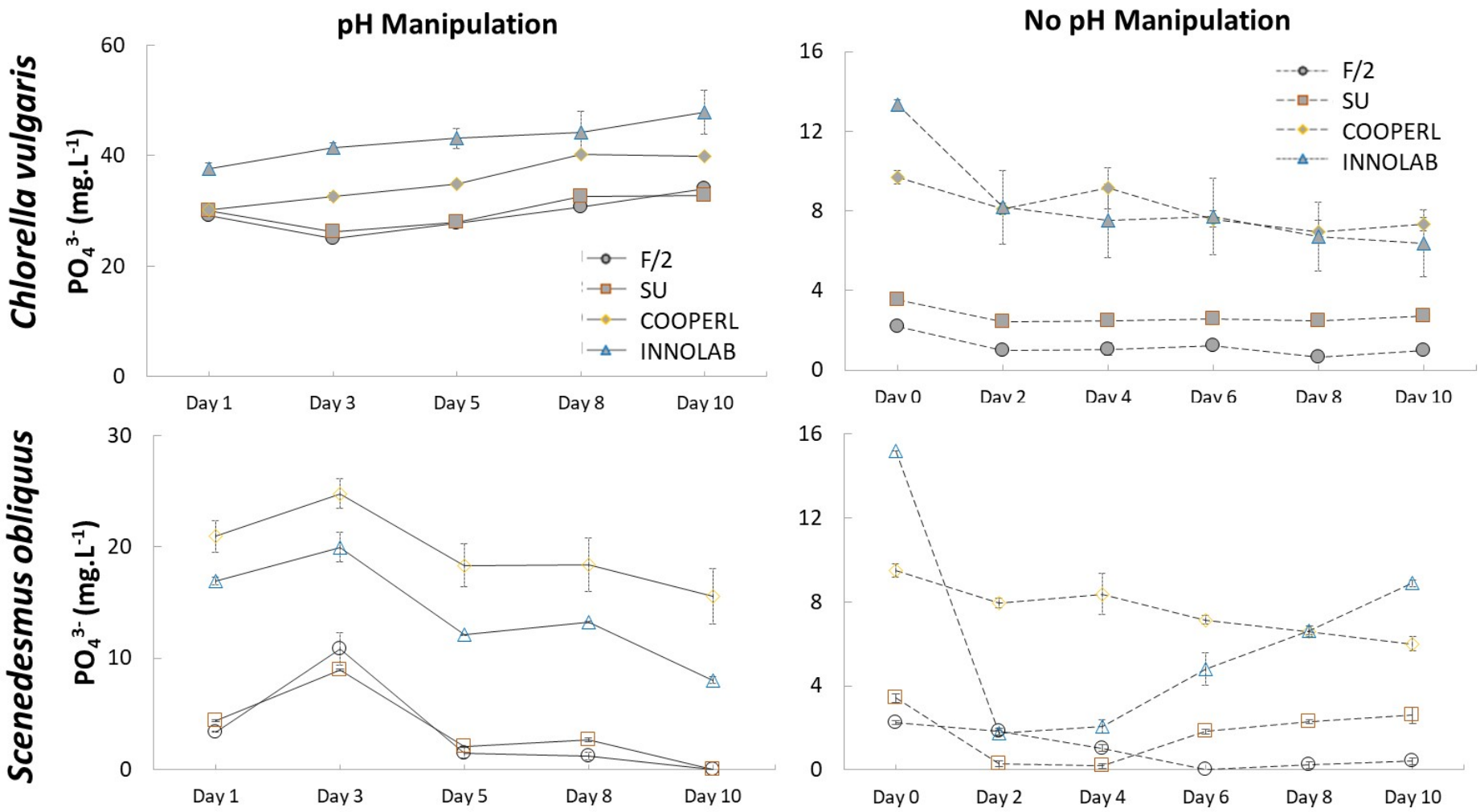

Figure 3.6: Concentration of phosphorus PO_4^{3-} in cultures of *C. vulgaris* and *S. obliquus* grown on three different digestates under pH manipulation and no pH manipulation for 10 days of experiment. Error bars represent the standard deviation on three replicates.

3.4.3. Composition analysis: fatty acids, proteins and carbohydrates

Analysis of the biomass composition was performed on the cultures where pH was manipulated. Indeed, cultures under pHM^+ yielded more biomass (**Figure 3.4**) and efforts were therefore concentrated towards analysing these specific samples.

In cultures of *C. vulgaris*, total fatty acids increased significantly over the time of experiment for all conditions tested ($p < 0.001$), with a most significant increase between day 6 and day 10 for the F/2 control and both SU and Innolab digestates, while FAs concentration was the highest after 6 days in the Cooperl digestate, reaching 4.91 ± 0.3 % DW (**Figure 3.7**). A similar trend was observed in the Total MUFAs and PUFAs concentrations, especially for the SU digestate, with a 5-fold increase between Day 0 and Day 10 (**Figure 3.8**). Additionally, most of the fatty acids measured as part of this work were found in higher concentrations in cultures grown using the different digestate than in the F/2 control ($p< 0.001$) (**Figure 3.8**).

In cultures of *S. obliquus*, the highest concentrations of fatty acids were measured on Day 6 of the experiment for all tested conditions. Additionally, the highest fatty acids content was recorded in cultures grown on the Cooperl digestate (6.94 ± 0.33 % DW), however, there was no significant differences between the F/2 control and the digestates conditions on Day 6 ($p>0.05$, **Figure 3.7**). Finally, statistical analysis showed that the total fatty acid content was not significantly different between the two tested strains at the beginning of the experiment, but that over the course of the study, concentrations of fatty acids were significantly higher in cultures of *S. obliquus* ($p<0.001$).

The protein content generally decreased over time or maintained a constant concentration for all digestates studied and the control for both cultures of *C. vulgaris* and *S. obliquus*. Apart from the odd increase on Day 6 of the experiment, the protein content after 10 days of culture was significantly lower ($p<0.001$) or similar ($p>0.05$) to the protein concentration recorded at the beginning of the experiment. For example, in the Cooperl digestate, protein content decreased from 46.8 ± 0.001 to 27.8 ± 0.001 % DW in cultures of *C. vulgaris*, and from 43.5 ± 0.001 to 29.6 ± 0.001 % DW in cultures of *S. obliquus* (**Figure 3.7**). The observed results were in accordance with the low nutrient consumption described previously and shown in Figure 3.3. Additionally, there were no significant differences between the protein content of the two strains ($p>0.05$).

Carbohydrates increased during the experiment in both cultures and for all conditions tested. In cultures of *C. vulgaris*, the most significant increases occurred between Day 6 and Day 10 of the experiment, in the F/2 control and cultures grown on the Cooperl and Innolab digestates ($p<0.001$), with the highest carbohydrate concentration of 33 ± 2 % DW measured in the F/2 control (**Figure 3.7**). In cultures of *S. obliquus*, an increase in carbohydrate content was the most significant between Day 0 and Day 6 ($p<0.001$) for the F/2 control and the SU and Innolab digestates. In cultures grown on the Cooperl digestate, carbohydrates increased the most between days 6 and 10 and reached the highest recorded carbohydrate concentration of 28.8 ± 1.0 % DW. Statistically, carbohydrates were significantly higher in cultures of *C. vulgaris* in the F/2 control ($p<0.001$, **Figure 3.7**), however, no significant differences were found between the two strains in the digestates conditions ($p>0.05$).

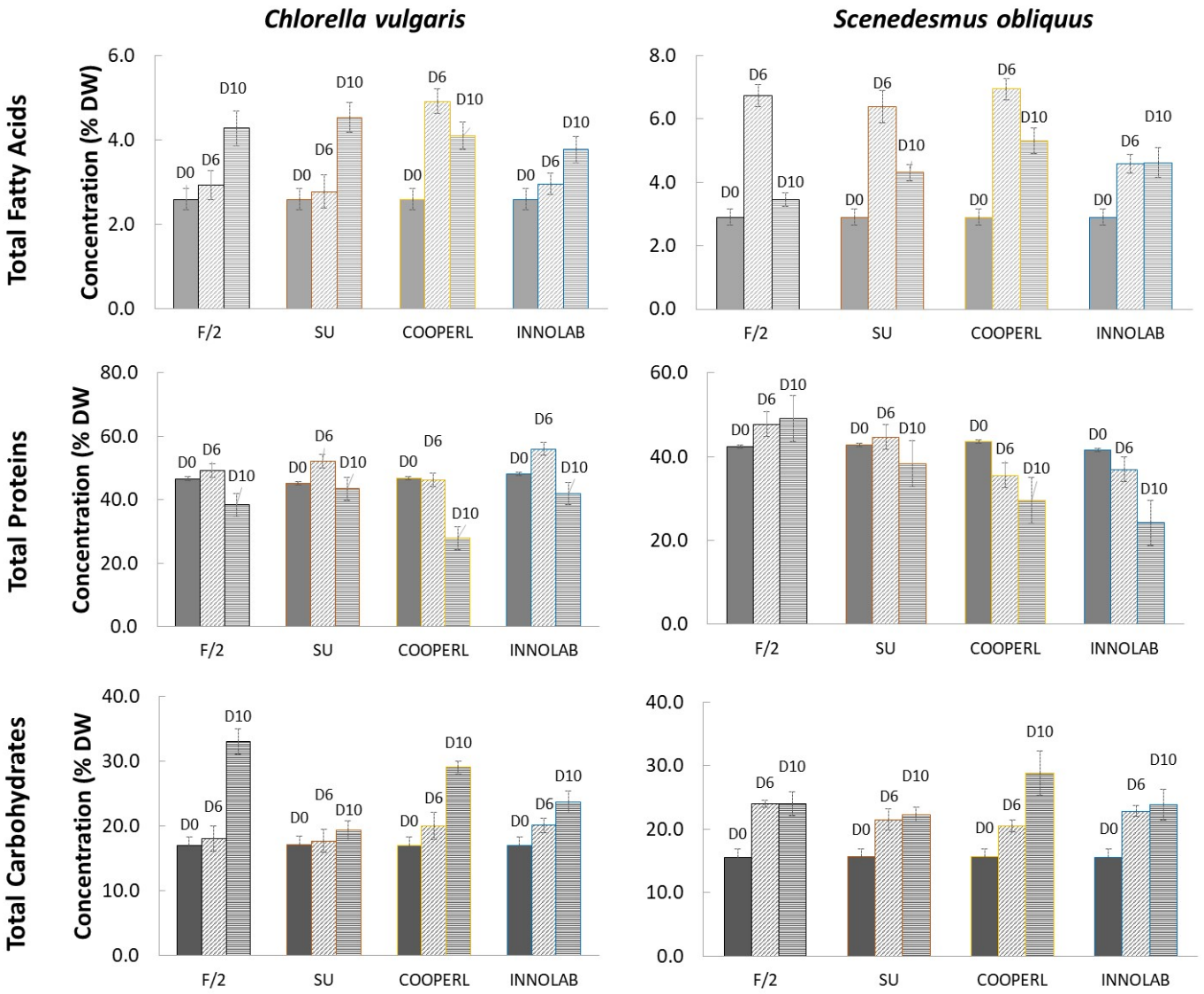

Figure 3.7: Total Fatty acids, proteins and carbohydrates content in cultures of *C. vulgaris* and *S. obliquus* grown on three different digestates under pH manipulation on day 0, 6 and 10 of the experiment. Error bars represent the standard deviation on three replicates.

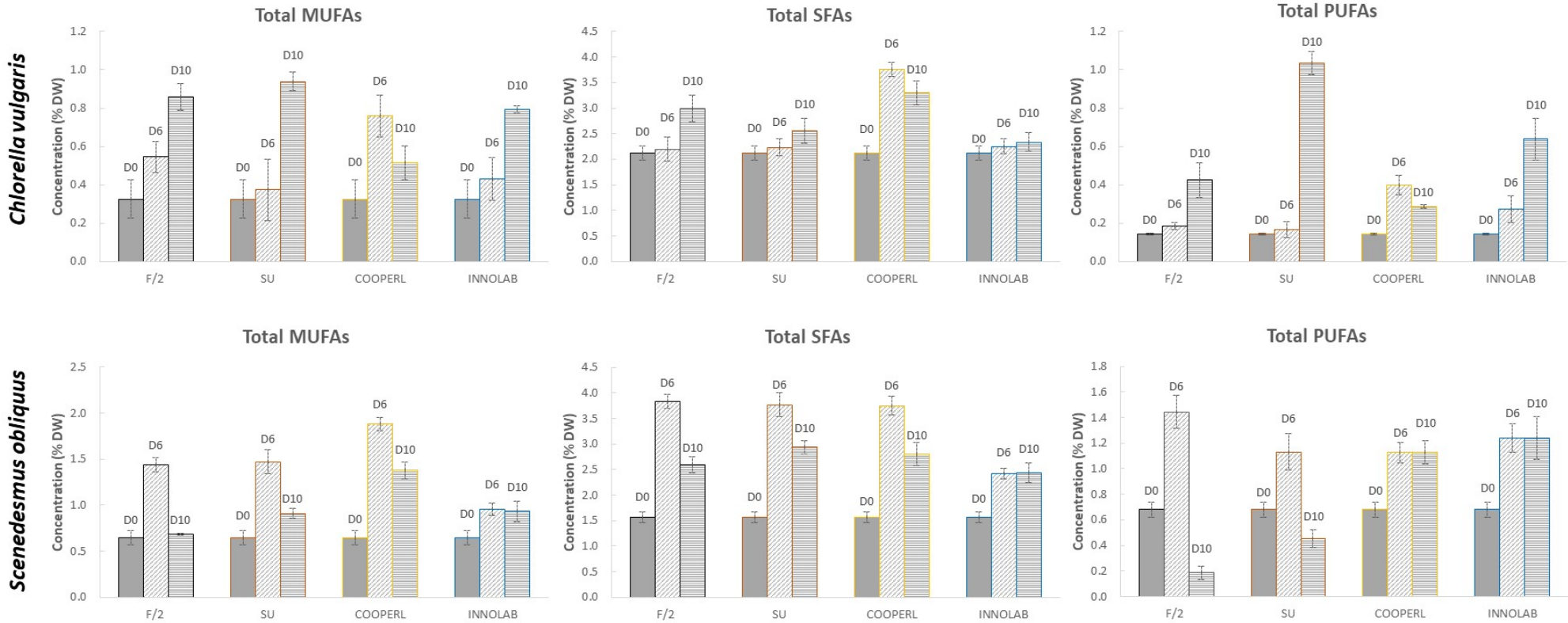

Figure 3.8: Total MUFAs, SFAs and PUFAs in cultures of *C. vulgaris* and *S. obliquus* grown on three different digestates under pH manipulation on day 0, 6 and 10 of the experiment. Error bars represent the standard deviation on three replicates.

3.5. DISCUSSION

In this study, two microalgal strains were grown on three digestates resulting from the anaerobic digestion of different feedstocks. Digestates were treated to suit microalgal needs and a concentration of 2% of the three digestates was used to grow *C. vulgaris* and *S. obliquus* for 10 days (digestate optimisation based on Fernandes et al., 2020). Due to the volatile nature of ammonium, especially in high pH, which is a characteristic of digestate, pH was manipulated to reach acidic values of 6-6.5 and a comparison was made against cultures where pH was not modified.

Results showed that pH manipulation to limit NH_4^+ evaporation from the different tested media generated better performances from the microalgae *S. obliquus*, with an overall better growth rate and dry weights recorded across all digestates. The same pH manipulation did not necessarily yield better growth in cultures of *C. vulgaris*, which demonstrated signs of stress, mainly via clumping of cells. However, dry weight was higher when the pH was maintained to be acidic in comparison to no pH manipulation. Additionally, nutrient data revealed that evaporation likely took place when the pH was not manipulated, indeed the observed decrease in ammonium during the experiment did not coincide with a corresponding gain in the biomass dry weight. A delayed consumption of ammonium was observed for *S. obliquus* under an acidic pH and the gain in dry weight was more in accordance with recorded ammonium data. The slow consumption of ammonium by both *S. obliquus* and *C. vulgaris* could be the result of an acclimation to the ammonium present in digestate; the same acclimation occurred in the modified F/2 control where the nitrogen source was supplied by ammonium chloride, demonstrating that ammonium was the main factor resulting in the observed acclimation and that digestate did not present other toxicity to both microalgal strains. Acclimation to a newly introduced source of nitrogen has been described in the literature, for example, Chuka-ogwude et al.(Chuka-ogwude, Ogbonna, & Moheimani, 2020a) showed that 6 weeks of acclimation time to high ammonium concentration was needed for both *C. vulgaris* and *S. obliquus*. In the present study, *C. vulgaris* did not appear to consume ammonium from digestate after 10 days of experiment, however *S. obliquus* consumed ammonium almost instantly after 8 days of acclimation, showing better acclimation capabilities in the presented work. Phosphorus was mainly consumed under no pH manipulation, except in decaying cultures, where increasing

concentrations of phosphorus were recorded and likely linked to a release from dying cells (Brown & Shilton, 2014; Su et al, 2021). In cultures of *C. vulgaris*, an acidic pH induced a phosphorus release via the dissolution of digestate particles, thus the observed increase of the compound over the course of the experiment (Gerardo et al., 2015; Mukherjee et al., 2015; Su et al., 2016). However, in cultures of *S. obliquus*, a decrease of phosphorus was observed after several days, showing a consumption by the microalgae. In environments with high concentrations of phosphorus, the phenomenon of P luxury uptake can occur (Sforza et al., 2018; Solovchenko et al., 2019), and phosphorus is accumulated under the form of polyphosphate in microalgal cells (Eixler et al., 2006; John & Flynn, 2000). Polyphosphates facilitate the incorporation of metal ions and consequently can contribute to the remediation of heavy metals in waste streams (Nishikawa et al., 2003), additionally, the excess phosphorus uptake by microalgae promotes phosphorus removal from waste (Powell et al., 2011). In the presented work, an acidic pH seemed to promote luxury uptake in *S. obliquus*, demonstrating the relevance of pH control in waste bioremediation systems.

In this study, an acidic pH provided better growth performances, especially in cultures of *S. obliquus*, this was in accordance with findings from Guedes et al.(Guedes et al., 2011) who demonstrated that the highest growth rate and biomass productivity in *S.obliquus* were associated with a pH of 6. The better growth performances in acidic pH could be linked to better nutrient availability as discussed above. Indeed, while an acclimation phase occurred, ammonium was available for the microalgae to uptake beyond this acclimation, rather than evaporating into the environment. This conveyed a significant advantage in terms of reducing ammonium leakage into the atmosphere, especially when considering a larger scale for industrial applications of waste remediation by microalgae. Furthermore, a higher ammonium availability allowed for greater biomass accumulation, which was reflected in the dry weight recorded at the end of experiment. Bouras et al. (2022) found that a neutral pH of 7 provided the best performances in terms of growth and DHA accumulation in *Schizochytrium limacinum*, however, they also tested a pH of 6 which yielded the second-best performances in cultures. On the contrary, higher pHs of 8 and 9 resulted in less growth as well as a rapid ammonium decrease from the medium, linked to evaporation and the authors directly linked the lesser growth to the lack of ammonium availability in the medium following evaporation.

The presented work demonstrated that an acidic pH had clear advantages in terms of improved nutrient availability and uptake, resulting in more consistent and higher biomass production from microalgae grown on digestate, and this result could be further improved by looking at acclimation strategies to novel sources of nitrogen. Due to the naturally high pH of digestate, efforts should be made towards controlling this factor to ensure that ammonium recovery is mainly performed via microalgal growth, rather than evaporation. This is particularly true when considering the larger-scale system, for which abiotic parameters are more difficult to control and evaporation can easily occur.

Another aspect of this study was to assess if there was any specificity between microalgal strains and digestates, in an effort to further optimise remediation by tailoring the combination of digestate and strain. Regardless of pH manipulation, both microalgal strains yielded less growth on the modified F/2 control in comparison to the three tested digestates. This could be explained by ammonium chloride being less available to microalgae in comparison to ammonium from digestate. Additionally, digestate can be enriched with dissolved CO_2 generated during the methane fermentation process, CO_2 that can be utilised for photosynthesis (Khanh et al., 2013). Under no pH manipulation, *C. vulgaris* grew better on the SU digestate, while growth was higher in the Innolab digestate when an acidic pH was maintained. In cultures of *S. obliquus*, better performances were recorded for SU and Cooperl digestates in both pHM^+ and pHM^-, which was reflected in the absorbance and dry weight data. Therefore, it can be assumed that certain types of digestates can be favourable to some microalgal strains, however, this can be modified by environmental factors, and in the case of the presented study, by pH manipulation, which was particularly true for *C. vulgaris*. It could hence be assumed from previous results that an acidic pH had the potential to modify digestates properties, notably by increasing nutrient availability, hence shifting strain performances from one digestate to another. Similarly, digestates yielding less growth could have inhibitory factors specific to the tested strains (Al-Mallahi & Ishii, 2022). The literature focuses on ammonium toxicity (Jiang et al., 2021; Park et al., 2010; Q. Wang et al., 2021), but digestate composition can be extremely complex and other inhibitory elements can come at play. Another well-known property of digestate is its dark colour, limiting light availability for photosynthesis, and this has been mainly counteracted by dilution (including in this study) (Cheunbarn & Peerapornpisal, 2010; Tan et

al., 2016; Uggetti et al., 2014; Zuliani et al., 2016). The transmittance of the different digestates used in this work could explain why the two microalgal strains yielded different growth rates on the three tested digestates. Indeed, while the three digestates were diluted to obtain similar ammonium concentration, transmittances were different as the digestates did not have the same particle composition and were produced from different feedstocks. Wang et al.(L. Wang et al., 2010) demonstrated that microalgal growth was negatively correlated to the digestate transmittance, therefore light availability could be one of the factors behind the different growth rates observed in the present experiment. The presented work demonstrated that microalgal strains can perform differently on different types of digestates, with a clear influence of environmental factors such as pH or light transmittance. Thus, when considering microalgae for digestate remediation, it is crucial to implement an initial strain screening, to optimise digestate uptake and consequently bioremediation.

