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Investigating the bioavailability of dissolved organic nitrogen using freshwater algae

Investigating the bioavailability of dissolved organic nitrogen using freshwater algae

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Abstract

Nitrogen (N) inputs to freshwaters are increasing, largely due to increasing human impacts in the environment. The role of Dissolved Organic N (DON) as a nutrient resource in freshwaters is poorly understood, even though it is an important and, in oligotrophic waters, dominant N source. Its role as a bioavailable nutrient resource is examined here. Six algal isolates were obtained from two streams: a eutrophic farmland stream, and an oligotrophic peatland stream. Each site was well-characterised with detailed data available on stream N, P and C composition. The isolates were cultured in the laboratory, identified using DNA barcoding (via ITS2), including largely cosmopolitan species with differing associated microbiomes (via 16S), and tested experimentally to determine their ability to utilise different DON chemistries (urea, glutamate) alongside inorganic N (nitrate, ammonium) compounds. Species-specific differences were observed in the rates of N uptake, even between closely related species. Compound-specific differences in uptake rates were also observed for individual isolates. In most sites and species, there was preference for nitrate uptake over ammonium. Some species utilised DON compounds (urea or glutamate) in preference to inorganic N compounds. Algal species ability to use DON as a nutrient resource was not obviously linked to stream character. Finally, dual-labelled ^{13}C, ^{15}N stable isotopes of urea and glutamate were used to trace uptake into algal biomass using a recently isolated *Chlorella vulgaris* strain. This showed bulk uptake of N in both treatments, with C uptake in the glutamate treatment, suggesting different metabolic pathways for differing DON chemistries. This research demonstrates that algae will respond and grow on DON compounds, in some circumstances at faster rates than on inorganic N compounds, and that there is both species-specific preference and compound-specific difference in uptake rates.

Acknowledgments

A thesis is not a piece of work in isolation, even though it may feel isolating at times. I have worked alongside a range of people, learned new skills and been able to share my skills along the way. There have been many inputs into my PhD experience, direct and indirect, that have helped in the creation of this thesis.

Thank you to my supervisors; Penny Johnes, Patricia Sanchez-Barcaldo, Richard Evershed and Stephen Maberly. Without your input and guidance this PhD would not be here. I appreciate the collective time and effort that have improved my PhD and thesis, from the rough initial plans to completion. This has been helped by additional direction and input from Fanny Monteiro, Martyn Tranter and others. I very much appreciate having had the opportunity to complete a PhD, which has been funded by a NERC GW4+ DTP studentship (NE/L002431/1). Without this support I would not have been able to complete a PhD, thanks to Sara Tonge and Emeliana Palk for running the studentship and supporting their students.

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Covid impact statement

The final experimental stage of this thesis, covering the entirety of Chapter 6 (isotope enrichment experiment), was planned to take place March-June 2020. Due to the COVID-19 lockdown in effect in the UK from 26 March 2020, this could not go ahead. Special permission was obtained to occasionally enter University buildings when necessary to perform culture maintenance, ensuring cultures remained viable. Experimental work was not permitted during this time and all other access to facilities was removed.

An extension to the PhD due to COVID-19 disruption was granted, and when the laboratories and culture rooms were re-opened by the university it then proceeded. Permission to re-enter university buildings was requested and approved. Cultures were grown to the required biomass. The experimental set-up was prepared and then the experiment was performed, with various samples collected and prepared for analysis. All work had to comply with local COVID-19 requirements in the laboratories and buildings of the university. These included reduced numbers of people working in an area at any one time, additional PPE requirements and time-consuming cleaning protocols. Time needed to be spent organising access to rooms and analytical machines to comply with social distancing requirements. In addition, a number of the analytical instruments needed to complete the planned analyses were functioning incorrectly after the long period of lockdown, requiring additional time for instruments to be serviced, and creating a further backlog in the queue of researchers needing to access them to complete their analyses. This meant that all experimental and analytical work took longer than it was planned pre-pandemic, and cuts had to be made to the planned work to complete the thesis within even the extended deadline.

While all bulk isotope analyses for Chapter 6 was completed, fewer analyses were undertaken than originally planned to enable quicker processing of samples. Samples for compound-specific stable isotope analysis were collected during the experiment, and prepared for analysis, but it was not possible to complete these alongside the bulk isotope analyses as originally planned. As these samples were very low biovolume and analysed using a recent method developed at Bristol, testing was needed to ensure samples would be above the limit of detection and behave as expected. This took time, and it was found that samples needed additional handling as they were around the limit of detection for this method. The method is time intensive, and this point was less than 6 months before the end of the PhD. To allow enough time to complete writing, the prepared samples were therefore placed in storage and the analyses were not performed. These samples will be processed in a post-doc position after PhD submission.

The impact of these missing compound-specific stable isotope analyses reduced the type of mechanistic interpretations of DON uptake possible, reducing the scope of result interpretation based upon experimental data, and is described in more detail in Chapter 6.

Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: .....C. E. Bayliss ..... DATE:.....16/06/2021.....

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Abbreviations

BNF	Biological nitrogen fixation
C	Carbon
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
DON	Dissolved organic nitrogen
DOP	Dissolved organic phosphorus
HMW	High molecular weight
ITS	Internal transcribed spacer
LMW	Low molecular weight
N	Nitrogen
Nr	Reactive nitrogen
P	Phosphorus
RNA	Ribonucleic acid
SIP	Stable isotope probing
TDN	Total dissolved nitrogen
TDP	Total dissolved phosphorus
TN	Total nitrogen
TP	Total phosphorus
WFD	Water Framework Directive

Chapter 1 – Dissolved organic nitrogen in freshwaters – current understanding and research challenges

1.1. Introduction

Nitrogen is an essential element for life and growth. It forms part of the nucleobases essential for RNA and DNA (Watson and Crick, 1953) and the amino acids and proteins they create, as well as many other essential components of living cells. However, an excess of nitrogen in the environment compared to normal ecosystem levels can lead to extreme changes to an ecosystem. An overabundance can cause eutrophication (Smith, Tilman and Nekola, 1999; Smith and Schindler, 2009; Dodds *et al.*, 2009; Pacheco, Roland and Downing, 2014), harmful algal blooms (Paerl *et al.*, 2001; Olson *et al.*, 2020; McHau *et al.*, 2019; Grattan, Holobaugh and Morris, 2016) and drinking water issues (Jardine, Gibson and Hrudey, 1999; Ward *et al.*, 2018), as well as altering biodiversity (Cook *et al.*, 2018; Lin *et al.*, 2014), ecosystem function and resilience (Rabalais, 2002). These tend to negatively impact local human activities or economy, though are not always negative for other species present in the ecosystem.

Aquatic nitrogen is present in a variety of forms, including dissolved organic nitrogen (DON), nitrate, nitrite, ammonium and some dissolved N_2 (James *et al.*, 2011; Loken *et al.*, 2016), along with particulate bound nitrogen. The effect of increased DON in oceans has been studied for its role in global carbon and nitrogen cycles, however the primary producer interactions, bioavailability and subsequent impact of increasing DON on related freshwater systems are not as well understood at present, lacking the same depth and breadth of knowledge (Schindler and Hecky, 2009). This section covers the current research background to understanding freshwater DON processing, assessing the currently available evidence on the role of nitrogen within DON as a nutrient resource to photosynthetic algae in freshwaters, identifying some of the knowledge gaps that need to be addressed.

1.2. Global nitrogen cycling and nutrient limitation

1.2.1. *Global nitrogen cycle*

Though nitrogen is required for all biological growth and comprises 78% of Earth's atmosphere, nitrogen gas is relatively unreactive (Lide, 2005). This is partly due to the strong, triple

covalent bonds between the molecules (NCBI, 2020). Significant energy is needed to break these bonds before the individual nitrogen atoms can form new compounds. Unreactive nitrogen gas is turned into reactive nitrogen forms (N_r) that are more accessible to biological activity through two natural mechanisms – microbial fixation (Kuypers, Marchant and Kartal, 2018; do Vale Barreto Figueiredo *et al.*, 2013) and lightning (Noxon, 1976; Hill, Rinker and Wilson, 1980; Shepon and Gildor, 2008). Microbial fixation is also known as biological nitrogen fixation, as an alternate method of anthropogenic chemical fixation is used by humanity to fix nitrogen on a large, industrial scale via the Haber-Bosch process. Lightning fixation of nitrogen gas also occurs, but at a much lower rate (~4 Tg N y^{-1}) than natural biological nitrogen fixation (~218 Tg N y^{-1}) or anthropogenic fixation through Haber-Bosch (~124 Tg N y^{-1}), and so is not discussed further here (Ciais *et al.*, 2013; IPCC, 2013).

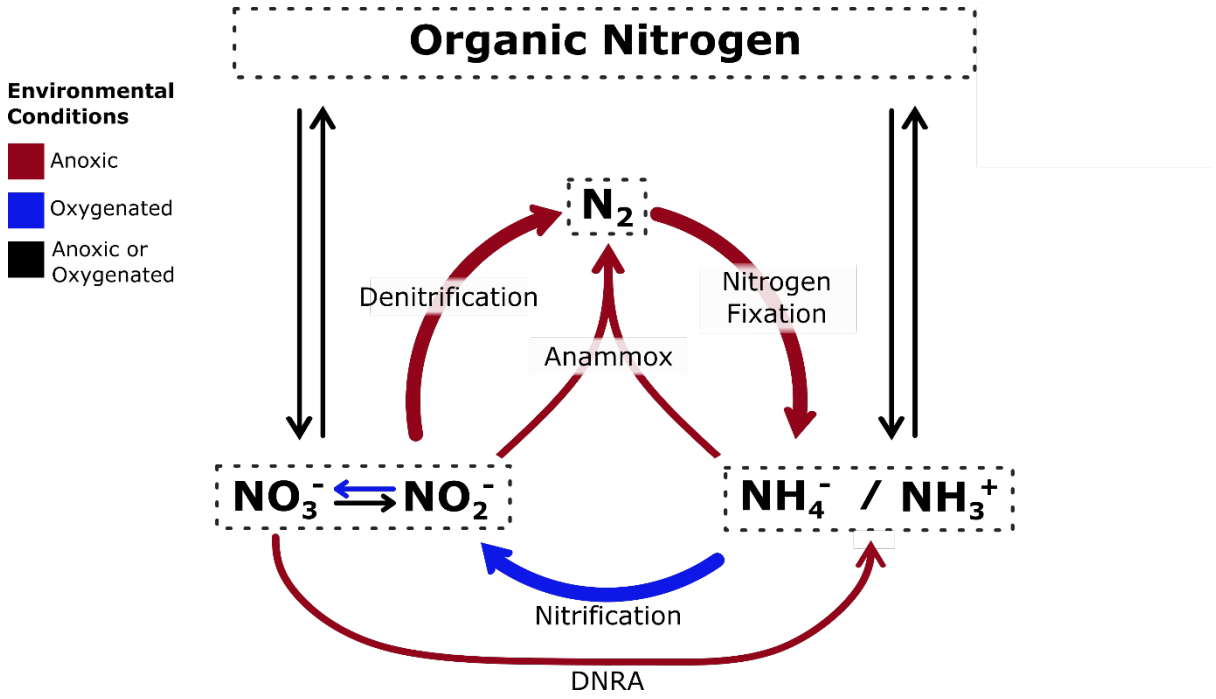


Figure 1.1. Simplified diagram of nitrogen cycling alongside organic matter conversions to and from the inorganic section of the cycle, including anaerobic ammonium oxidation (AnAmmOx) and Dissimilatory nitrogen reduction to ammonium (DNRA). See Appendix 1 for chemical reactions.

A simplified diagram of the global nitrogen cycle is shown in Figure 1.1, with the chemical reactions presented in Appendix 1. Some bacterial species can 'fix' atmospheric nitrogen (N_2) into

ammonia (NH_3), notably cyanobacteria and nitrogen fixing rhizome bacteria (do Vale Barreto Figueiredo *et al.*, 2013). This reactive nitrogen can then be used to create organic compounds in that cell, which can then become available to the wider food-web. Ammonia or ammonium is oxygenated to nitrate and nitrite by a wider range of organisms (Butterbach-Bahl *et al.*, 2011; Sutton *et al.*, 2011). The nitrite/nitrate can be denitrified under anaerobic conditions by bacteria to produce atmospheric nitrogen gas (Bouwman *et al.*, 2013). Anaerobic ammonium oxidation (anammox) is a relatively recently discovered process that returns nitrogen to the atmosphere (Strous *et al.*, 1999; Arrigo, 2005; Kuenen, 2008). Research is still underway into known anammox species (Schmidt, 2009) and there are believed to be many more uncharacterised species capable of anammox (Jetten *et al.*, 2009). Dissimilatory nitrogen reduction to ammonium (DNRA) converts nitrate to ammonium as an anaerobic method of respiration (Tiedje, 1988; Rütting *et al.*, 2011; Scott *et al.*, 2008). The anaerobic requirements of both anammox and DNRA processes mean they tend to occur in specific environments, including aquatic sediments and boundaries of aquatic environments (Seitzinger *et al.*, 2006).

Locations where reactive nitrogen is present are also where this nitrogen can flow further into organic nitrogen pools, via local biological activity. This occurs during the creation of cellular compounds and growth, organism death and the transfer of nutrients through the food web. Nitrogen atoms trickle through the cycle into various compounds, inorganic and organic, and various organisms before eventually returning to atmospheric nitrogen or leaving the ecosystem (Newbold *et al.*, 1981; Prior and Johnes, 2002; Schade *et al.*, 2011).

As in other global processes, humans have had a massive impact on the nitrogen cycle. Before human intervention in the nitrogen cycle, natural biological nitrogen fixation and denitrification/anammox were generally balanced globally, maintaining a stable amount of reactive nitrogen in the environment globally (Butterbach-Bahl *et al.*, 2011). Though nitrogen-rich material (e.g. guano, saltpetre) have been used as a human nitrogen source for centuries, the major change to the cycle has come from the innovation of the Haber-Bosch process, patented in 1909 (Erisman *et al.*, 2008; Galloway *et al.*, 2003). Another anthropogenic change with increasing impact on the nitrogen cycle is from atmospheric deposition of nitrogen via acid rain and similar processes (Bergström and Jansson, 2006).

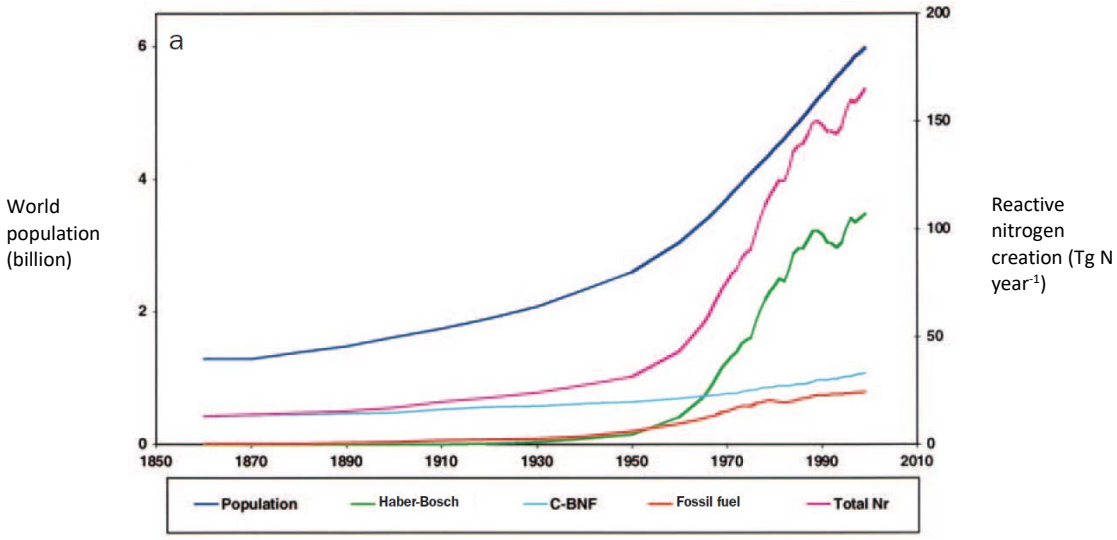


Figure 1.2. Reactive nitrogen production (right axis) and world population (left axis) from 1850 - 2000 showing contribution of Haber-Bosch, fossil fuel and biological nitrogen fixation (BNF) to global yearly reactive nitrogen production (total N_r). After Galloway et al. (2003).

The Haber-Bosch process to fix nitrogen allowed the large-scale production of fertiliser and explosives in the early 20th Century. This has supported the growth in world population through increased food production while also being directly linked to 100-150 million deaths during conflict in the twentieth century (Erisman *et al.*, 2008). The process has continually added extra reactive nitrogen (N_r) into global ecosystems (Figure 1.2), concurrently increasing the DON pool size in rivers, lakes and oceans (Osburn *et al.*, 2016; Dittman *et al.*, 2007).This has largely been through the run-off and leaching of inorganic fertiliser application and organic compounds created by biota, including agricultural waste products (Durand *et al.*, 2011; Osburn *et al.*, 2016).

Each atom of nitrogen can be part of multiple compounds, in various locations, before it eventually returns to the atmosphere. This can be over short periods of time (hours to days) to long term, geological time scales. This 'cascade' of nitrogen from the atmosphere, to soil and into streams, rivers and lakes, then though estuaries and wetlands to the ocean is a known biogeochemical process (Galloway *et al.*, 2003; Fowler *et al.*, 2013). However, the details of many parts of this pathway are not well understood. Some work has been done on the inorganic soil and freshwater pools and cascades between them (Osburn *et al.*, 2016; Smith, Tilman and Nekola, 1999; Thomas *et al.*, 2016). Less is known about the organic nitrogen side and how that is processed through the freshwater environment, though recent work has sought to address this (Yates *et al.*, 2019; Durand *et al.*, 2011; Mineau *et al.*, 2013; Seitzinger and Sanders, 1997; Webster *et al.*, 2009).

1.2.2. *Impact of excess nitrogen in freshwater environments*

An overabundance of N_r in the environment alters an ecosystem from the typical state. Species with faster uptake and growth are the first to make use of this extra resource, exporting altered forms of N_r that other organisms can utilise. Changes to nitrogen inputs over time can alter the species present that utilise locally found nitrogen compounds, with consequences for species composition, ecological function and ecosystem services (Dunck, Felisberto and de Souza Nogueira, 2019; Lamers, Tomassen and Roelofs, 1998; Vilmi *et al.*, 2015). The excess of reactive nitrogen can have a negative impact; high levels of nitrate are hazardous to human health (e.g. blue baby syndrome) as well as ecosystem health and stability (Wolfe and Patz, 2002; Duncan, Kleinman and Sharpley, 2012). Eutrophication is an extreme example of a negative impact – it can cause algal blooms that attenuate light, produce organic matter that can be degraded by bacteria causing a decrease in oxygen concentrations that can cause animal deaths (fish, invertebrates etc.) as well as local loss of species after recovery (Dodds *et al.*, 2009; Dorgham, 2014; Duncan, Kleinman and Sharpley, 2012). Excessive N_r also contributes to nitrogenous greenhouse gases and global climate change (IPCC, 2013), which are increasingly being recognised in freshwater ecosystems (Lauerwald *et al.*, 2019).

Increased reactive nitrogen can also cause other issues; changes to biodiversity, altered food web dynamics and species dominance (Suddick *et al.*, 2013; Porter *et al.*, 2013), pH changes (Gao *et al.*, 2012), and harmful or problematic changes to drinking water quality if the location is used for drinking water abstraction (Wolfe and Patz, 2002). Secondary effects on the ecosystem function can also occur – for example, changes in plant cover can destabilise river margins and riverbed, increasing bank vulnerability to erosion and sediment loss (Xu *et al.*, 2009; Jansson *et al.*, 2000; Panagos *et al.*, 2015).

Rivers and lakes respond differently to increasing N_r. Rivers tend to have higher flow, larger edge margins to volume ratios and a higher potential interaction of the water with the sediment and hyporheic regions (Allen *et al.*, 2010; Caruso, Ridolfi and Boano, 2016). Lakes, by virtue of having a longer retention time of water and nutrients, allow more interactions between the water chemistry and the local biota. Deep lakes also tend to be stratified, with more heterogeneous environmental conditions than rivers and differing concentrations of nitrogen and other nutrients with more *in situ* DON cycling (Collos, 1992; Jones *et al.*, 2005; Carstens *et al.*, 2013). There has been more DON research on lake systems, as mentioned in section 1.3. However, the negative impacts of

excess N_r are similar on both systems. This increased retention time in lakes compared to rivers means that local phyto-organisms have greater access to the nutrients and increased possibility of remediating them from the open water, but also when excessive nutrients are present it increases the stress and biological competition from high uptake, high growth rate organisms (Elser *et al.* 2007). A variety of factors affect the response of lake systems to nutrient changes, including turbidity, internal and external sources of nutrients and catchment morphology and geology (Durand *et al.* 2011), some of which also apply to rivers. Lake ecology and dynamics have been more studied within the literature than riverine ecosystems, especially mid- and head-water locations.

Rivers have a different relationship with N_r than lakes; the flow is much higher, usually linked in some form to local rainfall and soil conditions, mitigated by the local geology and affected by interactions with the riverbed and human impact on water flow (Wagenschein and Rode, 2008; Borsuk, Stow and Reckhow, 2004). UK rivers are smaller than many European, American or other rivers worldwide (e.g. Rhine, Danube, Mississippi), generally having a low flow with few UK rivers having a natural flow regime. Flow affects nutrient retention, as well as the types and diversity of the biota present. Many lakes are hydrologically fed by river input (Håkanson, 2006; Chen and Olden, 2018; Vinnå *et al.*, 2018), with rivers impacting lake systems and the eventual nutrient output to coastal regions. Understanding of how N_r is processed in rivers, especially organic nitrogen, contributes to a more holistic view of freshwater interactions with nitrogen and the impact of excess nitrogen.

In river systems, the type of algal blooms that are seen due to increased nutrients and N_r in lakes occur less often due to the higher flow of water and short retention time – though this doesn't prevent eutrophication of river systems as noted in recent UK news (Colley, 2020). Riverine algae tend to form biofilms on available surfaces and filamentous algae may form long streamers, as well as planktonic autotrophs moving with the water flow. These interact with the nutrients flowing past, both removing and contributing to the N_r and DON pools, in turn affecting water quality downstream.

The continuous flow of water through a river system may initially seem as if there would be low interaction between the biota and N_r. However, though a short river reach may not have significant impact on N_r pools or nutrient retention, the cumulative effect can mean that a majority of nutrients are retained by the river system (de Klein 2008). De Klein (2008) noted that this has implications for decreasing nutrient export to marine environments by increasing river retention times, e.g. floodplain creation or re-creation. Such changes to increase river retention times would also reduce nutrients moved further downstream to lakes or water abstraction points. More needs

to be known about the interactions in river systems between DON part of the N_r pool and the autotrophic base of the food web. This information would help to inform decisions on reducing nutrient loads to sensitive or important areas, as well as future mitigation projects to counter legacy flow reduction, increased nutrient input and land use changes affecting rivers (Garrick *et al.*, 2009; Heathwaite, 2010; Nicholson *et al.*, 2009; Stevens and Quinton, 2009; Sutton *et al.*, 2011; Turner, Morse-Jones and Fisher, 2010).

The biological properties of a water system will impact the nutrient cycling, retention and export taking place, as noted by Aguilera *et al.* (2013). Abiotic processes are often investigated or monitored to indirectly measure biological potential, as they are usually easier and quicker to measure, such as pH, oxygen content, etc. For a location to be 'good' ecologically, this tends to involve a relatively natural assemblage of species, natural ecosystem functions and the visual and chemical impact of these effects clear (e.g. clear gravel bed streams, functioning wetlands removing sediment, active native zoological and botanical species).

Increased biological diversity amongst algal species can lead to increased nitrate uptake, as shown by Cardinale (2011) who found algal biomass increased and water quality improved in flumes that were heterogeneous in habitat compared to homogenous trials. This pattern is likely to apply to DON uptake as well as inorganics. Cardinale (2011) noted that increased niche partitioning may occur with different species targeting certain nutrient forms, and when the heterogeneous habitat was removed, species assemblages collapsed to a single dominant species. Similar effects are seen in areas where biodiversity is reduced due to anthropogenic changes, including eutrophicated systems (Sayer *et al.*, 2010; Kononen, 2001; Vadeboncoeur *et al.*, 2003). Such areas of low biodiversity are often associated with poor water quality. The increased nutrients that lead to eutrophication, species changes and ecosystem changes are likely compounded by the loss of biodiversity once eutrophication or similar large scale events have occurred. This has large impacts especially in freshwater of industrialised countries, with heavily altered water systems and degraded ecosystems, as the degradation and reduced biodiversity means remaining dominant species will have a lower nutrient uptake than a more diverse ecosystem. Reduction of nutrients, reversing current trends, may be inadequate to dislodge dominant species, preventing other algal species filling more varied ecological niches. The extent to which dominant species use DON would be crucial knowledge in restoration work, understanding of how it impacts different types of river sections.

The status of rivers is under increasing UK media focus, largely due to a number of negative reports about the water quality of river systems (Laville, 2020b; News, 2020). Generally these

involve reports about poor chemical status based on inorganic and total measures of nitrogen and phosphorus (Laville, 2020a; TheRiversTrust, 2020), as well as biological indicators of river condition and other environmental issues like microplastics (Laville, 2021).

1.2.3. *Nutrient limitation and assumptions*

Many of the negative impacts on water quality are due to a generally sudden removal of a limiting factor on photosynthetic growth (Bechtold *et al.*, 2012; Domingues *et al.*, 2011; Elser *et al.*, 2007; Halterman and Toetz, 1984; Mineau *et al.*, 2013; Sharp, 1991). Limiting factors are varied. There may be a dearth of a particular element (e.g. nitrogen, phosphorus or trace metals), but growth may also be limited due to abiotic conditions (e.g. light or oxygen). Until recently, much of the focus of limiting nutrients causing eutrophication has been on phosphorus (Durand *et al.*, 2011; Read *et al.*, 2014; Schade *et al.*, 2011; Wang *et al.*, 2015; Yao *et al.*, 2011). The focus of nitrogen as a limiting element in freshwater systems is less prevalent, and even less so the impact of DON as part of nitrogen limitation.

It is assumed that the addition of a limiting nutrient in a natural system will result in eutrophication as it de-limits the productivity of the system, and in freshwater systems this is often assumed to be phosphorus (Ji and Sherrell, 2008; Johnes, 2007; Wang *et al.*, 2015; Yao *et al.*, 2011). A variety of studies have shown that N limitation and co-limitation with phosphorus occur in many European water bodies; though for diatoms silica may be a more limiting nutrient (Maberly *et al.*, 2002; James, Fisher and Moss, 2003; Elser *et al.*, 2007). Total phosphorus (TP) tended to predict chlorophyll *a* concentration of over 1000 largely North European lakes better than total nitrogen (TN) (Phillips *et al.*, 2008), even though both TN and TP tend to have common sources. Response differences were seen between shallow and deeper lakes depending on the alkalinity of the lake. This effect of alkalinity on the ability of a system to use nutrients (in this case total P and N) would affect transport downstream, and human impacts on alkalinity may alter bioavailability of nutrients and 'move' eutrophication events downstream.

Freshwater ecosystems are often more complex than lacking a single limiting factor preventing excessive growth of algae and negative impacts. Co-limitation occurs, including co-limitation of nitrogen and phosphorus (Bechtold *et al.*, 2012; Bergström and Jansson, 2006; James *et al.*, 2011; Maberly *et al.*, 2002; Thrane, Hessen and Andersen, 2017). Nitrogen limitation can have a strong impact on photosynthetic organisms, as proposed for plants by Kang *et al. (2015)* who

noted that nitrogen limitation may have had a strong impact on the genomic potential of photosynthetic plants in harsher environments. Legislation to improve freshwater resources has more recently attempted to be more holistic in approaches to which nutrients need control to reduce the negative impacts on freshwater ecosystems (Hering *et al.*, 2010).

Different sections of lakes may have photosynthetic based communities under a variety of limiting conditions, either at the same time or over the course of a year (Durand *et al.* 2011). The nutrient cycles in the different locations will all contribute to the overall nutrient cycling of that lake system and the eventual outflow of nutrient to the rest of the river system. Differences in nutrient limitation may also be temporal; summer N depletion in lakes due to previous algal blooms, plant growth and denitrification; autumn or winter influx of nutrients in temperate zones after storm and high flow conditions. Some river systems will also have meltwater nutrient influxes, or other local conditions affecting water flow (e.g. dams) and nutrient movement (Broadley *et al.*, 2020).

The reason why this thesis is investigating nitrogen is because phosphorus has received more focus as a limiting nutrient in freshwater ecosystems. Nitrogen and the DON pool is receiving more recent focus for its part in alterations to freshwater quality and biodiversity, and comprising potentially large sections of the total nitrogen pool (Willett *et al.*, 2004). There is an assumption that most DON is largely not bioavailable, but more research in this area is needed.

1.3. Nitrogen cycling in freshwaters

As N_r molecules progress downstream, denitrification reverts some molecules back to N_2, some is removed to less reactive nitrogen forms, and other reactive nitrogen compounds join the pool. The amount of N_r reaching river outlets to the continental shelves can be 63% to 6% of the nitrogen input on land, depending on the ability of the river and wetlands to denitrify, based on estimates of denitrification maxima and minima by Galloway *et al.* (2003). Human impacts of decreasing channel width and altering river residence time (e.g. through canalisation of rivers) impact on nitrogen residence time in freshwater (Abril *et al.*, 2015; Malcolm *et al.*, 2010). This affects the potential rates of denitrification and organic nitrogen conversion. The oxygenation of sediments is important as denitrification is an anaerobic process (Tiedje, 1988) so alterations to water oxygen content also impacts denitrification process, as well as pH and temperature (Castaldelli *et al.*, 2019).

The impact of nitrogen and cascading effects of inorganic nitrogen has gained recent attention partly due to the impact of greenhouse gas emissions, notably nitrous oxides (NO_x). NO

and NO_2 are produced partway through the denitrification process, as well as processes such as fossil fuel combustion producing NO_x compounds. The nitrogenous impact on climate change has more recently been appreciated, though estimates are not well constrained. The most recent IPCC report notes raising N_2O concentration at a rate of 0.73 ± 0.03 ppb yr^{-1} over the three decades prior to 2013 (Ciais *et al.*, 2013). The impact of nitrogen on other biogeochemical cycles could affect primary productivity in conjunction with elevated CO_2 concentrations (Dukes *et al.*, 2005; Finzi *et al.*, 2006; Luo *et al.*, 2004).

Anthropogenic changes in land use have also reduced denitrification rates in many ecosystems, notably wetlands, contributing to the increase in reactive nitrogen globally. A large portion of terrestrial denitrification takes place in freshwater systems (Seitzinger *et al.*, 2006). As such, changes to DON pools and knowing what proportion is available for use by local biota (bioavailable), is important to constrain nitrogen cycle understanding. As well as freshwater impacts, downstream impacts on coastal ecosystem health and marine nitrogen processing are also important.

Dissolved organic carbon (DOC) received more literature notice when the marine carbon pool became recognised as an important part of global carbon chemistry and cycling (Sharp 1991). The effect of and changes within the DON pool specifically has received much less research attention. Only the most recent IPCC report has taken account of this for the NO_x impacts on global warming, and notes the high uncertainty on both inorganic and organic processing of nitrogen (Ciais *et al.*, 2013). Reviews have highlighted the importance of freshwater nitrogen to marine ecosystems and the global nitrogen cycle (Voss *et al.*, 2011) as well as uncertainties on the impact organic nitrogen has on productivity (Voss *et al.* 2011). The amount and type of DON in freshwater systems is also uncertain, with a lack of monitoring compared to inorganic nitrogen (Durand *et al.*, 2011).

The number of species and the methods through which organic matter is used in the environment alter subsequent impact on ecosystems due to nitrogen increase. Biological nitrogen fixation (BNF) is limited, as only a few species can make use of N_2 to create bioavailable nitrogen. The transformations of organic nitrogen to inorganic and the reliance of plants on inorganic-only nitrogen have been challenged by recent experimental work (Jones *et al.*, 2005; Näsholm, Kielland and Ganeteg, 2009; Schmidt, Raven and Paungfoo-Lonhienne, 2013). If more species can make use of organic material more directly, this decreases the time nitrogen spends in pools of DON and increases the cycling rate in freshwater and terrestrial locations through different nitrogen pools.

As noted by Galloway *et al.* (2008), anthropogenically produced nitrogen is unlikely to be reduced due to global agricultural reliance on it for food production. However, Galloway *et al.* (2008)

also mentioned that the excess cascading into the environment and unequal distribution of fertiliser globally could be altered to limit the negative impacts and provide more widespread benefits to people more globally. Understanding the impacts of changes in DON would allow better targeting of locations at risk of serious negative impacts. Locations with an excess of nitrogen may not show in inorganic only measures until extreme impacts begin to be seen, depending on the processing rates and local use of liberated inorganic nitrogen. The processes by which DON can stimulate growth, with or without being converted into inorganic nitrogen, is also an essential part of understanding freshwater nitrogen conduits from land to ocean (Mackay *et al.*, 2020; Brailsford *et al.*, 2019). Mitigation practices to target the most effective locations for preventing ecosystem degradation and improving ecosystem health and function need this type of information.

1.4. Policy understanding and relevance of freshwater nitrogen

Current environmental targets are often a compromise between political social and technical factors, as well as the scientific recommendations available. Oenema *et al.* (2011) noted many of the targets set by countries for water quality monitoring are "based on their technical and political achievability or to set objectives for avoidance of adverse impacts" (*op. cit.*), rather than initial, marginal levels of damage to human health or to the functioning of an ecosystem that would bring greater benefits overall to society. Comparisons of EU-15 to newer EU-12 members by Oenema *et al.* (2011) showed that reductions were achieved due to the wider political and economic changes after 1989 rather than the various environmental policies in place. Some countries and groups have attempted to adjust monitoring policy to improve water quality, with varying success (Moss *et al.*, 2003; Moss, 2012; Hering *et al.*, 2010; Williams and Martin, 2009; Garrick *et al.*, 2009).

There is potential for antagonism between measures for reducing ammonium emissions and those for nitrate leaching, which may lead to a problem in pollution swapping (reduction in one reactive nitrogen species that increases another) (Christianson *et al.*, 2017; Stevens and Quinton, 2009). This would not lead to an overall reduction in N_r delivery to an ecosystem. The same is true for DON, especially as larger, less bioavailable molecules are likely to break down (via photo degradation, potential microbial cycling etc), resulting in larger pools of more reactive nitrogen downstream.

Environmental forms of wastewater treatment are being investigated for possible alternatives to current treatment facilities to meet policy water quality targets (Chinnasamy *et al.*,

2009; Kumar *et al.*, 2010; Su *et al.*, 2011; Xiao *et al.*, 2011). However this may again simply switch N_r forms in rivers and lakes. Unless more is known about the specifics of DON production and its bioavailability, the total N_r and the subsequent impacts will not lead to the improvements expected by policymakers. The bioavailability of DON strongly affects how these changes in N_r compound type would impact catchment water quality, and the mitigation policies needed to maintain good local and global ecosystem health. If a compound is noticeably a more bioavailable part of the N_r pool, it may be a quicker and more expedient compound to focus policy on minimising to improve water quality.

The European Water Framework Directive (2000/60/EC) (WFD), was originally envisioned to encourage cross-country participation in managing shared catchments. Moss (2008) noted that the WFD promoted ecology over monitoring of particular nutrient levels within the system as a measure of 'health' of a system, but this has been translated in various countries mainly towards adapting previous chemical monitoring rather than creating new WFD measures. Moss (2008) noted that many positives have come from the WFD implementation (e.g. Rhine management), but more could be done to improve EU freshwater resources and assemble a comprehensive ecologically based assessment of waterbodies under the WFD than is currently the case. Various countries have enacted dissimilar and non-comparable monitoring of freshwater for the WFD (Hering *et al.*, 2010), making outcome comparisons more difficult.

The use of DON by freshwater autotrophs is not included in most monitoring systems, which are generally based on inorganic N and P concentrations, or DOC concentrations for potential bacterial activity, such as the EU Nitrates Directive (Directive 91/676/EEC) regulating agricultural nitrate pollution impact on freshwater. Determination of DON and dissolved organic phosphorus (DOP) roles in the growth of freshwater autotrophs, plant and algal, would allow better restoration of waterways through a greater understanding of their impact to the ecosystem. The UK government body responsible for river water quality has also noted further measures are needed to improve water quality as;

"…current measures [to reduce nitrates] appear insufficient to deliver WFD objectives in the long term and a review of the policy options appears to be needed."

(EnvironmentAgency, 2019), pg 3

The monitoring of freshwater ecosystems with regards to the frequency of sampling has also been under discussion in recent years, mainly focussing on the inorganic compounds. Cassidy & Jordan (2011) found that only near-continuous monitoring of inorganic compounds reflects the rapid changes in concentrations that can occur during storm events or other high flow events. Other single point 'grab' samples, whether weekly or lower frequency, may miss these high nutrient input events. This is especially relevant to DON, as particulate matter may degrade quickly to DON and become a notable source of N_r in the lower reaches of a stream.

1.5. Dissolved Organic Nitrogen (DON) as a component of the total freshwater N pool

1.5.1. *DON Composition*

Organic nitrogen is a part of the global nitrogen cycle, but what is it specifically? The humus that makes up soil, plant and animal matter are examples of solid organic nitrogen. Dissolved forms include amino acids, tannins, humic and fluvial compounds amongst others. The focus here is on the dissolved fraction, as there is a gap in knowledge of how this behaves in freshwater systems and what impact it subsequently has on ecosystem health and function.

Dissolved organic nitrogen (DON) is practically defined as the filtrate of a water sample, usually 0.2um to 0.7 um pore size (Nimptsch *et al.*, 2014), that contains organic, nitrogenous compounds. It is important to note that DON is not the only dissolved organic fraction. All dissolved organic matter, by definition, contains carbon and so can be referred to as DOC (dissolved organic carbon). Some also contain nitrogen, or the other main building block of life, phosphorus amongst other elements in lower concentrations. Both nitrogen and phosphorus can be in present in one dissolved organic matter compound, meaning it could be referred to as DON, DOP or DOM. Figure 1.3 helps to show this overlap visually. If it contains N or P, it is DON or DOP respectively. If a compound contains both N and P, it is DON and DOP at the same time, along with more widely being classed as a DOC compound.

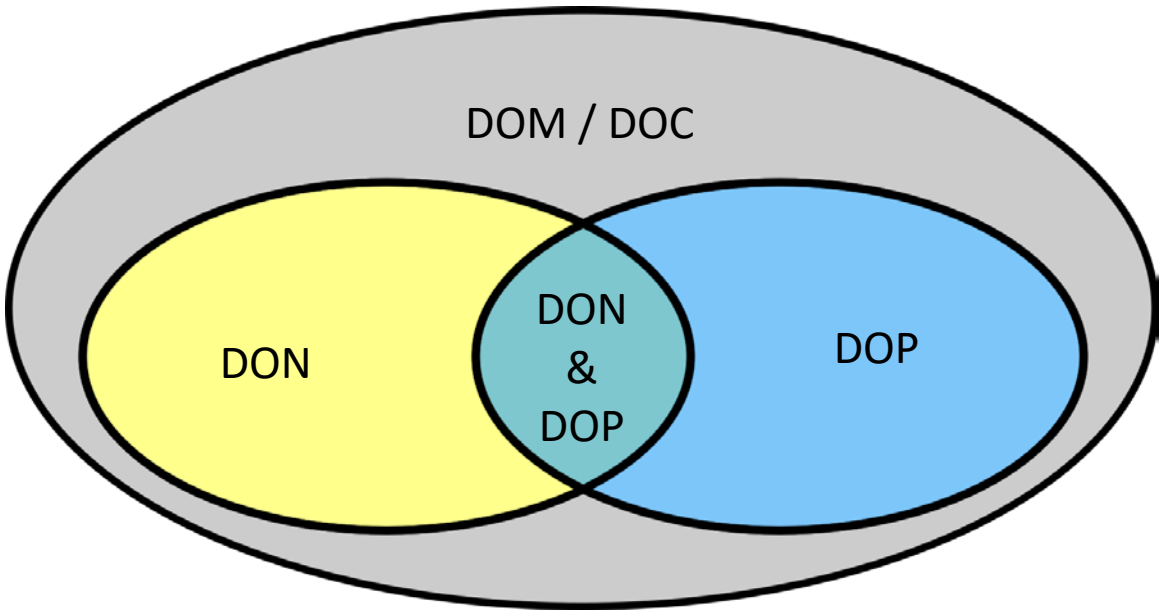


Figure 1.3. Dissolved organic compound pools and relation to each other.

The size of filter used in the literature to quantify DOM varies significantly (Norrman, 1993), for both natural samples as well as those taken in the laboratory. In general, the common filter sizes used does not affect subsequent DOM or DON analysis (Fellman, D'Amore and Hood, 2008) and a variety of pore sizes and types are used. Most literature considers filtered samples to be biologically inert. However Brailsford *et al.* (2017) found that ^{14}C and ^{33}P in amino acids and orthophosphate added to filtered samples showed depletion over time, suggesting small micro-organisms have a role in DOM cycling processes and that 'filtered samples' still contained micro-organisms. Though the authors added amino acids, they equated carbon removal with nitrogen use and though this is likely more work needs to be done. It shows that samples should not be considered 'sterile' or inert after filtering, though refrigeration and acidifying samples severely limits biological activity (*op. cit.*). This fact complicates attempts to compare DON measures where disparity between sampling time and analysis exist in literature.

Many papers refer to DOP or DON when only the larger DOC pool has been measured. This leads to a lack of clarity and understanding when investigating DON in literature. Some literature talks about DOM after only having measured dissolved organic carbon (DOC) and equivocated findings to DON and DOP pools in the same environment (Osburn *et al.*, 2001; Kamjunke *et al.*, 2008). The mechanics governing carbon transport and nitrogen transport are not necessarily the same, and may have different retention pools, uptake rates and processing locations (Fellman, Hood and Spencer, 2010; Ghosh and Leff, 2013; Medeiros *et al.*, 2015). DON is a carbon containing

compound but cannot necessarily be implied by DOC analysis alone, especially in environmental samples. Care is needed when reviewing literature, especially older literature that may conflate DOC with DON.

Given the definition of DOM and DON, various compounds can be classed as such. More precise identification varies depending on the analysis performed, as various chemical techniques are able to identify different sections of the DOM pool (Hockaday *et al.*, 2009), and tend to need targeting to the compounds of interest. Understandably, DOM analysis is often targeted to specific anthropogenic or natural compounds; pesticides, lignin, antibiotics, etc. However, many more compounds are present in our environment than the ones usually analysed. Work into a more complete analytical picture of DOM nature using untargeted approaches is underway (Pemberton *et al.*, 2019). New advances in analytical chemistry are allowing untargeted analysis to be more informative about changes in DOM, and subsequent DON pools (*op. cit.*). The large datasets produced require more recent computing techniques to draw meaningful information and conclusions out, so this is likely to increase in prevalence in future.

1.5.2. *Inorganic nitrogen vs organic nitrogen*

DON are organic compounds, as the name suggests. Other N_r compounds are inorganic, largely nitrate/nitrite and ammonium, commonly found in fertilisers. The distinction between inorganic and organic compounds and chemistry is based on the bonds and elements involved in the compound. There are cross over areas (Hoffmann, 1982) with research into the links between the organic and inorganic fields yielding new technological advances, such as solar panel technology (Berry *et al.*, 2015). Inorganic compounds tend to be smaller molecular weight compounds and generally lack carbon or C-H bonds. All organic compounds contain carbon, and most contain C-H bonds, tending to be larger molecular weight compounds than inorganics. There are exceptions to these trends though, for example diamond (pure carbon) is inorganic, and urea (DON) has no C-H bond.

There is a scale of riverine DON from small to large molecular weight compounds, though many are uncharacterised (Pemberton *et al.*, 2019). The elemental composition of DON tends to largely be carbon, hydrogen and oxygen, in varying proportions that can used to attribute broad chemical types (Figure 1.4). The smaller organic compounds chemically behave more like inorganic, and are more likely to interact with local biology.

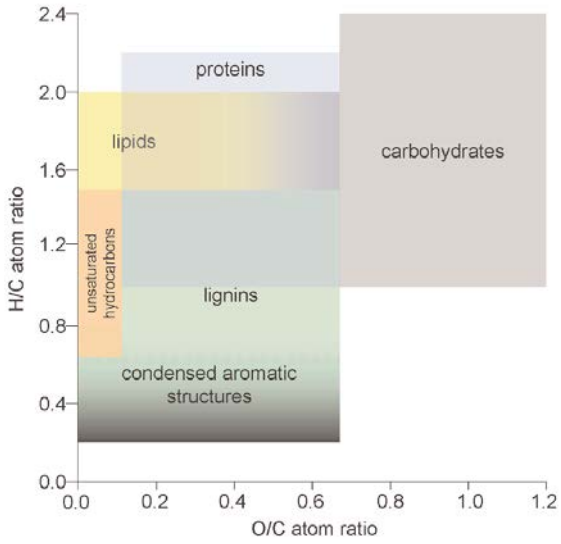


Figure 1.4. Model van Krevelen diagram of broad, natural DOM types separated by hydrogen/carbon (H/C) and oxygen/carbon (O/C) element ratios of various compounds. After Hockaday et al. (2009).

1.5.3. *Characteristics and classifications within DOM*

Within the DOM pool, different categories of DOM and DON exist that divide the large pools into more targeted, smaller groupings depending on compound function or trait. Often molecular weight is used of the compounds or pools under discussion, with two main distinctions. These are low molecular weight (LMW) and high molecular weight (HMW) compounds, though in reality this is a continuum from low to high and some papers use more classifications (Lee, Lee and Hur, 2019). The crossover point is not always mentioned in literature discussing LMW and HMW DOM. Some literature defines the separating point as 100 kDa, with above being HMW and below LMW (Ogura, 1977), and others do not define it using kDa (Aluwihare, Repeta and Chen, 2002).

Low molecular weight (LMW) compounds are relatively smaller, composed of fewer atoms per molecule and are generally more reactive. High molecular weight (HMW) compounds are much larger, generally less reactive and more biologically stable. HMW-DON compounds persist in the environment longer than LMW-DON, as smaller compounds tend to be more available to biological activity.

Separating DON groups based on aromaticity of compounds (number of aromatic rings within structure) is also present in literature (Zhou *et al.*, 2021; Amaral *et al.*, 2016). Low aromaticity is also considered to increase the bioavailabilty of DOM (Baken *et al.*, 2011). Bioavailability is also used as a functional way to separate the DON pool. There are also newer ways of characterising

DON, often making use of recent analytical techniques or deriving ratios for allocating categories to DON fractions using prevalent analyses (Lee, Lee and Hur, 2019). As DON comprises a wide range of compound sizes, further reactions vary, including the bioavailability of various compounds. This is investigated in section 1.4.4, as DON sources are discussed first.

The research presented in this thesis focuses on the smaller sized DON compounds, as they are more likely to be bioavailable than larger compounds, due to the reduced energetic cost in assimilating them.

1.5.4. *Bioavailability of DON*

The bioavailabilty/reactivity of freshwater DOM is different to soil measures. Assessments of the whole spectrum of freshwater DOM compounds has been attempted more recently (Mostovaya *et al.*, 2017), though the pool is very large (Pemberton *et al.*, 2019). The idea of a freshwater continuum of reactivity in DOM has been recently gaining literature support (Vähätalo, Aarnos and Mäntyniemi, 2010; Sierra, Harmon and Perakis, 2011; Koehler *et al.*, 2012), which would also apply to DON. It is largely based on size, with larger more complex compounds regarded as less bioavailable. This makes sense from a biological perspective, as organisms will take up and use compounds in order of ease, avoiding the most energetically expensive compounds where possible. There may be exceptions to this if the environment is limited in essential minerals or metals that may be bound in larger DOM compounds, altering the preferences of organisms, but not the bioavailability of the compounds.

A lake bioassay by Vähätalo *et al*. (2010) showed preferential uptake of DOM compounds, highlighting the variable bioavailability of DOM as a whole. As DON is part of this pool, it is likely similar variation in bioavailability will also be present.

1.5.5. *Storm and flush impacts on DON movements in the environment*

The origins of DON in the natural environment are still being understood (Raymond, Saiers and Sobczak, 2016; Thomas *et al.*, 2016) and DON movement through freshwater systems is not well understood. There are both point sources of N_r and DON, such as wastewater treatment plant outflow, as well as more diffuse sources outputting over a wide area, e.g. agricultural waste,

fertiliser application, peatland runoff (Newbold *et al.*, 2010; Ghosh and Leff, 2013; Osburn *et al.*, 2016; Mackay *et al.*, 2020).

Diffuse DON sources are harder to understand than point sources, where locations upstream and downstream of the point source can be compared. Diffuse sources of DON may contribute a large portion of total river nitrogen (Duncan, Kleinman and Sharpley, 2012; Yates and Johnes, 2013; Andersen *et al.*, 2014). Recent research has shown that large precipitation events, such as storms and heavy rain 'flush' much larger concentrations of DON into streams, following hysteresis patterns that are largely determined by river and catchment flow dynamics (Blaen *et al.*, 2016; Wagner *et al.*, 2019; Vaughan *et al.*, 2019). These flushing events are likely to also move DON tied up in the sediment and biomass of the river (sweeping away sediment, plant matter etc.) (Conte and Kucerik, 2016; Newbold *et al.*, 2010) alongside phosphorus (Bowes *et al.*, 2005). How long this input of nutrients persists within the stream versus exiting to the coastal region is likely to be variable, and highly catchment specific.

There is also likely a seasonal change to DON input (Willett *et al.*, 2004; Killberg-Thoreson *et al.*, 2021). Larger autochthonous inputs may occur in good weather during spring and summer. Seasonality is also present in the application of fertiliser, and maximum herd size during the year. Some of this DON will remain in stream, and the connection to output to ocean will not necessarily be immediate, nor well connected to DON inputs and movement within the freshwater ecosystem. Storm events may then mobilise 'pulses' of nitrogen through the system, of which some compounds may be disconnected in residence time within stream sections due to in-stream biological processing(Seitzinger and Sanders, 1997; Lillebo *et al.*, 2012; Loken *et al.*, 2016).