Composition analysis of *C. vulgaris* and *S. obliquus* showed a higher fatty acid content when the strains were grown on the different digestates, in comparison to the F/2 control. Similar findings can be seen in the literature (Koutra et al., 2018), especially when diluted digestate is used (which was the case in this work). Indeed, the lower nitrogen content of diluted digestate allows for an increase in lipid accumulation, and this was found for many microalgal species, including *Neochloris oleoabundan* (Olguín et al., 2015), *Synechocystis sp* and *Nannochloropsis salina* (Cai et al., 2013), *Chlorella sp, Scendesmus sp* and *Nannochlorpsis gaditana* (Zuliani et al., 2016). Additionally, fatty acid accumulation was different between the two microalgal strains studied, indeed, higher fatty acids concentrations were found after 10 days of experiment for *C. vulgaris*, while the same result was observed after 6 days of experiment in cultures of *S. obliquus*. Fatty acid accumulation in microalgal cells is mainly based on nutrient deprivation (Bouras et al., 2022; Xu et al., 2020), in this study, nitrogen results showed very little consumption of the ammonium from the digestates until the end of the experiment, due to an acclimation period. This could explain why *C. vulgaris* had the highest fatty acids content at the end of the experiment, as nutrients from the upscaling phase were depleted and the microalgae had not acclimatised to the ammonium from digestate yet. *S. obliquus* had the highest concentration of fatty acids at day 6, and ammonium results showed that the microalgae consumed ammonium

almost instantly between day 8 and day 10 of the experiment. Therefore, fatty acids were accumulated until day 6 when nutrients were depleted and decreased once *S. obliquus* acclimatised to the novel source of ammonium, shifting from nutrient depletion to nutrient consumption. However, the very limited consumption of nitrogen linked to the observed acclimation of both strains lead to a decrease of proteins over the time of experiment. Indeed, protein accumulation is directly correlated to nitrogen uptake (Kumar & Bera, 2020; Michelon et al., 2016), which was very minimal in the present study. Carbohydrates on the other hand increased significantly during the experiment, reaching maximum concentrations after 10 days for all digestates and the F/2 control. Similarly to lipid content, nitrogen starvation can increase the accumulation of carbohydrates (Depraetere et al., 2015; Massa et al., 2017; Michelon et al., 2016; Nwoba et al., 2016). Indeed, under nitrogen-depletion, microalgae can transform proteins into carbohydrates by shifting the use of photosynthetic carbon from the metabolic pathway of protein synthesis to carbohydrates synthesis, hence accumulating this compound as energy storage (Huo et al., 2011; Ledda et al., 2016). This was also in accordance with the observed decrease of proteins during the present experiment. Additionally, the synchronic increase in lipids and carbohydrates can be explained by their parallel synthesis pathways where energy is firstly stored as carbohydrates and then converted into lipids (BenMoussa-Dahmen et al., 2016). Similar results were found in the literature, for example, Bránykiová et al. (2011) reported a carbohydrate accumulation of up to 410 $g.kg^{-1}$ in cultures of *C. vulgaris* where nitrogen was depleted and Ji et al. (2011) obtained a carbohydrate content in *Tetraselmis subcordiformis* of 350 $g.kg^{-1}$.

In this study, the acclimation of *C. vulgaris* and *S. obliquus* to ammonium from digestate, which resulted in conditions of nitrogen-depletion, also conveyed advantages in terms of macronutrient accumulation, with significant increases of lipids and carbohydrates in the produced biomass. It can be further assumed that after acclimation and when ammonium consumption resumes, other compounds such as proteins could be in turn accumulated. Therefore, harvesting time of the biomass is crucial when targeting specific compounds from microalgae grown on digestate and different accumulation stages can occur, demonstrating the versatility of digestate to obtain a variety of high-value products.

3.6. CONCLUSIONS

The presented work demonstrated the importance of pH control, especially in terms of nutrient availability for microalgae, to ensure that bioremediation takes place via microalgal growth rather than by ammonium loss to the atmosphere. While looking at upscaling this technology, lower pHs of 6-6.5 should be maintained to minimise these ammonium losses which are bound to occur in larger scale systems. This study also emphasised the necessity for strain screening when considering digestate bioremediation, indeed strains can perform differently on different digestates from different feedstock, here a pig manure based digestate yielded better dry weights in both *S. obliquus* (0.23 $g.L^{-1}$) and *C. vulgaris* (0.49 $g.L^{-1}$). Additionally, environmental factors and specific inhibitors can also influence performances and should be carefully considered. Finally, this study shed some light on acclimation processes in microalgae and on the resulting biomass composition, showing some advantages in terms of lipids and carbohydrate composition, where nitrogen starvation resulted in 6.94% DW of fatty acids in *S. obliquus*. Harvesting time was also found to be an impactful factor when targeting high-value compounds, hence the importance of this work which revealed some of the dynamics linked to nitrogen uptake from digestate. The presented work showed promising results and current findings could be utilised to further the field of digestate bioremediation using microalgae, especially for larger scale applications and for high-value ingredient production within a circular economy.

3.7. STATEMENT OF WORK

This chapter is presented in "**Microalgae Cultivation on Nutrient Rich Digestate: The Importance of Strain and Digestate Tailoring under PH Control "** by Fleuriane Fernandes*, Alla Silkina, José Ignacio Gayo-Peláez, Rahul Vijay Kapoore, Denis de la Broise and Carole A. Llewellyn; published on the 27th May 2022 in Applied Sciences (Special issue "Advances in Microalgal Biomass Productions"). All the work was carried out by the author of this thesis apart from the following: Determination of total carbohydrates and protein; and determination of FAMEs which were performed by Dr. Rahul Kapoore. Other author contributions were of an advisory, supervisory, or proof-reading nature.

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CHAPTER 4. USE OF A WASTE-DERIVED MICROALGAL HYDROLYSATE AS A FEED INGREDIENT IN THE DIET OF NILE TILAPIA

4.1. ABSTRACT

Current utilisation of microalgae as ingredients in aquaculture feed is limited by their cost of production and palatability issues in some fish species. Using waste as a nutrient source for microalgal growth can contribute to tackle high production cost, and hydrolysation of resulting biomass offers an innovative solution to improve palatability but also increases availability of essential peptides and other compounds for fish nutrition. In this work, a hydrolysate from the green microalgae *Scenedesmus obliquus* derived from nutrient rich digestate was assessed as a feed ingredient in the diet of Nile Tilapia. A 10% inclusion level of the microalgal ingredient did not negatively impact individuals, but performances in terms of size and weight gain were less than ones recorded for a commercial control diet, which can be attributed to lesser nutrient availability of a "homemade" feed and to some changes in the gut microbiome of Nile Tilapia. Fatty acid composition of fish fillet, however, demonstrated a significant advantage of the microalgal ingredient with a total fatty acids content of 4.43% DW (against 3.4% DW in a control diet) and PUFAs reaching 1.95% DW (against 1.32% DW). Additionally, ω-3 to ω-6 ratio was also improved (with a value of 1.29) for human-related diseases such as cardio-vascular issues. This study demonstrated the feasibility of using a digestate-derived microalgal hydrolysate as a feed ingredient to provide added-value to Nile Tilapia.

4.2. INTRODUCTION

There is increasing demand in Northwest Europe for sustainable sources of fish, resulting in significant growth of the aquaculture industry in the past decades (FAO, 2021). The aquaculture sector, however, currently relies on feed formulated from fish by-products, namely fish meal which are rich in proteins, and fish oils rich in fatty acids. These fish by-products are sourced by the exploitation of natural stocks, hence increasing the threat on aquatic environments. Furthermore, the use of other conventional protein sources (soybean, wheat, corn, and animal by-products) is experiencing shortages linked to the competition between food and feed, and there is increasing sustainability concerns

regarding their supply and production chains (Sagaram et al., 2021). Consequently, alternative protein sources from crop plants (Kaushik and Hemre, 2008), insects (Henry et al., 2015), single cell proteins (bacteria, yeast, and microalgae) and filamentous fungi (Barka and Blecker, 2016; Jones et al., 2020) have, over the past two decades, been extensively investigated.

Microalgal cultivation has the benefit of not competing with agricultural crops for arable land and freshwater and they can be produced year-round (Pienkos and Darzins, 2009; Rösch et al., 2019). The use of microalgae as an ingredient in aquafeed has been investigated for several species of fish and different microalgal strains have been tested (Enzing et al. 2014; Camacho et al., 2019). Recently, *Schizochytrium sp.* was used to replace fish oils in the diets of salmon, tilapia, and shrimp (Miller et al., 2007; Sarker et al., 2018; Allen et al., 2019). Other microalgae from the genera *Nannochloropsis, Pavlova, Isochrysis, Porphyridium, Phaeodactylum, Nitzschia, Chaetoceros* known to accumulate EPA and/or DHA, have also been investigated for their potential to replace fish oils in aquafeed (Eryalçm et al., 2015; Madeira et al., 2017). Microalgae also contain molecules with immunostimulant properties, with the potential to provide benefits in terms of disease control and cost savings by avoiding extensive utilisation of antibiotics. Published studies report the use of microalgal ingredients resulted in positive results when compared to existing ingredients (Kissinger et al., 2016; Perez-Velazquez et al., 2018; Maderia et al., 2017; Wang et al., 2017), suggesting that microalgae-based products have potential to improve aquaculture practices in terms of sustainability.

The use of microalgae in fish feed, however, is currently restricted by their production cost, for which high investments cost are required when considering large scale activities. Using a free source of nutrients would provide a significant cost saving in microalgal cultivation systems. Nutrient rich digestate (NRD) produced from the anaerobic digestion (AD) of waste is mainly used as fertiliser, but is currently an underused resource across Northwest Europe, due to the European Nitrate Directive 91/676/CEE which limits the annual load of nitrogen applied onto farmland by designating Nutrient Vulnerable Zones (NVZs). NRD can provide a growth substrate for microalgae, and the combination of microalgal cultivation systems and AD technologies could contribute to a sustainable and economically sound aquaculture industry fitting within the context of a growing circular economy.

Another bottleneck to the full utilisation of microalgae as whole cells in aquaculture systems are linked to palatability and digestibility of these ingredients, as microalgae can have complex cell walls not necessarily easily digested, making valuable active compounds less available for animal development (Janssen et al., 2022; Muys et al., 2019). Additionally, compound extraction from microalgal biomass can be a costly and time-consuming endeavour, especially when considering large scale applications (Gifuni et al., 2019). Hydrolysis, which is the process by which enzymes are used to break down cell wall and metabolite structures into smaller compounds is a promising alternative to more expensive and complex processes to obtain some of the valuable compounds for the aquaculture industry; additionally, hydrolysis can alleviate issues linked to palatability and smells which can reduce feed uptake by animals. Hydrolysis has been investigated, mainly for the processing of fish by-products (Afreen & Ucak, 2020), and it remains a novel processing method for microalgae in aquaculture application as whole cells and algal oils are the two main microalgal ingredients currently found in fish feed formulations (Ahmad et al., 2022).

This study aimed to assess the feasibility of using a microalgal hydrolysate derived from a waste source as a feed ingredient in aquaculture. In this work, a hydrolysate from the green microalgae *Scenedesmus obliquus* (*S. obliquus*) grown on nutrient rich digestate was used as a feed ingredient in the diet of Nile Tilapia (*Oreochromis niloticus*). Focus was made on the potential of the hydrolysed *S. obliquus* ingredient to provide additional benefits to the aquaculture sector in terms of flesh quality and gut health, without compromising growth of Nile Tilapia.

4.3. MATERIALS & METHODS

4.3.1. Obtention of waste-derived hydrolysate of S. obliquus

Digestate treatment

Digestate was obtained from the AD plant Langage AD in Plymouth (UK) (PAS 110 certified). The digestate was the result of the anaerobic digestion of kitchen waste and waste from a local dairy factory. Due to a high dry matter content (5.5%) and dark colour, digestate was treated using ultra filtration at a molecular weight cut off (MWCO or pore size) of 0.1 μm. Optimisation of the digestate treatment was performed as described in Fernandes et al.

(2020), resulting in a treated digestate with an ammonium concentration of 3.1 $g.L^{-1}$ and a phosphorus concentration of 50 $mg.Kg^{-1}$.

Cultivation of S. obliquus

Biomass of *S. obliquus* was grown in a 5000 L photobioreactor at Langage AD over the course of 80 days to produce enough biomass for feed trials. Culture conditions included a temperature of 25°C and a pH of 7.5, maintained by automated CO_2 injections. *S. obliquus* was grown in batch, meaning that a weekly harvest of 25% of the total cultivation volume was performed, followed by an addition of new medium, represented here by water mixed with digestate to replenish nutrients. Additionally, digestate was added at 2.5% of the total cultivation volume, to avoid toxicity linked to the high ammonium concentration found in treated digestate, as optimised and described in Fernandes et al. (2020). Harvested biomass was dewatered using membrane filtration at a pore size of 0.1 µm and further centrifuged and frozen before undertaking further downstream processing. A total of 25 kg of wet biomass of *S. obliquus* were obtained after 80 days of cultivation using digestate as a nutrient source.

Hydrolysis of S. obliquus grown on digestate

Hydrolysis was performed on wet biomass of *S. obliquus* grown on digestate at the Langage AD production site. Following thawing, the biomass which had an initial pH of 7 was placed at 55°C with a 2% solution of Alcalase® 2,4 L FG (Novozymes), which is a serine endo-peptidase and mainly contains protease enzymes. Enzymatic incubation took place for 5 hours and an enzymatic inactivation was performed for 10 minutes at 90°C. After cooling, the resulting hydrolysate was freeze-dried to be used in the feed formulation. The process was performed at large scale at the site of the company IVAMER (France), but the experimental design was reproduced at Swansea University at laboratory scale to demonstrate reproducibility and allow a better understanding of the process thanks to microscopic observation of the biomass during hydrolysis (**Figure 4.1**). Hydrolysis resulted in 85 to 95% of peptides smaller than 3KDa (molecular weight analysis was performed using HPLC-UV by an external laboratory), while the raw material (i.e., *S. obliquus* biomass) had more than 50% of molecules with a molecular size of 6 to 35 KDa.

In this work, hydrolysis was selected to induce cell breakage and release of compounds of interest form *S. obliquus*, as well as for the breaking down of proteins into smaller metabolites. The use of a hydrolysate, and not the whole biomass, presented several advantages, the main one being that compound of interest such as peptides were more readily available and more easily absorbed by the digestive systems of animals used in feed trials. Furthermore, the use of a hydrolysate also alleviated constraints linked to smells and taste of the whole biomass, which can result in palatability issues and reduced uptake of formulated feed.

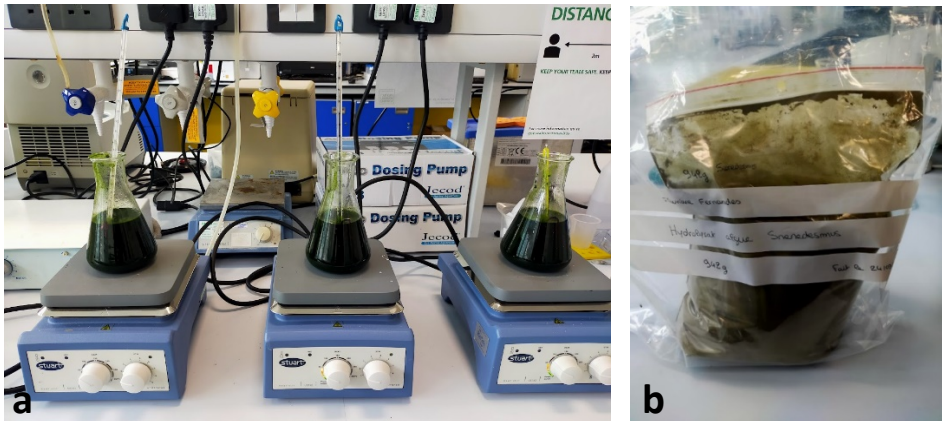

Figure 4.1: Reproduction of hydrolysis protocol at Swansea University (a) and resulting freeze-dried hydrolysate produced from *Scenedesmus obliquus* biomass (b).

4.3.2. Feed formulation

In this experimental work, 70% of the fishmeal usually found in commercial feed was replaced by the *S. obliquus* hydrolysate. Higher percentages of replacement were not studied here as animal welfare criteria required a minimum amount of the standard fishmeal to still be incorporated into the formulated feed. The pellet formulation is detailed in **Table 4.1**. After mixing all the ingredients at the required percentage, 30% of DI water was added to the mixture which passed through a meat grinder apparatus. The resulting strands were dried for 2.5 hours at 45°C. Once dried, strands were cut to the desired size using a blender (**Figure 4.2**). Pellets were 1.5 mm in size at the beginning of the experiment to suit juveniles of Nile Tilapia mouth size and size of pellet was progressively increased, reaching roughly 2.5 mm at the end of the experiment.

Table 4.1: Composition of experimental diet. Adapted from (El-Saidy & Gaber, 2002; El-Sheekh et al., 2014).

Ingredients	Percentage of inclusions %
Fish meal	5
Soybean meal	17.5
Fish/marine oil	7.5
Wheat bran	8
Wheat	19
Wheat gluten	10
Maize	13
Soya oil	5
***Scenedesmus obliquus* hydrolysate**	**10**
Vitamin and mineral mix	5

Figure 4.2: Pellet manufacturing process, left to right: ingredient mixture (a); strands produced by extrusion (b); dried and blended pellets (c).

4.3.3. Experimental design

A composition analysis carried out on the *S. obliquus* biomass grown on digestate and used in this experiment showed a protein content of 84%. Consequently, biomass grown on traditional nutrient media (*i.e.,* Guilliards' F/2 medium) was not tested as part of this study, as the protein content was lower, hence focusing effort on biomass grown on digestate only.

The feed formulation described above was compared to a standard commercial feed (Coppens) which had the following composition: 56 % Protein; 15% fat; 0.2% crude fibre; 13 % ash and 1.89% total P. Two replicates (or tanks) were tested per condition (algae feed and commercial feed), with 50 fish in each tank, totalling 4 tanks and 200 fish. Fish at the start of

the experiment were 6 weeks old and had an average size of 60 mm. Each tank had continuous water flow, set at 400 L.hour^{-1} for water renewal and to provide a current for the fish to swim against. Shading was added on top of each tank, alleviating stress due to lighting, and light intensity ranged between 30 and 35 µmol.m^{-2}.s^{-1}. Temperature was maintained between 27 and 29 °C and pH at 7. Parameters were recorded daily to ensure normal operation of the system (**Figure 4.3**).

Fish were fed twice a day, every day (morning and evening), at 4% of the total fish biomass, which was measured weekly, and feed quantities were consequently increased with the fish weight gain. The experiment was continued for 4 weeks, with an initial baseline sample and 2 subsequent sampling after 15 and 29 days. The experimental design was reviewed by an ethics committee with the ethics approval number SU-Ethics-Staff-230421/338 and the Animal Welfare Ethical Review Body (AWERB) reference IP-2021-09.

Figure 4.3: Experimental set-up showing tanks with shading system (a) and Nile Tilapia under described current conditions (b).

4.3.4. Sampling

A baseline sample of 20 fish was taken at the beginning of the experiment to obtain a representative initial sample. 10 fish per tank were sampled for the subsequent analysis. Fish anaesthesia was performed using phenoxyethanol at a concentration of 2mL.L^{-1} and

confirmation of death was performed by cutting the gills (exsanguination, according to Act 1986: Appropriate methods of humane killing for fish, schedule 1).

For each sampled fish, size (in mm) and weight (in g) were recorded (**Figure 4.4**). Gut samples of the stomach and whole intestinal track were taken by dissection, and flesh sample was taken by removing a fillet from the pectoral fin to the end of the dorsal fin. Samples were preserved at -80°C straight after collection for further analysis. Total fish biomass was measured in each tank weekly to assess the amount of feed necessary. This was performed by netting out roughly 20 to 25 fish per tank and recording the total weight of the sampled fish. Measured weight was then divided by the number of fish netted, providing a weight per fish and a total weight (or biomass) per tank (**Figure 4.4**).

Figure 4.4: size measurement (a)( ) and recording of fish biomass per tank (b) performed weekly to assess feed supplementation.

4.3.5. Husbandry and Welfare assessment:

Husbandry conditions were maintained during the experiment to ensure animal welfare and a range of abiotic parameters were monitored daily and maintained as described in **Table 4.2**. Additionally, initial fish density was low, at 50 fish per tank, avoiding overcrowding and related issues.

Table 4.2: Husbandry conditions maintained throughout the experiment

Parameter	Unit
Ammonia (NH_3)	<0.02 $mg.L^{-1}$
Nitrite (NO_2)	<0.2 $mg.L^{-1}$
Nitrate (NO_3)	<100 $mg.L^{-1}$
Dissolved oxygen (DO)	>5 $mg.L^{-1}$
Temperature	27-29 °C
Conductivity	>3000, < 4000 $\mu S.cm^{-1}$
pH	6-9
Photoperiod	12hrs light: 12hrs dark

Daily observations of the fish were performed, and the presence/absence of the following parameters were monitored: abnormal swimming, lethargy, gulping air at surface, fin clamping, observation of skin appearance, observation of the school behaviour (isolation of individuals, bullying). In addition, feeding was observed regularly to monitor the feed uptake and ensure that all fish were feeding properly throughout the experiment. Handling was also kept to a minimum (only at starting point and sampling time) by trained personnel.

4.3.6. Analysis of Fatty acids

Transesterification of fatty acids in tilapia fillets

Tilapia fillets sampled during the experiment were cut in 2 cm aliquots from consistent portion of sampled muscle and kept in 2 mL Eppendorf tubes under inert nitrogen gas. All procedures were performed on ice and fish fillets were immediately stored at −80° C until further fatty acid analysis. On the day of analysis, fillets were weighed (~5 to 7 mg) followed by direct transesterification as described elsewhere (Kapoore, 2014; Kapoore et al., 2019; Pandhal et al., 2017). Briefly, 290 µL of toluene, 300 µL of 0.5M sodium methoxide and 10 µL of hexane containing C13:0 (tridecanoic acid) as an internal standard were added to the weighed tilapia fish fillets samples, followed by incubation at 80°C for 20 min. After cooling to room temperature, 300 µL of 10% boron trifluoride in methanol was added and the mixture was incubated at 80°C for 20 min. After cooling to room temperature, 300 µL of water and 600 µL of hexane were added. The mixture was vortexed for 1 min and centrifuged at 10000 rpm at 4°C for 10 minutes. The organic phase was recovered, measured, and evaporated to dryness under inert nitrogen gas using a six-port mini-vap evaporator (Sigma-Aldrich, Dorset, UK). All chemicals and analytical reagents were of high-

performance liquid chromatography grade (Sigma–Aldrich, Dorset, UK) unless stated otherwise.

Fatty acid methyl esters profiling

The dried fatty acid methyl esters (FAMEs) were reconstituted in 300 µL hexane prior to identification and quantification on a GC-ToF-MS (Waters Corporation, Massachusetts, USA) using a TR-FAME capillary column (25 m × 0.32 mm × 0.25 µm). The sample volume of 1 µL was injected in split injection mode at 250˚C. The oven ramp was held at 150˚C for 1 minute and increased by 10˚C per minute and up to 250˚C, then held at 250˚C for 1 minute. Split injection was carried out at split ratio 50, split flow 75 mL per minute and carrier flow of 1.5 mL per minute. The GC-ToF-MS was operated in EI mode at a constant flow of 1.5 mL per minute of helium at an initial temperature of 150°C for 1 minute, followed by incremental increase of 10°C per minute and up to 250°C and held constant for 1 minute. Fatty acids were identified by comparing the obtained retention times with that of known standards (37 component FAME mix, Supelco™).

Identification and quantification of FAMEs

Initially, peak identities were ascertained using external standard 37 component FAME mix (Supelco™) (**Figure 4.5**). Peak identification and data acquisition were performed on a MassLynx (version 4.1) (Waters Corporation, Massachusetts, USA) with a mass range of 50 to 650.