1.6. Exploring organic nitrogen uptake by algae using experimental approaches

DON is in many environments, and as mentioned earlier, work has largely focused on organic carbon in marine systems. This is the area most covered by research, and more recently the freshwater environment been under more focus. Both in marine and freshwater ecosystems, photosynthetic organisms are the basis of the food web, providing the energy to the rest of the ecosystem. This sustaining importance is why they are the focus of this work. The most basic photosynthetic organisms are microalgae – single celled phototrophs. Planktonic microalgae are present throughout the ocean and are widely spread through the tree of life coming from a variety of phylogenetic backgrounds. Within freshwater systems they either live in the water column of

rivers or lakes (planktonic species), or live in and on the substrate (benthic species) (Wehr, Sheath and Kociolek, 2015). Benthic communities often form biofilms, with a sticky sheath of exopolysaccharides that prevents them being washed away in river locations, as well as creating a closely knit community of many bacteria and microalgae. Some species live in both locations, others are specialised to planktonic or benthic living.

Uptake of urea (DON) by microalgae has been shown in marine species, axenic and non-axenic (Antia, Harrison and Oliveira, 1991). An axenic marine diatom was found to take up isotope labelled urea and excrete ammonium that was then re-absorbed (Price and Harrison, 1988). This hints at direct DON uptake, perhaps for the carbon, and decoupled subsequent use of nitrogen by the species. Measures of particulate organic nitrogen were used, which cannot determine between adsorbed N (outside cells) or internal nitrogen. Three decades later, the advances in isotope analytical sensitivity and measurement methods for internal nitrogen pools can add more detail to microalgae DON use (Reay *et al.*, 2019b; Reay *et al.*, 2019a). Urea is one of the smallest DON compounds possible, and it may well be less stable in solution. Urea dissociating into ammonium may be caused by photolysis or other abiotic processes, as well as biological action. The classic, colorimetric measurement for urea is likely to over-estimate urea concentrations, especially in natural samples (Reay *et al.*, 2019c).

1.6.1. *Lake DON use*

Freshwater DON research focus has largely been on lakes. Organic nitrogen use by phototrophs was a focus of freshwater research in the latter half of the twentieth century and has seen a recent resurgence. The availability of advanced analytical techniques, both in DON quantification and genetic analysis of species coupled with an improved appreciation for nitrogen's role in climate change and greenhouse gas impact have played a part in this.

Single species cultures of lake microalgae have shown uptake of some DON compounds. Microalgae from freshwater Lake Kinneret, also known as the Sea of Galilee, showed growth on DON and DOP (Berman *et al.*, 1991), the effect varied by species suggesting different ecological niches for species. A more recent study using chlorophyll a as a proxy of growth also found lake microalgae monocultures utilised urea, natural organic matter and amino acids (Fiedler *et al.*, 2015). The more recent study also found different growth rates in their positive controls in successive experiments that could not be explained by the authors, suggesting a highly variable experimental

set-up or changes in the biological status of the microalgae. Riverine species of microalgae are likely to show similar patterns of response. The use of multiple growth measures could help define this as there can be problems with using chlorophyll *a* as a measure of biomass, especially when changing the nutrients provided (Kruskopf and Flynn, 2006; Bellinger and Sigee, 2010; Alvarez-Fernandez and Riegman, 2014). These issues include how the chlorophyll *a* to biomass ratio changes due to; different nutrient stresses, between species, changing light regimes of experiments. These can lead to a lack of stability in the relationship between chlorophyll *a* and biomass over time or between species under investigation.

1.6.2. *River DON use*

Most research on riverine DON has used mixed microalgae communities within the river of interest (Mackay *et al.*, 2020; Killberg-Thoreson *et al.*, 2021; Seitzinger and Sanders, 1997). While important, it is difficult to extrapolate the findings to other rivers with different communities of algae and bacteria present. Research into the global carbon cycle as part of global climate change has seen some research targeting DOC cycling in freshwaters.

Both the availability of DON and the nature of the compounds will change as they move downstream due to biological and abiotic processing. Different DON types enter the catchment and water, and those in stream are subject to more processes the longer they are in the stream. These include photo-degradation, biological interactions and chelation and others (Nalven *et al.*, 2020; Klapstein and O'Driscoll, 2018; Baken *et al.*, 2011; Osburn *et al.*, 2001). This results in changes to the complexity of DON the further downstream from headwater locations. The concept of stream spiralling of nutrient molecules through a system will also apply to DON, with changes to its availability occurring along the way, depending on the chemical and biological conditions the water passes through (Prior and Johnes, 2002; Griffiths *et al.*, 2012; Hall *et al.*, 2016). This suggests that the least complicated system to untangle the effects of DON on microalgae communities would be the headwater sections of rivers.

Natural riverine algal assemblages can change depending on the nutrients presented, implying that different members of the community specialise in different resources (Carlsson, Graneli and Segatto, 1999; Domingues *et al.*, 2011). This suggests a form of niche partitioning amongst riverine microalgal communities. Changes in algal taxonomy, dominance and biomass have been seen in mixed species microalgae communities on providing natural, riverine DOM (Frost *et*

al., 2007). Frost *et al.* (2007) found increased DOM increased algal C:P and N:P ratios -either a decrease in phosphorus or an increase in carbon and nitrogen would account for this. Other research has found 'hot' and 'cold' spots of nitrate uptake in headwater streams, as measured by field-deployed UV spectral analysis (Hensley, Cohen and Korhnak, 2014). This heterogeneity of uptake may partially reflect availability as well as species variation over small scales. There is likely to be the same heterogeneity of use in DON compounds, again supporting the assumption that various riverine microalgae species are likely to differ in ability to use DON compounds.

The uptake rate of glycine (DON) has been shown to be not significantly different to ammonium within a US headwater stream (Johnson, Tank and Arango, 2009). This suggests that LMW-DON, such as glycine, may be utilised by the riverine community. What part microalgae play in this is not clear, as the whole stream was involved. Were bacteria the main users of glycine? Or microalgae? Or plants? Quantification of individual riverine species responses and mechanisms in response to LMW-DON would allow generalisations to be made about other river systems, as it could then be related to the species present rather than the river community in that specific river system.

Phagotrophy by microalgae over geological timescales is generally thought to have led to the current algal phyla (Raven *et al.*, 2009; Falkowski *et al.*, 2004; McKie-Krisberg and Sanders, 2014). Current phagotrophy is a source of nutrition for microalgae, in some species the main method, and is a mechanism for DON uptake (Avrahami and Frada, 2020). The envelopment of DON for carbon or nitrogen alongside autotrophic growth may be considered mixotrophy. Many algae require vitamin B12 to be present in growth media (Schmidt, Raven & Paungfoo-Lonhienne 2013). This is a large molecule that must be absorbed from the media, as some algal species are auxotrophic and unable to synthesise it themselves, while otherwise being autotrophic to producing sugars. DOP and DON can be used by terrestrial plants with some species using exo-enzymes or direct transport of organic molecules (Durand *et al.* 2011; Schmidt, Raven & Paungfoo-Lonhienne 2013). This potential for direct uptake is present in algae, potentially as phagotrophy of particulate matter, encapsulation and vesicle formation of surface bound DON or direct transport across the membrane. Investigating the mechanisms of DON would help to understand how bioavailable compounds can be in different systems.

River communities more widely may be limited by trace metals and minerals more than nitrogen or phosphorus. Carbon has been found to be a limiting factor compared to nitrogen and phosphorus in headwater streams for the whole stream community (Johnson, Tank and Arango, 2009). Increased growth on supplying DON may be due to the use of carbon, and not necessarily

the nitrogen within the compounds, by either bacteria, algae or other organisms. Limitation of algae by trace metals and minerals can occur in rivers, as they are required for microalgae to uptake or to otherwise utilise DON (Antia, Harrison and Oliveira, 1991). Similarly, light is commonly a limiting factor in growth of algal species (Beardall *et al.*, 2001; Felip and Catalan, 2000).

Understanding of various individual riverine microalgae isolate responses will help to tease apart how different species use DON. Investigating in more depth if single species can take up DON, and differences between species would be important to confirm to enable extrapolation of DON bioavailability in freshwater. More detailed investigation into the mechanics behind the impact of DON on algae could provide more clarity on why some DON is more bioavailable.

1.6.3. *Experimental data to further DON modelling and global biogeochemical cycles*

Some work has modelled nutrient use within freshwater systems. Two linked papers, Flynn *et al.* (2015) and Suplee *et al.* (2015), modelled nutrient effects on part of the Yellowstone river algal biomass to help inform policy nutrient limits. Though they considered organic nutrients, they did not include data derived values on DOM use as the variable had unknown or circuitous links with nutrients. This highlights a need for more data on organic nutrient availability and use by riverine microalgae. Site specific information on algal composition and types of DON available would be needed for improved accuracy, but more general data on DOM bioavailability estimates would still help refine predictions made in this area, as well as impacting further policy and monitoring approaches.

A review of DOM modelling by Anderson *et al.* (2015) highlighted that "mechanistic approaches often remain tentative and speculative" rather than well defined. Anderson *et al.* (2015) also noted improved information is needed on the distribution and seasonal dynamics of DOM. This leads to uncertainty in model accuracy and predictions from them.

The Redfield ratio (Redfield, 1934) is linked to the elemental composition of marine microalgae, and assumes a preference for C:N:P of 106:16:1. This is sometimes used, alongside nutrient limitation assumptions previously mentioned, to link modelling of algal growth and available nutrients. In actuality, modelling is more complex and needs to account for the non-Redfield stoichiometry of DON compounds that could greatly affect the nitrogen budget (Somes & Oschlies 2015). This is particularly important when considering that modelling is often based around Redfield ratio stoichiometry, even though it is not suitable basis for accurate algal modelling (Flynn

2010). More definition is needed, based on experimental data, to constrain variables that impact the global forecasting of carbon and nitrogen cycling in the decades to come. This is particularly important as modelling often informs the policies required to mitigate severe negative impacts of excessive N_r on human, animal and plant life.

1.7. Genomic analyses of freshwater algal species

The reduction in cost and increase in ease of using genetic techniques has led to an exponential increase in the number and type of genomic information possible to collect (Ward *et al.*, 2005; Corse *et al.*, 2010; Darienko *et al.*, 2015; Guo *et al.*, 2015; Suriya *et al.*, 2020). The available information on whole genomes and chloroplast DNA of these groups have increased dramatically in recent years, leading to more genomic records (Figure 1.5). The more complete genomes analysed and available, the more the potential metabolic pathways for DON use can be analysed *in silico* to direct experimental work and for modelling purposes. A larger number of records to compare against also allows more accurate species identification. Previous morphological-based phylogenies are being overturned by genetic information on the relationships between previous species and clade definitions (Chakraborty *et al.*, 2014; Friedl and Lorenz, 2012; Siddiqui *et al.*, 2020; Trémouillaux-Guiller and Huss, 2007).

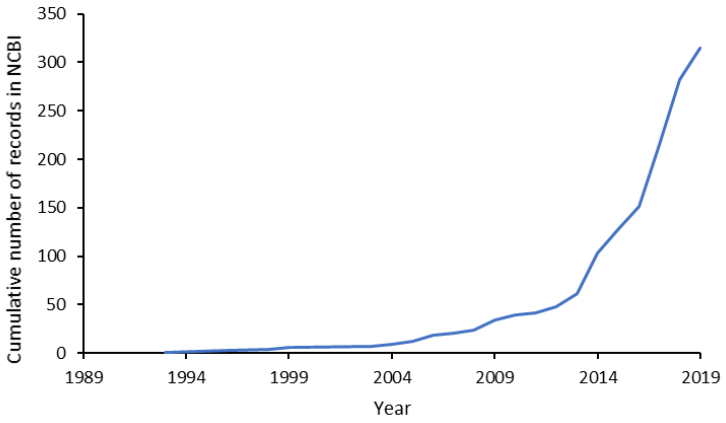


Figure 1.5. Cumulative number of records since 1990 of chloroplast DNA records in NCBI for green algae, red algae and protists to December 2019.

Species of commercial value or those heavily involved in the carbon cycle tend to be targeted for genomic analysis. These have historically tended to be more marine species, leaving less defined knowledge of freshwater species. The breadth of microalgal understanding is improving with the rising use of eDNA techniques – sampling all species present in a sample, usually for identification

purposes. However, this lacks the full genome sequencing needed to understand potential nitrogen cycling pathways in the environment. The number of complete genome assemblies for eukaryotic microalgae in particular is very small (Fig 1.6) less than 0.7% of the currently known 72,000 species (Guiry, 2012). This is by no means the total number of living microalgae species, many of which are difficult to culture and currently unknown to science.

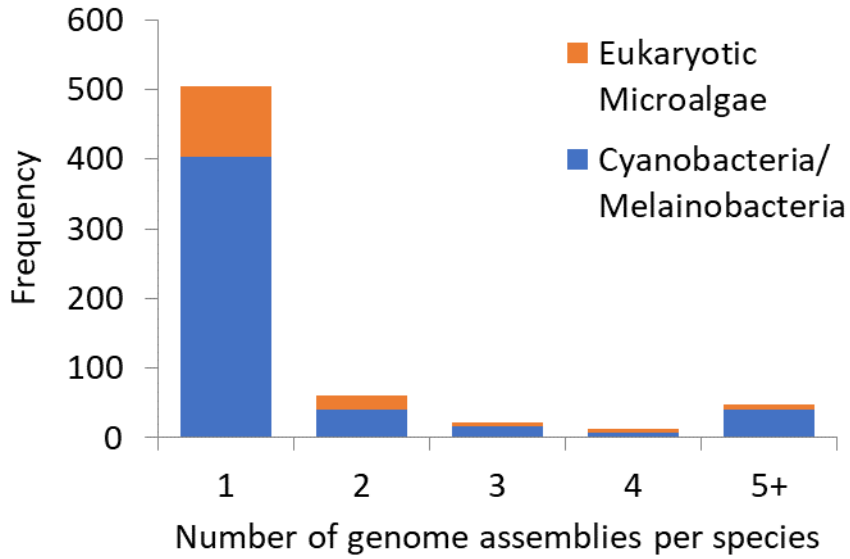


Figure 1.6. Histogram of number of genome assemblies per organism in NCBI, both cyanobacteria/ melainabacteria groups and eukaryotic algae (macro and microalgae).

The genomes of some microalgae indicate the ability to use organic compounds more directly than after full microbial processing back to inorganic compounds. Dyhrman *et al.* (2006) found a marine diatom from a nutrient poor ocean gyre is theoretically able to use DOP directly. This is due to the presence of a carbon-phosphate lysase pathway present in their genome. However, the purely theoretical knowledge needs to be tested through experimental research using the species in question. In practice the pathways may be internal, such as for re-using organic phosphate, as seen in dinoflagellates by Cui *et al.* (2016). The pathways present in genomes may show the possibility of uptake pathways, though the ability to use them may be internal only and not external, or highly dependent on local conditions. Genetic analysis needs to be combined with experimental use of DON to show which pathways are activated in normal algal functioning.

The potential use of DON by freshwater riverine microalgae needs to be investigated experimentally, and there are currently few genomes of freshwater species relative to the whole potential community. There is a lack of species-specific knowledge of freshwater DON impacts and

processing, which this thesis investigated through an experimental approach to DON combined with DNA barcoding to identify the species present.

1.8. Thesis research objectives and hypotheses

Freshwater systems are a conduit of reactive nitrogen from major terrestrial sources to the ocean and atmosphere, and an understudied part of global nitrogen biogeochemical cycling compared to marine sections of the cycle. Reactive nitrogen input is likely to increase in future largely due to anthropogenic activities, and will impact local ecosystems and human-freshwater interactions. There is a lack of understanding in how primary producers respond to DON, with experiments beginning to show DON compounds tend to increase autotrophic growth. The most basic primary producer in freshwater are algae, generally microscopic, single celled autotrophic organisms, though some freshwater species are multi-celled and macroscopic. It is likely algae can use DON for growth, with some mesocosm experiments showing this in bulk (Mackay *et al.*, 2020). However, species – specific understanding is lacking, which is needed to extrapolate responses to DON to other locations with similar species assemblages.

Objective 1: To determine whether riverine algae can use DON as a nutrient resource.

As DON composition changes and become more complex the further downstream, headwater catchments will be targeted to isolate microalgae species for testing in laboratory conditions to understand responses to DON. Isolation is required as most culture collection species have been grown for hundreds of generations on inorganic nitrogen and may have lost capacity to utilise DON as compared to wild strains.

Hypothesis 1 – Isolated algal species can utilise both inorganic and organic nitrogen sources for growth.

Objective 2: To investigate whether there is a preference for different DON compounds, and whether this is consistent between species groups

Presenting isolated species with various inorganic and organic nitrogen sources allows for basic preferences of chemical nitrogen for growth to be seen through the ability to utilise different

nitrogen forms. This is based on the niche partitioning idea, which extended to nitrogen compounds would mean different species vary in their ability to make use of the same nitrogen form, 'specialising' as it were in different nutrient sources to reduce resource competition.

Hypothesis 2 – Isolated algal species have different species-specific and compound-specific preferences for growth on inorganic and organic nitrogen.

Objective 3: To determine whether DON uptake varies between species isolated from streams with differing catchment characteristics

To follow the findings of Objective 1 and 2, a second headwater catchment will be investigated to determine if species from a catchment with a different DON history and land use follow similar patterns of DON use to those already found. The presence of different species due to the different river types will allow a greater chance of isolating and comparing evolutionarily diverse microalgae species.

Hypothesis 3 – Similar species isolated from differing headwater catchments show the same growth response to DON chemistry.

Objective 4: Investigating evidence of DON uptake into protein synthesis pathways in algal cells.

Though Objectives 1-3 investigate potential differences due to location and species, the mechanisms by which DON may stimulate growth are not well known. This objective aims to investigate how nitrogen is incorporated in cells grown on various nitrogen sources. Stable isotope tracing of carbon and nitrogen through the bulk and amino acids of the cells will help elucidate potential pathways, including how DON carbon and nitrogen arrives in the nitrogen pools within microalgal cells. This will provide more detailed, species specific process-understanding that is currently lacking in the literature, providing information to constrain models of microalgal growth and biogeochemical cycles. Carbon and nitrogen will be considered separately, as compounds may be cleaved before uptake by the microalgae.

Hypothesis 4 – Algae show isotopic enrichment in bulk biomass after growth on isotopically labelled inorganic and organic nitrogen compounds.

Hypothesis 5 – Algal amino acid pools will show enrichment after growth on isotopically labelled inorganic and organic nitrogen compounds.

Chapter 2 – Methods of isolation, experimental optimisation, analysis and genetic identification of species

2.1. Introduction

The methods used in this thesis are presented here in detail, with brief overviews of the specific analyses employed in each experiment outlined in each of the results chapter. Methods include approaches for algal isolation, set-up of experimental conditions, analytical techniques, genetic identification of species and bioinformatics. A schematic outlining the isolation and experimentation process is shown below in Fig. 2.1.

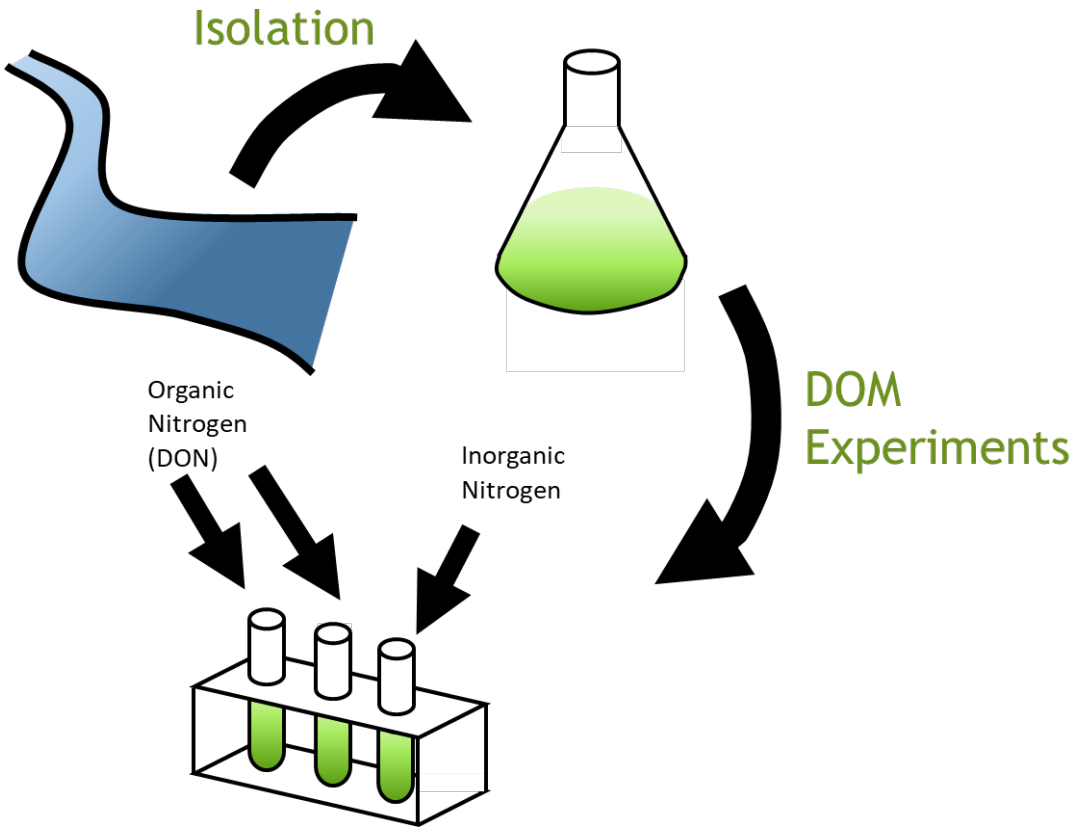


Figure 2.1. Schematic of isolation and experimental process.

2.2. Isolation media, techniques and conditions

This thesis explores the responses of riverine algae to different dissolved organic nitrogen compounds through a series of laboratory culture-based experiments or bioassays. Experimental work with microalgae typically involves commercially available species from culture collections. These are strains that have been isolated and cultivated in liquid media,

on agar plates or preserved in liquid nitrogen. Such culture collections are found globally and contain species from a variety of locations (UTEX, 2021; CCAP, 2021; SAG, 2021). Species have been isolated and maintained from the early 20th century, such as CCAP 45/2 (*Lobomonas rostrata*) isolated in 1930 by E.G. Pringsheim (CCAP, 2021), that is still available to culture. It takes time, money and staff to maintain culture collection strains, so collections usually prioritise historic, commercial or environmentally important strains. More recent cryopreservation techniques allow more strains to be kept by culture collections (Day *et al.*, 2007; Taylor and Fletcher, 1998; Brodie *et al.*, 2009) though this needs to be optimised for each species (Friedl and Lorenz, 2012). Due to the focus on the marine basis of the food-web and the carbon cycle, many marine cultured strains focus on these traits. Various freshwater strains are deposited in culture collections often due to their unique characteristics, isolation location or biological potential but not all known microalgae species are represented in these collections.

Culture collection algae are maintained using media containing a variety of nutrients, generally based on inorganic nitrogen and phosphorus with additional metals and minerals, though some strains cannot be cultivated in laboratory conditions on inorganic nutrients alone. Some cultured species have media that makes use of soil extracts to sustain growth (CCAP, 2021), which contains a range of trace metal, mineral and dissolved organic matter compounds (Belcher and Swale, 1982). More recent work has shown some algal cultures also depend on associated bacteria to gain essential vitamin B12 (Croft *et al.*, 2005), which may be why soil extracts were essential in isolating and maintaining some algal species.

Algal cultures which have been sustained on prepared media for decades may differ genetically from the wild strains. The generation time of most algae species is 1-7 days (Arin, Morán and Estrada, 2002; Nielsen, 2006) and without exposure to natural variety of nutrients, cultures strains may have lost or reduced their nutrient processing potential. As the ability to use DOM is not evolutionarily favoured when cultures are only exposed to inorganic nutrients in the culture media, culture collection species may no longer be representative of native species in riverine environments. Due to this, cultures were established in this programme through direct collection and isolation of species from natural riverine ecosystems. To allow investigation of the bioavailability of DON to riverine algae, and the ways in which this varies between heterogeneous stream systems, species were collected and isolated from two contrasting stream locations in two well-researched rivers, in peatland (Conwy catchment, Wales) and clay (River Sem, Hampshire Avon catchment, England) locations.

2.2.1. *Site Descriptions*

Sites were chosen based on those with a history of DON measurement. Sites used in the DOMAINE project work fitted this description and were known to be accessible. This also enabled further research to build upon work done as part of that project and their future findings, making use of other strands of research in the same locations (Mackay *et al.*, 2020; Brailsford *et al.*, 2019; Yates, Johnes and Spencer, 2019; Pemberton *et al.*, 2019).

Impacts on stream biology and DON pools gets more complicated and difficult to disentangle further downstream. For this reason, headwater catchments were chosen as isolation sites. Algal species present in these sites are likely to be swept downstream and form part of the lower reach communities (Power and Stewart, 1987; Rosen *et al.*, 2018; Nejadsattari, 1992). The species-specific ecological impact downstream of microalgae colonisation are not well known, but the biological response of headwater algal species should reflect similar patterns of response further downstream (Smith, 2003), and potential responses of the freshwater environment.

DON load, input of fertiliser and animal waste runoff to the stream were used to choose site 1. This was on River Sem, a tributary of the River Nadder and Hampshire Avon river in South England. This sampling point will be referred to as the Priors Farm sampling location (longitude 51.055, latitude -2.157). Sampling catchment covers 2.26 km^2, with a population density of 22.7 people per km^2 and is dominated by improved grassland land type (58.5%). The catchment at the sampling point is on a bedrock of clay-mudstone geology, with the later and larger catchment it feeds into (Hampshire Avon) being a largely chalk stream environment. Chalk stream environments are known for their global rarity and support an ecologically important array of species, especially aquatic animals and water plant species (e.g. water cress, native salmon species), that have been lost elsewhere (Natural England, 2016; Britton *et al.*, 2017). Samples at Priors Farm were taken in June 2017.

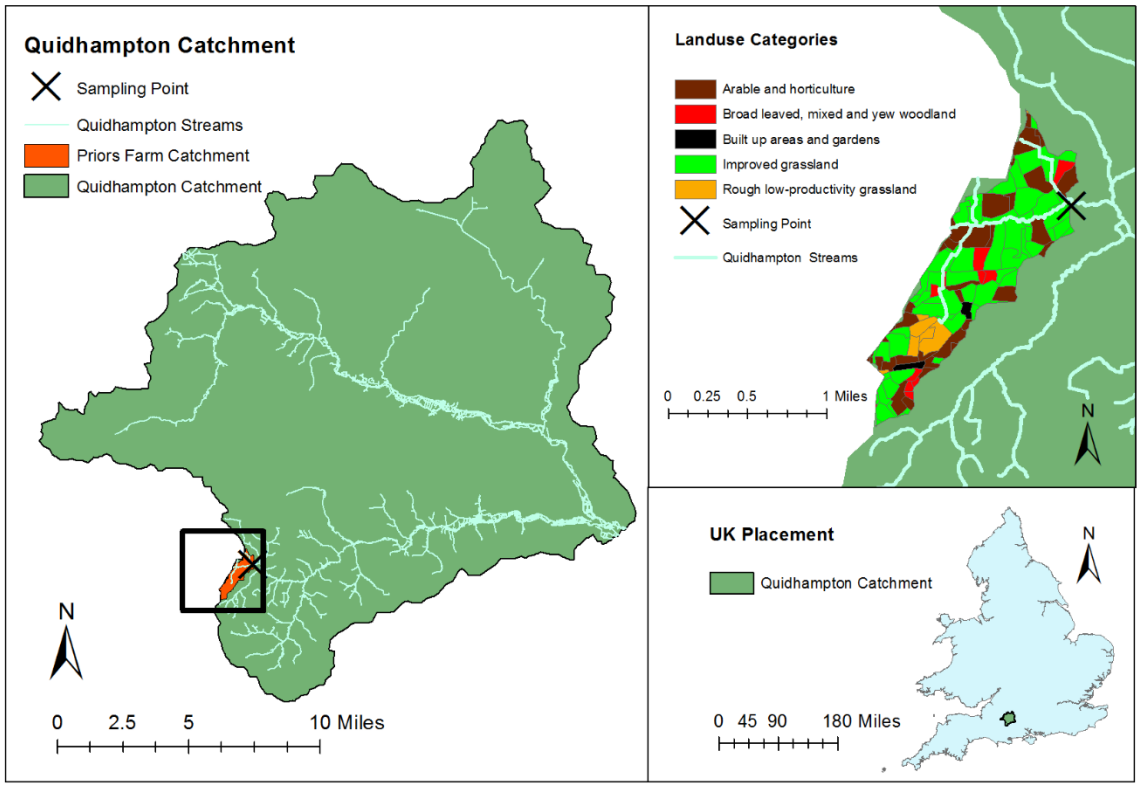


Figure 2.2. Map of clay sampling location, at Priors Farm on the River Sem, tributary of Hampshire Avon River, using land cover data from CEH (2007).

The second isolation site was chosen to contrast with the first, as a location with high DOM but low human nitrogen impact on the site. This ruled out catchments with largely improved or arable grassland land cover. The site chosen was in a peat bog area, with occasional roaming sheep present, no fertiliser input but high DOM levels in the river from the peat leached from the bog (Yates *et al.*, 2019). Sampling was close to headwater on the peat moorland, draining into Afon Llugwy, which is a tributary of Afon Conwy in North Wales (latitude 52.988, longitude −3.801). It is situated on the edge of Yr Wyddfa (Snowdon) national park and is site of special scientific interest for sphagnum moss and liverwort species amongst others (Natural Resources Wales, 2012). Sampling catchment covers 1.31 km^2, with no population density present and is dominated by blanket peatland land type (79.9%). The sample site catchment is over bedrock sandstone and mudstone, as is largely the rest of Afon Conwy with occasional igneous intrusions. The peat gives way to farmland, coniferous woodland and improved grassland lower in the catchment. This site will be referred to as Nant-Y-Brwyn. Samples from Nant-y-Brwyn were taken in May 2017.

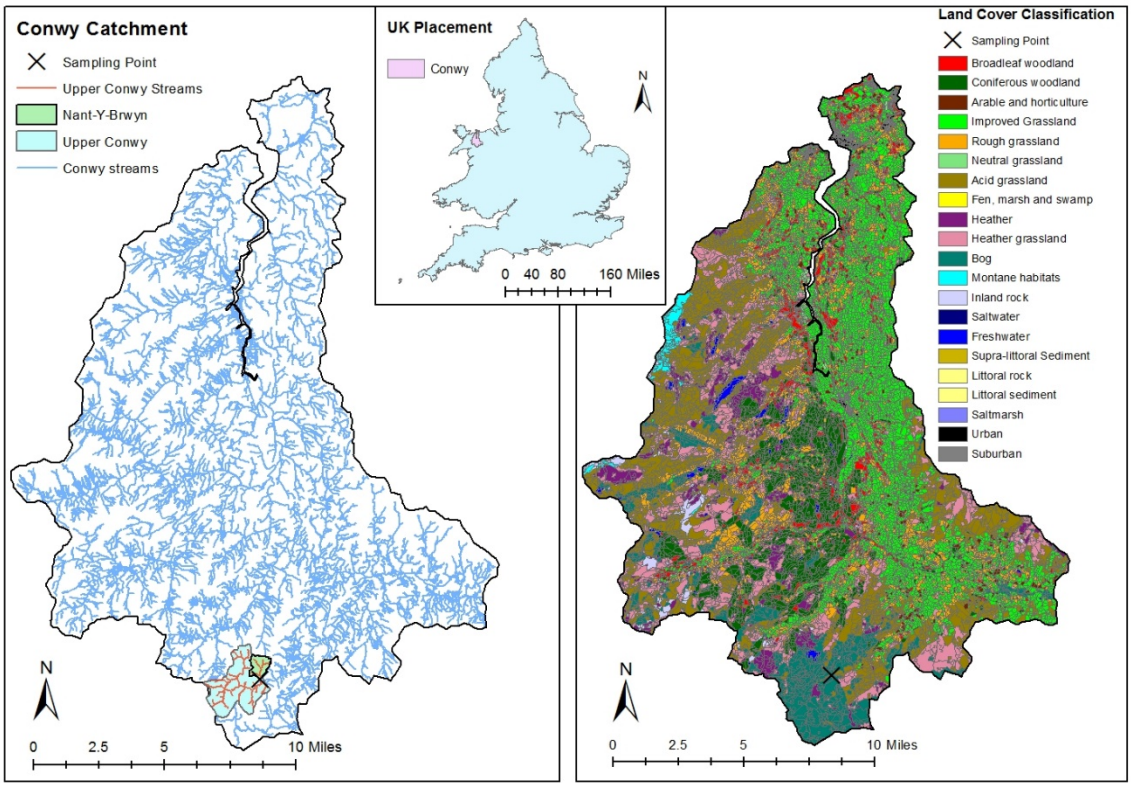


Figure 2.3. Map of peatland sampling location, at Nant-y-Brwyn on the Conwy River using land cover data from CEH (2007).

Both sites chosen were part of a long-term monitoring programme for dissolved organic nitrogen and dissolved phosphorus concentrations, amongst other compounds including inorganic nitrogen forms. The data for the relevant sampling locations are shown in table 2.1. Methodology and the full dataset is present in the original paper by Yates *et al*. (2019). This did not include pH data. For the Conwy site, pH is based on EA monitoring data at Nant-y-brwyn (Cooper *et al.* 2013) using monthly samples Sep 2006 - Mar 2011 with an average pH 5.45 ± 0.86 (± standard deviation, n= 75). For Priors Farm, pH data was obtained from the Hants Avon DTC consortium (2016) daily sampling between 26 Dec 2011 - 17 Mar 2014, with an average pH 7.81 ± 0.29 (± standard deviation, n = 504).

Table 2.1. Biogeochemistry of catchment at sampling locations from regular sampling over a year. Adapted from Yates et al. (2019). Samples are the mean ± standard deviation.

	Nant - y - brwyn (Conwy)	Priors Farm (Hampshire Avon)
No. samples	37	23
Sampling frequency	weekly	bi-weekly
NO_3-N (mg l^{-1})	0.0993 ± 0.367	2.33 ± 1.25
NH_4-N (mg l^{-1})	0.011 ± 0.007	0.237 ± 0.326
DON (mg l^{-1})	0.458 ± 0.168	1.25 ± 0.368
PO_4-P (µg l^{-1})	4.81 ± 8.93	144 ± 82.7
DOP (µg l^{-1})	11.7 ± 7.49	42.4 ± 24.4
DOC (mg l^{-1})	10.8 ± 6.14	13.7 ± 3.65
DOC:DON (molar)	26.6 ± 9.02	13.1 ± 2.41
DOC:DOP (molar)	2767 ± 1857	1227 ± 1491

2.2.2. *Sample collection*

At each site, two samples were taken – one from the midstream flow and a second from the surrounding vegetation and/or rocks. Mid-stream samples were to collect phytoplankton algae, vegetation and rock samples to collect benthic and biofilm algae. This enabled a wide range of material to collect diverse algal cells from. Samples for isolation were collected at each site in 1 L high density polyethylene sample bottles while wearing nitrile gloves. The bottles were pre-washed with 7% HCl and rinsed with milli-Q water in the laboratory before sampling visits. They were then rinsed with river water on site immediately prior to sample collection. Samples were kept in the dark, at stream ambient temperature and returned to the laboratory within 24 hours of sampling.

Mid-stream samples were collected using an automatic water sampler already installed at both sites, as part of the NERC DOMAINE Large Grant programme. These collected

water from the central portion of the river channel, at 60% of river depth. The autosampler was run for ~1 minute to clear and flush the sampling tube, then a fresh sample was collected.

The vegetation/rock sample was collected from the stream margins and vegetation at the same location as the autosampler. After rinsing with local river water, it was filled about 35% full. A range of nearby submerged vegetation was chosen. If rooted it was left in place, and a gloved hand used to gently squeeze/rub the surface and rinse in the sample bottle water. If un-rooted vegetation was present, a small amount was placed in the sample bottle, rinsed in the sampling water and the vegetation removed and placed back *in situ*. If sites had pebbles visible, these were gently scraped with a gloved fingernail and rinsed into the second sample bottle with river water. Bottles were filled no more than 2/3 full to allow an air space under the lid during transport to enable gas exchange. Any large, planktonic organisms were scooped out with a mesh filter at location.

2.2.3. *Isolation techniques and conditions*

Investigatory microscope samples were collected to look at sample diversity and condition upon arrival in the laboratory using a light microscope. Ciliates and amoeba were seen in all samples. In the laboratory each sample of river water was mixed by inverting. A sterile cotton tipped swab was dipped into the samples and used to streak onto an agar plate. Over 10 agar plates were initially created from each sample bottle. Additional agar plates were made from the initial river samples each week for the first month after collection, to attempt to maintain as much diversity as possible. After that month, diluted media (1/5 of agar media concentration) was added to sample bottles and they were left to create stable communities as an alternative backup to the agar plates, with the lids being opened for gas exchange a few times a week.

Various isolation techniques were attempted, including micro-pipetting, repeated dilution of samples and agar plate streaking. The most successful technique that produced the isolates used in this report was repeated agar plate streaking of cultures. Samples not grown on agar plates contained an abundance of predatory ciliates, rotifers and amoeba. Regardless of method, isolation growth occurred in a temperature-controlled culture cabinet (Model 1A-201, LMS Ltd., Kent, UK) set at 15°C, with light at ~50 µmol m^{-2} s^{-1} (photosynthetically available radiation) and a light:dark ratio of 16h:8h. Light was kept relatively low to prevent photodamage of dilute cultures. The media used is discussed below in section 2.2.4.

Agar plates were each left for 1-2 weeks to grow under the isolation conditions described above. Once growth was visible, morphologically different colonies as determined by microscopy were picked using sterile swabs, with each different type placed on separate agar plates. Once species remained visible on the agar plates after streaking, the swabbing timing changed depending on the species. Faster growing species were picked and re-swabbed more often, slower growing species had longer growth time between transfers. Dense or adhesive algal colonies were sometimes diluted with sterile liquid media to better enable single species colonies on the next plate.

Separation of colonies on agar plates continued until one morphologically distinct species was present on one agar plate. These cultures were maintained by sub-culturing monthly onto new agar. After about 6 months, about 20 lineages were obtained from each sampling location, including representatives of diatom, green algae and cyanobacteria at both the peatland and clay river sites. Cultures were not deliberately axenic, though microscope examination suggested some cultures did not have visible bacteria. Some cultures were morphologically similar to others isolated from that site, but all were kept in culture as separate isolate strains.

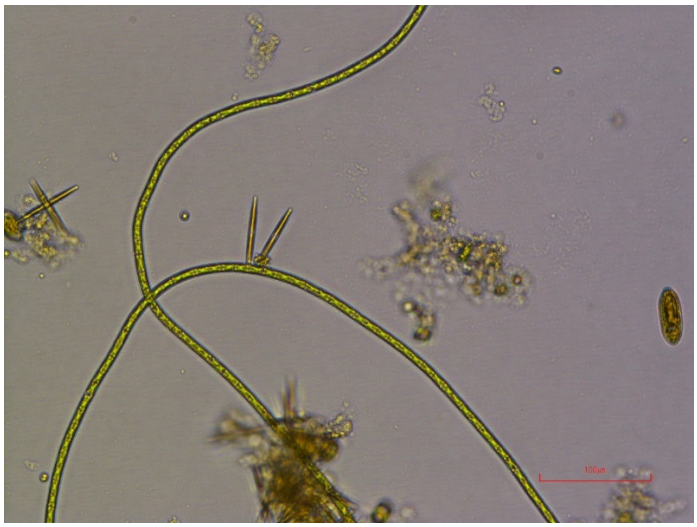
Fungal hyphae were occasionally seen in the initial phases of isolation. Microalgae were picked from hyphae-free locations where possible, and plates with large fungal presence were discarded completely. Due to the difficulty of fungal removal once well spread through a microalgae culture, this more cautious (though severe) approach was used to ensure final cultures were fungi free. Repeated, quick microalgae colony picking and sub-culturing were used to isolate as many species as possible from these more difficult plates. Not all species in the original samples were cultured, and it is recognised that the species isolated represent some but by no means all algae in the river locations sampled.

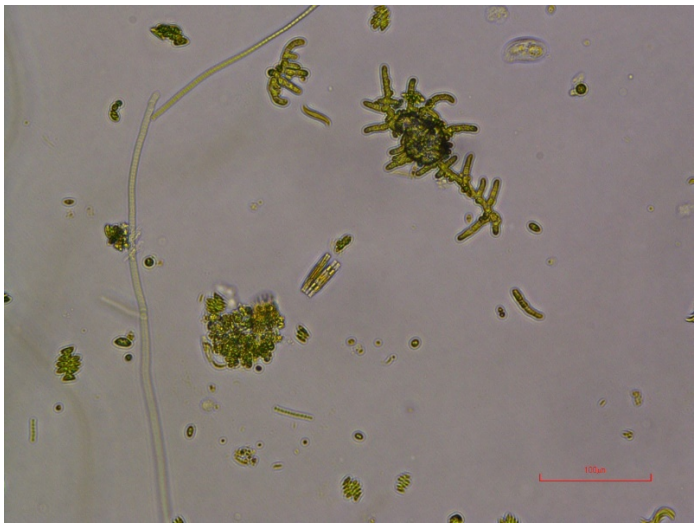
Though each morphologically distinct group is referred to as a single species, they were not necessarily axenic and were generally unialgal. One isolate was notably multi-species, consisting of a cyanobacteria and diatom that that were difficult to separate even under rapid sub-culturing. The diatom and cyanobacteria present went through cycles of dominance on plates over the months they were kept in culture, and a sustained effort was made to keep two plates with a majority of each species. All other isolates were unialgal at 400x microscopy. Most species were isolated alongside a bacterial community, which varied in amount from almost none to easily observable bacteria.

Isolated cultures were kept on agar as the 'master' cultures. These were sub-cultured once a month to new agar. The use of agar as the main isolation medium may have preferentially isolated benthic species instead of planktonic species. However, it should be noted that this is consistent with approaches in culture collections globally, where microalgae species are often kept on agar (or cryopreserved) to reduce the culturing maintenance required. This includes planktonic marine and freshwater species. That means that in this work, planktonic species should have been isolated alongside benthic, with other isolation conditions (media, light, temperature) impacting the range of species isolated. Some images of species not used in later experimental work were taken during the initial isolation phase from Nant-Y-Brwyn (Figure 2.4) and Priors Farm (Figure 2.5), and show the range of species present.

Having isolated the strains and developed these cultures, the individual species were then grown on liquid media for 1-14 days prior to use in the experiments in this thesis.

a) b)





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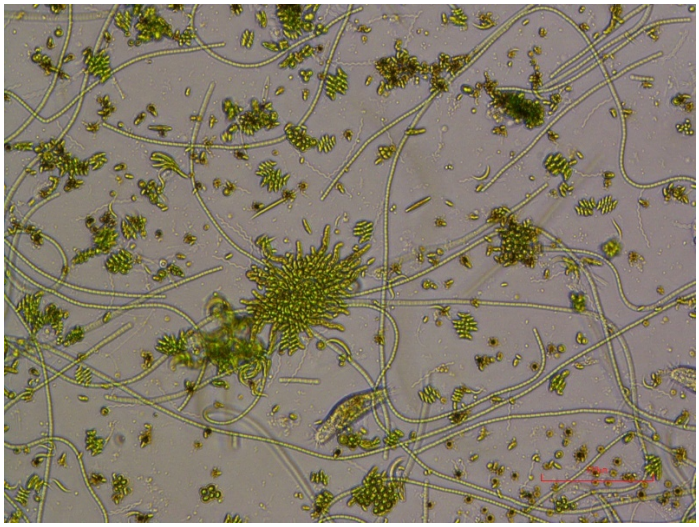


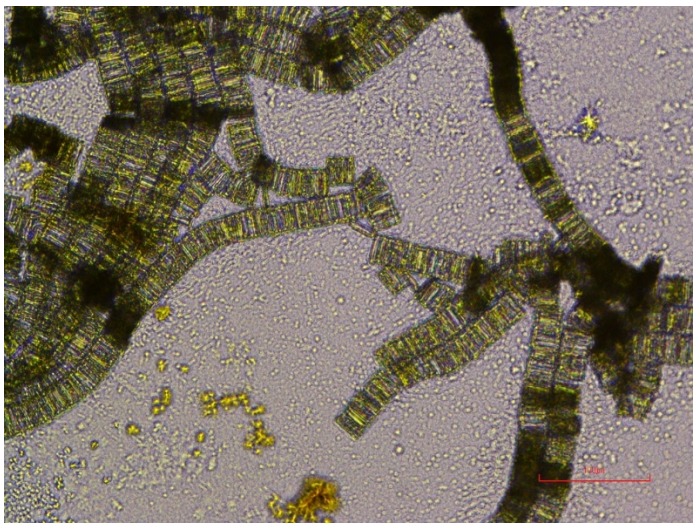


Figure 2.4. Images of some strains from the Nant-Y-Brwyn site at the isolation stage, taken with light microscopy at magnification of (a) x400, (b) x200, (c) x100 (d) x200.

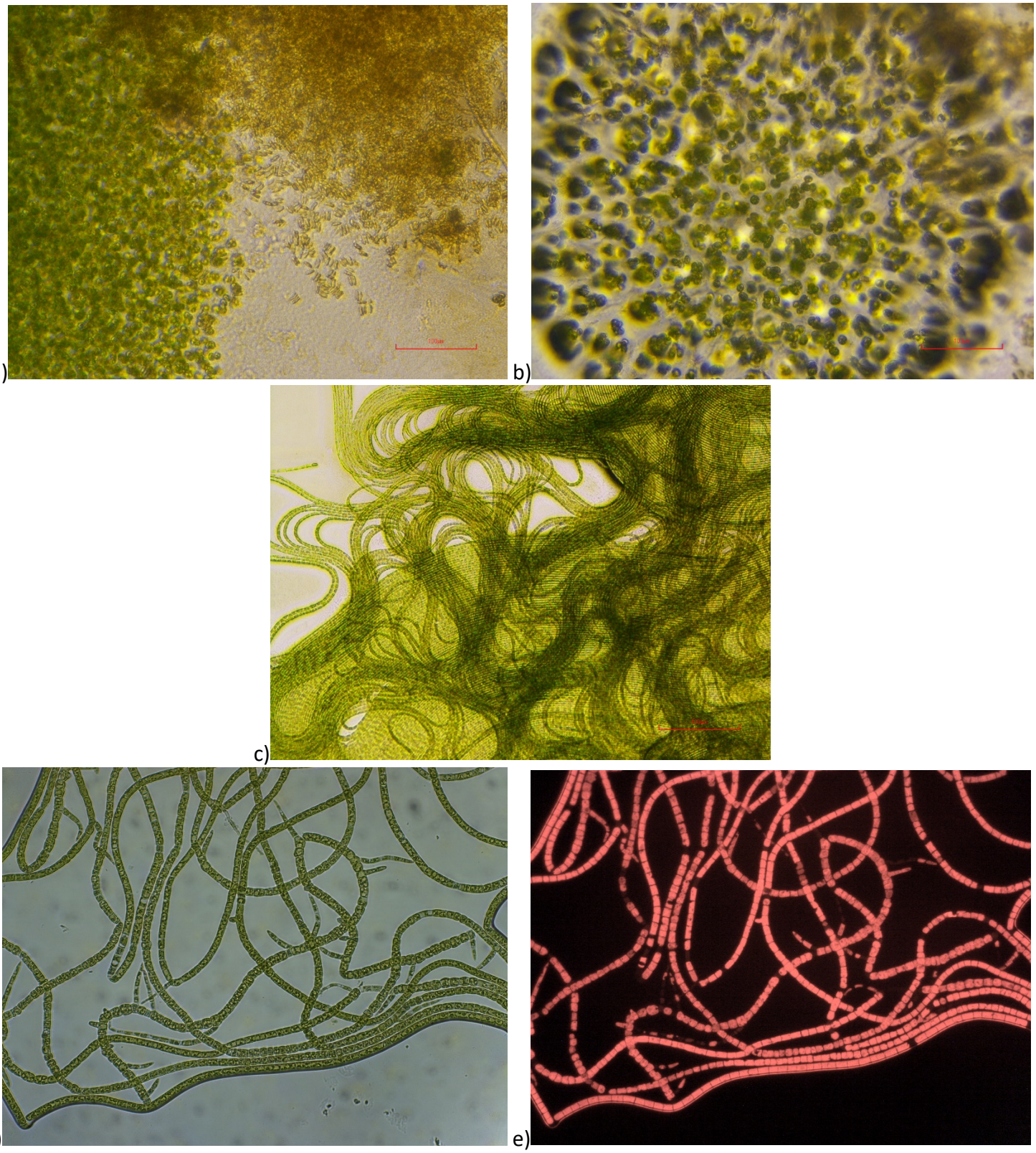


Figure 2.5. Images of some strains from the Priors Farm site at the isolation stage, taken with light microscopy at magnification of (a) x100, (b) x200, (c) x100 (d) x400 and (e) with chlorophyll fluorescence microscopy at x400 magnification.

2.2.4. *Isolation media*

Media choice is important in determining which species can be isolated or maintained in a laboratory culture. Textbook media, such as those used by culture collections, are usually tailored to the needs of a specific genus or group of algae. This project aimed to isolate as many species as possible, rather than specific algal groups, to enable exploring species-specific bioavailability and gain a better understanding of wider ecological responses possible. Comparisons would then be between species isolated in an identical manner at the same time. Research collaborators on linked projects have conducted work on community responses to DON compounds as these varied between sites of differing character (Mackay *et al.*, 2020) as well as chemical and bacterial responses (Yates *et al.*, 2019; Brailsford *et al.*, 2019). Here, the focus was on smaller scale, species-specific responses possible to these same DON compounds under controlled laboratory conditions, ideally using single-species cultures.

Culturable microalgae species are often outweighed by 'unculturable' microalgae in the environment. They may be unculturable due to a requirement for very specific conditions to survive, e.g. media, light or associated bacteria. Genetic investigations are beginning to uncover some of the unculturable diversity of algal species, as well as detecting cryptid species (Trémouillaux-Guiller and Huss, 2007; Krivina and Temraleeva, 2020; Kaczmarska *et al.*, 2014). The aim here was to isolate a wide range of culturable species, as 'algal' species comprise a diverse range of organisms, including diatoms, cyanobacteria, dinoflagellates and green algae. The media used was broad in scope to accommodate this. This also meant some algal species were lost in the isolation phase from the initial samples, as prioritising a wide range of species will favour generalist species more than specialist species.

Algal media generally contain sources of nitrogen and phosphorus alongside trace metals and vitamins, usually with chelating agents to maintain these in solution so they are bioavailable. The chemical form, concentration and breadth of compounds included in different culturing media varies between different media recipes. Media are often tailored to the species being cultured or isolated, which has led to the large array of media currently used in algal culture collections (CCAP, 2021; UTEX, 2021). Lists of commonly used media can be found from culture collections, for example in the UK (CCAP, 2021), Germany (SAG, 2021) or the US (UTEX, 2021).