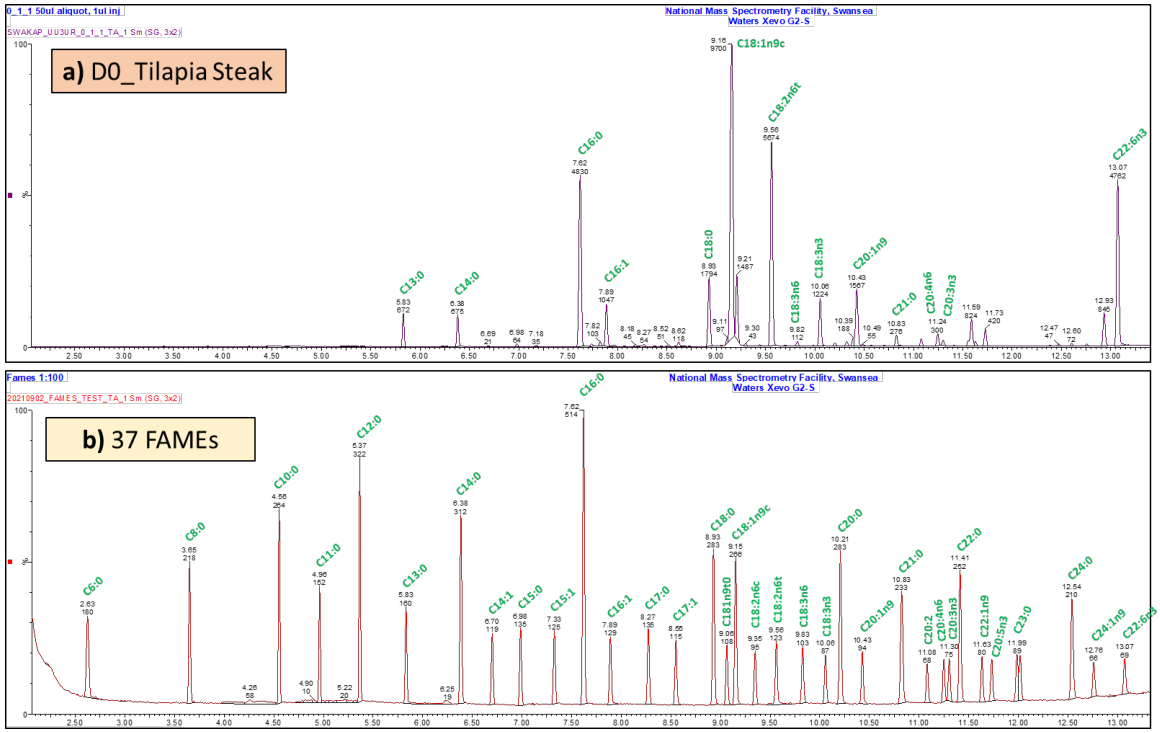

Figure 4.5: Representative GC-ToF-MS chromatogram of a) tilapia fish fillet (day 0) and b) 37 FAME mix (C8:0 – C24:0 from Supelco).

Data for identified FAMEs such as retention time, peak areas and peak height was collected manually from MassLynx (version 4.1) (Waters Corporation, Massachusetts, USA) and exported in Microsoft office Excel 2016 for further data analysis. Based on the known amount values of 37 FAME components, a ratio was established between the area and the amount. Briefly, a six-point calibration curve was initially generated using one internal (C13:0) and two external standards (C17:0 heptadecanoic acid and C19:0 nonadecanoic acid). For the calibration curve, the concentration of the internal standard (C13:0) was kept constant (10 µL from 1 mg/mL stock in hexane), whereas the concentration of spiked external standards (C17:0 and C19:0) varied from 0.1 µL to 40 µL. All generated standards (in triplicates) were evaporated to dryness under inert nitrogen gas and reconstituted in 300 µL hexane prior to identification and quantification on a GC-ToF-MS as described above. Separate calibration curves were generated using a ratio of internal (C13:0) to external standards (C17:0 and C19:0) peak areas. A representative example of the C17:0/C13:0 calibration curve is displayed in **Figure 4.6**.

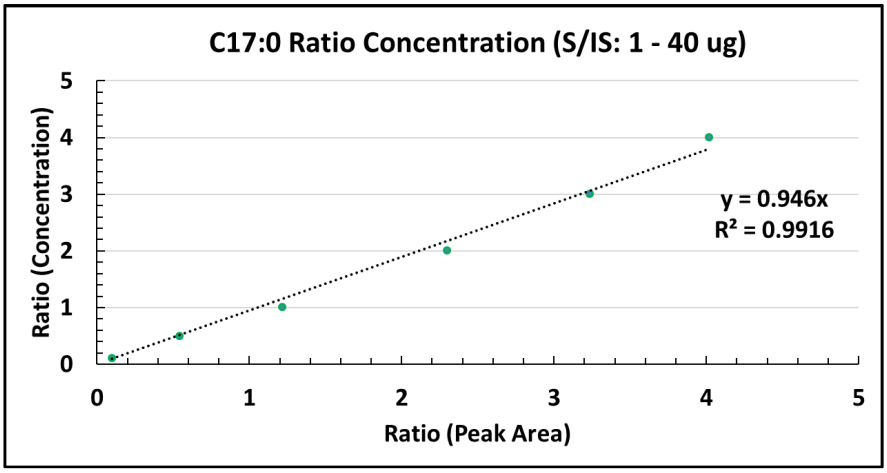

Figure 4.6: Representative example of the C17:0/C13:0 calibration curve

FAMEs in the fish fillet extract were then determined by their peak areas by generating a calibration curve for individual components and quantified by reference internal and external standards, as described above. In total, n=5 replicates (biological) were run, among which FAMEs identified only in 3 or more replicates were considered as true hits. The data was later normalised to the dry weight of the fish fillets and FAME's were reported on a percentage dry weight basis.

4.3.7. Analysis of gut microbiota using MinION sequencing:

DNA from gut samples was extracted after pooling replicates using a QIAamp Fast DNA Stool Mini Kit, according to manufacturer's instruction. The extracted DNA was QCed using a Nanodrop equipment and Qubit to assess organic and proteins contaminations as well as DNA concentration. 10 ng of DNA were amplified and multiplexed using a 16S barcoding kit (Oxford Nanopore Technologies – ONT thereafter). The following mixture was prepared: 10 µL input DNA (10 ng); 25 µL LongAmp Hot Start Taq 2x Master Mix; 5 µL nuclease-free water and 10 µL of 16S barcode. DNA was then amplified using the following PCR cycle: Initial denaturation at 95°C; 30 cycles: 95°C for 20s, 55°C for 30s, 65°C for 2 minutes; final extension at 65°C for 5 minutes. PCR products were then cleaned using AMPure XP (Agencourt) beads (80 µL) and quantified using a Qubit. Barcoded libraries were then pooled at equal ratios (10 ng) and incubated with a rapid adapter. The DNA library (11 µL) was mixed with sequencing buffer (34 µL), loading beads (25.5 µL) and nuclease free water (4.5 µL) and loaded into the MinION flow cell (FLO-MIN106, ONT). The flow cell was checked prior to analysis to ensure that enough pores were available and had not been degraded

during storage. Analysis was performed for 16 hours, during which real-time data acquisition and basecalling was performed by the MinKNOW software (ONT). Following sequencing, fastq files were analysed using the cloud based EPI2ME platform and the EPI2ME agent (ONT). The fastq 16S workflow was used based on the sequence-based bacterial identification with BLAST using an E-value of 0.01 which is considered as good hits for homology matches (Sović et al., 2016). The sequencing analysis resulted in 56777 reads classified at an average sequence length of 1412 nt. De-multiplexing of the barcoded samples was performed by using the filter function following the production of the EPI2ME report. No pipelines were used based on operational taxonomic Units (OTUs) as these techniques were developed for short-read data and not suitable for nanopore read lengths and error profiles (Ciuffreda et al., 2021).

4.3.8. Statistical analysis

Statistical analysis was carried out on the studied factors using the R project software (R 4.2.1, Core Team 2022). Two-ways ANOVAs were carried out on normally distributed data and normality was determined using Shapiro tests. Diet tested and time of sampling were used as parameters and tested on size and weight of the fish, as well as on fatty acid composition of the fish fillets. When significance was found, a post hoc Tukey test was implemented to determine the level of differences (Agricolae CRAN package, R). Sequencing data were analysed by calculating the Shannon's alpha-diversity index at genus level per sample using the Excel software (Microsoft). The impact of the sampling time and diet on the alpha-diversity, number of reads and number of identified genera and orders were assessed using a two-way ANOVA. Generalised Linear models (GLM) were then performed at the order level to determine the impact of the interaction between factors (treatment, sampling time and order identified) on the number of reads, to identify dominating orders in the different treatments and at the different sampling point implemented during the experiment. When a similar analysis was performed on genera, no clear trends were identifiable, and results are consequently not shown as part of this work. Additionally, as replicates were pooled before sequencing to economise on the analysis procedure, beta-diversity could not be calculated and analysed in this work.

4.4. RESULTS

Temperature, pH, and oxygen levels were monitored daily and remained stable throughout the experiment at the levels mentioned in **Table 4.2**. Additionally, no welfare concerns were observed during the experiment (no signs of cataracts, distressing behaviours, or observations of concern on the fish bodies).

4.4.1. Growth measurements

Measured individuals of Nile Tilapia had an average starting weight of 4.4 ± 1.8 g. Weight measurements performed weekly on the overall tank biomass, and bi-weekly on single individuals, showed a significant increase in the weight of fish fed with the commercial feed (p=0.0225*), reaching an average weight of 16.7 ± 0.12 g after 4 weeks (approximately an increase of 4 times compared to the starting weight) (**Figure 4.7**). The ALG-AD feed also resulted in an increase of the weight overtime, but the fish only doubled their weight, reaching 8.1 g after 4 weeks of experiment (p=0.0125*). A significant impact of the diet used was found statistically with a p-value of 0.00235**, and a Tukey test showed that weight was significantly higher for fish under the commercial diet.

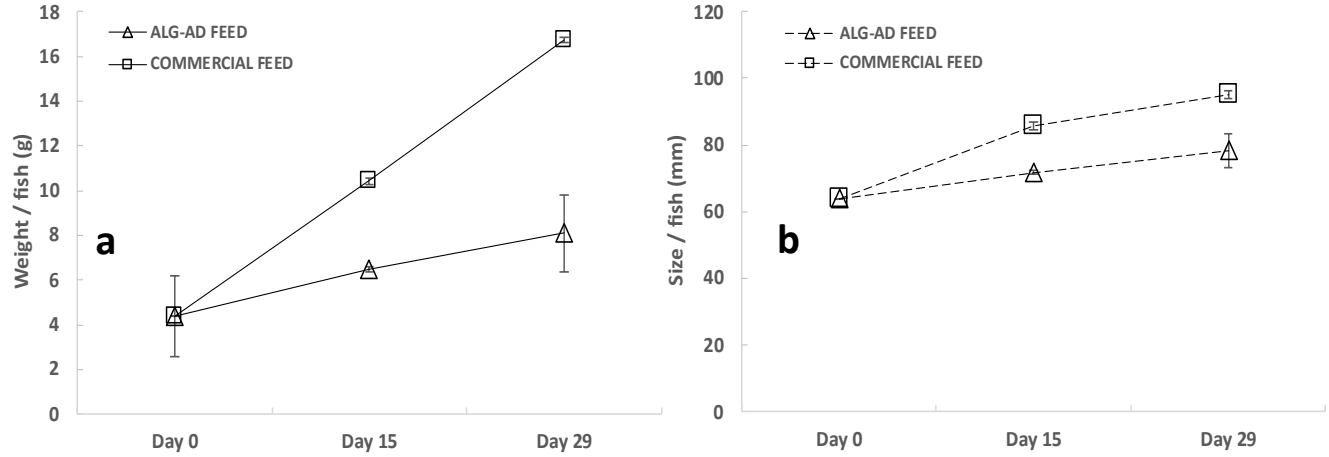

Figure 4.7: Average weight (a) and size (b) measured per fish in commercial feed and ALG-AD feed conditions at day 0, 15 and 29. Error bars represent standard error for 10 fish measured for 2 replicates. Standard error was below 0.2 when not seen on the graph.

Recording of the total biomass per tank also showed that a plateau was reached after 4 weeks in the tanks were the ALG-AD feed was used, while for a commercial feed, the total biomass seems to keep increasing after 4 weeks (**Figure 4.8**). Again, total weights recorded per tanks were significantly higher for the commercial feed (p=0.0189**); this was also true

for the calculated weight per fish (p=0.0191*, **Figure 4.8**). Initial size of fish was 63.7 mm and after being fed the commercial pellet, individuals reached a size of 95.1 mm, while fish fed with the ALG-AD feed reached 78.3 mm (corresponding to 1.5 and 1.2 times increases, respectively) (**Figure 4.7**). Statistical analysis, however, did not show significant differences between the size of fish under the two diets (p=0.187).

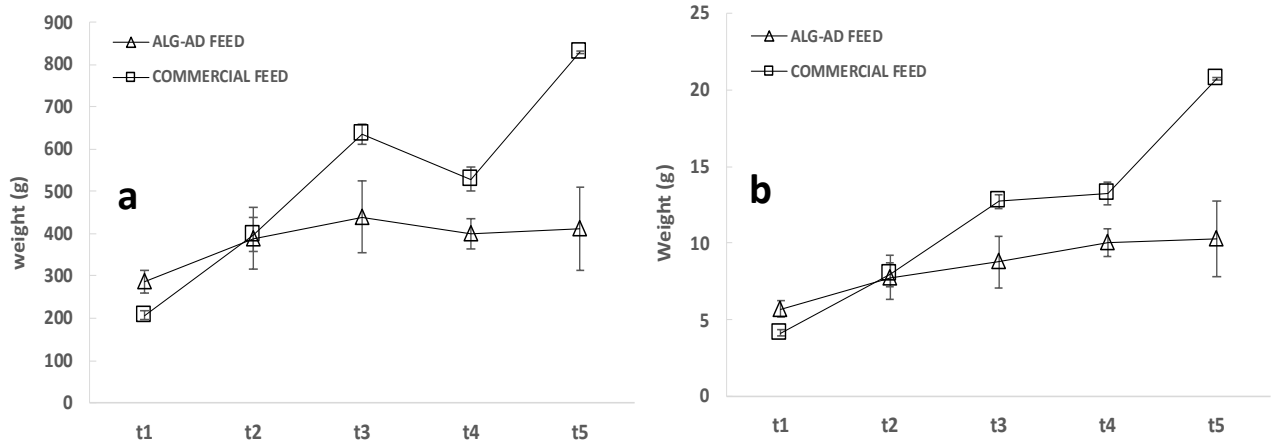

Figure 4.8: Average total weight measured per tank in commercial feed and ALG-AD feed conditions (a) and corresponding weight per fish calculated (b) from week 1 (t1) to week 5 (t5) of experiment. Error bars represent standard error for 2 replicates. Standard error was below 0.2 when not seen on the graph.

It should also be noted that across all tanks and conditions, bullying was commonly observed, with the occurrence of much bigger fish, showing aggressive behaviours to obtain more food, resulting in significant discrepancies in the size and weight of measured individuals during sampling, across all the tanks and tested conditions (**Figure 4.9**). These were likely to be sexually maturing males, however fish sexing was not performed as part of this experimental work.

Figure 4.9: Picture showing size difference between fish of the same age and under the same feeding regime, demonstrating the bullying effect. ([redacted])

4.4.2. Fatty acids analysis of fish fillets

The addition of microalgae to the diet of Nile tilapia altered the fatty acids (FAs) profile of resultant flesh fillets. Twenty-two FAs were found in sampled fillets of tilapia, including saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), with averages ranging from 3.27 to 4.79 % DW of total FAs (**Figure 4.10**).

Results show a decrease in the fatty acid content of tilapia fillets in both the control and in fish fed with the ALG-AD feed following 15 days of experiment (p=0.006*). However, after 29 days, total fatty acids (p=0.00675*) and PUFAs (p=0.00605*) were significantly higher for the ALG-AD feed, with 4.43% and 1.95% of the DW of the total fatty acids respectively, against 3.4% and 1.32% for the commercial feed (**Figure 4.10a**). Whilst similar levels of SFAs (p=0.141) and MUFAs (p=0.265) were observed after 29 days for both tested conditions, closer analysis showed higher levels of C16:0 (p=0.01594*) and C18:0 (p=0.0167*) (SFAs) as well as C18:1n9c (p=0.00603**) (MUFAs) in the fillets of fish fed with the ALG-AD feed (**Figure 4.10b & c**).

Analysis of PUFAs showed similar levels of ω-3 throughout the experiment in both the control and algae-fed groups, ranging from 0.62 to 0.72 % (p=0.242) (**Figure 4.11a**). Results of ω-6 analysis however, revealed a significantly higher content of C18:2n6t in the fillets of fish under the algae diet (p=0.00721**), with high values throughout the experiment, reaching 0.96 % at the end of experiment, while values decreased drastically in the control, reaching 0.4 % (**Figure 4.11b**). ω-6 were generally found in higher amounts for the ALG-AD feed in comparison to the control (**Figure 4.11c**).

Results showed that fillets of tilapia grown under the ALG-AD feed diet achieved similar or higher levels of fatty acids content in comparison to fillets from fish under a standard commercial diet, demonstrating the advantages of an algal feed to improve flesh quality and consequently commercial value of tilapia for the aquaculture sector.

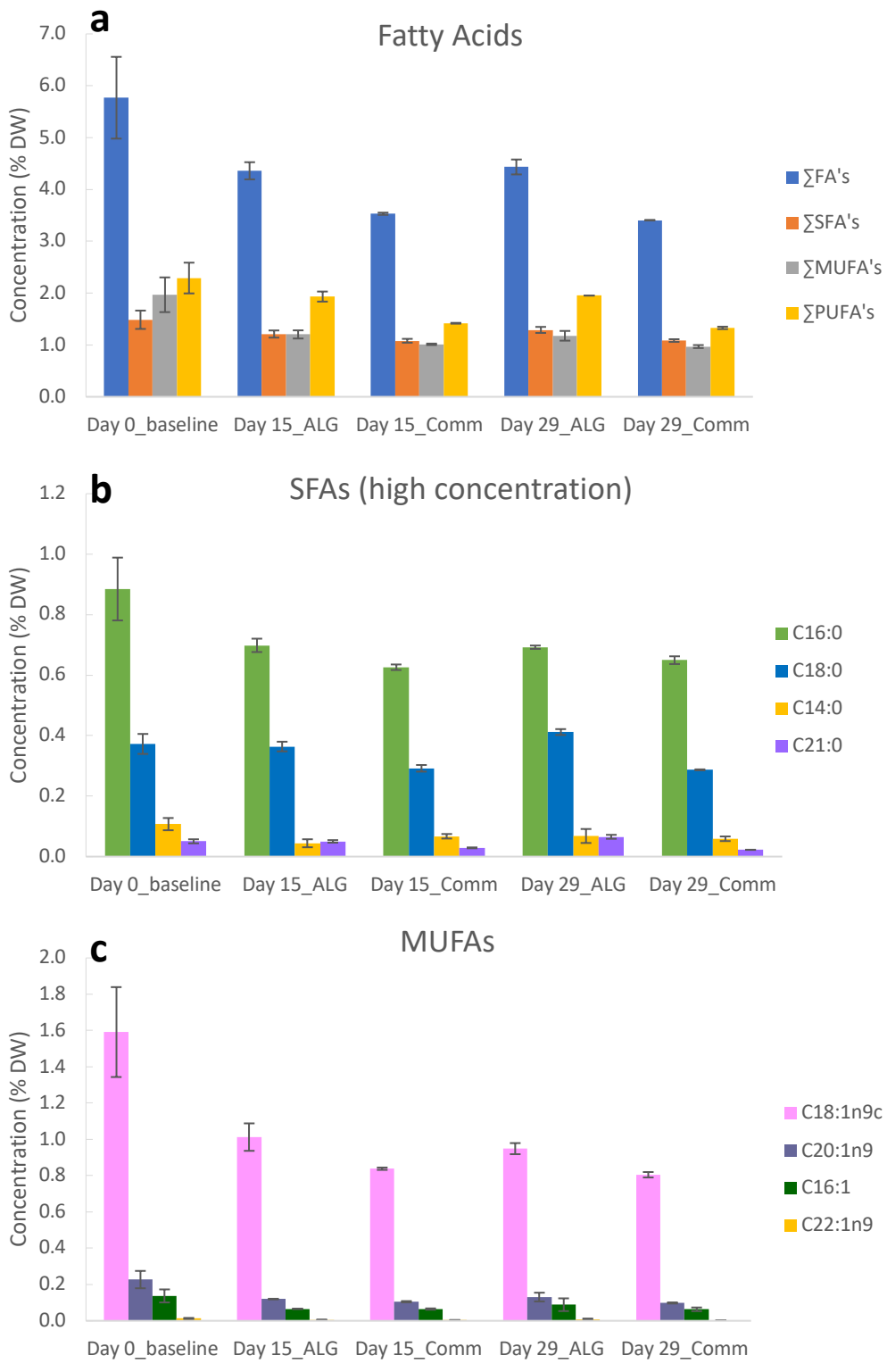

Figure 4.10: Influence of fish feed diets on recovery of a: different fatty acid classes (∑FA's = Total fatty acids, ∑SFA's = Total saturated fatty acids, ∑MUFA's = Total monounsaturated fatty acids and ∑PUFA's = Total polyunsaturated fatty acids), b: Saturated fatty acids (SFA's) and c: Monounsaturated fatty acids (MUFA's) from tilapia fish analysis. The X-axis represents fish feed diets and time series (in days), where Comm = Commercial feed and ALG = feed formulated from S. obliquus hydrolysate. Error bars represent standard deviation for 3 replicates.

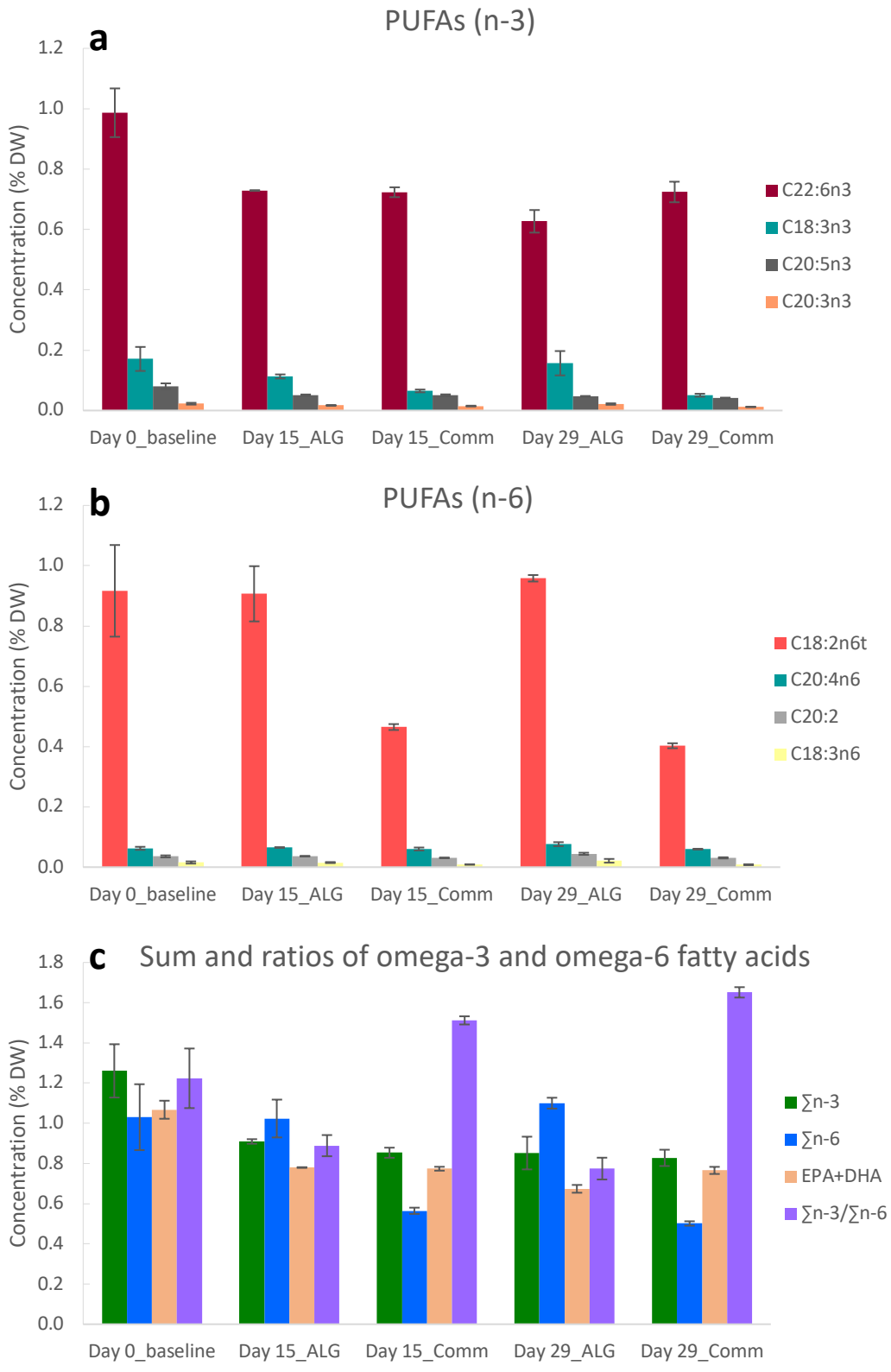

Figure 4.11: Influence of fish feed diets on recovery of a: omega-3 fatty acids, b: omega-6 fatty acids and c: sum and ratios of omega-3 and omega-6 fatty acids from Tilapia fish fillets. The X-axis represents fish feed diets and time series (in days), where Comm = Commercial feed and ALG = feed formulated from S. obliquus hydrolysate. Error bars represent standard deviation for 3 replicates.