Many traditional recipes were created decades ago to cultivate strains of marine and freshwater microalgae and are largely optimised for those individual species. As the potential

species to be isolated in this thesis were not known, a generalised medium was required, with silicon for diatoms. Deciding the concentration of trace metals to use is more difficult, as some species are very intolerant to the presence of a metal that other species may require. Nitrogen and phosphorus concentrations in the generalised medium were included at a higher concentration than those observed in the river environment to stimulate rapid algal growth, enabling algae to counter ciliate and bacteria growth that were not removed by the mesh during sampling.

Many microalgal culture media were produced before high resolution analytical characterisation of organic matter composition was possible to pinpoint which compounds were essential to the species targeted. Some cultivation media list soil, yeast extract or mammalian serum extracts that are essential to the continued cultivation of the microalgae grown on it, such as those in artificial sea water medium or Bold's Basal medium (CCAP, 2021). Trial and error during the initial era of research on algal culturing led to the incorporation of a range of these extracts into now classic culturing media recipes. These complex, organic compounds are substituting for natural compounds, potentially local trace metals, bacteria-produced vitamins and other organic compounds in the algal environment. Culture collection species are maintained as deposited, using these original media recipes. As this project is determining DON responses, media that contained unquantified organic extracts were avoided. Instead, the isolation media contained a wide range of trace metals, minerals and as well as vitamins at low concentrations. Media metal and minerals can inhibit the growth of some species but are required for growth by others, so to isolate most algal species only the most common metals were included at low concentrations.

A range of general-purpose culture media were compared in terms of compounds and concentrations, as shown in Table 2.2. Though F/2 is generally a marine medium, freshwater versions of it are used in aquaculture of microalgae. *Spirulina* medium is also generally a marine medium for cyanobacteria. The others were freshwater Jaworski, Walnes, BG11 and Bolds Basal Medium. More detailed compound comparisons can be seen in Appendix 2. Some media compounds contain nitrogen or phosphorus alongside metals, e.g. ammonium molybdate. These were avoided to ensure the same metal solution could be used during isolation and later in the experimental phase, where nitrogen compounds additions were altered. Both BG11 and *Spirulina* medium have nitrogen levels an order of magnitude higher than the other media, which is significantly higher than present in UK streams. To reduce the

growth of sulphur metabolising bacteria, large amounts of sulphur were avoided in the compounds used, though it is needed by the algal species as well.

The optimal stoichiometry (elemental ratio) for algal culturing is usually based on the Redfield ratio (Redfield, 1934), which is a ratio of elemental C:N:P of 106:16:1. This is intended to relate to the stoichiometric needs of marine algal species with a number of media based on supplying this ratio. However, algal species have considerable variation in their stoichiometry that also vary with nutrient stress conditions (Geider and La Roche, 2002; Meyer *et al.*, 2016). More recent oceanic data suggests a ratio of 163:22:1 instead (Alvarez-Fernandez and Riegman, 2014; Somes and Oschlies, 2015b). Others, such as Bolds basal medium have an altered N:P ratio with much more phosphorus relative to nitrogen than other media. This leaves Jaworski's, Walnes and F/2 media as potential bases for the isolation media.

Each potential media base differs in the metal and vitamin content present, so a combination of these was used at low concentrations in the isolation media. EDTA (ethylenediaminetetraacetic acid) is often used as a chelating agent for metals in commercial culturing media, enabling metals to remain in solution more easily. However, as EDTA contains nitrogen and the experimental treatments are focused on nitrogen, it was not included in the media. Also, mixed media solutions often interact when autoclaved together, with metals often precipitating out into insoluble and non-bioavailable forms. To prevent this without using EDTA, stock solutions of each were kept separated until use. Iron was autoclaved separately, with the other solutions (Table 2.3) being filter sterilised using sterile syringes and disposable filters (Filtropur S0.2 Polyethersulfone syringe filter, 0.2 µm, Sarstedt Ltd., Leicester, UK). To isolate diatoms that need silica to grow, silica was included based on F/2 metasilicate concentrations. After initial pilot testing, $NaHCO_3$ was added to provide buffering capacities to the media, based on the concentration in Jaworski's medium (Table 2.3). As it also provides carbon, it was initially left out. However, the resulting media was unbuffered and the pH changed to unfavourable levels in initial pilot tests.

Table 2.2. Comparison of major nutrients in generalist culture medium by ug of element per litre of medium from media lists at (CCAP, 2021), rounded to 3.sig fig. An empty box shows the compound / element is not in the media.

Element/ compound (ug L^{-1} media)	F/2 Medium (marine)	BG11 Medium	Walnes Medium	Bold Basal Medium (BBM)	Jaworski's Medium (JM)	Spirulina Medium (SM) marine
N	12400.0	247000.0	16500.0	41200.0	15500.0	412000.0
P	1120.0	16100.0	3970.0	120000.0	9500.0	257000.0
Fe	651.0	7280.0	269.0	1000.4	350.0	2710.0
EDTA	4160.0	1000.0	45000.0	50000.0	2300.0	84000.0
B		2860.0	33600.0	11400.0	2500.0	50.0
Ca		9800.0		6800.0	5760.0	2730.0
Co	2.5	14.9	5.0	150.0		1.5
Cl				15200.0		607000.0
Cu	2.5	26.0	5.1	407.0		0.1
K		8960.0		88500.0	3553.0	367000.0
Mg		7390.0		7390.0	4929.6	19700.0
Mn	50.0	499.0	111.0	389.0	388.4	2.2
Mo	2.4	159.0	0.7	467.0	77.7	2.0
Zn	5.0	45.5	10.1	2000.0		1.1
Vitamin B1	100.0		10.0		40.0	250.0
Vitamin B7	0.5		0.2		40.0	0.5
Niacin (Vitamin B8)						50.0
Vitamin B12	0.5		10.0		40.0	10.0
Folic acid						1.0
Inositol						2500.0
Thymine						1500.0

µg L^{-1} range	Colour
0 - 10	
11-100	
101-1000	
1001-10000	
10001 – 100000	
>100001	

Table 2.3. Isolation media used with 1% agar.

Primary element or compound	Chemical formula	Final media concentration (µg L^{-1})	Media origin	Stock solution group
N	$NaNO_3$	80000	JM	1
P	$Na_2HPO_4.12H_2O$	36000	JM	2
P	KH_2PO_4	12400	JM	3
Fe	$C_6H_5FeO_7$	1180	BG11	4
B1	$C_{12}H_{18}Cl_2N_4OS$ (Vit B1)	40	JM	5
B7	$C_{10}H_{16}N_2O_3S$ (Vit B_7)	40	JM	5
B12	$C_{63}H_{88}CoN_{14}O_{14}P$ (Vit B_{12})	40	JM	5
Ca	$Ca(NO_3)_2.4H_2O$	20000	JM	6
B	H_3BO_3	2500	JM	7
Mg	$MgSO_4.7H_2O$	50000	JM	7
Mn	$MnCl_2.4H_2O$	1400	JM	7
Mo	$NaMoO_4.2H_2O$	1180	BG11	7
Co	$CoCl_2.6H_2O$	20	Walnes	8
Cu	$CuSO_4.5H_2O$	20	Walnes	8
Zn	$ZnCl_2$	21	Walnes	8
Si	$Na_2SiO_3.2H_2O$	30000	F/2 + Si	9
CO_3	$NaHCO_3$	7800		10

2.3. Algal culture conditions

2.3.1. *Experimental conditions*

Experiments and isolation were performed in the same cabinet, a temperature controlled culture cabinet (Model 1A-201, LMS Ltd., Kent, UK) set at 15°C, with light at ~50 µmol m^{-2} s^{-1} and a light:dark ratio of 16h:8h. As the cabinet was only lit from the back panel, a more even light environment was needed to ensure experimental treatment effects were not confounded by light differences. A UV transparent acrylic panel was cut to required dimensions, holes drilled to support lighting from below and installed as a shelf in the culture cabinet. Two lights (LED Linkable Strip Light 277mm 4W 4000K, LMS Ltd., Kent, UK) were attached underneath the shelf (Figure 2.6). Light was diffused using thin paper to provide more even lighting to the base of the culture flasks. The experimental shelf was isolated from other light sources by thick foil above and below, and foil covered card at the back of the shelf (i.e. in front of cabinet lights). Light levels were re-measured within flasks of media on the shelf and averaged 50 µmol m^{-2} s^{-1} across the shelf.

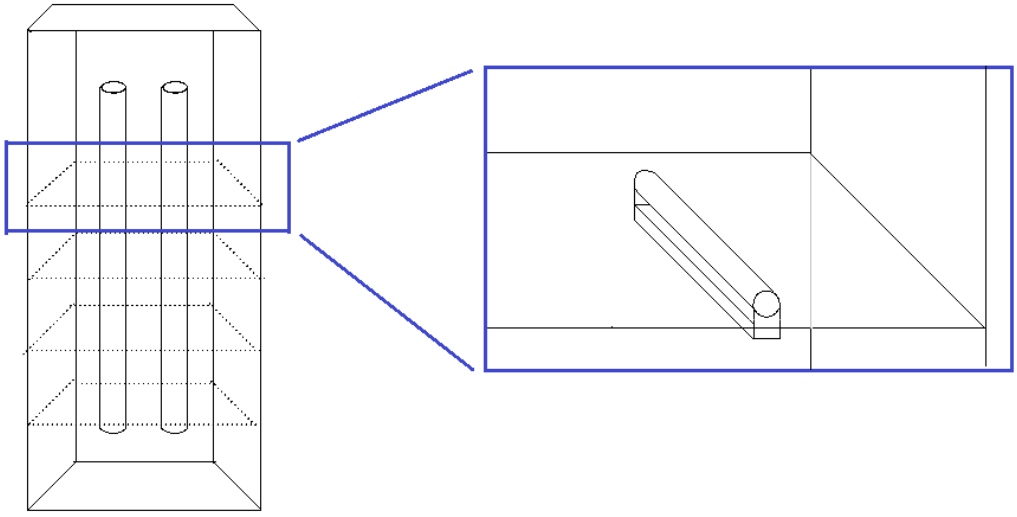


Figure 2.6. Diagram of culture cabinet and additional shelf detail.

Growth of experimental cultures took place in either plastic culture flasks (Chapter 4 and 5) or glass Duran bottles (Chapter 6). Plastic culture flasks were 50 mL with a vented cap (UltraCruz® Flask, vented lid with 0.22 um membrane, Santa Cruz Biotechnology Inc., Germany), filled to 30 mL or 40 mL culture volume. Glass bottles were 500 mL, clear borosilicate glass (DURAN™ Laboratory Bottle, Square, Clear, 500 mL, 78 mm, polypropylene lid), filled to 400 mL culture volume. The

placement of flasks and bottles on the shelf during experimental growth is shown in Figure 2.7. Experimental treatments lasted for at least 14 days. This allowed cultures to adjust to experimental conditions and the different nitrogen compounds before showing a response.

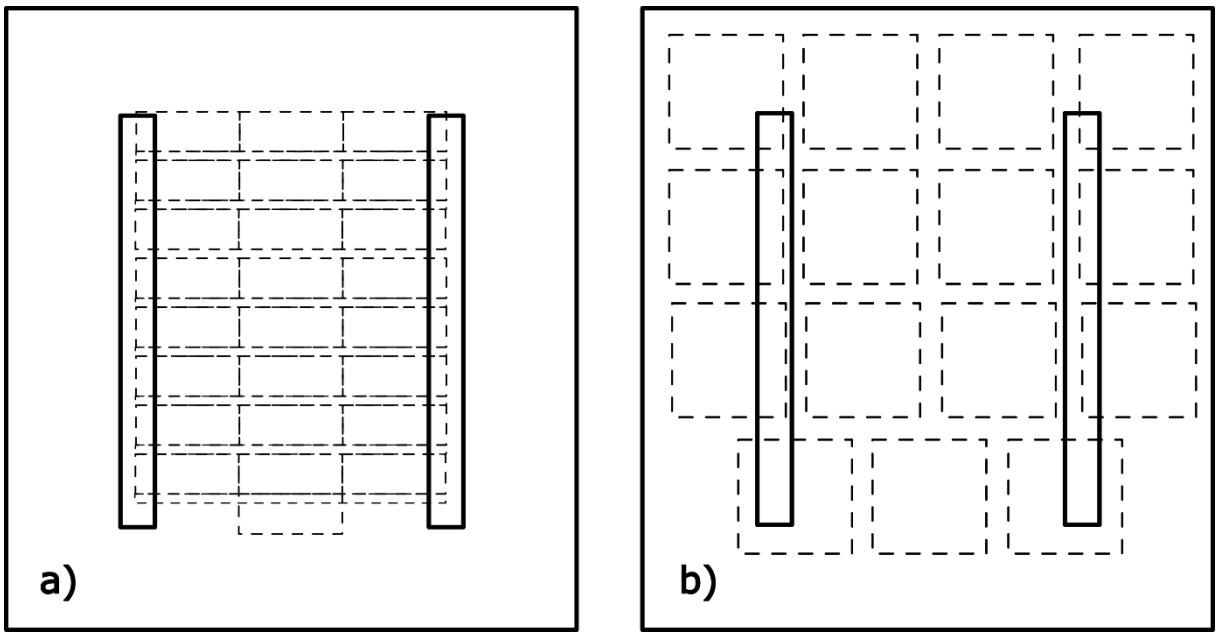


Figure 2.7. Diagram of experimental flask layout for (a) small, vented flasks and (b) large glass bottles. Dotted lines are flasks, solid inner lines are shelf lights.

2.3.2. *Experimental media*

Experimental media was based on the isolation media, a modified Jaworski's medium as previously described in section 2.2.4. The medium shown in Table 2.4 was derived from pilot experiments, based on a 1/10 concentration of the isolation medium. An experimental medium closer to natural concentrations of N and P at each site was used to give more representative results, based on observational data for each site from the NERC DOMAINE programme, as reported in Yates *et al.* (2019). As in the isolation medium, compounds (Table 2.4) were weighed, diluted, and then either passed through a 0.2 µm filter (Filtropur S0.2 Polyethersulfone syringe filter, Sarstedt Ltd., Leicester, UK) to sterilise the stock solutions or autoclaved (sodium bicarbonate and iron solution). Stock solutions of 1000 times concentration were created, usually in 50mL volumes kept at 4˚C in the dark while not in use. The iron citrate solution was sterilised by autoclaving, otherwise large amounts of acid were required to dissolve the compound (which resulted in a low pH in the media). Media was buffered used $NaHCO_3$ and adjusted to pH 7 with 1M HCl. Vitamins were added to most

experimental treatments at the concentrations shown in Table 2.3. As they contain nitrogen they were initially omitted, but determined to be essential for some species in the pilot experiments (section 2.6) and were added to all subsequent trials.

Stock solutions were added aseptically to autoclaved, deionised water via sterile pipetting within a laminar flow hood. The resulting media was stored at 4°C until required or disposed of after 2 months. Stock solutions were kept for up to 6 months at 4°C, with vitamins kept frozen at -18˚C. All chemical compounds used were analytical grade, sourced from Sigma Aldrich (UK) or Scientific Laboratory Supplies (UK), unless otherwise noted.

Table 2.4. Growth medium. Different numbers represent stock solutions made separately. Treatments correspond to A (control), B (nitrate), C (ammonium), D (urea), E (glutamate).

Name	Chemical Formula	Amount in media (mg L^{-1})	Treatment
Iron citrate (1)	**$C_6H_5O_7$**	**1.18**	**A, B, C, D, E**
Boric acid (2)	*H_3BO_3*	*0.5*	*A, B, C, D, E*
Calcium chloride dihydrate (2)	*$CaCl_2.2H_2O$*	*14.7*	*A, B, C, D, E*
Magnesium sulphate (2)	*$MgSO_4.7H_2O$*	*9.6*	*A, B, C, D, E*
Manganese chloride (2)	*$MnCl_2.4H_2O$*	*0.27*	*A, B, C, D, E*
Sodium molybdate VI dihydrate (2)	*$NaMoO_4.2H_2O$*	*1.18*	*A, B, C, D, E*
Zinc chloride (3)	**$ZnCl_2$**	**0.004**	**A, B, C, D, E**
Cobalt chloride (3)	**$CoCl_2.6H_2O$**	**0.004**	**A, B, C, D, E**
Copper sulphate (3)	**$CuSO_4.5H_2O$**	**0.004**	**A, B, C, D, E**
Sodium bicarbonate (4)	*$NaHCO_3$*	*7.8*	*A, B, C, D, E*
Sodium phosphate (5)	**$Na_2HPO_4.12H_2O$**	**4.3**	**A, B, C, D, E**
Sodium Nitrate (6)	*$NaNO_3$*	*15.3*	*B*
Ammonium chloride (7)	**NH_4Cl**	**9.6**	**C**
Urea (8)	*$CO(NH_2)_2$*	*5.4*	*D*
Glutamic acid monosodium salt (9)	**$C_5H_8NNaO_4 \cdot xH_2O$**	**30.4**	**E**

2.4. Analytical methods

2.4.1. *Sampling overview*

Cultures were sampled at the end of each growth period (generally 14 days), and a range of chemical concentration data were determined, as outlined in Figure 2.8. Filters were immediately frozen at -20˚C. Filtrate for determination of nutrient and DOC concentrations was stored in the dark at 4˚C until analysis. Samples for cell counts were preserved using 1% glutaraldehyde and stored at 4˚C. Cell counts took place as soon as possible after sampling, usually within 24 hours. Segmented flow N and P analyses were undertaken within 24 hours of sampling, though this was occasionally not possible owing to instrument failure. DOC analysis also took place within 24 hours where possible. Filters for combustion or isotope analysis were prepared and analysed in larger batches, as they were considered stable while frozen.

2.4.2. *Replication*

Experimental treatments for Chapter 4 and 5 involved 5 replicates of each treatment; control, nitrate, ammonium, urea and glutamate. Experimental treatments for Chapter 7 involved three replicates of each treatment; control, nitrate, ammonium, urea and glutamate. A single sample from each replicate was taken for cell number counting. One sample was taken from each replicate for dissolved nutrient fraction estimation via segmented flow analysis (for N and P fractions) and NPOC (for DOC). Two samples from each replicate were taken for bulk N and C analysis. For isotopic enrichment analysis in Chapter 7, two additional samples were taken for amino acid specific analysis.

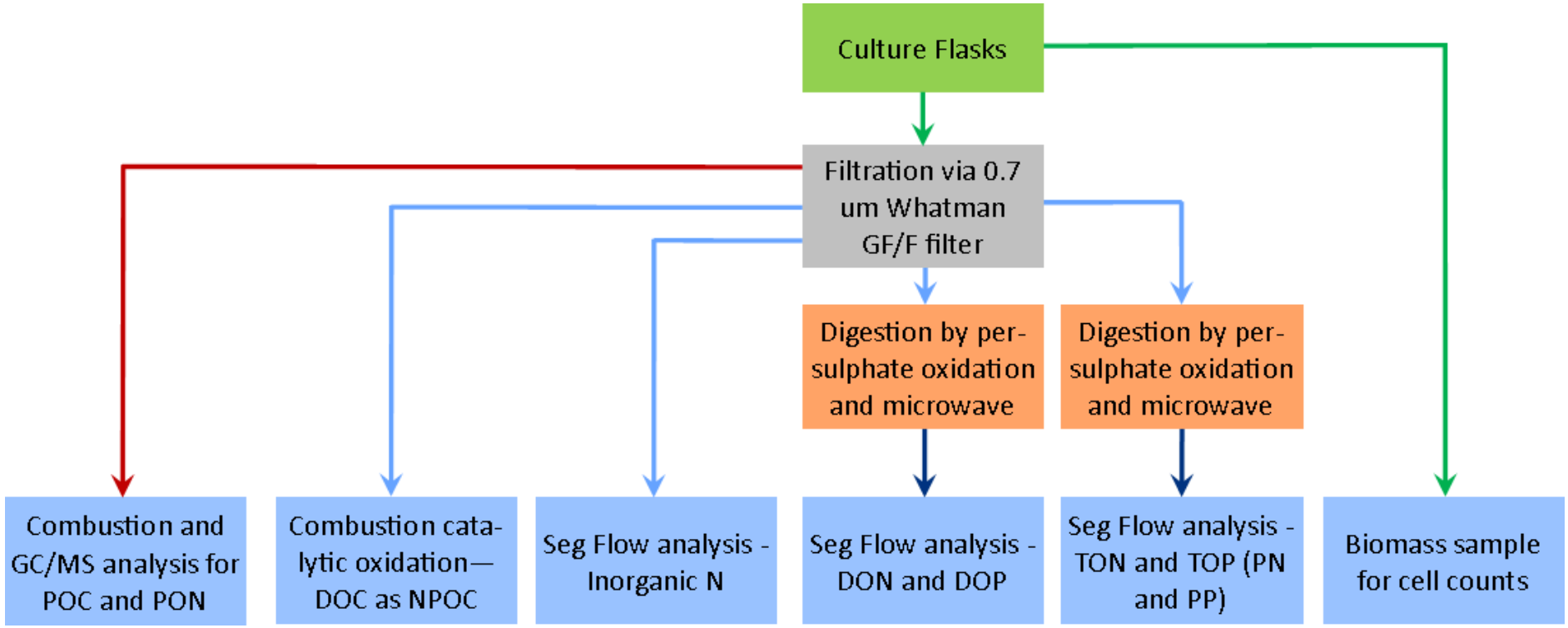


Figure 2.8. Diagram of sampling procedure for experimental analyses after Lloyd et al. (2019). Lines denote type of sample; green - direct from culture, blue - filtrate, dark blue - digested filtrate, red – algae retained on glass filter.

2.4.3. *Cell counts*

Cells were counted manually using Kova™ Glasstic™ haemocytometer counting slides (KOVA™ 22270141, 0.9 µL counting grid, Kova International Inc., US) on an inverted microscope (Nikon Eclipse MA100N, Nikon Metrology Ltd.). Each sample was counted until either 500 cells overall were counted or 3 full counting chambers (3.6 µL total) were counted. Where separate chamber counts were less than 500, these were combined to give one reading. This was repeated for the starting time point at least 3 times (n=3). Each treatment replicate was counted once (n=5). Cell counting was based upon literature approaches to counting algae (Bellinger and Sigee, 2010) and cell counts are presented as average ± standard error (Rier, Stevenson and LaLiberte, 2006).

2.4.4. *Colorimetric determination of dissolved nutrient concentrations*

Dissolved inorganic nitrogen and phosphorous fractions, comprising total oxidised nitrogen (TON = nitrate + nitrite), nitrite (NO_2-N), total ammonium (NH_4-N) and soluble reactive phosphorus measured as orthophosphate (PO_4-P) were determined by colorimetric methods. Nitrate was calculated by difference (TON - NO_2-N = NO_3-N). All concentrations were reported in mg L^{-1}. These inorganic nutrient fractions were measured on filtered samples, which had been passed through a 0.7 µm glass fibre filter. Analyses were performed on a Skalar San ++ (Skalar B.V., Netherlands) multi-channel continuous flow analyser linked to a 1050 series sampling unit. Raw absorbance data was collected and analysed by Skalar San ++ FlowAccess™ V3 software package for Windows®. Five channels were run concurrently for each sample to determine these inorganic nitrogen and phosphorous concentrations, together with the concentration of urea (or ureido-N, as defined by Reay *et al.*, 2019). Samples were split into each parallel channel on the instrument, and then followed the appropriate method module timings, heating and reagent additions as per the Skalar methods for the modules used (see detail below). Standards were used to create calibration curves during each run, and concentrations were reported as mg N L^{-1} or mg P L^{-1} as appropriate. Calibration curves were checked manually and an r^2 of >0.99 used to determine a suitable calibration result before continuing with analysis. All calibrations were 1st order lines. The methods are based on those reported in Yates *et al.* (2016).

An unfiltered sub-sample and a filtered sub-sample were separately digested. These were to breakdown and analyse all particulate and organic compounds in the unfiltered sub-sample, and to analyse all dissolved organic compounds in the filtered samples. For non-dissolved and organic

compounds, a persulfate oxidation in a microwave digestion unit as was performed as outlined below (after Johnes and Heathwaite, 1992; as modified in Yates *et al.,* 2016; 2019). The unfiltered sample digests allowed determination of Total Nitrogen (TN) and Total Phosphorus (TP) concentrations on the Skalar San ++ instrument. The filtered sample digests were similarly analysed to determine Total Dissolved N (TDN) and Total Dissolved P (TDP) concentrations in each sample. This allowed calculation of organic nitrogen and phosphorus compounds within the samples as follows:

$$Particulate\ Organic\ N\ (PON) = TN - TDN$$

$$Particulate\ P\ (PP) = TP - TDP$$

$$Dissolved\ Organic\ N\ (DON) = TDN - TON - NH_4\text{-}N$$

$$Dissolved\ Organic\ P\ (DOP) = TDP - PO_4\text{-}P$$

Reagents were made as per the Skalar method for the relevant analysis and module used (reagent grade chemicals, Sigma Aldrich, Germany). All analyses of time zero media were performed in triplicate and end point treatment experimental replicates measured once per replicate, resulting in different numbers for start point (n=3) and end point (n=5) averages. System autosampler timing was set to the time required for the longest method required (urea); 90 seconds sampling time and 75 seconds wash time between each sample or standard measure. After every 10 samples a drift and wash were added. These were used to drift correct and baseline correct each section of a run. These were also used to compare reagent effectiveness and potential contamination present between runs of the machine on separate days. All glassware and other equipment used were cleaned by soaking in 5% HCl for 24 hours, then triple rinsed with MilliQ water before drying.