4.4.3. Analysis of gut microbiome

Table 4.3: Number of reads identified using MinION sequencing and calculated Shannon alpha-diversity at Days 0, 15 and 29 of the experiment for the Commercial and ALG-AD feed at genera and order levels.

	Commercial feed			**ALG-AD Feed**		
Genera level	*Number of reads*	*Number of genera identified*	*alpha-diversity*	*Number of reads*	*Number of genera identified*	*alpha-diversity*
Day 0	36004	81	1.29	36004	81	1.29
Day 15	12233	70	2.04	1448	38	2.85
Day 29	5351	38	1.23	1741	35	1.88
p-value	*alpha-diversity*		*Number of reads*		*Number of genera*	
Feed	0.188		0.565		0.999	
Day	0.112		0.441		0.452	
Order level	*Number of reads*	*Number of orders identified*	*alpha-diversity*	*Number of reads*	*Number of orders identified*	*alpha-diversity*
Day 0	36004	29	1.10	36004	29	1.10
Day 15	12233	23	1.16	1448	23	1.99
Day 29	5351	18	1.31	1741	13	1.08
p-value	*alpha-diversity*		*Number of reads*		*Number of orders*	
Feed	0.598		0.442		0.104	
Day	0.552		0.139		0.235	

DNA sequencing resulted in a total of 56777 good reads (e-value<0.01) across the conditions tested and at the different time points sampled, with 142 genera and 38 orders identified. Some of the samples' quality did not allow for optimal DNA extraction using the mini stool kit, consequently all the good reads obtained were utilised in the statistical analysis to obtain the best representation; however, this results in the following analysis being qualitative rather than quantitative when comparing conditions and time points for which samples were collected. Statistical analysis showed that there was no significant impact of the feed studied or of the time of sampling on the alpha-diversity, nor on the number of reads, genera, or orders (**Table 4.3**). A multifactorial GLM performed at the genus level showed a significant dominance of the *Plesiomonas* at day 0 ($p<2.10^{-16}$***) as well as the *Romboustia* ($p=4.67.10^{-12}$***). The sample collected at Day 0 was a baseline sample performed before separating individuals into different diet conditions. The same analysis performed at the order level showed a significant dominance of the Fusobacteriales ($p=2.52.10^{-12}$***) and the Enterobacteriales ($p=1.29.10^{-6}$***) orders. The Clostridiales order

was also present at Day 0 (16.28%, **Figure 4.12**). In the microbiome of fish under the commercial feed, the *Plesiomonas* genus remained dominant after 15 days (p=0.0043**) and 29 days ($p<2.10^{-16}$***) of experiment; at the order level, similar results were observed with the Fusobacteriales and Enterobacteriales dominating at day 15 (p=0.00055**; p= 0.000953*** respectively) and day 29 ($p=1.97.10^{-9}$***; p=0.000115**, respectively). Additionally, the Aeromomadales order was also present at the end of the experiment with 20.27% of reads identified for fish under the commercial diet (**Figure 4.12**). At the genus level, statistical analysis of the microbiome composition of fish under the ALG-AD feed did not reveal any genera dominating the population at day 15 significantly (p-value of the interaction between day 15 and the ALG-AD feed was 0.298), however after 29 days the *Plesiomonas* genus was significantly dominant ($p<2.10^{-16}$***). At the order level, Enterobacteriales and Fusobacteriales were significantly dominating at day 15 ($p=1.05.10^{-8}$***; $p=5.09.10^{-8}$***, respectively) as well as at day 29 (p=0.0311*; $p= 8.06.10^{-5}$***, respectively) for fish under the ALG-AD diet, however it could be observed that the relative abundance of Enterobacteriales was higher at day 15 in comparison to the abundance of Fusobacteriales (38.69% against 21.71%, **Figure 4.12**); while this observation was reversed at day 29 with 64.92% of Fusobacteriales against 20.87% of Enterobacteriales (**Figure 4.12**). Additionally, the relative abundance of several orders such as Bacillales (13.34%), Pseudomonadales (4.89%), Plesiomonas (3.11%) or Propionobacteriales (2.89%) seemed higher at day 15 in fish under the ALG-AD feed in comparison to the ones under the commercial feed after the same amount of time. Overall, the main differences between diets were observed after 15 days of experiments while the microbiome composition appeared somewhat similar after 29 days (**Figure 4.12**). The same observation was made at the phylum level, where Proteobacteria were dominating after 15 days of experiment in fish under the ALG-AD diet, but the Fusobacteria phylum abundance returned to similar levels in comparison to the ones of recorded at Day 0 and after 29 days of experiment for a commercial feed (**Figure 4.13**).

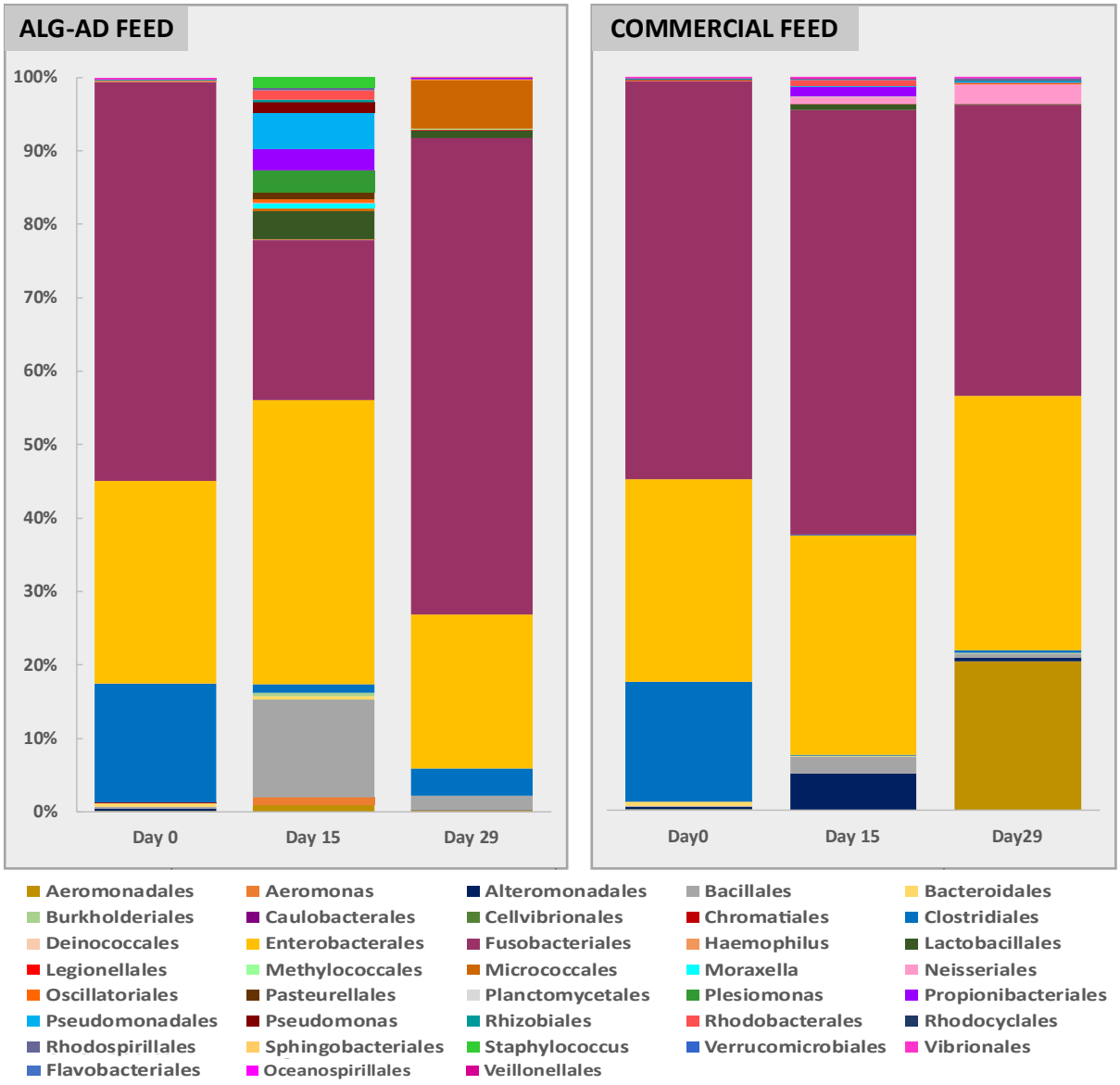

Figure 4.12: Order-level composition of the microbiome of Nile Tilapia from Day 0 to Day 29 of experiment (horizontal axis). Samples are grouped by feed tested. Each bar represents the relative abundance of identified orders, expressed as percentages (vertical axis).

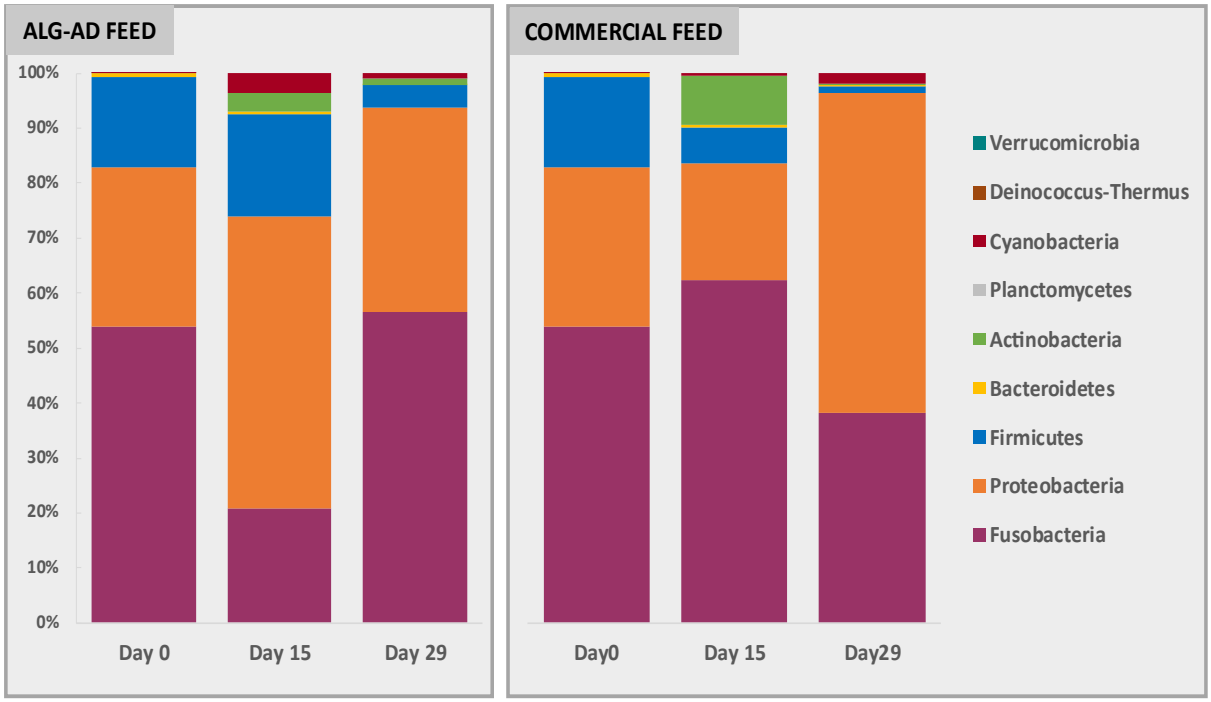

Figure 4.13: Phylum-level composition of the microbiome of Nile Tilapia from Day 0 to Day 29 of experiment (horizontal axis). Samples are grouped by feed tested. Each bar represents the relative abundance of identified orders, expressed as percentages (vertical axis).

4.5. DISCUSSION

4.5.1. Growth performances of Nile Tilapia

In the present work, growth rate of Nile Tilapia fed with a commercial feed was better when compared to the algae feed, however no adverse effect of the algae-based feed was observed on the health, behaviour or morphology of the fish. As part of this work, the algae-based feed was formulated in-house in the laboratory, while the commercial diet was provided by a feed company with established processes and a thorough quality control. Consequently, it could be argued that the nutrient availability of the algae-based diet was less so than the commercial diet, or that nutrients could have leached from the homemade feed, resulting in reduced weight gain during the experiment. During the experiment, welfare observation did not show any adverse effects of the algal feed on Nile Tilapia, no deaths were recorded, and no signs of ill health were observed. Indeed, the pellet formulated from *S. obliquus* was adapted from formulations established in El-Saidy & Gaber (2002) and El-Sheekh et al. (2014), and the microalgal hydrolysate was used to replace the fishmeal at a level of 70 %. In the studies mentioned above, a complete replacement of fish meal by the spirulina *Arthrospira platensis* did not show adverse effect on tilapia, and a

replacement of the fish meal of up to 75% resulted in an increased feed conversion ratio, as well as a higher protein value in carcass composition, and higher immunity translated in an increased white blood cell count (El-Sheekh et al., 2014). Similar results were obtained in other studies, where replacing 50 % of the fish meal by *Chlorella sp* and *Scenedesmus sp* biomass improved growth performance, feed conversion ratio and protein production values (Badwy et al., 2008). Sarker et al. (2018) also demonstrated that 33% inclusion of *Nannochloropsis oculata* as a fish oil replacement in the diet of Nile Tilapia yielded similar growth, feed conversion and survival rate as for a control diet. Consequently, and based on previous work, algae-based diet can provide increased or similar growth performances, but it is paramount to use feed formulations and manufacturing technics matching commercial ones to improve competitiveness of novel algal feed in aquaculture. Additionally, this work could serve as an indication for aquaculture feed manufacturer on the level of incorporation of microalgal ingredients into aquafeed.

4.5.2. Flesh composition in fatty acids

In the present study, fatty acids were overall found in higher concentrations in the fillets of Tilapia grown under the ALG-AD feed in comparison to a commercial feed; this was in accordance with the literature where the utilisation of microalgae in Tilapia's diet provided similar growth when compared to a standard diet but resulted in better quality and higher lipid content of the fish flesh. Several studies have investigated the use of algae-based diets to increase fatty acid content of several species, and it has been shown that supplementation of microalgae in diets can modify the lipid profile and body chemical composition in fish (Chisti, 2007; Richmond, 2004). Takeuchi et al. (2002) showed that a spirulina-based diet increased the polar lipid content in Tilapia, the authors believed this increase was the result of the metabolization of linoleic acid and g-linolenic acid (GLA) found abundantly in Spirulina. Stoneham et al. (2018) demonstrated that an algae meal replacement in the diet of Tilapia resulted in a three-fold increase in the omega-3 fillet content, and an improved omega-3:omega-6 ratio of 1.29 (against 5.19 in a commercial feed). Feed conversion ratio, protein retention efficiency and PUFAs were also better in Gbadamosia and Lupatsch (2018) when Tilapia was under a *Nannochloropsis*-based diet; furthermore, they demonstrated that the fatty acid content of fish body was directly influenced by the fatty acid composition of the diet. The increased fatty acid content

observed in our study can hence be related to the inclusion of *S. obliquus* in Tilapia's diet, as the microalgae can accumulate large amounts of alpha-linoleic acid (Chen et al., 2021; Darki et al., 2017), in turn metabolised in different lipids measured in this experiment. Tilapia fed with the ALG-AD feed clearly demonstrated added nutritional value in terms of fatty acid content of the fillets, indeed an omega-3:omega-6 ratio of nearly 1 was recorded after several weeks of experiment, which conveys lower risks of developing allergies, inflammatory and cardio-vascular diseases (Duarte et al. 2021). Hence, this work demonstrated that the inclusion of microalgal ingredients developed from digestate had potential to bring added value to products provided by the aquaculture sector.

4.5.3. Microbiome analysis

In the presented work, Fusobacteria and Proteobacteria were the two dominating phyla found in fish under the commercial diet and the ALG-AD diet after 29 days of experiment, which was in accordance with previous findings from the literature in which similar bacterial compositions were found (Yu et al., 2019; Bereded et al., 2021; Pinheiro de Souza et al., 2020; Tan et al., 2019; Li et al., 2018). Fusobacteria are known to possess immunomodulatory and anti-inflammatory properties due to their capacity to ferment amino acids and carbohydrates into butyric acid (Terova et al., 2016), and some species of these bacterial group are also able to produce vitamin B12 which provides dietary advantages to Nile Tilapia (Tsuchiya et al., 2008). Enterobacteriales are very common in aquatic ecosystems (Das Neves et al., 2022) and while they can cause intestinal diseases (Stone et al., 2007), they are also very important to the fabric of fish microbiomes. The identification of these two major bacterial groups on Nile Tilapia as part of this study was consequently in accordance with previous findings. In our study, differences were however observed between the two diets tested at 15 days of experiment, with Enterobacteriales dominating in the ALG-AD diet, while Fusobacteriales were dominating in the commercial diet. These changes were less apparent after 29 days of experiment and microbiome composition were similar between the two diets. Previous studies have shown that gastrointestinal microbiota is directly responsible for a fish's ability to absorb nutrients and digest food efficiently (Nayak, 2010; Banerjee and Ray, 2016), and consequently impacts fish's weight and size (Clements et al., 2014). Several studies report that changes in the gut microbiota are a direct response to changes in diets (Adeoye, 2016; Hai, 2015), however, a

change in microbiota does not necessarily induce adverse effect on nutrient uptake. In the present study, it appears that an acclimation of the fish to a new diet (*i.e.,* algae-based diet) might have resulted in the shifts in abundance observed after 15 days. Additionally, while all equipment was cleaned between sampling of different tanks under different conditions, and diets were kept separate, microbe contamination between tanks might have occurred, and contributed to the similar microbiome populations observed between diets at the end of the experiment. The observed changes in the gut microbiome and assumed acclimation could be the cause of the slower weight and size gain observed in fish fed with the ALG-AD feed. Indeed, a disturbance of the gut microbiome due to the introduction of a new feed may have caused digestibility and nutrient absorption issues, likely to be linked to the lower growth observed as part of this experiment.

Furthermore, while looking at microbiome composition at the order level, the relative abundance of several orders was higher under the algae-based diet in comparison to the control diet after 15 days of experiment in this work. For example, Pseudomonadales, Bacillales, Lactobacillales and Cellvibrionales were all found in higher abundance in the ALG-AD diet in comparison to the commercial one. Microbial diversity plays an important role in the health of fish, as gut microbiota supplies enzymes helping in the digestion of specific compounds, hence improving nutrition. Gut microbiota can also improve immunity capacities, for example, Carballo et al. (2019) showed that an increased microbial diversity in the Senegalese sole reduced the proportion of Vibrio in the fish and improved overall immunity to diverse pathogens. Another study from Bravo-Tello et al. (2017) found that including *Tetraselmis sp*., and *Chlorella sp.* to the diet of zebrafish significantly improved the survival rate of individuals infected by the pathogen *Edwardsiella tarda*. Other studies have investigated the adverse effects of plant-based ingredients, such as soy meals which can increase the presence of harmful pathogens in the fish gut microbiome, Madeira et al. (2017) and El-Ghany et al. (2020) demonstrated that microalgae inclusion balanced the negative effects of plant-based ingredients and provided an indirect advantage in terms of growth and productivity. The supplementation of microalgae was also proven to enhance immunity. In the present study, acclimation of Nile Tilapia to the algae-based diet may have caused slower growth which could be related to modification of the microbiome composition in the animal's digestive systems, however after acclimation similar bacterial

groups were observed in both diets; showing that the disturbance in the microbiome was only temporary with no long-term adverse effects on the fish.

4.5.4. Use of hydrolysate in fish feed

Hydrolysates have been used in previous studies as feed ingredients in aquaculture. Hydrolysis, using a variety of enzymatic cocktails, is mainly performed on aquaculture and fisheries by-products, such as viscera, heads, fins, skin, or smaller fish not destined for human consumption (Afreen & Ucak, 2020). Hydrolysates have been investigated in the literature as they allow for a better availability of bioactive peptides, which can stimulate defence mechanisms and achieve better growth (Siddik et al., 2020). They can be produced via different mechanisms, but enzymatic hydrolysis is widely implemented as it conveys a better retention of the nutritive value of the source protein, and avoids the production of residual organic solvents or toxic chemicals in the end-product (Najafian & Babji, 2012). Leduc et al. (2018) demonstrated that hydrolysate from white shrimp and Nile Tilapia increased the final body weight and specific growth rate of the European sea bass, with no effect on the rate of feed intake, and similar results were found in the literature when using fish by-product hydrolysates in Barramundi (Chaklader et al., 2020; Siddik et al., 2018, 2019), Japanese flounder (Zheng et al., 2012, 2014) or Atlantic Salmon (Hevrøy et al., 2005). Little interest has been dedicated to hydrolysate from microalgae for aquaculture feed, and studies focus on the incorporation of whole cells, however microalgal biomass can have high levels of soluble polysaccharides fibres which can trap proteins in the cellular matrix, making them less available within feed formulation (Marrion et al., 2005). Consequently, hydrolysis presents a relevant advantage for microalgal biomass processing and enzymes such as cellulase or alcalase (used in this work) are commonly used to hydrolyse microalgal biomass (Fu et al., 2010). A study by Sarker et al. (2020) showed that replacement of fish meal and oil by defatted *Nannochloropsis sp*. and whole cells of *Schizochytrium sp.* in the feed of Nile Tilapia resulted in better growth, weight gain and feed conversion ratio. Additionally, the study showed an increased quality of the flesh with higher levels of lipids and DHA, which was in accordance with the results presented in this work. While hydrolysis was not used to treat the microalgal biomass as part of this specific study, the authors still demonstrated a higher in-vitro protein hydrolysis and protein digestibility of microalgal biomass using enzymes from Tilapia's stomach. This result supports the relevance of using an already

hydrolysed biomass to improve digestibility and protein availability, as was demonstrated by the present work.

4.5.5. Use of Microalgae from waste sources for aquaculture feed

Microalgae grown using waste nutrients have been investigated for their potential to be used as feed ingredient within a concept of circular economy. Several studies have shown successful remediation of aquaculture wastewaters by microalgae, for example, Hawrot-Paw et al. (2020) achieved 88% removal of nitrogen and 99% removal of phosphorus by *Chlorella minutissima* grown on salmon aquaculture wastewater. Similar results were found for *Chlorella sorokiniana*, *Ankistrodemus falcattus* and *Scenedesmus obliquus* (Ansari et al., 2017), as well as *Chlorella ellipsoidea* and *Scenedesmus dimorphus* grown on shrimp aquaculture wastewater (Kim et al., 2019) . While remediation of waste by microalgae has been successful, a comprehensive assessment of incorporating the resulting biomass into aqua feed is still needed from a safety and regulatory aspect, indeed bacterial communities can also develop alongside microalgae used for waste remediation, and can contain toxic elements which could impair animal health (Mishra et al., 2022). Additionally, regulatory bodies still consider the re-introduction of waste-derived biomass into an animal feed as a health concern, even if the use of microalgae is still novel in the field and not enough studies are available to fully assess safety concerns. Digestate, which often results from the anaerobic digestion of animal waste, also falls in this category of waste for which re-utilisation in feed is not fully covered by the regulation. A recent study by Soudant et al. (2022) used biomass of *Aurantiochytrium mangrovei* grown on digestate from pig manure in the feed of sea bass juveniles and larvae. The authors demonstrated that 15% of inclusion of the microalgal ingredient resulted in similar growth compared to a control diet in both juveniles and larvae; additionally, livers of juveniles had higher proportions of 22:6n-3, 22:5n-6, and 20:4n-6 lipids, which was in accordance with the results presented in this study. In the presented work, microalgal biomass was derived from a digestate, which was the by-product of the anaerobic digestion of kitchen waste. Results did not show adverse effect of the feed on the fish with no deaths or signs of ill health recorded. Digestate was first processed by the microalgae for its growth and the resulting biomass was then hydrolysed, consequently it could be assumed that the multi-step processing of the digestate had potential to alleviate any toxicity related to bacteria or other compounds

found in the digestate such as heavy metals. Additionally, as part of the ALG-AD project, which supports this work, a safety analysis was performed on the algal biomass grown on digestate and no bacterial species of concern were found (Keita et al, 2021). While a thorough review of the current regulatory background, associated to technico-economic assessment of the implement technology are needed, the utilisation of a microalgal hydrolysate derived from digestate for aquaculture feed appears as a promising and novel way to implement a circular economy.

4.6. CONCLUSIONS

The use of a waste-derived hydrolysate from the microalgae *Scenedesmus obliquus* in the diet of Nile Tilapia resulted in lesser performances in terms of growth in comparison to a commercial feed. These lesser performances could be attributed to lower nutritional value or availability of the algae-based feed. Analysis of fatty acids content of fish fillet, however, resulted in a higher fatty acids content in fish grown under the algae-based diet. Tilapia, as most fresh-water fish, has low content of α-linolenic (ALA, 18:3 omega-3), eicosapentaenoic (EPA, 20:5 omega-3) and docosahexaenoic (DHA, 20:5 omega-6) fatty acids, compared to marine fish (Global aquaculture Advocate – Report 2006). Consequently, there is an interest for the aquaculture sector to increase fatty acid content in Tilapia, to provide added nutritional value and improve marketability of produced fish. Additionally, fatty acid composition was of particular interest for health applications in human nutrition, as the omega 3 to omega 6 ratio in fish under the algae-based diet was more in-line with health recommendations to tackle cardio-vascular related diseases. Using a waste stream such as digestate to produce an algal ingredient did not show, as part of this study or other previously published work, adverse effects on the overall welfare of Nile Tilapia or other species of relevance in the aquaculture sector, and the utilisation of a hydrolysate demonstrated a novel approach to increase digestibility of microalgal compounds. Regulation around the circular economy concept for animal feed applications is still in need of updating to keep up with the developed innovation, however the present work has demonstrated the potential of using waste-derived microalgal ingredients to bring added-value to the aquaculture feed sector, providing adequate feed formulation and nutritional availability of the feed.

4.7. STATEMENT OF WORK

All the work was carried out by the author of this thesis apart from the following: Determination of FAMEs which was performed by Dr. Rahul Kapoore, hydrolysate production performed by the company IVAMER. Dr Alla Silkina and José Ignacio Gayo-Peláez assisted the author with sample collection. Other author contributions were of an advisory, supervisory, or proof-reading nature.

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CHAPTER 5. THE INFLUENCE OF NUTRIENT AVAILABILITY ON THE BACTERIAL COMMUNITY ASSOCIATED TO CHLORELLA VULGARIS – POTENTIAL IN DIGESTATE BIOREMEDIATION

5.1. ABSTRACT

The interaction between microalgae and bacteria is a complex and dynamic relationship, influenced by an array of factors, and showing promises in the current development of microalgal biotechnologies in a diversity of fields. In this study, I attempted to assess changes in the bacterial population associated to the microalgae *Chlorella vulgaris* when cultures were subjected to low, medium and high nutrient supplementation, in an effort to identify the nature of the interaction between the two populations within a context of growth performance and biochemical composition. Results showed continued growth of *Chlorella vulgaris* under all three nutrient regimes, with better performances under medium and high nutrition media (final OD 750 nm of 3.62 ± 0.38 and 2.54 ± 0.02 after 21 days of experiment, respectively). Flow cytometry analysis showed a strong correlation between the abundance of microalgal and bacterial cells for most of the experiment, however, bacterial count was still increasing when cells of *C. vulgaris* declined, which could be explained by bacteria taking advantage of decaying microalgal cells by using released organic carbon. Lipid profile analysis revealed a significant increase of PUFAs, which doubled by the end of the experiment for all three levels of nutrient investigated. Finally, 16S DNA sequencing using a MinION equipment (determined at day 0, 2, 7, 11, 14 and 16 of the experiment) allowed for the identification of the Pseudomonadales, Caulobacterales, Sphingomonadales, Burkholderiales and Xanthomonadales orders, dominating at different stages of the experiment and for the different nutrient treatments investigated. Some of these groups were found to have potential growth-promoting effect on C. vulgaris and results allowed for some elucidation of the microalgae-bacteria relationship in oligotrophic conditions and growth-promoting effects were discussed; a positive effect of bacterial communities on *Chlorella vulgaris* was demonstrated, emphasising the importance of such systems for biotechnology applications, such as waste remediation.

Keywords: Chlorella vulgaris; bacteria; interaction; nutrient; growth-promoting; waste remediation

5.2. INTRODUCTION

Bacteria, commonly found within microalgal cultures, have been considered as contaminations, especially when their abundance reaches uncontrollable levels leading to culture decline in cultivation systems (Zhang et al., 2020). The production of axenic microalgal cultures (*i.e.,* bacteria-free cultures) has had very little success in laboratory environments with contaminations re-occurring rapidly after isolation (Fernandez-Valenzuela et al., 2021; Yao, 2018) and is simply not achievable at higher scales (Xu et al., 2009). Consequently, increasing attention has been dedicated in the past decade to understand the relationship between microalgae and bacteria, to investigate the potential of associated bacterial communities to provide a beneficial function to microalgal cultures. The interaction between microalgae and bacteria has been studied and most studies have focused on the dynamic of compound exchange between the two kingdoms, and the use of available resources, leading to either competition, mutualism or commensalism being the main nature of the interaction between microalgae and bacteria (Natrah et al., 2014). The specificity of the microalgae-bacteria relationship, however, has yet to be fully elucidated, which demonstrates the complex nature of the association (Amin et al., 2012; Stock et al., 2019), but many studies have eluded to the phycosphere (habitat directly surrounding microalgal cells and formed by microalgal exopolysaccharides) being the centre-stage of the microalgal-bacteria interaction. (Mühlenbruch et al., 2018a; Wirth et al., 2020; Zhang et al., 2020).

Chlorella vulgaris (*C. vulgaris*) is an extensively studied freshwater green microalgae used in the food and feed sectors, but also in cosmetics or biofuel production. Microalgae have also received growing interest in the recent years for their capacity to remediate waste from different industries. While there is abundant knowledge on the microalgae itself, attention for its bacterial community and its potential ability for beneficial functions, has only been investigated in the recent years. Indeed, advances in DNA sequencing have allowed to shed some light on the bacterial community associated to *C. vulgaris,* with species of the *Brevundimonas, Sphingomonas, Blastomonas, Porphyrobacter, Mesorhizobium* and *Letolyngbya* genera being identified (Lakaniemi et al., 2012; Tate et al., 2013; Thi Vu et al., 2010), however more recent studies have discussed that the level of specificity of the association between the identified bacteria and *C. vulgaris* was still unclear (Zhang et al.,

2020) and that the role of external factors on the association was not fully elucidated (Tait et al., 2019).

One of the environmental factors likely to impact the microalgae-bacteria interaction is nutrient source and availability, as several studies have shown that exchanges of nutrients are at the basis of the mutualistic association between microalgae and bacteria. Indeed, bacteria can supply compounds such as vitamins, siderophores and even nitrogen to microalgae (Buchan et al., 2014), and it has further been showed that nitrogen-mediated interactions can occur between the two kingdoms, especially in oligotrophic environments (Zhang et al., 2020). These studies have however investigated nutrient-driven interactions between microalgae and bacteria in natural environments, and fewer studies have looked at the dynamic of the interaction in more controlled laboratory settings, in an effort to pinpoint functions of interest for diverse applications, such as in the field of waste remediation.

The presented study evaluated the growth performances of *C. vulgaris* under 3 nutrient regimes (low, medium and high nutrient supply) as well as its lipid, protein and carbohydrate composition. Additionally, the cell abundance of *C. vulgaris* and associated bacteria were also investigated overtime using flow cytometry, in an effort to highlight any correlation in the dynamic of the two populations. Sequencing using nanopore technologies was used to identify the main bacterial groups present within cultures of *C. vulgaris,* after isolating and culturing of these groups, to assess if they were modified by the nutrient regimes implemented in culture. Additionally, discussion was made regarding the potential of this relationship to be utilised for improving waste remediation by microalgae.

5.3. MATERIAL AND METHODS

5.3.1. Culture of Chlorella vulgaris

A strain of *C. vulgaris* (strain CCAP211/21A) was obtained from the Culture Collection of Algae and Protozoa (CCAP, SAMS Ltd.) and was upscaled at Swansea University. Cultures were grown into conical flasks in an incubator, where temperature was maintained at 25°C. Culture agitation was created by a shaking platform running at 110 rpm and by injection of filtered air (0.22 µm). Illumination was provided using white light at an intensity of 70 $\mu mol.m^{-2}.s^{-1}$ at a photoperiod of 12L:12D (**Figure 5.1**). Cultures were upscaled on the BG11

medium or the F/2 medium, allowing for acclimation of cultures prior to the experiment. In the case of the treatment where no nutrients were added, cultures were grown on the F/2 medium. Upscaling was conducted over 40 days and occurred in increments of 10 days with subsequent medium addition. The experiment started after 10 days of the last nutrient addition to allow for nutrient consumption prior to experimental inoculation.

5.3.2. Experimental design

C. vulgaris was grown in triplicate under three nutrient supplementation regimes. Firstly, cultures were inoculated into autoclaved deionised water, with no medium addition. This treatment represented a low nutrient treatment (referred thereafter as treatment Low-N), and the only nutrient available were the ones likely to remain from the upscaling phase. A nutrient treatment at a medium supplementation level was also applied, using the F/2 medium (Guillard, 1975) with a N:P ratio of 1 and a nitrate concentration of 75 $mg.L^{-1}$ (**Table 5.1**) (thereafter referred as Med-N). Finally, a high nutrient treatment was implemented using the BG11 medium (blue-green microalgae medium, NutriSelect® Basic), with a N:P ratio of 47 and a nitrate concentration of 1.5 $g.L^{-1}$ (**Table 5.1**) (thereafter referred as High-N). Additionally, the BG11 medium was also selected as its nitrogen concentration was the closest to digestate, which is a by-product of the anaerobic digestion of waste and is investigated as a nutrient source for microalgal cultivation in bioremediation purposes, and has a nitrogen concentration ranging from 1 to 4 $g.L^{-1}$ (Xia & Murphy, 2016). Additionally, the two different media had some advantages over each other, with BG11 containing additional carbon under the form of disodium carbonate, while F/2 contained vitamins, however within the context of this study, the difference in nitrogen levels was the main focus.

All replicates for all tested conditions were inoculated at a starting absorbance at 750 nm ranging between 0.5 and 0.6 (corresponding to a 20% inoculation). F/2 and BG11 media additions were performed at a concentration of 10 $mL.L^{-1}$, and both media were filtered (0.22 µm) and UV sterilised before addition in the cultures. 1L Duran bottles were used for cultivation and a 900 mL volume of culture was prepared using autoclaved de-ionised water and 20% of *C. vulgaris* cultures. Cultures were grown under the same conditions as for the upscaling phase described in 5.3.1. Samples were taken through a sampling point allowing minimum manipulation of the bottles during sampling, bottles remained closed for the time

of experiment and were moved during the experiment to allow for a similar light penetration in all the bottles. All bottles, tubing system, connections and materials used for the cultures inoculation and aeration were autoclaved and UV sterilised before use. Cultivation of *C. vulgaris* under the different nutrient regimes was performed for 21 continuous days (**Figure 5.1**).

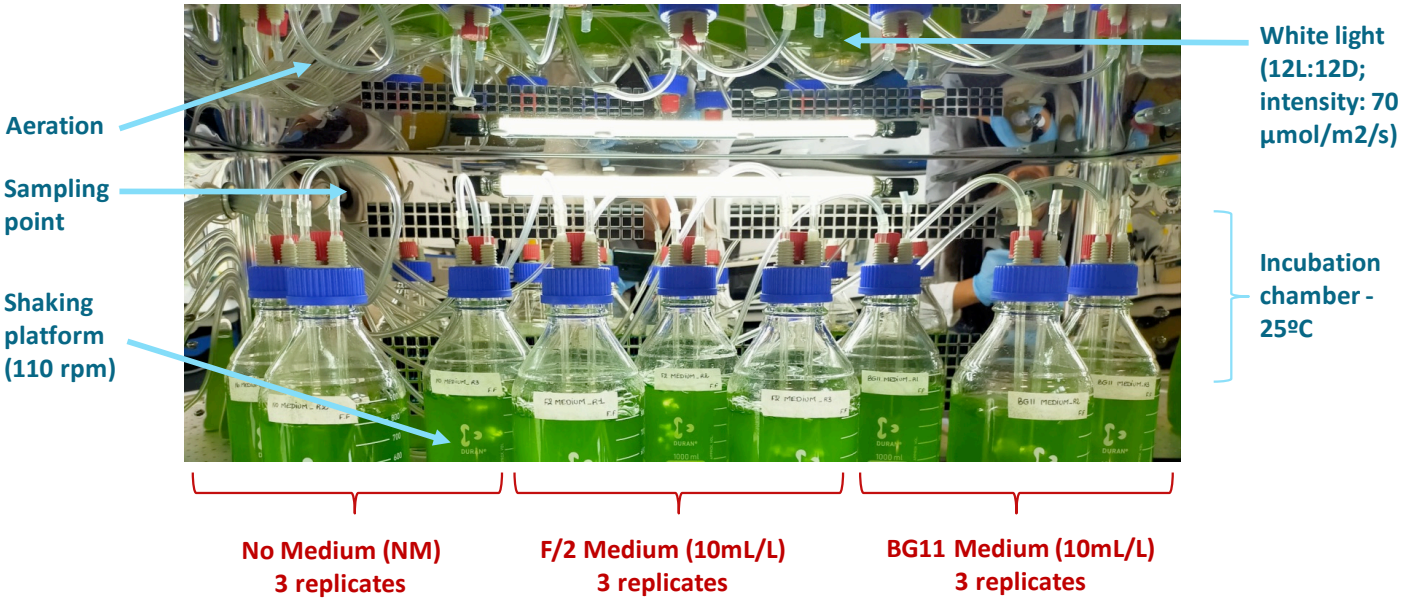


Figure 5.1: Experimental set-up in the incubation chamber for three replicates of *C. vulgaris* grown in NM, F/2 and BG11.

Table 5.1: Compositions of BG11 and F/2 media

BG11 medium		F/2 medium	
Compound	Concentration mg.L^{-1}	Compound	Concentration mg.L^{-1}
$CaCl_2H_4O_2$	36.7	$NaNO_3$	75
$C_6H_8O_7$	5.6	$NaH_2PO_4.2H_2O$	75
K_2HPO_4	31.4	NA_2 EDTA	4.16
$C1_0H_{12}MgN_2Na_2O_8$	1	$FeCl_3.6H_2O$	3.15
$C_6H_8FeNO_7$	6	$CuSO_4.5H_2O$	0.01
$MgSO_4$	36	$ZnSO_4.7H_2O$	0.022
Na_2CO_3	20	$CoCl_2.6H_2O$	0.01
$NaNO_3$	1.5 g.L^{-1}	$MnCl_2.4H_2O$	0.18
		$Na_2MoO_4.2H_2O$	0.006
		Cyanocobalamin (Vitamin B12)	0.005
		Biotin	0.005
		Thiamine HCl (Vitamin B1)	0.1

5.3.3. Analysis of C. vulgaris growth

Culture aliquots (1 ml) were taken daily to measure *C. vulgaris* growth rate in culture. Absorbance at 750 nm was recorded using a spectrophotometer (SPECTROstart^{Nano}, BMG Labtech). This specific wavelength was used as it avoids light absorption by pigments, and can be treated as a light scattering measurement, hence a direct measurement of biomass in culture (Chioccioli et al., 2014). Growth rate was calculated using the absorbance at 750 nm and the following equation:

$$\mu\ (day^{-1}) = ln\ (A_2/A_1) / (t_2-t_1)$$

(Where μ is the specific growth rate, A_1 and A_2 are the Absorbance measured at 750 nm at time 1 (t_1) and time 2 (t_2) respectively).

Additionally, dry weight (in $g.L^{-1}$) was measured at the beginning and the end of the experiment, providing a direct assessment of biomass increase in cultures. Cultures samples of 20 mL were processed on pre-dried and pre-weighed filters (Whatman 47 mm GF/C glass microfiber filters, pore size: 1.2µm) and oven dried for 24 hours at 80°C. Dry weight was calculated as the weight difference between the dried-filtered sample and the pre-weighed-filter.

5.3.4. Flow cytometry: cell count and analysis of microbial populations

Flow cytometry analysis of the cultures were performed daily on a 1 mL sample using a BD Accuri C6 plus equipment (BD instrument). The flow cytometer was equipped with an argon-ion excitation laser (488 nm), four fluorescence detectors corresponding to wavelength intervals of 530 -533, 540-585, 670 and 625-675 nm, and forward and side scatter detectors. Culture samples were vortexed for 15 seconds prior to analysis to separate any microalgal clusters and improve measurement accuracy. The total cell count (or number of event) per µL was recorded for each sample and later processed using the De Novo Software FCS Express to separate cell populations. Based on their forward scatter (FS, indication of size), side scatter (SS, indication of cell structure) and their autofluorescence properties, the distinction between populations of *C. vulgaris*, bacteria and debris was made (Peniuk et al., 2016). Debris did not show any fluorescence emission and presented extremely low FS and SS. Bacteria showed some auto fluorescence in the green excitation wavelength and presented a low FS, indicating a small cellular size, however, differentiation between groups

of heterotrophic bacteria could not be made using this technique (Peniuk et al., 2016). *C. vulgaris* cells, presented the highest levels of autofluorescence as well as FS and SS.

5.3.5. Phosphorus and nitrate analysis

Samples of 10mL of cultures were taken every other day and filtered using 0.22 µm syringe filter to collect supernatants, on which phosphorus and nitrate analysis were performed. Phosphorus was measured using a phosphorus reagent kit (Spectroquant®), based on a colorimetric quantification (method analogous to EPA 365.2+3, APHA 4500-P E, and DIN EN ISO 6878). The absorbance of treated samples was measured at 410 nm according to supplier instructions and ran against a calibration curve for phosphorus concentration determination. Nitrate was measured using a hack kit (Visicolor®) and absorbance was recorded at 450 nm and analysed against a calibration curve.

5.3.6. Biochemical analysis of microalgal biomass – FTIR and FAME

A biomass sample of 10 mL was collected at Day 0, 14 and 16 and centrifuged for 35 minutes at 4000 rpm, the pellet was washed with 2 mL of DI water and further centrifuged for 1 hour at 14 000 rpm. The washing step prevented interference of the media compounds on the analytical process, as well as the removal of debris and other particles from the biomass. Pellets were stored at -80°C before freeze drying for 24 hours and a fine powder was obtained.

Fourier transformed infra-red spectrometry (FTIR)

The total lipid, protein and carbohydrate content of the collected biomass was assessed by FTIR analysis (Fourier Transformed Infra-red Spectrometry) using a PerkinElmer Model Spectrum Two instrument. The FTIR analysis was performed in triplicate for each sample and the FTIR attenuated total reflectance (ATR) spectra were collected. The instrument was equipped with a diamond crystal ATR reflectance cell with a DTGS (Deuterated Tri Glycine Sulfate) detector scanning over the wavenumber range of 4000–450 cm^{-1} at a resolution of 4 cm^{-1}. The pressure applied upon the sample on the diamond was 145 ± 1 N. Background correction scans of ambient air were made prior to each sample scan. Scans were recorded using the software Spectrum (v10. PerkinElmer, Germany).

The total lipid, protein and carbohydrate concentrations were then assessed as proxy of the calculated area of each peak corresponding to the different biochemicals. Peak areas were calculated based on Yao et al. (2012), where carbohydrates, proteins and lipids were recorded in the spectral regions ranging from 1150 to 950 cm^{-1}, 1712-1481 cm^{-1} and 3020-2800 cm^{-1}, respectively.

FAME analysis

FAME analysis was performed as described in (Fernandes et al., 2022). Briefly, between 1 and 2 mg of freeze-dried biomass from the samples outlined above were weighed and processed by direct transesterification. The recovered organic phase was measured and dried by evaporation under inert nitrogen gas and reconstituted in hexane prior to identification and quantification on a GC-ToF-MS equipment. Fatty acids were identified by comparison to 37 known standards and via peak identification. Quantification of FAME in the biomass extract was then determined by comparing experimentally derived component peak areas with the calibration curve generated by the reference standards. Total fatty acids (FAs), saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) concentrations were reported as a percentage of dry weight.

5.3.7. Identification of bacterial community – MinION sequencing

An aliquot of 100 µL was sampled from each culture on alternate days over a 16 days period, a serial dilution was performed and 100 µl were inoculated aseptically onto plates containing a non-selective nutrient agar medium with the following composition: 1 $g.L^{-1}$ of Lab-lemco powder, 2 $g.L^{-1}$ of yeast extract, 5 $g.L^{-1}$ of peptone, 5 $g.L^{-1}$ of sodium chloride and 15 $g.L^{-1}$ of Agar. A non-selective medium was chosen to grow bacteria, in an attempt to provide an environment that would allow for the most bacterial growth. Additionally, culturing of bacteria was performed to ensure that enough biological material was available for DNA extraction, as not enough bacterial DNA was obtained when performing extractions directly on microalgal cultures. Indeed, cultures of *C. vulgaris* were grown in relatively sterile conditions before the experiment, and direct DNA extractions on microalgal cultures would have been dominated by microalgal DNA. Bacterial cultures were kept in the dark and incubated at 37°C to enable fast growth for 48 hours. Bacterial cultures were not grown in

the light as a previous study demonstrated almost no growth of bacteria under these conditions after isolation from *C. vulgaris'* cultures (Tait et al., 2019).