2.4.4.1. Standard solutions

Stock standard solutions were made up monthly and working solutions prepared daily. Stock solutions were individual compounds: nitrite (sodium nitrite, $NaNO_2$, 100 mg N L^{-1}); nitrate (sodium nitrate, $NaNO_3$, 1000 mg N L^{-1}); ammonium (ammonium chloride, NH_4Cl, 100 mg N L^{-1}); urea (urea, CH_4N_2O, 100 mg N L^{-1}) and phosphorus (potassium dihydrogen phosphate, KH_2PO_4, 1000 mg P L^{-1}). Reagents were dried for >24 hours in a desiccator before stock solutions were weighed out. Working solutions were mixed standard solutions and covered the ranges; 0 - 10 mg N L^{-1} nitrate, 0 - 1 mg N L^{-1} nitrite, ammonium and urea and 0 - 1 mg P L^{-1} orthophosphate.

2.4.4.2. Total oxidised nitrogen

Total oxidised nitrogen (TON) comprises nitrate as NO_3-N and nitrite as NO_2-N, and is based upon the hydrazine reduction method as per Henriksen and Selmer-Olsen (1970). Nitrate is reduced by hydrazinium sulphate to nitrite, and the combined nitrite (previously present and reduced nitrite) is measured by converting to a diazo compound using sulphanilamide and N-(1-napthyl)ethylenediamine dihydrochloride which forms a coloured, pink azo dye. This colour is measured spectrophotometrically at 540 nm. An additional dilution with a buffer solution occurs at the start of the module for higher concentration samples. The sample is then run through a membrane alongside sodium hydroxide after the buffer is added and heated to 40°C before the colour reagent is added.

Reagents were prepared weekly. The buffer contained potassium sodium tartrate ($C_4H_4O_6KNa.4H_2O$, 33 g L^{-1}), tri-sodium citrate ($C_6H_5O_7Na_3.2H_2O$, 24 g L^{-1}) and Brij 35 (30%) (3 mL L^{-1}). The sodium hydroxide reagent contains sodium hydroxide (NaOH, 6g L^{-1}) and Brij 35 (30%) (3 mL L^{-1}). The hydrazinium sulphate solution consisted of hydrazinium sulphate ($N_2H_6SO_4$, 2.3 g L^{-1}) and 1.5 mL of a stock copper (II) solution [Copper (II) sulphate ($CuSO_4.5H_2O$, 12 g L^{-1}) made monthly]. The colour reagent contained o-Phosphoric acid (85%) (H_3PO_4, 150 mL L^{-1}), sulphanilamide ($C_6H_8N_2O_2S$, 10 g L^{-1}) and N-(1-Naphthyl)ethylene diamine dihydrochloride ($C_{12}H_{16}Cl_2N_2$, 0.5 g L^{-1}). Water and acid were added first to the colour reagent flask, then thoroughly coated internally with the slightly diluted acid before adding the other reagents, as otherwise a colour is prematurely developed and analysis is affected.

2.4.4.3. Nitrite and Nitrate

Nitrite (as NO_2-N) is determined using a colorimetric method similar to total oxidised nitrogen as described above, but excluding the reduction step. It too is based upon the method by Henriksen and Selmer-Olsen (1970). After passing through a membrane dialysis, samples are reacted with sulphanilamide to produce a diazo dye, coupled with N-(1-naphthyl)ethylenediamine dihydrochloride under acidic conditions to produce a pink-purple colour. This is then measured spectrophotometrically at 540 nm as per the Skalar manual (Catalogue number 467-323 issue 080714/MH/99290607).

Reagents were prepared weekly. Buffer contained potassium sodium tartrate ($C_4H_4O_6KNa.4H_2O$, 33 g L^{-1}), tri-sodium citrate ($C_6H_5O_7Na_3.2H_2O$, 24 g L^{-1}) and Brij 35 (30%) (3 mL L^{-1}). The colour reagent contained o-Phosphoric acid (85%) (H3PO4, 150 mL L-1), sulphanilamide ($C_6H_8N_2O_2S$, 10 g L^{-1}) and N-(1-Naphthyl)ethylenediamine dihydrochloride ($C_{12}H_{16}Cl_2N_2$, 0.5 g L^{-1}). Water and acid were added first to the colour reagent flask thoroughly coated internally with the slightly diluted acid before adding the other reagents otherwise a colour is prematurely developed.

Nitrate (NO_3-N) concentrations are calculated by subtracting nitrite concentrations from TON concentrations.

2.4.4.4. Ammonium

Total ammonium (as NH_3-N plus NH_4-N) was determined using a modified Berthelot reaction, based on Crooke and Simpson (1971). Samples were buffered and ammonia chlorinated to monochloramine which reacts with salicylate to 5-aminosalicylate. After oxidation, a green coloured compound is formed. This is then measured spectrophotometrically at 660 nm. This method is detailed in the Skalar manual (Catalogue number 155-318w/r issue 080714/MH/99290607).

Reagents were prepared weekly. The buffer solution consisted of potassium sodium tartrate ($C_4H_4O_6KNa.4H_2O$, 33 g L^{-1}), tri-sodium citrate ($C_6H_5O_7Na_3.2H_2O$, 24 g L^{-1}) and Brij 35 (30%) (3 mL L^{-1}). Dichloroisocyanuric reagent consisted of dichloroisocyanuric acid sodium salt ($C_3N_3O_3Cl_2Na.2H_2O$, 2 g L^{-1}). Salicylate solution contained sodium hydroxide (NaOH, 25 g L^{-1}) and sodium salicylate ($C_7H_5NaO_3$, 80 g L^{-1}). The nitroprusside solution used sodium nitroprusside alone ($Na_2[Fe(CN)_5NO].2H_2O$, 1 g L^{-1}).

2.4.4.5. Urea (ureido-N)

Urea determination used a diacetyl monoxime reaction (Skalar manual, Catalogue number 612-001 issue 080714/MH/99290607). Each sample was diluted in a sodium chloride solution. An acid catalyst was then added, then the diacetyl monoxime colour reagent. The sample was heated to 90°C where urea and diacetyl monoxime combines and produces a colour that is intensified by semicarbazide. Urea concentrations were then measured spectrophotometrically at 520 nm. There has been evidence that this method may produce colour in the presence of compounds other than urea, specifically other ureido group compounds as noted by Reay *et al.* (2019c). However, there should be fewer ureido group compounds initially present in the experimental media compared to

soil (*op. cit.*). As the method is not urea specific, urea measured in this way is referred to as 'ureido-N' in this work.

Salt solution and acid reagent for this ureido-N method were prepared bi-monthly, while the colour reagent was made weekly. The saturated salt solution contained sodium chloride solution (NaCl, 300 g L^{-1}) and Brij 35 (30%) (3 mL L^{-1}). The acid reagent consisted of sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$, 50 g L^{-1}) and sulfuric acid (95%) (H_2SO_4, 588 mL L^{-1}). Acid was added carefully while continuous cooling took place, resulting in a c.a. 60% H_2SO_4 acid solution. The colour reagent contained diacetyl monoxime ($C_4H_7NO_2$, 16.5 g L^{-1}), semicarbazide hydrochloride (CH_6ClN_3O, 0.2 g L^{-1}), manganese (II) chloride ($MnCl_2.4H_2O$, 52.3 g L^{-1}) and potassium nitrate (KNO_3, 1 g L^{-1}).

2.4.4.6. Orthophosphate

Soluble reactive phosphorus measured as orthophosphate (PO_4-P) was determined using a molybdate reaction based on Murphy and Riley (1962) and the Skalar manual (Skalar methods, Catalogue number 503-382w/r (+P3) issue 080714/MH/99290607). Ammonium heptamolybdate and potassium antimony tartrate in an acidic medium react with orthophosphate in the sample to create an antimony-phosphate-molybdate compound. Ascorbic acid is used to reduce it to a strong blue colour that is measured spectrophotometrically at 880 nm.

Reagents were made fresh weekly, with the ascorbic acid checked daily as it is less stable than the other reagents (even when stored overnight at 4°C in the dark). As it decays it develops a colour, and this can lead to unstable output on the orthophosphate channel. The ammonium heptamolybdate reagent is made with sulphuric acid (95%) (H_2SO_4, 40 mL L^{-1}), ammonium heptamolybdate $(NH_4)6Mo_7O_{24}.4H_2O$, 4.8 g L^{-1}) and Skalar proprietary phosphate-free surfactant (FFD6, 2 mL, Skalar Analytical). A stock potassium antimony tartrate solution was made of potassium antimony (III) oxide tartrate ($K(SbO)C_4H_4O_6.3H_2O$, 3 g L^{-1}). The ascorbic acid reagent contained L(+)Ascorbic acid ($C_6H_8O_6$, 18 g L^{-1}) and 20 mL of the stock potassium antimony tartrate solution.

2.4.4.7. Total dissolved nitrogen and total dissolved phosphorus

The concentrations of total dissolved nitrogen (TDN) and total dissolved phosphorus (TDP) using filtered samples was determined through a persulphate digestion, and similarly total nitrogen

(TN) and total phosphorus (TP) were determined on unfiltered samples. This followed the method developed by Johnes and Heathwaite (1992) as modified by Yates and Johnes (2013) and used by Yate *et al.* (2019). It relies on presenting the samples with an oxidising reagent, and digesting them at high temperature and pressure in a microwave digestion unit, releasing all nitrogen in the form of TON, and all phosphorus in the form of PO_4-P. These are then determined colourimetrically on the Skalar San ++ as described above. The digestion solution contained sodium hydroxide (NaOH, 4.5 g L^{-1}) and potassium peroxodisulphate ($K_2S_2O_8$, 22.5 g L^{-1}). This is difficult to dissolve at room temperature and was therefore heated gently on a magnetic stirring plate at <40°C to dissolve. The digestion reagent was generally prepared on the day of use, but occasionally was kept refrigerated overnight, and gently re-heated (<40°C) before use.

Sample volume of 6 ml was placed in a microwave digestion tube (55 mL MARSXpress Vessels, TFM, CEM Microwave Technology Ltd., Buckingham, UK) together with 3 ml of the digestion reagent. A pressure cap and lid were added and tightened immediately. Each was then placed into a carousel and placed in the CEM MARS Xpress Microwave Digestion Unit (MARS 5, CEM Microwave Technology Ltd., Buckingham, UK). Tubes were heated and held above 170˚C for 30 mins, then cooled. Cooled samples were then analysed on the Skalar San ++ as described above, and the resultant dilution (0.666 of original concentration) is accounted for in the results presented.

2.4.4.8. Determination of organic nitrogen and organic phosphorus

The TDP and TDN after microwave digestion and the inorganic nitrogen and phosphorus fractions were used to determine the organic fraction in the media. This was determined as shown below for each filtered sample.

$$DON = TDN - TON - Ammonium$$

$$DOP = TDP - PO4$$

2.4.5. *Dissolved organic carbon*

Dissolved organic carbon (DOC) was determined on sample filtrate as non-purgeable organic carbon (NPOC) by acidification with HCl and high catalytic oxidation (680˚C), as measured using a Shimadzu TOC-L analyser (Shimadzu corp., Kyoto, Japan). Each sample (100 µL) was injected 3 to 5

times, until the software determined <2% variation between sample injections and measures. Standard concentrations of DOC were used to create calibration curves, using a 1st order line and r^2 value of >0.999 to determine a suitable calibration. Standards were diluted TOC certified stock standards (1000 mg C L^{-1}, Sigma TraceCERT®, Sigma Aldrich).

Samples were run in triplicate for the start point of each treatment, and each experimental replicate run separately (start n=3, end n=5 per treatment). Results were output as mg C L^{-1}. Glassware used for dissolved organic carbon was soaked in 5% HCl for 25 hours, then triple rinsed with ultra-pure (18 MΩ) water before being placed in a furnace at 450°C for 4 hours. Caps were cleaned by soaking in 5% HCl for 24 hours and triple rinsing with ultra-pure (18 MΩ) water. Foil was used directly from the packaging. This is based on previous work using this TOC analyser (Holland *et al.*, 2019).

2.4.6. *Nutrient analyses on algal biomass*

2.4.6.1. Elemental carbon and nitrogen analysis

The sampling method was based on techniques used in Silkina *et al.* (2015) and others (Mayers, Flynn and Shields, 2013). The method measures the total elemental carbon and nitrogen present. Samples were collected on glass filters (13 mm, 21 mm, 25 mm diameter, GF/F ~0.7µm pore size Whatman® Filters from Sigma Aldrich, Germany) using a vacuum filtration holder (Sterifil® Aseptic Holder, Merck Millipore, UK) and filter holders (13mm polypropylene, Swinnex™ Filter Holders, Merck Millipore, UK). Later in the project, 13 mm GF/F filters were no longer available. To maintain the same pore size and ensure samples between experiments were comparable, filters larger than 13 mm were die-cut to size before use and then prepared as normal. Filters were pre-combusted (450 °C, 4 hrs) before use to remove any residual carbon.

Samples were rinsed with deionised water, then frozen at -20*C and analysed in batches. Samples were gently dried at 45°C for 24hrs, and then wrapped in tin disks (16 mm tin disks, Elemental Microanalysis Ltd, Okehampton, UK). Samples were analysed on an elemental analyser (Vario PYRO cube, Elementar Analysensysteme GmbH, Hanau, Germany) calibrated with sulphanilamide (N: 16.26%, C:41.81%, S: 18.62%). Analytical grade gases (O_2, N_2, CO_2, He) were used for analysis (BOC Ltd, UK). The proprietary software, IonOS, was used to run the machine for CHNS analysis. Briefly, samples were combusted, fed through a water trap and copper reduction tube before a CO_2 trap. N_2 was measured first, then CO_2 released via heating and measured second. Each

sample was run in duplicate unless low sample concentration meant two replicates were run together as one sample to aid accurate detection.

During each run, isoleucine and sulphanilamide check standards were run, both to verify the low algae concentrations measured and the linearity of machine at this level. Sulphanilamide was weighed on a microbalance (mg balance, 4 decimal place) into tin cups (8 x 5 mm tin capsules, D1009, Elemental Microanalysis Ltd, Okehampton, UK) and folded. Sulphanilamide check standards ranged between 0.1 mg to 2.5 mg. To check concentrations of nitrogen and carbon below this, isoleucine was used. Standards were analysed every 10-15 samples in a run.

An isoleucine ($C_6H_{13}NO_2$) stock solution of 9.37 g per mL was pipetted into tin capsules (12 x 8mm tin capsules, D4094, Elemental Microanalysis Ltd, Okehampton, UK) using micropipettes to cover a range of nitrogen and carbon values per capsule. These ranged from 2 µg N – 100 µg N, 12 µg C – 600 µg C. Capsules were held in a sterile microwell plate while filling, and dried gently at 45°C for 24 hours. They were then folded and analysed identically to samples. Drift and blank correction by software was manually checked. Blanks used in the analysis included empty tin disks, empty capsules, unused filters in tin disks and DI washed filters in tin disks. Each batch of filters combusted concurrently before analysis were analysed independently. Only one set of DI washed filters showed nitrogen or carbon significantly above background levels. This value was removed from samples using that batch of filters during post-processing of data.

2.4.6.2. Stable isotope methods

Isotope enrichment is normally reported in delta notation. Absolute elemental ratios are not reported, but the isotope ratios relative to international standards are used. This represents the change in samples as compared to an international standard of isotopic ratios. The analytical standard used for analytical work in this thesis is atmospheric nitrogen for $\delta^{14}N/\ \delta^{15}N$ values, and USGS standards 61, 62 and 63 (caffeine) for $\delta^{12}C/\delta^{13}C$, allowing comparison to δ_{VPDB} (the Vienna Pee Dee Belemnite fossil carbon isotope ratio). Calculating sample values from these standards are shown below in equation 1 and 2. These equations are based on definitions by McKinney *et al.* (1950) and subsequently modified for ease of use as per mille (‰) by Farquhar *et al.* (1989). The $\delta^{13}C$ and $\delta^{15}N$ are reported as per mille (‰) but remain a unit-less ratio of sample compared to the standard. As changes tend to be low numerically compared to standards, using per mille allows easier comparison of differences.

$$\delta\ ^{13}C_{sample} = \left(\frac{^{13}C_{sample} \,/\, ^{12}C_{sample}}{^{13}C_{standard} \,/\, ^{12}C_{standard}} \right) - 1$$

Equation 1

$$\delta\ ^{15}N_{sample} = \left(\frac{^{15}N_{sample} \,/\, ^{14}N_{sample}}{^{15}N_{standard} \,/\, ^{14}N_{standard}} \right) - 1$$

Equation 2

Equations for calculating $\delta^{13}C$ and $\delta^{15}N$ ratios, after Farquhar et al. (1989) and Mook (2000).

The main isotope of nitrogen is ^{14}N, with ^{15}N used here as a stable isotope in the treatment compounds used in the experiments reported in this thesis (nitrate, urea, ammonium and glutamate). In each inorganic and dissolved organic N compound used here, all nitrogen atoms within a molecule were labelled with the heavier isotopic fraction. The major fraction of carbon is ^{12}C, with ^{13}C used here as the stable isotope within organic treatment compounds (urea and glutamate). All carbon atoms within isotopic DON compounds are labelled.

Labelled compounds did not constitute 100% of the substrate presented to experimental algae cultures, as fully labelled compounds tend to alter the uptake and processing of compounds (Andriukonis and Gorokhova, 2017). Instead, 5 at% of nitrogen was used to calculate concentrations of labelled versus unlabelled substrate. Of the total nitrogen substrate concentration, 5% of nitrogen was isotopically labelled and 95% of nitrogen remained unlabelled.

For bulk stable isotope analysis, an Isotope Ratio Mass Spectrometer (IRMS) was used (Elementar Isoprime Precision, Elementar Analysensysteme GmbH, Hanau, Germany). This was linked to the elemental analyser (EA) previously described in section 2.4.6.1 for elemental carbon and nitrogen analysis. Samples for stable isotope analysis were analysed immediately via IRMS after the same sample had undergone elemental carbon and nitrogen analysis. The output valve of the EA machine fed directly into the IRMS. As such, samples and standards were prepared as previously described previously.

The IRMS was calibrated against international reference standards (caffeine: USGS61, USGS62, USGS63, USGS, United States). The proprietary software, IonOS, was used to run the machine for IRMS analysis. A lower limit of analysis was not provided by the manufacturer. As sample values of IRMS detector current approached 0 μA, variance increased substantially. Analysis of a range of reference standards and in-house isoleucine standard determined most variance to increase markedly below ~0.5 μA of detector current. Due to this, sample values below 0.5 μA were considered unreliable, and the bulk isotope results were omitted. This is mentioned in later results, where applicable.

2.4.7. *Sampling times*

Experiments in Chapter 4 and 5 all had end point sampling only. These varied slightly, but samples were usually at taken on day 0 and day 14 of each experiment, and are mentioned in each results section in the relevant chapters. Samples were only taken at the start and end of each experiment for these chapters.

The experiment in Chapter 6 involved a time course over seven days. The timings of each sample taken in this period are shown in table 2.5. These would ideally have been taken at more grouped time points, e.g. all at 1 h, then 3h etc.. Physical constraints on sampling meant this was not possible, and samples were taken as fast as physically possible. All data for this chapter have been plotted with their respective sampling times.

Table 2.5. Sampling time by treatment for Chapter 6, isotope enrichment experiment.

	Treatment sampling point (h)				
Day	Control	Nitrate	Ammonium	Urea	Glutamate
1	00:00	00:00	00:00	00:00	00:00
1	01:10	01:35	02:15	02:35	03:00
1	03:40	04:05	04:30	05:05	05:35
1	06:15	06:40	07:00	07:30	07:55
2	23:55	24:20	24:50	25:20	25:55
3	47:35	48:10	49:00	49:20	49:55
7	126:00	126:30	127:10	127:45	128:15

2.4.8. *Genetic analysis*

As the cultures used for experimental results in later chapters were unknown isolates, genetic testing was used to identify which species had been isolated. The bioinformatic analyses and sample results are shown in Chapter 3.

2.5. Statistical analysis

All data was blank corrected before use. Statistical analyses were undertaken using RStudio software (RStudioTeam, 2016). One way ANOVA was used for each type of results among one species or experiment. When comparing multiple experiments, a two-way ANOVA was used. For both, post-hoc multiple pairwise testing was carried out using Tukey's post-hoc multiple pairwise testing. Data were tested for normality and homogeneity of variance using the Shapiro-Wilk and Levene's tests respectively (Brailsford *et al.*, 2017; Fuentes-Grünewald *et al.*, 2015; Fernández-González and Marañón, 2021). If the conditions for normality were not met, a Kruskal-Wallis test was performed. If homogeneity was not seen, a Welch's test was used with a Games-Howell post-hoc test (Brailsford *et al.*, 2021). Error bars in charts show mean values ± SEM (standard error of the mean). The number (n) in each group is reported separately in each case, as they alter by experiment and analyses.

Many biological experimental treatments use 3 treatment replicates and are not always clear in explaining why that number is chosen. This impacts on the statistical power of the experiment and results (Krzywinski and Altman, 2013). More replicates were considered here due to a potentially small difference in growth between treatments. Difficulties with slow responses of the cultures or growth of negative viruses or amoeba which could potentially occur, could be mitigated by including larger replicate numbers to avoid substantial bias resulting from the loss of one culture replicate. As cultures were isolated from the environment, the likely responses to experimental conditions were unknown before experiments took place. The variability within the experiment was also unknown, as there was no prior research at the same laboratory to estimate from. A power analysis of the sample size for a test comparing 2-tailed means supported a sample size of 5 for most experimental treatments (*Power and Sample Size Calculators*, 2021; Bate and Clark, 2014). This was based on mean cell numbers, estimated to start at 20,000 cells per mL and modestly increase to 50,000 cells per mL after two weeks in positive treatments.

2.6. Pilot experiments

Algal isolation and cultivation in sufficient abundance for replicated bioassay experiments is time consuming. In order to ensure that the programme of experiments would be successful, and to optimise the experimental design for the main experiments, an initial set of pilot experiments were set up with three treatments; a negative control, nitrate addition and urea addition. All pilot treatments were given the same trace metal and phosphorus solutions to ensure that growth was not limited by P or micronutrient availability.

Pilot experiments did not all go smoothly, and issues with algal growth occurred in some of them. These were then resolved or adjusted through alteration of the methodology used, and is explained alongside each pilot experiment that resulted in a change to the methodology. This led to the final methodology used for the main experimental results. A summary of each pilot experiment is given in table 2.6. Pilot experiments 1-3 resulted in cell growth and showed differences between treatments. These are shown later in Figures 2.9-2.11. All pilot experiments had 5 replicates for each treatment.

Four experiments resulted in no cellular growth. Potential reasons for this are listed in table 2.6, and adjustments were made to subsequent pilots/experiments. As the data from these isolates was not complete nor comparable, they were not prioritised for genetic identification. Unless they were later used for experiments and identified through genetic analysis, species are labelled c.f. with an identification at genus level based on morphology alone.

Pilot experiments 1-3 showed that isolates could grow on nitrate and urea, generally utilising media nitrogen and phosphorus to do so. Most showed an increase in media DOC, possibly due to cellular exudates or cellular breakdown. Light levels, pH, media volume, cell preservative, metal media solutions were all altered due to these pilot experiments, resulting in the final experimental design used. These methodology adjustments included;

- Addition of a $NaHCO_3$ buffer to media.
- Autoclaving some metal solutions to remain in solution.
- Addition of vitamins B1, B7 and B12 to media.
- Increase in initial inoculation cell count.
- Reduction in light level to 50-80 µmol m^{-2} s^{-1}.
- Increase of culture volume to allow for more replicates and larger sample volume.

Table 2.6. Summary of each pilot experiment. Changes made are cumulative, with new changes being added to the existing experimental protocol. Cell counts are rounded to nearest 1000. Experiments with multiple treatments had a negative control with either two nitrogen treatments (nitrate and urea), or four nitrogen treatments (nitrate, ammonium, urea and glutamate).

Experiment	Species	Duration (days)	Inoculation count (cells mL^{-1})	Start date	Treatments	Changes made for this experiment	Problems
Pilot 1	PF7a	29	300,000	30/05/2018	3	Single media nutrient samples for each treatment.	Low replicate number, more culture volume needed.
Pilot 2	PF 6	23	100,000	10/07/2018	3	All 5 treatment vials sampled for nutrients, analysed as in main experiments.	Issues with light, clustered biomass in corners of vessels
Pilot 3	cf.Euglena sp.	19	50,000	10/08/2018	3	Light levels lowered with paper strips on lights.	Cells shrinking in glutaraldehyde – difficult re-counting cells.
Pilot 4	PF 6	28	213,000	13/08/2018	2	Growth curve to check end point is in stationary phase, triplicate samples of control and nitrate.	Not all nitrogen treatments used.
Pilot 5	PF 6	4	224,000	13/09/2018	5	Using Lugols to preserve cells and cell counting within 24 hours. Media expanded and two solutions acidified to dissolve. Media adjusted to pH 7 to start.	High pH levels after a few days.
Pilot 6	cf.Euglena sp.	5	71,000	19/09/2018	5	Use of autoclaved media for iron and calcium chloride.	No growth seen
Pilot 7	cf.Euglena sp.	30	245,000	05/10/2018	5	Media buffered with $NaHCO_3$, autoclaved iron and calcium stocks.	No growth seen.
Pilot 8	Filamentous cyanobacteria	6	21,000	05/11/2018	5	Likely need for higher inoculation level. Potentially lacking in vitamins, and so isolation media vitamins are subsequently added.	No growth seen.