Following bacterial growth, replicates were pooled together and all the developed colonies for each tested conditions were picked to obtain a representative sample. Resource limitation did not allow to analysis replicates separately and it was assumed that replicate pooling would provide sufficient robustness. Colonies were then preserved in 20% glycerol and stored at -80°C before sequencing experiments.

DNA extraction from the collected samples was carried out using a DNeasy blood and tissue kit, following supplier instruction. The extracted DNA was quantified, and quality controlled using a Nanodrop spectrophotometer, and a Qubit, to ensure that there was enough DNA (10 ng) to amplify and multiplex samples using a 16S barcoding kit (Oxford Nanopore Technologies – ONT thereafter). The following mixture was prepared: 10 μL input DNA (10 ng); 25 μL LongAmp Hot Start Taq 2x Master Mix; 5 μL nuclease-free water and 10 μL of 16S barcode. DNA was then amplified using the following PCR cycle: Initial denaturation at 95°C; 25 cycles: 95°C for 20s, 55°C for 30s, 65°C for 2 minutes; final extension at 65°C for 5 minutes. PCR products were then cleaned using AMPure XP (Agencourt) beads and quantified using a Qubit. Barcoded libraries were then pooled at equal ratios (10 ng) and incubated with a rapid adapter. The DNA library (11 μL) was mixed with sequencing buffer (34 μL), loading beads (25.5 μL) and nuclease free water (4.5 μL) and loaded into the MinION flow cell (FLO-MIN106, ONT). The flow cell was checked prior to analysis to ensure that enough pores were available and had not been degraded during storage. Analysis was performed for 48 hours, during which real-time data acquisition and basecalling was performed by the MinKNOW software (ONT).

Following sequencing, fastq files were analysed using the cloud based EPI2ME platform and the EPI2ME agent (ONT). The Fastq 16S workflow was used based on the sequence-based bacterial identification with BLAST using an E-value of 0.01 which is considered as good hits for homology matches. The sequencing analysis resulted in 6,840,729 reads classified at an average sequence length of 1394 nt. De-multiplexing of the barcoded samples was performed by using the filter function following the production of the EPI2ME report and further exported into Excel (Microsoft). Operational Taxonomic Units (OTUs) and Amplicon Sequence Variants (ASVs) were not considered in this work as these analysis were

developed for short-read data and are not suitable for nanopore long reads, as they can lead to overestimation of taxa (Ciuffreda et al., 2021).

5.3.8. Statistical analysis

Statistical analysis was carried out on the studied factors using the R project software (R 4.2.1, Core Team 2022). Two-ways ANOVAs were carried out on normally distributed data and normality was determined using Shapiro tests. Treatment and time were used as parameters and tested on the growth variables, as well as the nutrient composition of the supernatant and the biochemical composition of the sampled biomass of *C. vulgaris*. When significance was found, a post hoc Tukey test was implemented to determine the level of differences (Agricolae CRAN package, R). Additionally, correlation between algal and bacterial populations were calculated using the Pearson test on the flow cytometry data.

Sequencing data were analysed by calculating the Shannon's alpha-diversity (Shannon, 1948) index at genus level per sample using the Excel software (Microsoft) to assess diversity between samples; however beta-diversity was not analysed as limited resources did not allow for replicates to be sequenced using nanopore technology and replicates were pooled as described in *5.3.7.*, additionally, long reads generated as part of this study allow for a better taxonomic and functional analysis due to the higher level of information that they contain, and it was assumed that analysis of samples provided a level of robustness sufficient to the purpose of this study.

The impact of the sampling time and treatment on the alpha-diversity, number of reads and number of identified genera were assessed using a two-way ANOVA. Generalised Linear models (GLM) were then performed at the order level to determine the impact of the interaction between factors (treatment, sampling time and order identified) on the number of reads, to identify dominating orders in the different treatments and at the different sampling point implemented during the experiment. GLMs were used as order data were not normally distributed (Shapiro p-value < 0,01), and this model allowed for the study of the factorial interaction.

5.4. RESULTS

5.4.1. C. vulgaris growth in the different media

C. vulgaris grew steadily during the course of the experiment, and data of absorbance at 750 nm were still increasing after 21 days of recording for all three treatments. Absorbance at 750 nm was significantly impacted by the nutrient treatment ($p=4.69\ e^{-10}$***, Tukey: High-N=Med-N>Low-N). Absorbance at 750 nm reached a maximum of 3.62 after 21 days in Med-N cultures, while High-N cultures reached an absorbance of 2.54 at the end of the experiment. Low-N cultures reached a final absorbance of 1.75 (**Figure 5.2a**). Dry weight yielded similar results, with a significant increase overtime ($p=4.53\ e^{-13}$***) and a significant difference between the treatments ($p=4.78\ e^{-10}$***, Tukey= Med-N>High-N>Low-N). A final dry weight of 0.417 $g.L^{-1}$; 0.282 $g.L^{-1}$ and 0.160 $g.L^{-1}$ was reached after 12 days of experiment for the Med-N, High-N and Low-N treatments, respectively (**Figure 5.2b**). Calculated growth rate was also the highest in Med-N cultures, reaching 0.09 day^{-1}.

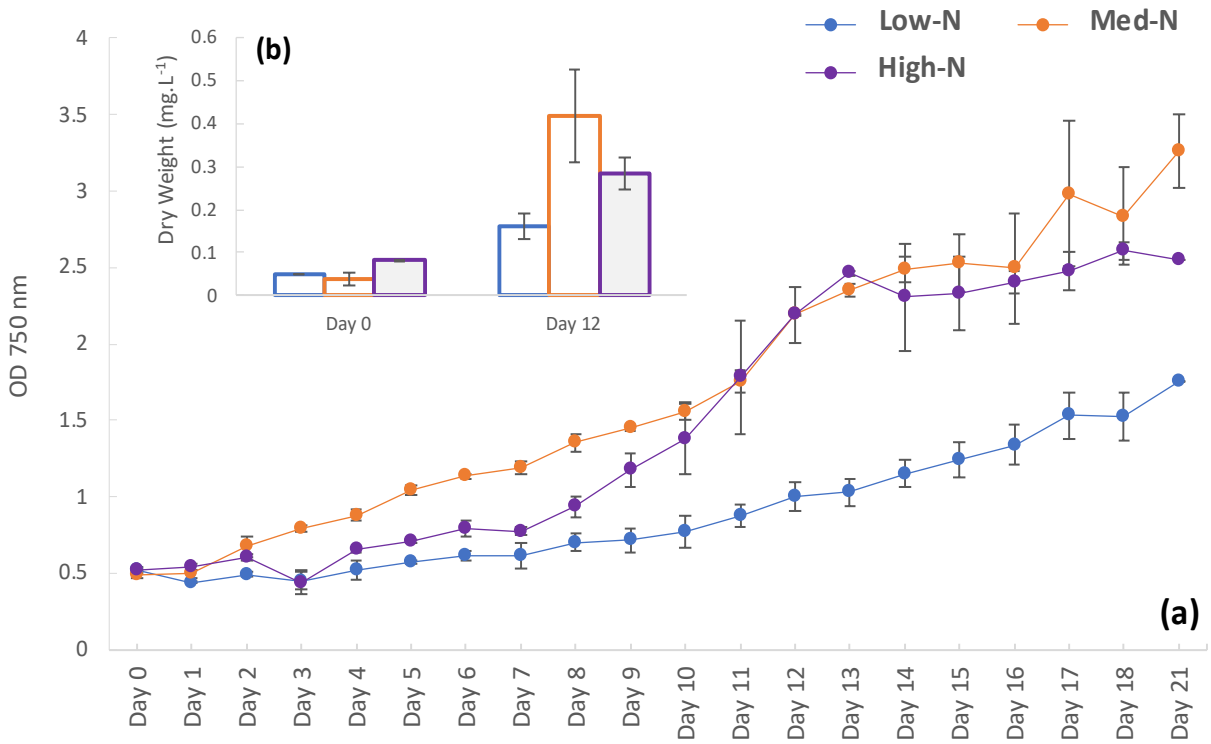


Figure 5.2: (a) Daily Absorbance at 750 nm measured in cultures of *C. vulgaris* grown in Low-N (blue line), Med-N (orange line) and High-N (purple line) treatments; (b) dry weight in $mg.L^{-1}$measured at Day 0 and Day 12 for the three nutrient treatments. Error bars represent the standard error for three experimental replicates.

5.4.2. Cell count evolution of C. vulgaris and bacterial populations

In all three conditions tested, there was a significant increase of the cell count of both *C. vulgaris* and bacterial populations ($p=4.36\ e^{-08}$***; $p=5.48\ e^{-08}$***, respectively and from Day 0 to Day 21). Statistical analysis revealed a significant impact of the treatments on both populations, and the cell count of *C. vulgaris* and bacteria were significantly higher in the Med-N treatment, however there were no significant differences between Low-N and High-N nutrient regimes (*C. vulgaris*: $p=1.09\ e^{-09}$***, Tukey: Med-N>High-N=Low-N; bacteria: $p=2.20\ e^{-05}$***, Tukey: Med-N>High-N=Low-N).

For all three treatments, flow cytometry analysis revealed that the increase in the *C. vulgaris* cell count, was followed by an increase of the bacterial count (**Figure 5.3**). The trends in both bacterial and microalgal populations were similar up to 15-17 days of trial, with a positive correlation between the two populations (Pearson=0.87; 0.91 and 0.82 in Low-N, Med-N and High-N, respectively) (**Figure 5.3a, b & c**). After 17 days, a drop in the cell count of *C. vulgaris* was observed in all treatments, however bacterial populations kept increasing beyond this point. Additionally, concentration of debris also increased significantly during the trial (p=0.03*), potentially being the result of microalgal cell decay in aging cultures.

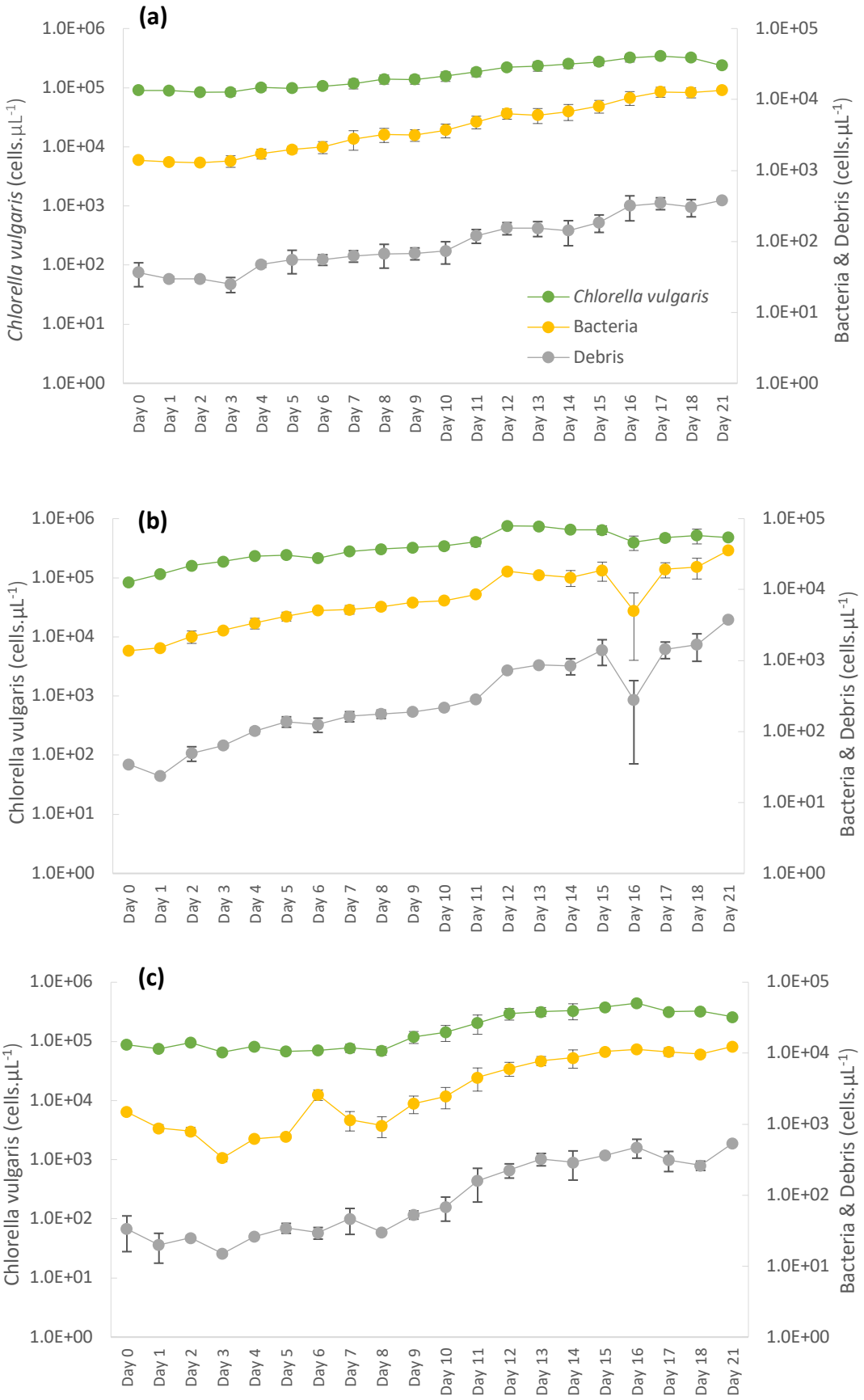


Figure 5.3: Event count analysed by flow cytometry (log scale) for populations of *C. vulgaris* (green line, left vertical axis), bacteria (yellow line, right vertical axis) and debris (yellow line, right vertical axis) for (a) Low-N, (b) Med-N and (c) High-N treatments. Graphs show counts in cells.uL^{-1} for 21 consecutive days of experiment. Error bars represent the standard error for three replicates.

5.4.3. Nutrient Analysis

Nitrate was detected at extremely low levels in the supernatant of Low-N cultures, showing that there was little nutrient remaining from the upscaling phase prior to the experiment (**Figure 5.4a**). Nitrate decreased significantly after 2 days in cultures of *C. vulgaris* in the Med-N and High-N treatments ($p=7.05\ e^{-05}$***; $p=6.89\ e^{-06}$***, respectively), demonstrating consumption of nutrients by cultures. A decrease was also observed for the first 5 days of experiment; however, this was followed by a peak in nitrate concentration at Day 7 in both Med-N and High-N treatments. Nitrate consumption soon resumed and was fully depleted after 12 days of experiment (**Figure 5.4a**). Growth measurements presented above were in accordance with the observed nutrient consumption as nutrients are usually depleted after 10 days of cultivation and stored by microalgal cells to continue their growth.

No phosphorus was detected in cultures of *C. vulgaris* in the Low-N treatment, and phosphorus did not change significantly ($p=0.26$) in the Med-N treatment. A significant increase was measured in High-N cultures overtime ($p= 6.91e^{-05}$***) with a maximum concentration of 74.23 $mg.L^{-1}$ after 12 days of experiment (**Figure 5.4b**). This could be explained by *C. vulgaris* cell decay over the course of the experiment as microalgal cells are known to store phosphorus in their cells which can then be released during cell degradation (Solovchenko et al., 2019), this is particularly true when cultures are aging, which is what was observed in this piece of work.

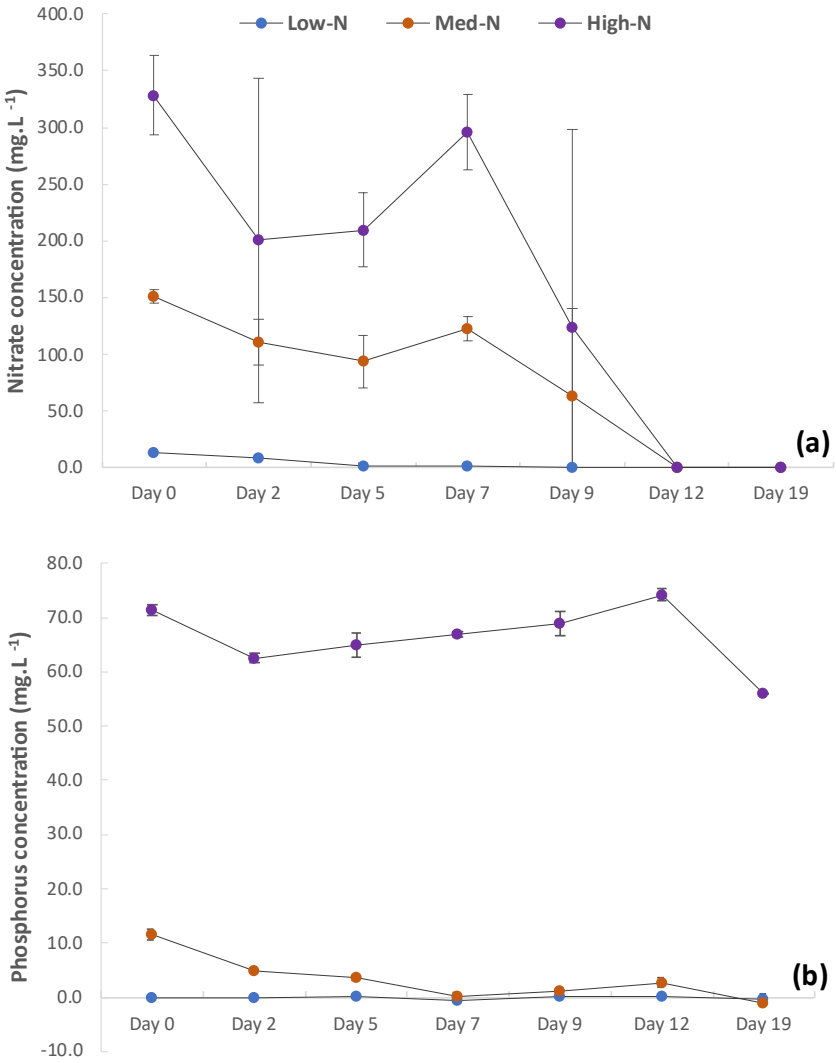


Figure 5.4: Nitrate (a) and phosphorus (b) concentrations in mg.L^{-1} measured in the culture supernatant of *C. vulgaris* grown under Low-N, Med-N and High-N supplementation. Error bars represent the standard error for three biological replicates.

5.4.4. Microalgal biomass composition analysis

FTIR analysis of *C. vulgaris* biomass showed that there were no significant changes in the lipid profile of *C. vulgaris* between the start and end of experiment (p=0.27), regardless of the nutrient regime implemented during the experiment (p=0.08) (**Figure 5.5**). Proteins were significantly impacted overtime (p= 5.87e^{-06}***) and decreased in the Low-N and Med-N treatments, which was in accordance with nutrient depletion and nitrogen not being available for protein biosynthesis. However, the protein profile remained the same in the biomass grown under a High-N treatment. Finally, carbohydrates did not significantly change over the time of the experiment (p= 0.21), (**Figure 5.5**).

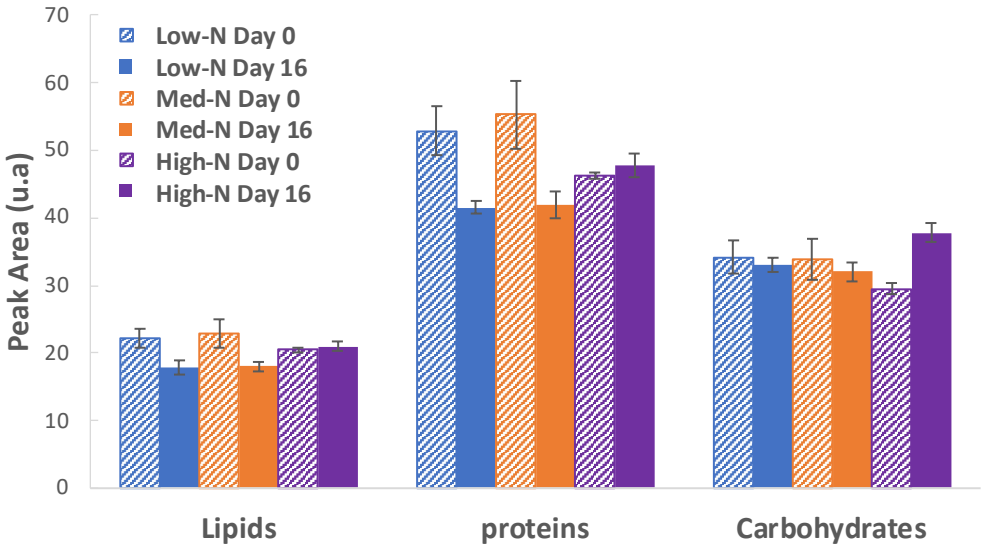


Figure 5.5: Peak areas measured by FTIR and used as proxy for the representation of lipids, proteins and carbohydrates profiles recorded at day 0 and day 16 in biomass samples of *C. vulgaris* grown under Low-N (blue bars), Med-N (orange bars) and High-N (purple bars) treatments. Error bars represent the standard error for three replicates.

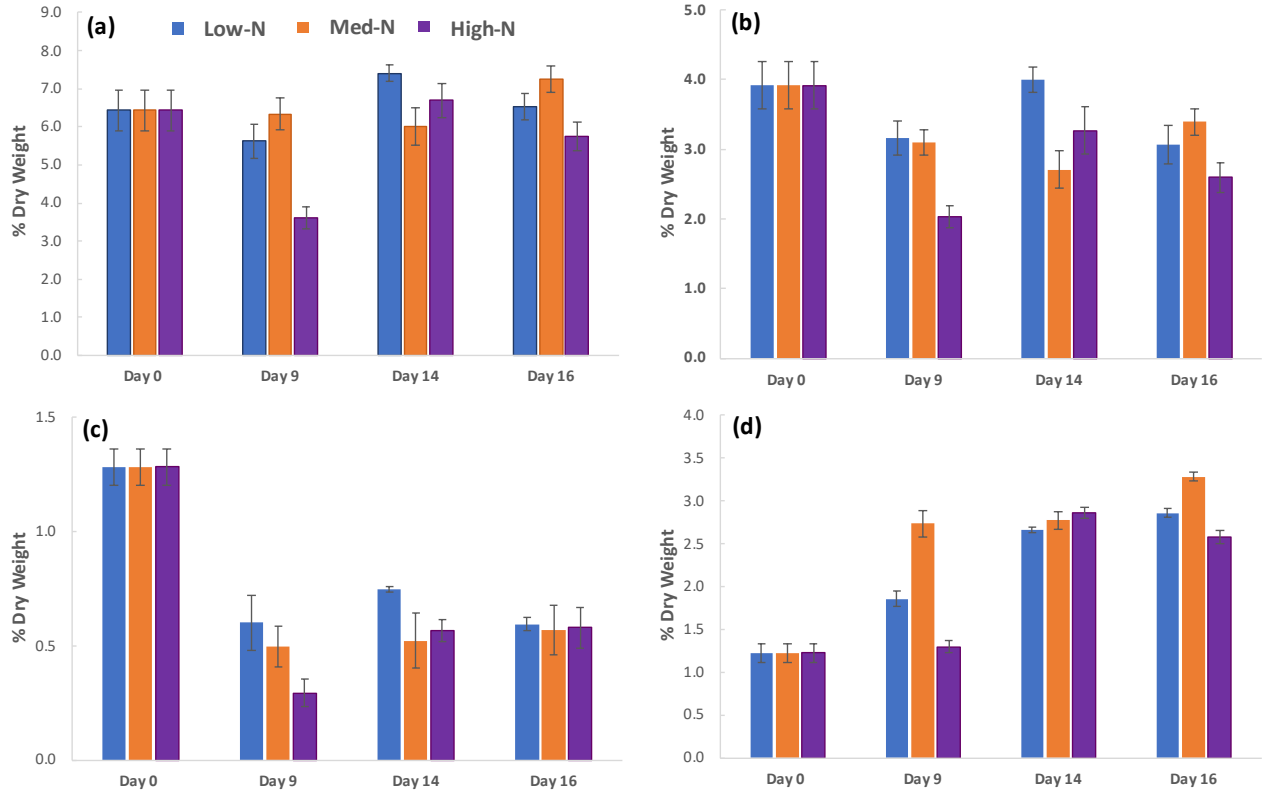


Figure 5.6: Percentage of dry weight of (a) total fatty acids (FAs), (b) total saturated fatty acids (SFAs), (c) total monounsaturated fatty acids (MUFAs) and (d) total polyunsaturated fatty acids (PUFAs) in cultures of *C. vulgaris* grown under Low-N (blue bars), Med-N (orange bars) and High-N (purple bars) treatments from Day 0 to Day 16 of experiment. Error bars represent the standard error for three biological replicates.

Analysis of total fatty acids using GC-MS showed no significant differences in the biomass content during the experiment (p=0.20) or between treatments (p=0.29), which was in accordance with FTIR data presented above (**Figure 5.5 & 5.6**). A more detailed analysis of the fatty acids, however, revealed a significant decrease in the concentrations of SFAs ($p=7.64e^{-04}$***) and MUFAs ($p=8.59e^{-05}$***) between Day 0 and Day 16 for all treatments (**Figure 5.6b & c**). The opposite was recorded for concentration of PUFAs in biomass of *C. vulgaris*, and a significant increase was observed after 16 days of experiments (p=0.003**), for all treatments, but this was more apparent in the Med-N treatment (**Figure 5.6d**).

5.4.5. Identification of bacterial communities using DNA sequencing

Table 5.2: Number of reads and genera identified using MinION sequencing and calculated Shannon alpha-diversity at Days 0, 2 7, 11, 14 and 16 of the experiment for the three nutrient treatments (Low-N, Med-N and High-N).

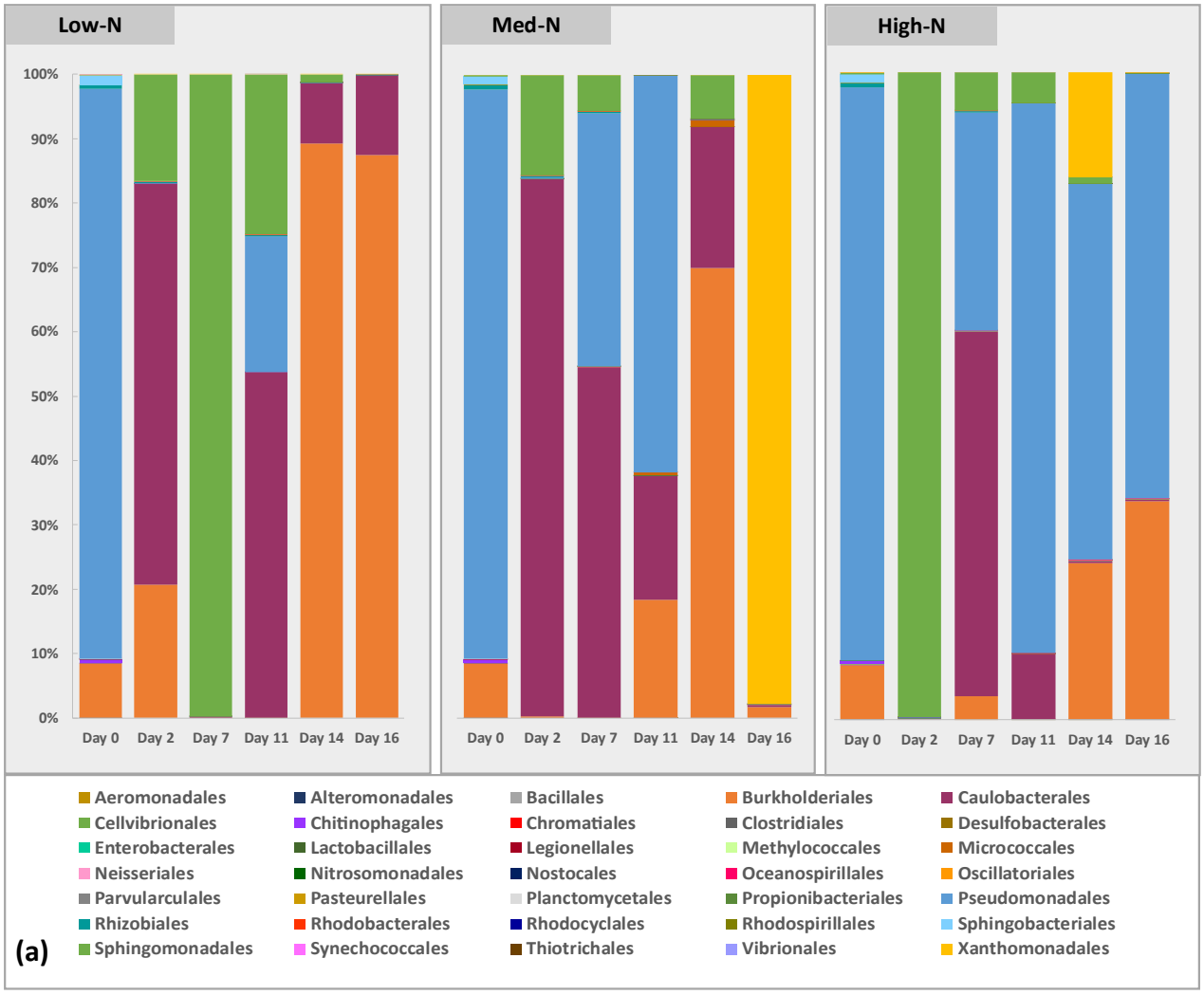
	Low-N			**Med-N**			**High-N**		
	Number of reads	*Number of genera identified*	*alpha-diversity*	*Number of reads*	*Number of genera identified*	*alpha-diversity*	*Number of reads*	*Number of genera identified*	*alpha-diversity*
day0	194842	138	0.488	194842	138	0.488	194842	138	0.488
day2	392722	268	0.898	343306	127	0.278	170586	119	0.289
day7	51306	51	0.274	373921	200	0.842	272017	172	0.960
day11	447700	183	0.939	363498	228	0.991	445697	166	0.464
day14	408043	159	0.427	383033	209	0.813	585979	254	1.123
day16	302950	105	0.448	305308	112	0.201	481239	193	0.711

p-value	*alpha-diversity*	*Number of reads*	*Number of genera*
Treatment	0.873	0.087	0.588
Day	0.610	0.663	0.787

DNA sequencing resulted in a total of 5.5 million good reads (e-value<0.01) across the conditions tested and at the different time points sampled, with 396 genera identified. As a different number of reads was used for the different samples, the analysis performed on sequencing data was qualitative rather than quantitative. Statistical analysis showed that the treatments used during the experiment, or the time of sampling did not have a significant impact on the alpha-diversity, nor on the number of reads or genera (**Table 5.2**). As analysis on the order level showed the strongest trends, which were also better visualised, therefore, results description was focused on the order level thereafter.

A cross-factor analysis performed using a generalised linear model showed that the Pseudomonadales were significantly dominant at day 0 (p= 0.025241*), for all conditions

tested with 87.72% of reads identified ((**Figure 5.7 a & b**), most abundant strain: *Pseudomonas extremaustralis*). This was followed by a shift in the dominating orders, with a significant increase of the Caulobacterales (p= 0.008530 **, dominating strain: *Brevundimonas aurantiaca*) and Sphingomonadales (p= 0.000729 ***, dominating strain: *Sphingopyxis terrae*) orders at Day 2 and 7, across the three nutrient regimes tested (**Figure 5.7**). Finally, at day 16, the Burkholderiales order was dominant in the Low-N treatment, with 87.30% of identified reads (p= 0.004040**, dominating strain: *Cupriavidus metallidurans*), while Xanthomonadales were the most abundant in the Med-N treatment with 97.65% of identified reads (p= 0.000729 ***, dominating strain: *Stenotrophomonas maltophilia*) (**Figure 5.7**). The Pseudomonadales order remained the dominating one in the High-N treatment with 64.95% of sequenced reads (p=0.021423*). Additionally, it is relevant to note that Burkholderiales were also present at the end of the experiment in the High-N treatment with 33.35% of identified reads (**Figure 5.7b**).



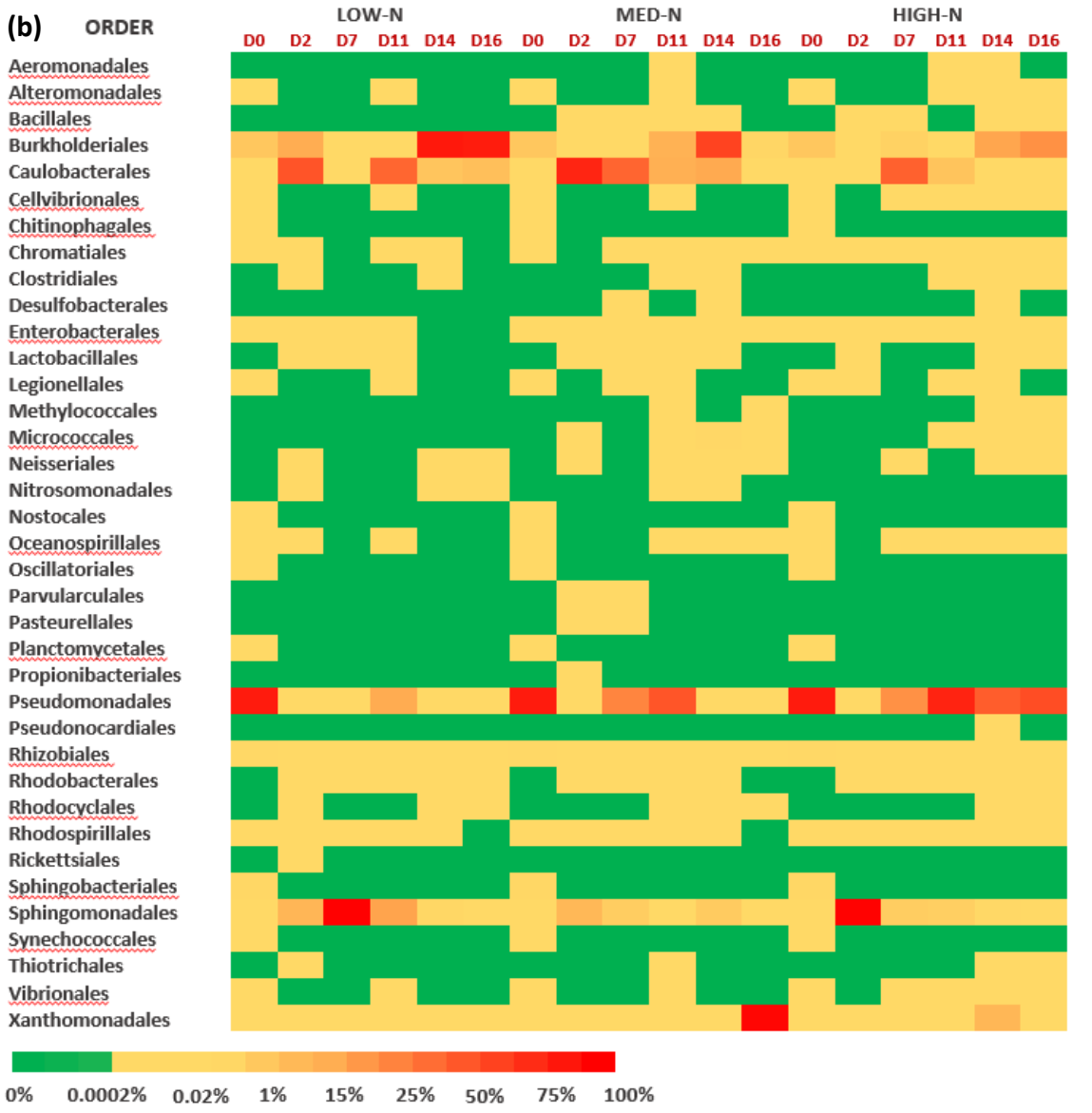


Figure 5.7: (a): Order-level composition of the microbiome associated to *C. vulgaris* from Day 0 to Day 16 of experiment (horizontal axis). Samples are grouped by treatment. Each bar represents the relative abundance of identified orders, expressed as percentages (vertical axis). (b) Heat map summary of the order-level composition from Day 0 to Day 16 (D0,D2,D7,D11, D14,D16) grouped by nutrient treatments.

5.5. DISCUSSION

5.5.1. C. vulgaris growth and nutrient consumption

C. vulgaris showed better growth performances when cultured under a medium or high nutrient supply (the nitrate concentration in High-N was 20 times higher than in Med-N), while growth still occurred (to a lesser extent) under low-N for the duration of the

experiment. Additionally, optical density at 750 nm indicated that *C. vulgaris* was still growing towards the end of the experiment despite nutrients being depleted after 10 days, as shown by nutrient analysis. Cells of *C. vulgaris* are able to accumulate nitrogen, phosphorus, as well as other compounds such as lipids and carbohydrates reserves that can be used to maintain growth when nutrients are depleted (Eduardo et al., 2016; Pavel et al., 2012; Ratomski, 2021). These reserves would not have been measured in this work as nutrient analysis were only performed on cultures supernatants, but it can be assumed that the continued observed growth of *C. vulgaris* in a nutrient depleted environment (in Low-N or after 10 days of experiment in Med-N and High-N) could be attributed in part to this ability of the microalgae to build up nutrient reserves in its cells (de Farias Silva & Sforza, 2016; Kong et al., 2021; Pavel et al., 2012). Flow cytometry analysis, however revealed a decrease in the cell number of *C. vulgaris* at the end of the experiment (after 17 to 20 days). Therefore, it is likely that the nutrient reserves mentioned above might support microalgal growth for a limited time (*i.e.,* 10 days in this work).

Flow cytometry analysis of cultures resulted in a higher cell number in cultures grown under Med-N, while cell counts were comparable between Low-N and High-N treatments. This can be explained by the level of cell division occurring at a faster rate under the Med-N treatment, while division occurred at a much slower rate in the other treatments. Less cell division under Low-N can likely be attributed to a low nutrition of the treatment, not supporting this cell activity (Paes et al., 2016). *C. vulgaris* might have diverted energy and reserves for cell maintenance rather than exponential growth in this nutrient-depleted environment. However, a similar cell count was observed under the High-N treatment, for which nutrients were available to support cell division. Consequently, it could be assumed that in a highly nutritious environment, *C. vulgaris* favours accumulation of reserves and growth of cells in terms of biovolume rather than cell division. For example, (Paes et al., 2016) found that a high nutrient supply in cultures of *Chlorella sp*. resulted in a higher biovolume, that could be linked to nutrient accumulation. Additionally, the low division rate can also be a result of nutrient toxicity, indeed, previous works have shown that high nitrogen, and nitrate concentration can cause medium toxicity for green microalgae, resulting in lower growth or even cell decay (Sanz-Luque et al., 2015; Xia & Murphy, 2016). However, while division rate appeared to be slower, growth occurred for 17 continuous

days, with a decrease in cell number recorded on the last day of experiment. This result was supported by phosphorus analysis in the culture supernatant of *C. vulgaris* under the High-N treatment, indeed phosphorus increased overtime which can be linked to cell decay as microalgal cells are known to store phosphorus, which is released during cell degradation (Powell et al., 2011; Solovchenko et al., 2019). This is particularly true when cultures are aging, which was in accordance with the observations made during this work.

5.5.2. C. vulgaris biomass composition

FTIR analysis showed no change in the lipid profile according to nutrient regime, but there was a decrease in the protein content in Low-N and Med-N treatments, which was consistent with the measured nutrient depletion, resulting in nitrogen availability being reduced for protein synthesis (Amorim et al., 2021; Ördög et al., 2012). In the High-N treatment, however, proteins increased overtime and it could be assumed that the higher supply of nitrogen enabled protein synthesis until the end of the experiment. This result supports the assumption discussed above of nitrogen supply being used for other usage than cell division, for example accumulation of compounds to create reserves (Jiang et al., 2012; Lavín & Lourenço, 2005).

Analysis of fatty acids using GC-MS showed a decrease of SFAs and MUFAs but a consistent increase of PUFAs across all nutrient regime tested. Several studies have shown an increase in PUFAs in cultures of *C. vulgaris* but also other microalgal species when nutrients were depleted (B. Fernandes et al., 2013; Klok et al., 2013; Yang et al., 2018). For example, Agirman and Cetin, 2015 showed that a 50% nitrogen depletion resulted in a 80% increase in the lipid content of *Chlorella vulgaris*. Consequently, the observed results were likely attributed to nutrient depletion measured during the experiment.

5.5.3. Bacterial Analysis – Abundance of bacterial populations

Analysis of bacterial population in cultures of *C. vulgaris* using flow cytometry showed a higher number of bacterial cells under the Med-N treatment, while bacterial abundance in Low-N and High-N treatments was comparable. Additionally, correlation analysis on populations of *C. vulgaris* and bacteria resulted in a strong correlation between the two communities for 18 days of experiment. This observation could be attributed to bacteria inhabiting the close vicinity of *C. vulgaris'* cells, otherwise known as the phycosphere.

Indeed, the phycosphere which is mainly composed of exopolysaccharides excreted by microalgae is believed to be the main environment where exchanges occur between microalgae and bacteria (Samo et al., 2018; Wirth et al., 2020). Additionally, exopolysaccharides provide a habitat for bacteria (Mühlenbruch et al., 2018b). It can be further assumed that bacteria found in correlation with *C. vulgaris* as part of this work could have provided a function to the microalgae and vice-versa, resulting in a certain level of positive cooperation as found in several studies (Qu et al., 2014; Ramanan et al., 2016; Sepehri et al., 2020). Indeed, it is well known that bacteria can provide CO_2 to microalgae via respiration, and that oxygen resulting from photosynthesis can be used in return by bacteria (Zhu, 2015). Additionally, denitrifying bacteria can also play a role in this positive interaction, by reducing nitrate into more available sources of nitrogen for *Chlorella sp.* as demonstrated in Chen et al. (2019) in which growth and nutrient removal yields by *Chlorella sp*. were improved by interactions with denitrifying bacteria. In this study, similar mechanisms could have taken place, resulting in a strong positive correlation between the two populations. However, after 17 to 20 days (depending on treatment), *C. vulgaris* abundance decreased while bacteria kept increasing in all nutrient regime tested. Therefore, it could be assumed that the relationship between algae and bacteria within this work was not a strict interaction as bacteria could develop independently from *C. vulgaris*. Furthermore, the observed increase in bacteria could also be attributed to their ability to utilise carbon from decaying cells of *C. vulgaris* (Fuentes et al., 2016; Yao, 2018). This could lead to a shift from a positive interaction between the two populations to a more competitive relationship, in which bacteria can take advantage of cell decay. This would demonstrate that in low nutrient environment and in healthy cultures of *C. vulgaris*, the relationship between the two communities can be positive with a high likelihood of compound exchange and other benefits mutually provided; however, when nutrients are fully depleted, and when cultures start showing signs of decay, there is a shift in the relationship in which bacteria can take advantage of degrading cells. For example, Liu et al. (2012) showed that there was a complementary relationship for phosphorus demand between microalgae and bacteria, but only in carbon-depleted environment, indeed, the addition of glucose in their experiment resulted in a decline of the microalgal population, as bacterial nutrient demand increased with carbon availability, leading to competition between microalgae and bacteria for nutrients. Consequently, it appears that the

interaction between algae and bacteria is highly dependent on nutrient availability and is not an obligatory relationship, as the present study also showed that bacteria could maintain growth without *C. vulgaris*.

5.5.4. Bacterial Analysis – Sequencing of the bacterial population

Community analysis of the bacterial population associated to *C. vulgaris* showed that the Pseudomonadales order was mainly found at the beginning of the experiment in all nutrient treatments. For reminder, cultures of *C. vulgaris* were upscaled using different media to acclimate cultures to the nutrient treatments before the experiment, and nutrients were never depleted during this stage. Consequently, despite different media being used, the same bacterial order was found at the start of the experiment when nutrients were available. Pseudomonadales have been found in association with *C. vulgaris* in several studies (Guo & Tong, 2014; Haberkorn et al., 2017; B. H. Kim et al., 2014; Tait et al., 2019). Hence, it could be assumed that in a nutrient-abundant environment, Pseudomonadales are commonly found in association with *C. vulgaris*. Additionally, Tait et al. 2019 discussed that *Pseudomonas sp*., had inhibitory properties on other bacterial groups, demonstrating a strong interaction between this strain and *C. vulgaris,* and it could be argued that a certain level of specificity exists between the microalgae and bacterial strains of the Pseudomonadales order, as species of this order seem to have developed specific mechanisms to remove other bacterial species and beneficiate primarily of an association with *C. vulgaris.*

Results also showed an increase in the abundance of the Caulobacterales and Sphingomonadales orders after several days of experiment, which was observed across all three nutrient treatments, showing that changes occurred overtime in the bacterial population but with no clear implication of the level of nutrients available between tested conditions. However, at the end of the experiment, differences between dominating orders were more pronounced, with Burkholderiales being the most abundant in Low-N, Xanthomonadales dominating in Med-N and finally Pseudomonadales and Burkholderiales being the most present in High-N. Furthermore, an array of factors was also likely to influence bacterial communities, including abiotic factors but also contaminations (Grossart & Simon, 2007; Grover, 2000; Guo & Tong, 2014). For example, Quijano et al., 2017 discussed that pH, light intensity, mixing, and temperature could change the interactions

between microalgae and bacteria as well as the community structure of the consortium. In this work, it appears that nutrient levels might have influenced the bacterial community in association with *C. vulgaris*, however it also appears that the described bacterial groups might have developed due to a nutrient depletion occurring over the course of the experiment, rather than because of the different nutrient levels implemented.

5.5.5. Potential impact of bacterial population on C. vulgaris grown under different nutrient regimes

One of the main results of this experiment was that cultures of *C. vulgaris* maintained growth under very low concentrations of nutrients available. Nutrient reserves might have played an important role in the prolonged growth of *C. vulgaris*, however observed patterns in biomass composition as well as from the sequencing analysis of the bacterial population allow for a few speculations regarding the role of bacterial communities associated to the microalgae *C. vulgaris*.

Firstly, despite measurements showing a depletion of nutrients after 10 days of experiment, nutrients might have been present and not measured by the method used in this work. Indeed, intracellular reserves have been discussed above, but there is also an argument to be made regarding denitrifying properties of bacteria, potentially increasing nutrient availability for *C. vulgaris* and prolonging culture growth. For example, Fallahi et al., 2021 discussed that bacteria could perform denitrification in microalgal cultures during the night, when dissolved oxygen is low, and that nutrients were then used during the day for photosynthesis. It can be further assumed that microalgae could favour denitrifying bacteria, however there is little knowledge about microalgae favouring these bacterial groups as part of a specific relationship or if microalgae and bacteria have evolved concomitantly taking advantages of these same mechanisms (Amin et al., 2012)

Several studies have shown growth promoting effects of certain bacterial groups on microalgae and more particularly on *C. vulgaris*. For example, bacteria of the *Brevundimonas* genus (part of the Caulobacterales order) were found to have growth-promoting effects on *Chlorella sp*., by prolonging culture life. Park et al., 2008 demonstrated that *Brevundimonas nasdae* and *vesicularis* increased growth of the microalgae *Chlorella ellipsoidea* by 3-fold and were responsible for a prolonged lifetime of the cultures.