2.6.1. *Pilot experiment 1*

The first pilot experiment used a spherical, single-celled eukaryotic green algae species isolated from the River Sem, Hampshire Avon catchment. Conditions were largely as described in the methodology, apart from the following. It ran for 29 days from 30 May 2018, with three treatments used (negative control, nitrate addition and urea addition). The iron solution was acidified to remain in suspension, and no additional vitamins or $NaHCO_3$ were added. Light levels were 50-120 µmol m^{-2} s^{-1} across the shelf, with a thin piece of paper directly above the lights and the rest of the shelf left uncovered. Culture volume in each treatment was 25 mL. The algal growth and nutrient chemistry response to each treatment is presented in Figure 2.9.

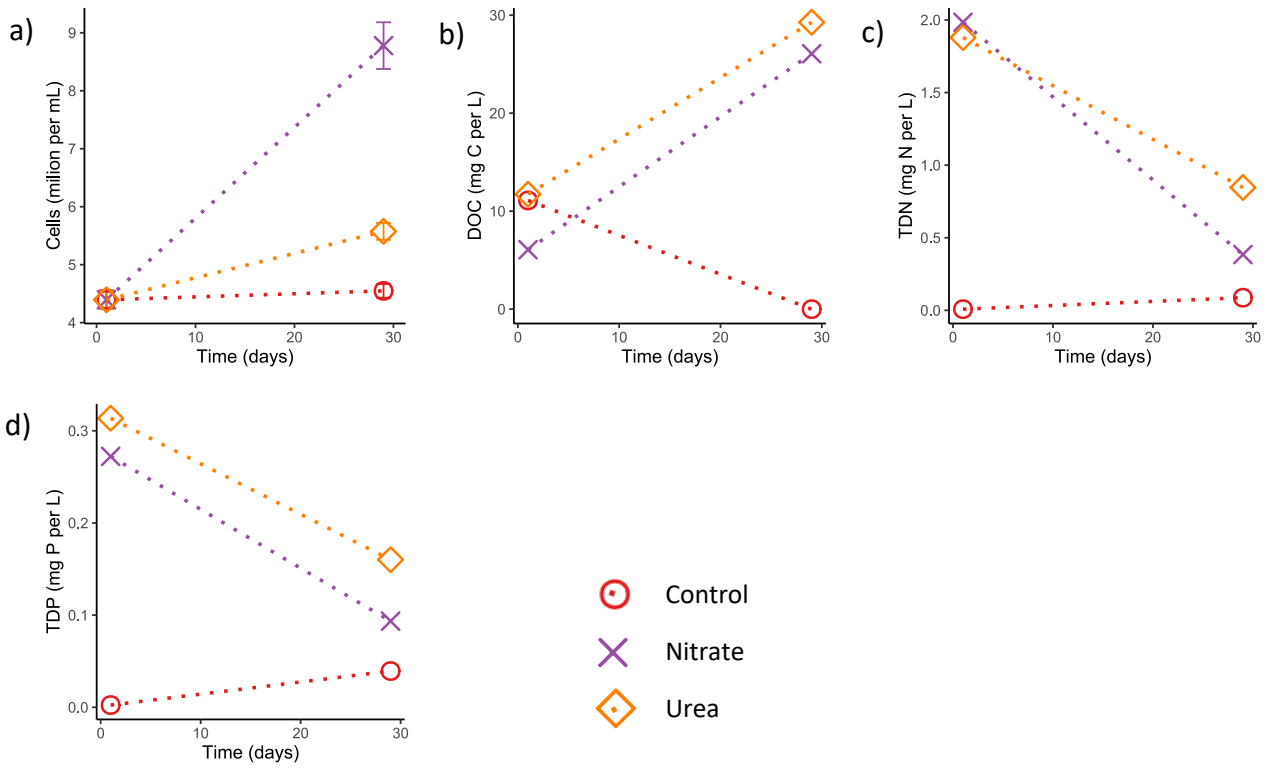


Figure 2.9. Pilot 1 experimental results showing cell counts (a), DOC (b), total nitrogen in media (c) and total phosphorus in media (d). DOC, TN and TP points are all n=1 for each point. Cell counts are n=5 with error bars showing standard error.

Cell numbers increased in the nitrate treatment as compared to the control and starting numbers in each treatment (Figure 2.9, a). Cell numbers also increased in the urea treatment, though less than that shown by nitrate addition treatment. Both nitrogen addition treatments showed an increase in DOC (Figure 2.9, b), and a decrease in TDN (Figure 2.9, c) and TDP (Figure 2.9,

d) concentrations in the media. This suggests that both nitrogen and phosphorus were removed from suspension and used to support cellular growth. Increased DOC is seen, likely due to the grow of algae causing carbon fixation, and a leak or loss of DOC compounds to the media from the living cells. More replicates were needed for TDN and TDP analyses, and culture volume needed to be increased to do so.

2.6.2. *Pilot experiment 2*

The second pilot experiment used 30mL cultures, with a different, single celled green eukaryotic species isolated from the River Nadder (c.f. *Chlorella spp*.). The experiment ran for 23 days from 10 July 2018 with 30mL total culture volume.

Though morphologically similar to the species used in the Pilot 1 experiment, this species seemed to be more motile during the course of the experiment. Biomass visibly accumulated in the corners of culture vessels, perhaps seeking shading from the thicker edges of the plastic culture flasks or preferring to live amongst other cells in a more 3D environment.

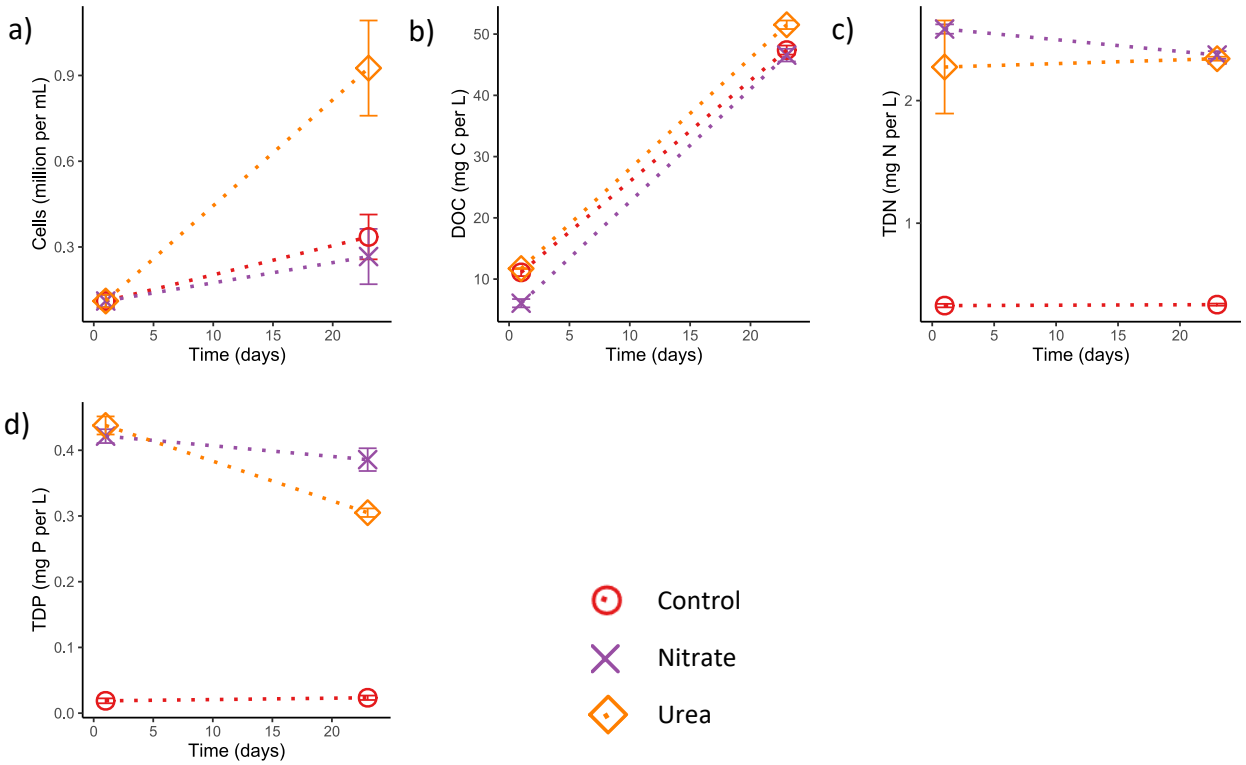


Figure 2.10. Pilot 2 experimental results showing cell counts (a), DOC (b), TDN in media (b) and TDP in media (b). Day 0 points are n=3, day 23 points are n=5, with error bars showing ± standard error.

Cell counts at the end of the experiment were higher in the urea treatment than the nitrate or control treatments (Figure 2.10, a). This showed a different response to the first pilot experiment, perhaps due to being a different species, environmental differences or different starting cell numbers. DOC increased for all treatments, including the control. This may be due to a response to the change in conditions causing carbon-based exudates from all treatments. The increase in DOC may also be as a stress response to the change in conditions. No treatments showed much change in total nitrogen (Figure 2.10, c). Nitrogen is needed for cell growth, so this highlights that internal stores of nitrogen were likely to have been used instead and a longer acclimation period was needed to deplete internal stores before experimental use of cultures. TDP notably decreased from initial concentrations in the urea treatment, potentially to support the growth seen in the treatment.

As neither nitrogen or phosphorus was reduced to control levels in either nutrient treatment, it does not appear these were limiting algal growth. Literature points to rapid uptake of limiting nutrients (ref). Light stress was considered a possibility, and lighting reduced to 50-70 µmol m^{-2} s^{-1} with different thickness of paper. Later experiments revealed a necessity of vitamins for some species.

2.6.3. *Pilot experiment 3*

The second pilot experiment used 30mL cultures, with a highly motile and flagellated Euglenoid species isolated from the River Sem, Hampshire Avon catchment. The experiment ran for 19 days from 10 August 2018 with 30mL total culture volume.

Both nitrate and urea treatments saw growth above the control and of a similar magnitude to each other (Figure 2.11). The urea treatment started at a higher DOC concentration, and all three treatments increased in DOC to a similar end point. TDN and TDP concentrations in the media decreased over the experiment to levels approaching the control. This suggests nitrogen and phosphorus were taken up by the cultures and were used for growth. However, the control treatment media increased slightly from around 0.01 mg P L^{-1} to 0.08 mg P L^{-1} during the course of the experiment, suggesting the algae or bacteria in the culture had a store of phosphorus that leaked to the media over time, potentially during cell lysis or excretion of phosphorus containing compounds.

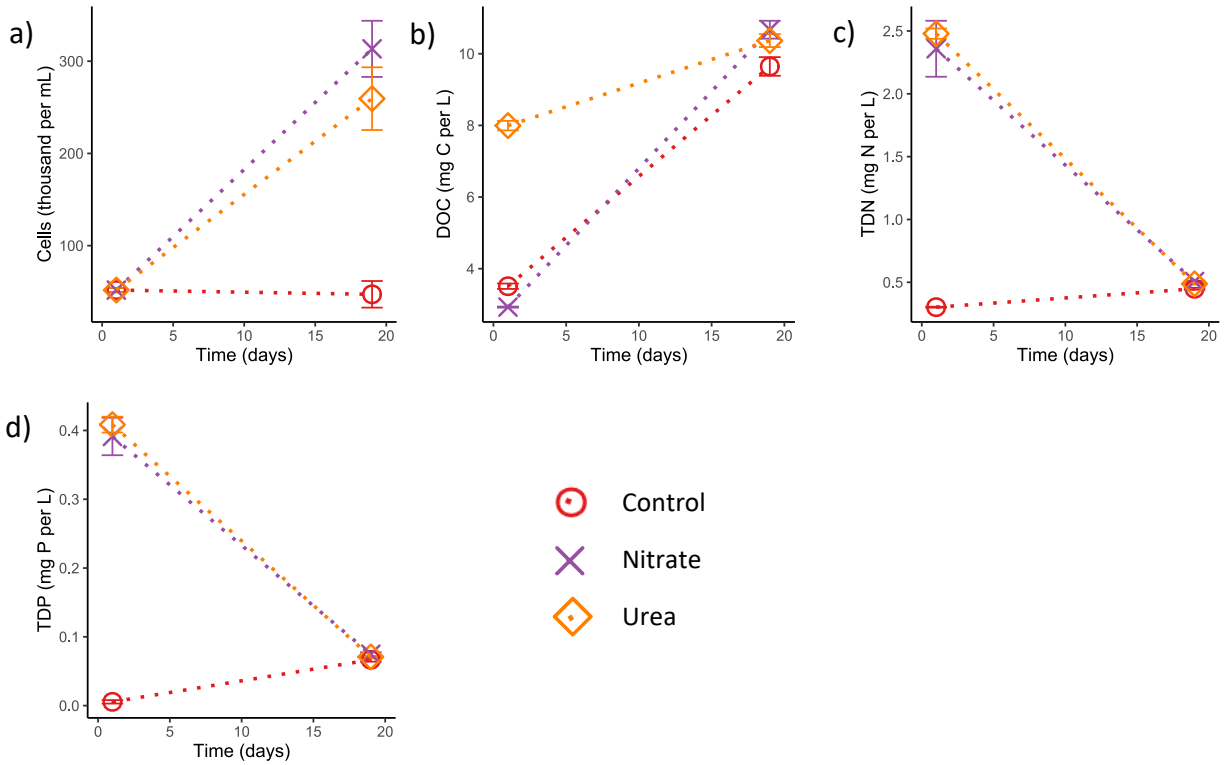


Figure 2.11. Pilot 3 experimental results showing cell counts (a), DOC (b), TDN in media (b) and TDP in media (b). Points are n=5 with error bars showing standard error.

There were issues with using glutaraldehyde to store cell counting samples. Both buffered and unbuffered solutions altered the shape and reduced the size of the cells, making counting more difficult and less accurate. Lugol's iodine solution was used instead for later experimental samples.

2.6.4. *Pilot experiment 4*

This experiment refined the timeline required for experimental treatments. It used a single-celled green algae (species PF6) and ran for 28 days from 13 August 2018. As end-point sampling was intended, this checked that the timing for the stationary phase sampling was appropriate. This used 30 mL cultures, in triplicate. Samples were only taken for cell counts, and are shown in figure 2.12. This confirmed that this species reached stationary phase after around day 11. As some species may vary in growth rates, or the lag phase may differ, growth of at least 14 days was later used to ensure end point sampling took place in the stationary phase.

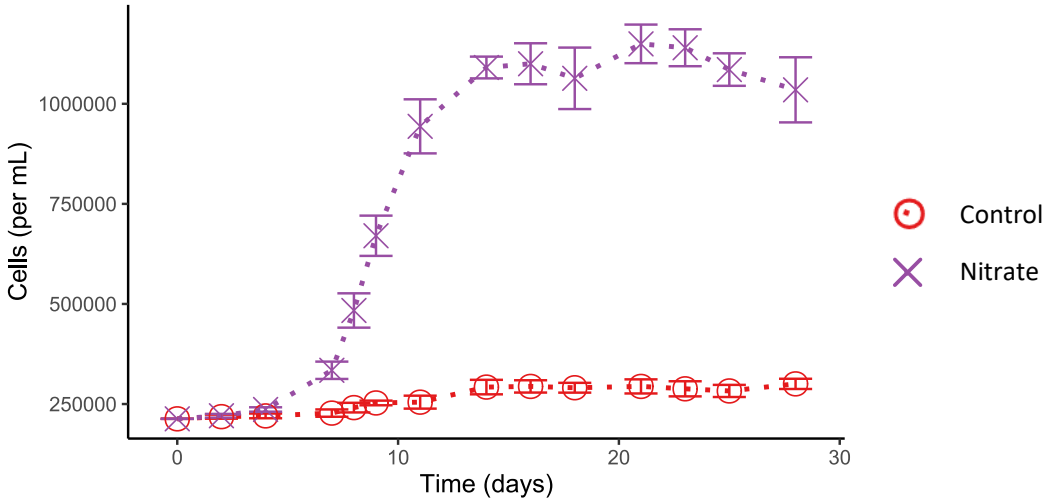


Figure 2.12. Pilot 4 experimental results showing cell counts. Points are n=3 with error bars showing standard error.

2.6.5. *Pilot experiments 5-8*

These experiments all ended in low or no growth of algae. In each case a problem with liquid media or local conditions was considered, altered and then another experiment run. This is summarised in table 2.6 at the start of this section and involved changes to media pH buffering, autoclaving metal solutions and ensuring starting biomass was high enough. These iterations led to the eventual experimental methods (Chapter 4, 5 and 6) used in the later experiments.

Chapter 3 - Species composition for experimental treatments: genetics methods, analyses and findings

3.1. AimsThe species isolated as described in Chapter 2 are unknown isolates from two locations. To better understand experimental results, taxonomic identification was needed. As algal cultures were not axenic, identification of their associated microbiome was also undertaken to allow a wider understanding of DON processing potential. Genetic identification techniques were used to determine algal taxonomy and associated microbiome for each algal strain. Most of the algae isolates are eukaryotic, with cyanobacteria and associated microbiome being prokaryotic. Two approaches were used to identify the different groups, with the aim of identifying the variety of algal and bacterial species present in each isolate culture.

3.2. Introduction

Genetic approaches have been developed to study species taxonomy and diversity, with DNA 'barcoding' techniques as defined by Hebert *et al*. (2003), are now commonly used during species identification studies (Chakraborty *et al.*, 2014; Darienko *et al.*, 2015; Hollingsworth, Graham and Little, 2011). DNA barcoding analyses short sections of genetic material of an unknown organism. These are compared to a database of known organisms to identify what species was analysed, similar to a 'barcode' for each taxon (Kress and Erickson, 2012). Locations used for genetic barcoding need a conserved section for a primer to bind to, to start replicating the DNA/RNA, with the subsequent fragment covering a more variable region that can be used to distinguish between species.

Due to the differences in genetic material within organisms across the tree of life, DNA barcoding locations differ between organisms. Techniques have improved rapidly during recent decades, with the base approach being adapted and improved upon over time (Suriya *et al.*, 2020; Schander and Willassen, 2005). There are 'universal' primers, which tend to be appropriate for large groups of organisms, often targeted at identifying animals (Hajibabaei *et al.*, 2007), plants (Kress, 2017), fungi (Stielow *et al.*, 2015; Schoch *et al.*, 2012) or bacteria (Chakraborty *et al.*, 2014). Various DNA regions are used, often based on nuclear, mitochondrial or chloroplast DNA. In this chapter, DNA barcoding was used to identify species and study their associated microbiome.

Based on morphological identification using microscopy (x40), the species isolated here covered a range of prokaryotic and eukaryotic organisms. Various DNA barcoding regions are used for the study of eukaryotic algae and prokaryotes, as mentioned above. Eukaryotic species generally have nuclear DNA with significantly larger genomes than prokaryotes. Isolated cultures varied in the biomass of bacteria present, so both bacterial and eukaryotic markers were used to allow investigation of associated microbiome along with the algal species (Mußmann *et al.*, 2013; Moitinho-Silva *et al.*, 2017; Franasiak and Scott, 2017).

With either prokaryotic or eukaryotic species, primers are often tailored to the genus or group in question to get more detail on separating species and sub-species apart. As the isolates used in this thesis were unknown and microscopy determined a wide range of species present, primers were not targeted to specific species. Universal primers were used, as appropriate to each group. Due to resource constraints, two primers needed to be chosen; one for the eukaryotic algal species and one for the bacterial/prokaryotic microbiome present.

Prokaryotic species have a plastid (circular) genome. Amongst prokaryotes, the ribosomal subunit is often used for prokaryote identification, specifically the ribosomal 16 subunit (16S) location (Walters *et al.*, 2016; Caporaso *et al.*, 2011; Chakraborty *et al.*, 2014). There are other universal primers for bacteria, but 16S is very often used in literature and microbiome studies (Bukin *et al.*, 2019; Edgar, 2018; Pichler *et al.*, 2018; Franasiak and Scott, 2017; Moitinho-Silva *et al.*, 2017). This was needed as isolates were known to not be axenic, and any bacteria present may affect the isolate ability to process DON compounds.

Primer choice for eukaryotes is more difficult, as there is no single primer that performs well for all eukaryotes. Eukaryotic species generally have nuclear DNA with much larger genomes (more base pairs) than prokaryotes. As well as nuclear DNA, eukaryote cells often contain varied additional DNA within organelles (mitochondria, chloroplasts etc.). Eukaryotic primers are varied between sub-groups and can target different cellular components. The mitochondrial cytochrome c oxidase region (COI) is widely used in DNA barcoding of animals (Kucera and Saunders, 2012; Kerr *et al.*, 2007; Hajibabaei *et al.*, 2006; Ward *et al.*, 2005), but is not variable enough in algal species to tell species apart. Chloroplasts primers are used in land plants, which are more closely related to algae (Janzen and CBOL, 2009; Sherwood, 2008; Hollingsworth, Graham and Little, 2011). However different groups of algae vary considerably in type and structure of any chloroplast present, meaning it is not appropriate for a wide range of completely unknown algal species when using a single primer.

The ribosomal 18 subunit (18S) region is used for some eukaryotes (Corse *et al.*, 2010; Chakraborty *et al.*, 2014; Łukomska-Kowalczyk *et al.*, 2016) and could be used here. As a single primer 18S is not always adequate to identify algal species as it is not variable enough, and is often combined with other barcoding locations for algae identification (Zou *et al.*, 2018; Radha *et al.*, 2013). A eukaryotic primer that is more variable than 18S is the nuclear internal transcribed spacer (ITS) regions of rDNA (Kress, 2017; Moniz and Kaczmarska, 2010; Youn Cho *et al.*, 2007) used for identification of fungi and algae (Siddiqui *et al.*, 2020). The ITS2 region is a highly variable region between the 5.8s and 28s ribosomal DNA in eukaryotes. Darienko *et al.* (2015) showed ITS2 alone can identify algae, along with its secondary structure if needed. ITS2 has been used for green algae identification (Hadi *et al.*, 2016) and identifying other eukaryotic microalgae (Samanta, Ehrman and Kaczmarska, 2018; Radha *et al.*, 2013). It is also present in fungi, and will also amplify fungal species if any are present in the samples (Kryukov *et al.*, 2020). The ITS barcoding database is smaller than the 18S, but still covers a wide enough range of algal species to identify most common algal and fungal species. As only one eukaryotic primer was used, ITS2 was chosen due to the slightly better identification of various algal species.

The DNA regions targeted for barcoding are amplified through primers that attach to the region of interest, and then undergoes repeated polymerase chain reaction (PCR) cycles. The subsequent samples containing multiple copies of the amplified barcode regions are then prepared for sequencing, in this case on an ILLUMINA MiSeq. The sequences obtained from this require bioinformatic processing to identify what species were present in the original sample compared to the reference database, and this is described in detail below. There are a number of databases to compare barcoding sequences to, the main one used in literature cited above is GenBank, maintained by the National Center for Biotechnology Information (NCBI) (National Center for Biotechnology Information (NCBI), 2021) and freely searchable through the NCBI BLAST portal (NCBI Resource Coordinators, 2018). This produces a percentage identity with unknown samples, and enables the construction of a phylogenetic tree to investigate relationships between samples and references spanning the likely taxonomic groups involved.

Not all sequences in NCBI are peer reviewed sequences, so care needs to be taken to check similar sequences quality and origin. This approach also relies on the database containing sequences from the sample species. DNA barcoding is being more widely used as costs decrease, and most common and well-researched algal species will have representative sequences in the database. Less common species, if they are present in the isolates, may only be identifiable to genus level. However as more known type material is sequenced, these could be identified to species level in future or

show species groups that need more sequencing research. A phylogenetic tree can be constructed to support sequence matches to the database (Edgar, 2018).

3.3. DNA Extraction

Isolation and purification of DNA from the six distinct species was attempted (DNeasy PowerSoil and PowerWater kits, Qiagen). Initial attempts repeatedly led to low-quality DNA/RNA after extraction, as ascertained by a nanophotometer (NanoPhotometer NP80, Implen, Germany). DNA purity and yield were highly variable. DNA purity that should ideally be 1.7-2.0 as measured by the ratio between absorbance at 260 nm and absorbance at 280 nm ratio ($_{A260/A280}$). Samples were 0.7-2.0 ($_{A260/A280}$) and rarely above 1.7 ($_{A260/A280}$). A high level of contamination by non-DNA/RNA was generally present, as measured by absorbance ratio <0.2-1.0 ($_{A260/A230}$), when 2.0 is considered high purity. Yields were consistently low (<4 ng DNA / µL).

The DNA extraction required optimisation or alternate extraction methods to obtain reliable extraction from the samples. The differences in species and cell construction meant optimisation of extraction was needed, requiring extended laboratory time and expertise in DNA extraction. Due to this, samples were sent for external extraction, primer addition, PCR and next generation analysis by Microsynth AG (Switzerland) in April 2020. Samples were extracted and primers as detailed below were added. Samples underwent repeated PCR and were then sequenced on an Illumina MiSeq v2, (2x250 base pairs). Output of demultiplexed reads as zipped `.fastq` files (sequence data files) was provided from the company. All further bioinformatic analysis was done as part of this thesis. An overview of the process of DNA extraction and bioinformatic analysis is summarised in Figure 3.1. Samples were taken during the late exponential phase where possible.