Additionally, authors showed that bacterial growth was also improved by the microalgae, revealing potential for symbiotic effects, which was then confirmed by electron microscopy showing direct adhesion of the bacteria to the surface of *Chlorella ellipsoidea*, which was in favour of a compound exchange-based relationship. Similar results were found in Tait et al. (2019), where *Brevundimonas sp* increased the optical density of *C. vulgaris* in culture. Consequently, it is possible that a similar growth-promoting effect of the Caulobacterales, identified as part of this study, could have contributed to the extended culture life of *C. vulgaris*. Additionally, the dominating strain found in the Caulobacterales order was *Brevundimonas aurantiaca,* which strengthens the link with findings from Tait et al., (2019). Burkholderiales and Xanthomonadales were also found in this study, and both orders have been shown to produce indole acetic acid and gibberellins which are plant-growth promoting hormones (Dao et al., 2020; Vo et al., 2018). The production of growth hormones by these two orders could explain, in part, the continued growth of *C. vulgaris* in Low-N, but also after 10 days in the Med-N and High-N treatments, when nutrients were depleted. Additionally, one could hypothesise that there are microalgal mechanisms in place favouring growth-promoting bacterial orders in low nutrient environments. Rier and Stevenson (2002) expected competition between algae and bacteria under oligotrophic conditions, however competition was not observed, and authors hypothesised that bacteria were not using free organic nutrients but rather nutrients from algal–released carbon (external layer of polysaccharides), demonstrating the importance of the positive interaction in a low nutrient environment. Additionally, they determined that the interaction was mainly based on the utilisation of the algae by bacteria as a colonisation substrate. Liu et al. (2012) also suggested that bacterial growth was probably dependent on DOC released by microalgae, and that a mutualistic relationship between the two kingdoms was favoured under conditions of phosphorus and carbon deficiencies.

Some bacterial species are also known for their impact on microalgal metabolism. For example, Xue et al. (2018) have shown that *Stenotrophomonas maltophilia* (from the Xanthomonadales order) increased the growth rate, maximum productivity and lipid content of *C. vulgaris*. As part of the presented work, *Stenotrophomonas maltophilia* was identified in cultures of *C. vulgaris* grown under the Med-N treatment and it could be assumed that a similar influence of this bacterial strain on the PUFAs content of *C. vulgaris*

could have occurred. FTIR analysis showed a slight increase of the carbohydrate content in the biomass of *C. vulgaris* in the High-N treatment. This increase could be the result of Exopolysaccharide production by *C. vulgaris*, which could have been a mechanism to attract bacteria and favour beneficial compound exchange.

A shift in the interaction between microalgae and bacteria has been discussed in other studies, looking into the influence of abiotic and biotic parameters. For example, the relationship between the two populations has been shown to change from cooperation or cohabitation to competition and further to cell decay induced by one or the other group (Grossart & Simon, 2007; Grover, 2000; Guo & Tong, 2014). In this study, the microalgae-bacteria interaction appeared to be a positive one, where in a low nutrient environment, bacteria might have contributed to support microalgal growth and prolong culture life. Additionally, the phycosphere seems to play an important role in this dynamic interaction, by providing a habitat for bacteria within the direct vicinity of microalgal cells, thus facilitating compound exchange.

5.5.6. Scope for microalgae-bacteria consortium for digestate remediation

In this study, the potential for a growth-promoting effect of the bacterial community associated to *C. vulgaris* in a low nutrient environment has been discussed. In this following section, I discuss how this ability of bacteria to support *C. vulgaris* growth provides scope for the engineering of microalgae-bacteria consortium for digestate remediation. Indeed, Sforza et al. (2018) showed that the symbiotic relationship between *Brevundimonas diminuta* and *Chlorella protothecoides* improved remediation of nitrogen, phosphorus and COD from wastewater, mainly due to improved growth of *C. protothecoides* by the bacteria. Additionally, several papers showed that *Pseudomonas sp.* could also improve remediation capacities by *Chlorella sp*. by promoting growth and nutrient uptake (Iocoli et al., 2017; Rizwan et al., 2018). The engineering of artificial consortium of algae and bacteria, to favour specific interactions that would facilitate waste remediation by microalgae, as consequently been investigated in the past years. For example, some studies have shown that co-culture of *Chlorella sp.* with the growth-promoting bacteria *Azospirillum brasilense* significantly improved nutrient-removal efficiency, mainly due to the ability of the bacteria to produce indole acetic acid (De-Bashan et al., 2008; Perez-Garcia et al., 2010). Additionally it could be further assumed that modification of trophic regime could favour development of targeted

bacteria bringing specific advantages and functions to microalgal cultures. Kim et al. (2015) showed that the association between *C. vulgaris* and *Microbacterium sp*. increased dry weight of the microalgae by 65.7% when grown in treated livestock wastewater. Additionally, the chlorophyll a content was also increased by 78,8%, demonstrating commercial value of the produced biomass for different applications. This study was a practical example of how to increase biomass production by modifying the population of symbiotic bacteria. Consequently, consortium of microalgal and bacterial population present a tremendous potential in the field of algal biotechnology and waste remediation.

5.6. CONCLUSIONS

In this study, growth performances of *C. vulgaris* under three nutrient regimes was assessed, while studying the microalgae biochemical composition and attempting to identify the bacterial community developing concomitantly in cultures. Recorded growth and some changes in the lipid profile of *C. vulgaris* could be linked to its association with bacterial groups identified in this work, which may result in a beneficial interaction, especially under oligotrophy, which was in accordance with the existing literature. A full elucidation of the relationship between *C. vulgaris* and its associated bacteria is yet to be performed, due to the complexity of this dynamic system, likely to rely on underlying mechanisms such as quorum sensing, signalling molecules and compound exchange which were not studied in this work. As high scale production of microalgae gains interest worldwide, the exploitation of natural or artificial bacterial-microalgal consortium represent a promising and novel approach in microalgal biotechnologies, especially in the field of waste remediation.

5.7. STATEMENT OF WORK

All of the work presented in this chapter was carried out by the author of this thesis apart from the following: Determination of FAMEs which were performed by Dr. Rahul Kapoore. Other author contributions were of an advisory, supervisory, or proof-reading nature.

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CHAPTER 6. GENERAL DISCUSSION AND CONCLUSIONS

This thesis aimed **to evaluate the use of microalgae for the valorisation of nutrient rich digestate in a context of circular economy**. To do so, a thorough study of digestate was performed in **chapter 2**, evaluating processing methodologies to optimise the waste as a nutrient source for microalgal cultivation, which was then conceptually proven at laboratory and pilot scales. Cultivation of microalgae on nutrient rich digestate was then refined in **chapter 3** by assessing how tailoring of digestate sources and microalgal strains could improve digestate uptake as a nutrient source by microalgae, while implementing abiotic factors such as pH to optimise the overall process. The resulting biomass, for which production was performed according to findings from the studies described above was utilised in **chapter 4** as a feed ingredient in the diet of Nile tilapia, hydrolysation was investigated as a novel way to incorporate microalgal ingredients into aquafeed and the potential of waste-derived microalgal ingredient to be re-introduced into the supply chain within a feed was consequently assessed and discussed within the circular economy concept. Finally, the potential of microalgae-bacteria consortium for digestate bioremediation was also discussed in **chapter 5** by studying the influence of nutrient availability on the bacterial community associated with the microalgae *Chlorella vulgaris*, which represent significant commercial interest in the field of microalgal biotechnologies. Circular economy concepts and re-utilisation of underused waste into the supply chain was explored in this thesis and the potential of microalgae to valorise digestate was demonstrated.

6.1. NUTRIENT RICH DIGESTATE: TREATMENT AND POTENTIAL TO SUPPORT MICROALGAL GROWTH

Nutrient rich digestate, or NRD, is a by-product of the anaerobic digestion of various waste streams and is an underused and undervalued source of nutrients. The current route for valorisation of NRD lies in its utilisation as a fertiliser on arable land, however soil eutrophication limits its spreading, and the implementation of nutrient vulnerable zones, on the rise across Northwest Europe, results in millions of tons of excess digestate (Fuchs & Drosg, 2013; Guilayn et al., 2019). To answer this challenge, **chapter 2 investigated routes for the valorisation of excess digestate and aimed to assess digestate potential to support**

microalgal growth to develop the initial step of a circular economy by utilising an underused source of waste. Digestate was obtained from the Langage AD plant (Devon, UK) and thoroughly studied for its nutrient composition, but also its physical properties, to assess its potential to support microalgal growth. Raw (or untreated) digestate contained 4.016 $g.L^{-1}$ of NH_4^+-N and 665 $mg.kg^{-1}$ of P, which are the two main nutrients used to support photosynthesis (Luo et al., 2017; Stiles et al., 2018), and dry matter content as well as dark colour emphasized the need for treatment prior to microalgal cultivation (Marcilhac et al., 2014). Mechanical treatments of digestate were implemented. Dilution combined with settlement provided a low-cost solution for the processing of digestate, allowing for release of valuable compounds for microalgal development, however, membrane filtration, used in chapter 2 as a multi-step approach combining micro, ultra and nano-filtration, was the most efficient way to recover nearly 95% of nitrogen and phosphorus, hence demonstrating significant potential for nutrient tailoring in microalgal cultivation (Adam et al., 2018; Gerardo et al., 2015). As membrane filtration can be a costly process, coupling this method with microalgal cultivation is essential to bring added-value and competitiveness at large scale. In this chapter, growth of *Chlorella vulgaris* at laboratory scale and pilot scale using micro-filtered digestate as a nutrient source was demonstrated. 2.5% of digestate supplementation supported the growth of the microalgae for 28 continuous days in a 800 L photobioreactor, culture decline was then observed and was attributed to accumulation of ammonium in the culture which caused toxicity (Uggetti et al., 2014; Xia & Murphy, 2016). **While chapter 2 demonstrated the feasibility of using treated digestate for cultivation of microalgae**, there is scope to improve uptake of nutrients provided by digestate, especially ammonium which accumulates in cultures when not utilised, by tailoring strains and digestates sources, but also by manipulating certain abiotic factors important in microalgal cultivation to optimise culture conditions and growth. **Chapter 3 subsequently built on the findings of chapter 2, looking into improving the digestate uptake by microalgae by investigating biotic and abiotic factors likely to influence the bioremediation process.**

6.2. STRAIN AND DIGESTATE TAILORING UNDER pH CONTROL TO INCREASE DIGESTATE UPTAKE

Ammonium is the main source of nutrient from digestate used by microalgae, indeed ammonium is more readily available to microalgal metabolism as no reduction step is

needed as part of the nitrogen assimilation process (Mandal et al., 2018; Salbitani & Carfagna, 2021). In high concentrations, however, ammonium causes toxicity to microalgae (Jiang et al., 2021; Wang et al., 2021); in addition, digestate's naturally high pH (average pH of 8 to 9) causes ammonium evaporation, reducing its availability to microalgae (Chuka-ogwude et al., 2020). Consequently, fine tuning of ammonium present in digestate is essential for optimal uptake and subsequent growth. Tailoring of strains to digestate sources is also paramount, as digestates (and other waste streams) can have very different nutrient compositions (Häfner et al., 2022; Lamolinara et al., 2022), and not all microalgae have similar abilities to utilise these nutrients. **Chapter 3 aimed to improve uptake of digestate by microalgae, to increase their bioremediation potential further improve the scalability of valorising digestate within a circular economy**. To reach this aim, growth performances and metabolite compositions of the green microalgae *Chlorella vulgaris* and *Scenedesmus obliquus* were assessed for three digestates under pH control (pH=6.5**). The maintenance of a lower pH during the experiment increased ammonium availability from digestates** in general and a digestate resulting from the anaerobic digestion of pig manure generated better growth in both species studied, **demonstrating the importance of tailoring digestate sources to microalgal strain**. While ammonium was shown to be more available to microalgae under low pHs, higher concentrations of digestate should be tested in the future to fully optimise digestate uptake. During this work a fixed digestate concentration of 2% was used and additional optimisation should be considered to improve further remediation. This work also revealed that **acclimation of microalgae to novel nutrient sources was essential to obtain maximum growth, but nitrogen starvation was also demonstrated to provide advantages in terms of biomass composition**. Indeed, lipid composition of *Scenedesmus obliquus* was much improved when nutrients were depleted (6.94% of DW after nitrogen depletion, compared to ~2.5% DW when nutrients were available). Thus, harvesting time was deemed to be an impactful factor in the targeting of high-value compounds, and future research should focus on this dynamic, to provide additional value to the biomass produced from digestate, which is again highly relevant to allow scalability and sensible costing of the technology developed in this work. **After demonstrating growth of microalgae on digestate in chapter 2 and identifying relevant factors for increased bioremediation in chapter 3, my work focused on using the resulting biomass for applications relevant to the implementation of a circular economy by re-**

incorporating the initial waste source (digestate) into the supply chain. Chapter 4 consequently investigated the use of microalgal biomass resulting from digestate processing by using it as a feed ingredient.

6.3. MICROALGAL INGREDIENT FROM WASTE IN THE DIET OF NILE TILAPIA

An essential step to the implementation of a circular economy is the re-introduction of a waste or waste-derived product into the supply chain. My thesis has demonstrated so far that microalgae could be used to remediate digestate and that their resulting composition could be of interest for several applications. Using microalgae in animal feed is highly promising, thanks to their protein, lipid and other high-value compounds providing health and growth benefits (Madeira et al., 2017; Yaakob et al., 2014). Additionally, using microalgae grown on digestate contributes to reducing production cost and conveys an additional interest to use this resource as a feed ingredient. Consequently, **chapter 4 aimed to assess the feasibility of using a microalgal hydrolysate derived from digestate as a feed ingredient in aquaculture, consequently closing the loop on the developed circular economy by re-introducing waste-derived biomass into a supply chain.** In this chapter, focus was made on hydrolysed biomass as a novel processing technique to alleviate palatability issues but also to potentially remove some of the concerns linked to waste re-introduction into animal products. To answer the objectives of chapter 4, hydrolysed biomass of *Scenedesmus obliquus* grown on digestate was included in the diet of Nile Tilapia at a level of 10%. The waste-derived microalgal feed performed less well in terms of growth, as nutrient availability was likely to be reduced by the manufacturing of the feed which was different to a standardised commercial feed, and changes in the gut microbiome of Nile Tilapia were not conclusive in terms of benefits or detrimental impact conveyed by the microalgal feed. Fatty acid composition of fish fillet, however, demonstrated a significant advantage of the microalgal ingredient with a total fatty acids content of 4.43% DW (against 3.4% DW in a control diet) and PUFAs reaching 1.95% DW (against 1.32% DW). Additionally, ω-3 to ω-6 ratio was also improved in regard to human-related diseases. The utilisation of digestate as nutrient source for the microalgal ingredient did not appear to have detrimental impact on the health of the fish, but the current regulation remains a bottleneck for the full usage of a waste derived ingredient in animal feed, consequently other waste streams, such as carbon waste or other wastewater and digestate from plant

sources should be considered to support growth of microalgae to alleviate this regulatory risk (Hopwood, 2020). **Overall, chapter 4 demonstrated the implementation of a circular economy concept by re-introducing a waste-based product into the supply chain, here under the form of an aquafeed.** While chapters 2,3 and 4 showed the feasibility of developing a circular economy based on the remediation of digestate, my work also highlighted some of the bottlenecks to the implementation of this technology, the main one being the uptake of digestate by microalgae for commercial applications, demonstrating the need for further research and innovative thinking. **Consequently, chapter 5 focused on a different approach, by attempting to assess how other microorganisms, present within microalgal cultures could contribute to digestate remediation.**

6.4. BACTERIAL COMMUNITY ASSOCIATED TO CHLORELLA VULGARIS – PROSPECTS FOR DIGESTATE REMDIATION

This thesis has demonstrated so far that digestate was a suitable source of nutrients to sustain microalgal growth, providing optimisation and tailoring to increase uptake, and that the resulting biomass was of relevant interest as an ingredient in animal feed, with a regulatory caveat. As discussed above, increasing digestate uptake by microalgae is essential to increase viability of the circular economy concept presented in this thesis. **Consequently, chapter 5 investigated the potential of microalgae-bacteria consortium for this purpose; to do so focus was made on the bacterial community associated to *Chlorella vulgaris* and the influence of nutrient availability on this dynamic association**. A strong correlation was found between the two microbial populations when nutrients were available, and while the range of interactions and growth-promoting effects could not be fully assessed, oligotrophy appeared to result in cooperation between algae and bacteria, even for a limited period of time, which was in accordance with findings from the literature (Liu et al., 2012; Tait et al., 2019). Additionally, lipids, and more specifically PUFAs seemed to benefit from the bacterial interaction with *Chlorella vulgaris*, bringing an additional argument for added value of biomass while exploiting such consortia (Cho et al., 2015; Ramanan et al., 2016). It was posited that mechanisms favouring the attachment of specific bacteria to the cells of *Chlorella vulgaris* were at play within this complex interaction (Mühlenbruch et al., 2018; Wirth et al., 2020), however future work is needed to fully comprehend the role of compound exchange, but also signalling processes and quorum sensing in the dynamic of

the algae-bacteria relationship**. The literature and part of the work presented in chapter 5 demonstrated the likelihood of growth-promoting and other advantages conveyed by bacteria growing concomitantly with microalgae and it can be argued that this relationship can be exploited in remediation systems** as recently investigated in the literature (Sforza et al., 2018). *Azospirillum brasilense*, for example, which is a bacterial strain studied for its potential to be associated to microalgae for waste remediation has been shown to improve nutrient removal by microalgae due to its production of the growth hormone indole acetic acid (Amavizca et al., 2017; De-Bashan et al., 2008; Perez-Garcia et al., 2010). **Future research should be pursued to test algae and bacteria combinations to remediate digestate, as bacteria have potential to improve digestate uptake by microalgae. Additionally, as described in previous chapters, tailoring of strain and digestate would be essential to optimise the overall process and maximise biomass yields.**

6.5. CONCLUSIONS AND RESEARCH PROSPECTS

In this thesis, the circular economy concept was demonstrated. **I showed that digestate could be used as a nutrient source to support microalgal growth up to pilot scale after novel mechanical processing** (chapter 2) and that **tailoring of digestate and strains, alongside manipulation of abiotic factors were paramount to improve uptake of digestate by microalgae** (chapter 3). Additionally, **I demonstrated the feasibility of using a waste-derived microalgal ingredient in an aquafeed, under the form of a hydrolysate which was highly novel** (chapter 4), with some improvement of flesh quality, hence **creating a circular economy from an underused waste**. My findings also **highlighted scope for optimising the level of digestate uptake by microalgae**, and additional research in the field of algae and bacteria consortium was performed (chapter 5); **and I demonstrated the potential of bacterial communities to increase microalgal growth and subsequent digestate remediation properties.** To my knowledge, no previous studies have performed the work carried out in my thesis, *i.e.*, investigated the different steps required to the implementation of a circular economy as a whole, and previously published work has rather looked into the separate aspects of digestate remediation using microalgae. Additionally, no previous studies have investigated the use of a microalgal hydrolysate derived from digestate in an aquafeed, which was a highly novel aspect of my work.

The circular economy has seen growing interest in the past decade, with a significant will from stakeholders, governments, and regulatory bodies to limit the input of waste generated by diverse industries, to align with the net zero objectives of the UK government, but also alleviate pressures on local and wider environments within a context of accelerating climate change and global warming (Ahmad et al., 2022; Giwa et al., 2022; Olabi et al., 2023; Stiles et al., 2018). Digestate was the focus of this thesis, but microalgae have potential to remediate many other waste streams. Current works are investigating the utilisation of carbon waste from brewery fermentation and brewery wastewaters to support the growth of *Spirulina platensis*, which is the main source of microalgal protein currently on the market (Papadopoulos et al., 2022; Pereira et al., 2022; Song et al., 2020). Wastewaters of many industries, such as dairy factories or distillery are also utilised as nutrient sources for microalgae cultivation (Doušková et al., 2010; Labbé et al., 2017). Consequently, there is tremendous scope and interest to develop the technology described as part of this thesis to optimise remediation of waste, however as this work demonstrated, there is also a need to perform extensive background research at the laboratory scale to increase the potential of microalgae to remediate waste. It is particularly essential as microalgal production requires high scales applications to be viable economically, even when using a waste to reduce production costs (Venkata Subhash et al., 2022; Wan Mahari et al., 2022). Production performances also need to be reliable and high yields are essential to justify investment costs, which is why there is a growing body of evidence for mixotrophic cultivation of microalgae, *i.e.,* the association of phototrophic growth during which photosynthesis takes place and the addition of an organic carbon source to increase production yields. This new cultivation technique has shown promising results with, for example, production yields of *Chlorella sorokiniana* were 10 times higher than the ones recorded in photoautotrophic growth when supplemented with glucose (Dragone, 2022). There are however concerns linked to the development of bacterial contamination within such growing systems as the source of organic carbon also constitute the perfect substrate for bacterial growth. This however, also presents opportunity, indeed as discussed in this thesis algae-bacteria consortium can provide advantages in terms of waste remediation and the engineering of such populations appears as a new line of research of interest in the field, even if recommendations should be made in terms of regulation especially when considering food and feed production systems (Mubashar et al., 2021; Patel et al., 2020). Additionally,

mixotrophic systems could also take advantage of waste carbon sources, such as spent grain from brewing processes which do not present regulatory issues for their use in feed as they are already part of feed formulations for agricultural purposes. Another line of research that could be explored is the use of microalgal consortium, *i.e.,* a combination of several microalgal strains. Indeed, microalgae-bacteria consortium have been discussed in this thesis, but microalgal consortium also present advantages to commercial remediation of waste, as cooperative interactions between strains can lead to higher nutrient uptake from waste and consortium cultivation systems are more robust to environmental fluctuations (Gonçalves et al., 2017). Consortium cultivation is already applied in open cultivation systems (*e.g*., raceways) for waste remediation purposes, however the produced biomass is not necessarily used for further applications (Zhu et al., 2019). Consequently, there is a scope to apply this cultivation technique to closed systems (*e.g*., PBR), in which higher control during cultivation allows for better production of high-value compounds which could then be used in animal feed, as implemented in this thesis, or in other applications. Further research in this field could allow for improved scalability of microalgae cultivation, especially in the UK, where natural light and temperature conditions are major bottlenecks for the industry. Finally, hydrolysis of microalgal biomass was introduced in this thesis, and additional research on this biomass processing technique would be highly beneficial to study how different enzymatic cocktail would help targeting different functional compounds found in microalgae for diverse applications. Consequently, there is still a significant gap in the field of waste remediation by microalgae, mainly linked to the scalability and commercialisation of the process, as well as regulatory bottlenecks. The different lines of research discussed above could contribute separately, or as a combination, to bridge this gap and allow for the sustainable development of a microalgal industry in the UK and globally. The use of microalgae to valorise waste within the circular economy presents tremendous potential for the implementation of new research, with a clear pathway to minimise generation of various waste products by our current industrial practices as exposed in this thesis.

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