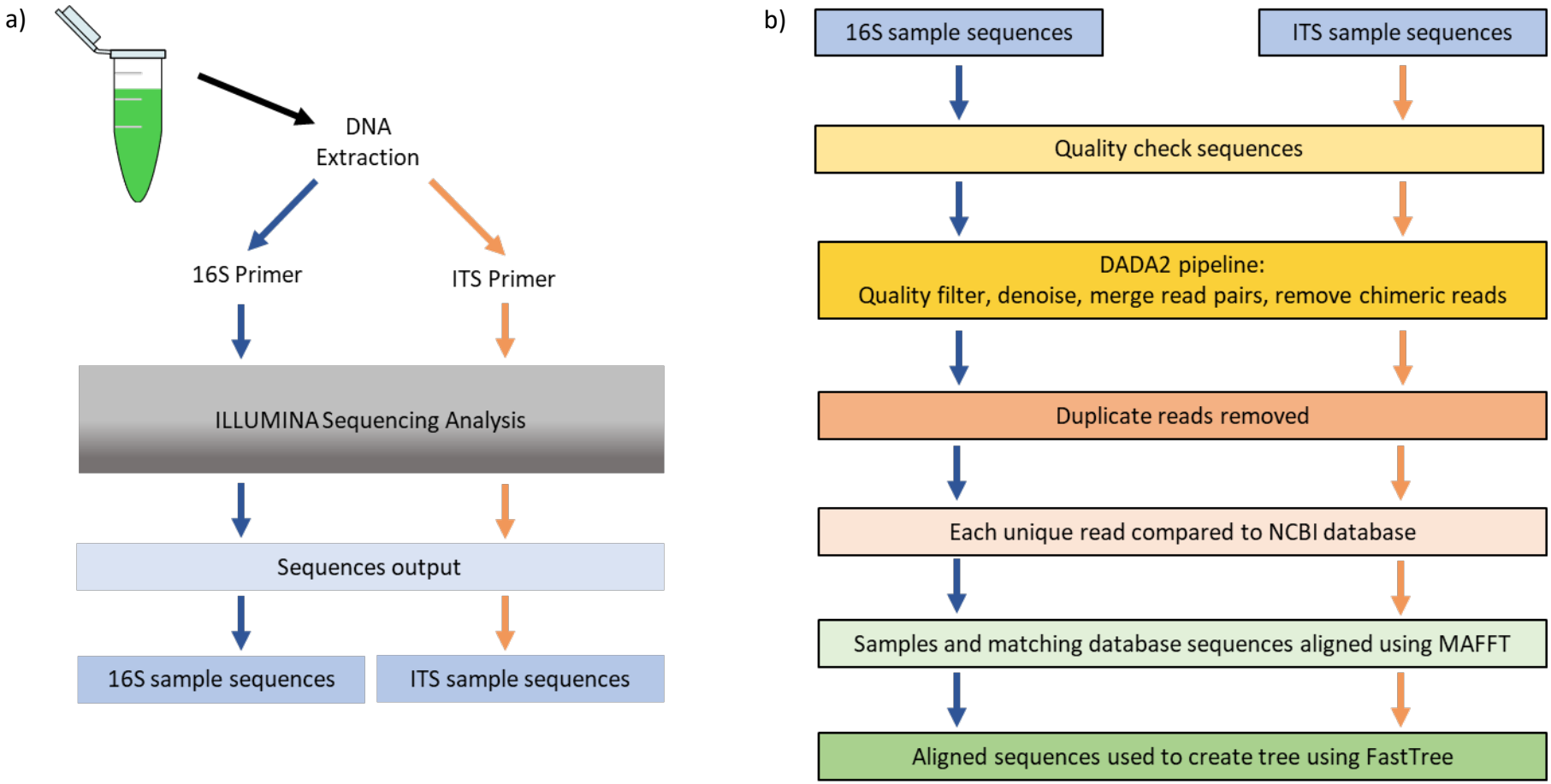


Figure 3.1 Overview of (a) DNA extraction & sequencing, and (b) bioinformatics anlaysis performed .

3.4. Primers sequences

For bacteria, the widely used 16S V34 region was targeted. The forward primer was the 16S 341F universal primer, 5'-CCTACGGGNGGCWGCAG-3', the reverse primer was the 16S 802R universal primer, 5'-GACTACHVGGGTATCTAATCC-3', as initially used by (Caporaso *et al.*, 2011) and further modified by Apprill *et al.* (2015).

For eukaryotic algae, primers for identification were based upon White *et al.* (1990) and the regions within the ITS2 of ITS3/ITS4. These were ITS3-2024F 5'-GCATCGATGAAGAACGCAGC-3' and ITS4-2409R (reverse primer) of 5'-TCCTCCGCTTATTGATATGC-3'. Ideally the use of both ITS and 18S would allow better species identification, but budget limitation meant only one could be used at this stage.

3.5. Bioinformatics analysis

Once sequencing data was received, bioinformatics analysis to determine sample species identities was undertaken, and overview is in Figure 3.1 (b). The general commands used for all steps are shown in Appendix 3. The software used are shown in text at each relevant step. Each single sequence is termed a read.

The primers used attach and amplify any organisms present in a sample. Therefore the data output contains the algal species sequences, as well as any bacteria (by 16S) present or fungi (by ITS) present. Universal primers were used, though it is possible that some species present may not be represented in the sample sequences. This would especially be if they were in very low numbers or in a dormant state, e.g. species that have cyst or resting stages. The data was used to ascertain the presence and identity of sequences obtained, not the relative amount of each species present.

Samples resulted in multiple PCR sequences based upon any genetic material within the sample, algae or bacterial. Both forward and reverse reads were present, based on the locations of the two primers in the 5' and 3' direction along the strand. These are then paired at the overlap to give a longer sequence to identify the sample species. More reads are generally found of the most abundant organisms in a sample, and more copies of identical sequences. An overview of the initial counts received from the sequencing company shows the different total sequence reads for each sample (Table 3.1), including duplicates.

Table 3.1. Sequence counts of data provided post-sequencing via Mi-Seq analysis of ITS2 and 16s regions.

Sample ID	ITS sequencing facility original reads	16s sequencing facility original reads
Nant Y Brwyn sample 1	45065	41995
Nant Y Brwyn sample 2	59089	42248
Nant Y Brwyn sample 3	21517	29232
Priors Farm sample 1	41682	43680
Priors Farm sample 2	45602	31875
Priors Farm sample 3	42632	35223

3.5.1. *Importing data*

Bioinformatics data is usually large in size and not processed by hand. As seen in Table 3.1, each sample generally contained >40,000 reads. A variety of computer programs are used to handle bioinformatics data and analysis. One of these is QIIME2 (Bolyen *et al.*, 2019), which has previously been used for 16S and ITS analysis (Prodan *et al.*, 2020).

Read data was input to QIIME2 (Bolyen *et al.*, 2019) using the Casava file format input for paired end sequences with quality scores and primers were removed. This was done for both the ITS and 16S sample sequences. The Nant-y-brwyn *spp.* 3 was noted by the company as difficult to extract good quality DNA from. To investigate if the quality of reads differed for this compared to the other samples, it was imported into QIIME2 separately from the other samples. Average forward sequence read counts were much lower for Nant Y Brwyn species 3 (c.a. 21,000 reads) compared to all other samples (c.a. 40,000 + reads). Later quality checks showed Nant Y Brwyn species 3 ITS reads to be of poor quality, and this was also imported separately to the other data.

3.5.2. *Quality checking*

Read sequences consist of a string of genetic bases detected at each location along the length. Ideally, a single base would be 100% of the signal at a location, but in practice other bases can also be detected. At any location multiple bases could be detected, and the most likely or

strongest signal is used to determine the base reported. Other signals recorded at the location are not discarded once a base is determined, but contribute to the quality score at that position along the sequence. Quality score generally start at the most accurate, and tend to lose accuracy toward the end of a sequence.

The output '.fastq' files consist of sequences and the quality scores at each location for each read. The quality scores determine the error present in determining the base present. They are related to the probability of the base being incorrectly assigned. Table 3.2 gives an overview of this. Samples here are comparable though over long periods of analysis between different machines quality scores can differ (equipment, software, chemistry changes etc.). A quality score of 20 or less is considered poor quality, though scores tend to be Q30-Q40 when good quality (Ewing *et al.*, 1998).

Table 3.2 Base call quality scores and error probability, with inferred accuracy of base calls.

Quality Score	Error Probability	Inferred Accuracy
Q40	0.0001	99.99%
Q30	0.001	99.9%
Q20	0.01	99%

The imported ITS Nant-y-Brwyn sample 3 quality scores were compared to the other five species analysed (Appendix 3, Table C.2). Though the Nant-y-Brwyn sample 3 had fewer reads than the other ITS samples, read quality was much better than the average of the other five samples. The other samples were investigated individually, and Nant-y-Brwyn sample 2 was found to largely contributed to the poor-quality reverse reads. Figure 3.2 show this clearly by the drop below Q20 after only ~80 base pairs, as compared to the expected ending quality drop around base pair 210 in the other five samples.

The other ITS reads with species Nant y Brwyn sample 2 removed were of better quality for longer than when all 6 samples were combined (Appendix 3). For the remaining five ITS samples, forward reads started to decrease in quality largely after 220 bp (Figure 3.2 a), with the reverse reads being of better quality (though still worse than the forward reads) with median read quality decreasing after 200 bp (Figure 3.2 b). The removal of Nant y Brwyn sample 2 lead to improved

reverse read qualities for the remaining 5 species, and much more constrained quartiles on reverse read quality.

The 16S samples were also checked for quality of both forward and reverse reads, and quality visualisation summaries are shown in Figure 3.3. Reverse reads were of better quality in the 16S dataset than the ITS datasets. Quality tailed off slightly in the last few base pairs of both forward reads (around 228 bp) and reverse reads (around 223 bp).

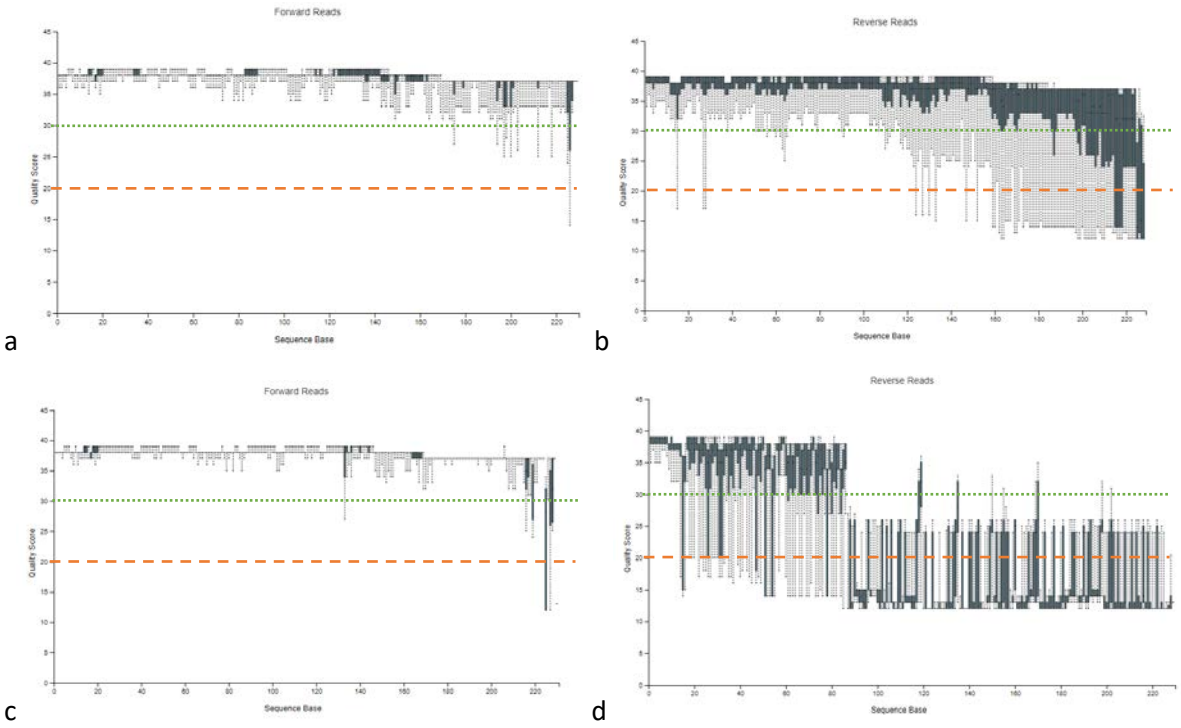
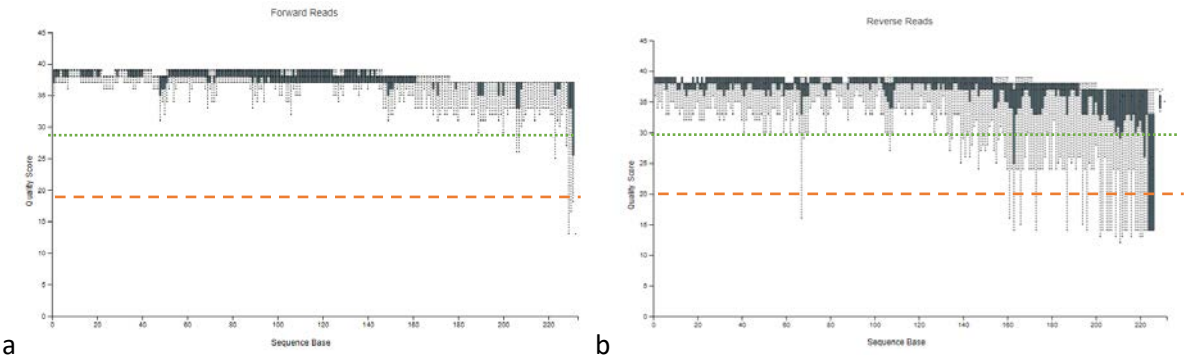


Figure 3.2. Quality scores of raw data forward and reverse ITS V3/V4 reads of 5 samples forward (a) and reverse (b) and ITS reads of quality outlier Nant-y-Brwyn sample 2 forward (c) and reverse (d). Lines show quality scores of 30 (green dotted line) and 20 (red dashed line). Boxes are the interquartile range (25%-75%) with whiskers showing 10% and 90% points and central line the median quality.



a b

Figure 3.3. Quality scores of forward and reverse 16S reads of all 6 samples forward (a) and reverse (b). Lines show quality scores of 30 (green dotted line) and 20 (red dashed line). Boxes are the interquartile range (25%-75%) with whiskers showing 10% and 90% points and central line the median quality.

3.5.3. *Sequence denoising, pairing and removing chimeras*

Reads were put through a DADA2 pipeline in QIIME2 for quality checking and pair assembly. Briefly, DADA2 uses the input command to determine trim lengths, drops much shorter reads, denoises data, merges pairs and removes notably chimeric sequences. The 16S sequences were trimmed to remove low quality base pairs at the end of the forward and reverse reads. Based upon Figure 3.3, trimming lengths were determined on the median quality decrease point as 228 base pairs (bp) in forward reads and 223 bp in reverse reads. This removes some of the lowest quality reads from the ends of each read, in theory allowing for more accurate matching of the paired data and subsequent identification. The amount of reads that were removed at each stage of this pipeline are shown below.

The 16S reads were trimmed to 225 bp in forward reads, and 223 base pairs in reverse reads (based on quality profiles above). The percentage of reads passing through the entire pipeline is shown in table 3.3. Full versions of the DADA2 comparison tables are shown in Appendix 3.

Table 3.3. Summary of 16S reads passing through DADA2 pipeline after trim, denoise and merging processes. Processing occurs left to right.

16s DADA2 pipeline with trim - sample-id	Input count	% of input passed filter	% of input denoised	% of input merged	% of initial input non-chimeric
Nant Y Brwyn sample 1	41995	91.28	91.18	91.00	86.27
Nant Y Brwyn sample 2	42248	86.04	85.09	82.53	69.91
Nant Y Brwyn sample 3	29232	90.24	89.60	86.88	73.25
Priors Farm sample 1	43680	86.96	86.80	86.53	83.64
Priors Farm sample 2	31875	79.53	79.21	78.32	69.16
Priors Farm sample 3	35223	88.20	88.08	88.00	87.9

To check the trimming lengths were appropriate, this pipeline was then re-done with no trim function involved (Table 3.4). Most samples had similar percentage of reads making it to the end as each other. Two had more reads going through pairing and found to be non-chimeric – Priors Farm sample 1 (+10%) and Priors Farm sample 2 (+6%). Due to this no trimming was applied to the 16S sequences in the DADA2 pipeline.

Table 3.4. Summary of 16S reads passing through DADA2 pipeline without trimming, and with denoising and merging reads. Processes occur left to right.

16S DADA2 pipeline, no trimming - sample-id	Input count	% of input passed filter	% of input denoised	% of input merged	% of initial input non-chimeric
Nant Y Brwyn sample 1	41995	91.44	91.29	91.05	86.37
Nant Y Brwyn sample 2	42248	86.47	85.57	82.65	70.24
Nant Y Brwyn sample 3	29232	90.19	89.58	86.66	73.24
Priors Farm sample 1	43680	93.25	93.16	92.75	89.65
Priors Farm sample 2	31875	91.76	90.81	88.63	79.24
Priors Farm sample 3	35223	88.35	88.20	88.12	88

ITS reads were not trimmed during the DADA2 stage, as ITS is a highly variable region in terms of length. Trimming would exclude reads shorter than the trim length that are biologically shorter than the expected length due to its variable nature between species, not due to sequencing, poor PCR or poor DNA quality. The results of the input are shown in table 3.5. Generally, a similar percentage of reads were retained as compared to the 16S samples, apart from Nant-y-Brwyn sample 2. Less than 2% of initial reads were retained for this sample.

Table 3.5. Summary of ITS reads passing through DADA2 pipeline without trimming, and with denoising and merging reads. Processes occur left to right, and the anomalous results are highlighted.

ITS DADA2 pipeline (2ee) sample-id	Input count	% of input passed filter	% of input denoised	% of input merged	% of initial input non-chimeric
Nant Y Brwyn sample 1	45065	85.25	85.24	85.23	85.19
Nant Y Brwyn sample 2	59089	3.09	3.05	1.82	1.82
Nant Y Brwyn sample 3	21517	75.02	74.54	60.03	59.47
Priors Farm sample 1	41682	85.06	85.05	85.05	85.05
Priors Farm sample 2	45602	88.79	88.78	88.77	88.77
Priors Farm sample 3	42632	86.32	86.32	86.32	86.32

Due to this removal of most reads, Nant-y-Brwyn sample 2 was investigated further. As seen in table 3.5, very few reads were passing the initial quality stage. As no additional trimming factors were input at this stage, it is likely the error rates and quality of these sequences were lower. The same DADA2 denoising pipeline was used, with more relaxed filters for the error present. The default for this parameter is 2, and it was increased in steps to 10 before any major change in read retention for Nant-y-Brwyn sample 2 was seen (Table 3.6). This very relaxed approach to permissible error rates resulted in other samples also having lowered qualities when first input. This resulted in retaining almost all initial reads to the denoising stage, though some were removed in the merging stage. The Nant-y-Brwyn sample 2 still had <10% of initial reads retained at the end of the process, and other samples were likely to have retained more error with the relaxed filter.

Table 3.6. Summary of ITS reads passing through DADA2 pipeline without trimming, and with denoising and merging reads, with a relaxed error rate (10 ee). Processes occur left to right, and the anomalous result is highlighted.

ITS DADA2 pipeline (10ee) sample-id	Input count	% of input passed filter	% of input denoised	% of input merged	% of initial input non-chimeric
Nant Y Brwyn sample 1	45065	100	99.97	90.57	90.52
Nant Y Brwyn sample 2	59089	99.99	99.94	9.21	9.09
Nant Y Brwyn sample 3	21517	99.96	99.22	70.29	69.66
Priors Farm sample 1	41682	100	99.97	87.89	87.89
Priors Farm sample 2	45602	100	99.97	90.76	90.76
Priors Farm sample 3	42632	100	99.97	93.13	93.13

Due to the issues with the sample, the ITS sequences were separated into the Nant y Brwyn sample 2 (ITS-NB2) and the other five ITS samples (ITS-5). These two groups (ITS5 and ITSNB2) were then imported and visualised as previously described. As previously shown in Figure 3.3, the forward reads of NB2 are of reasonable quality (Figure 3.3 a), but the reverse reads are of very poor quality,

especially after 80 bp (Figure 3.3 b). This is unlikely to be useful when trimming and pairing reads, so only forward reads were used for ITS-NB2 sample. These were imported using a single-end Casava style import and single-end DADA2 pipeline. This resulted in 97% of forward reads being retained (Table 3.7). These forward only reads are referred to as ITS-NB2 from this point.

Table 3.7. Summary of ITS forward only reads for Nant y Brwyn sample 2, after passing through DADA2 pipeline without trimming, and with denoising and merging reads and the default error rate. Processes occur left to right.

ITS NB2 DADA2 pipeline, forward reads only	Input count	% of input passed filter	% of input denoised	% of initial input non-chimeric
Nant Y Brwyn sample 2	59089	97.18	97.16	97.05

After all groups were put through the DADA2 pipeline, each output included a list of unique sequences within each group and other statistics on the process. The highest frequency of sequences is seen over a small number of sequences, with frequency decreasing as the number of unique sequences increases (Figure 3.4). A few sequences comprised the majority of counts in each group, and these are likely the algae sequences in the ITS groups and associated bacteria or chloroplasts in the 16S group. Each unique sequence was associated with an alphanumeric key by DADA2. These were ordered by the amount of each sequence found, and changed to reflect the number of reads and group. So 16s_seq_001 is the 16S sequence with the most reads in the group, ITS5_seq_02 the second most reads in the ITS-5 group.

The ITS-NM2 unique sequences were checked against the unique sequences from the other 5 species samples. Of the 11 unique ITS-NB2 sequences, 5 were identical to sequences in the ITS-5 group and none were the most prevalent two sequences in terms of frequency (reads identical to ITS-5 group <2% filtered reads by count).

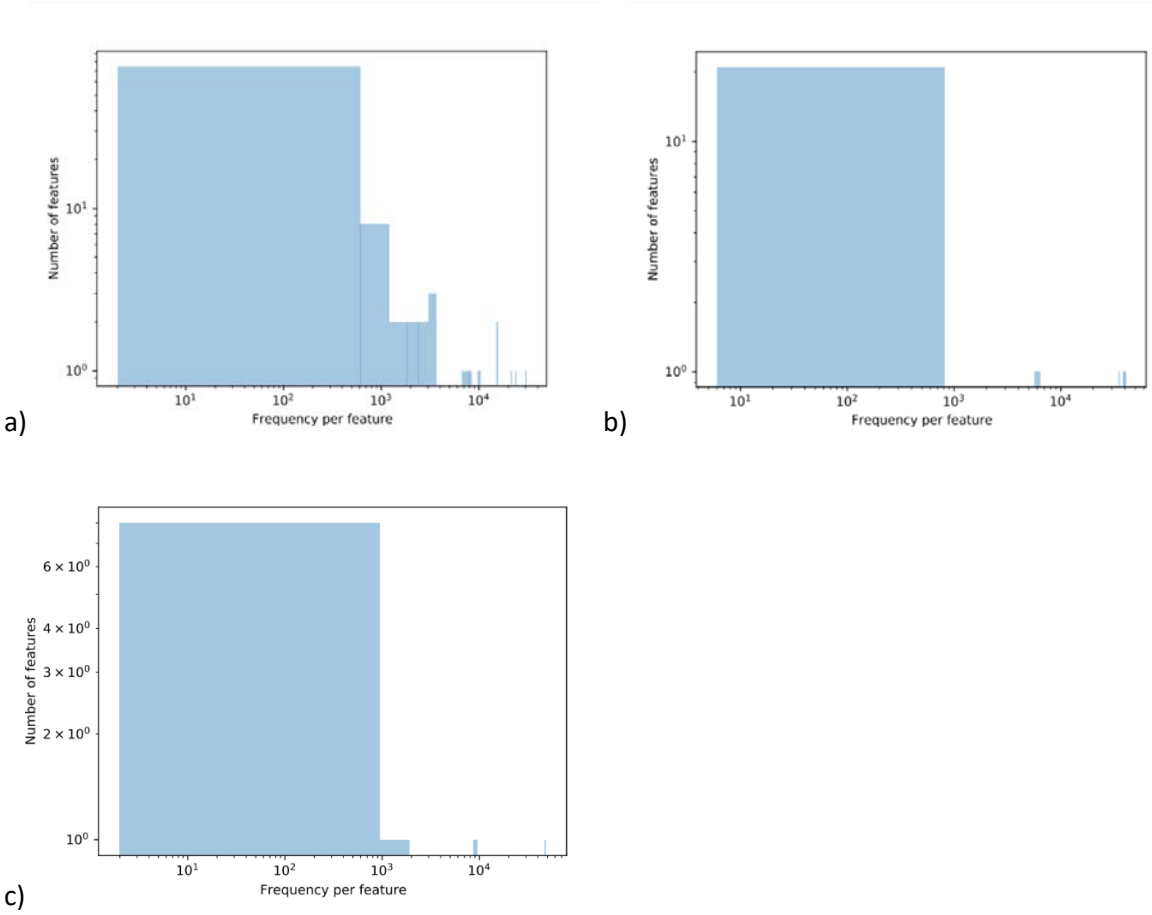


Figure 3.4. Counts of each unique feature and frequency of feature, 16s (a), ITS5 (b) and ITSNB2 (c), with a log scale of frequency per feature and feature count.

3.5.4. *Database search of unique sample sequences*

Each unique sequence from 16S, ITS-5 and ITS-NB2 groups were searched for in the NCBI website (BLASTn search, non-specialised database). Default parameters were used and the non-specific nucleotide database. Where more than 50 matches were found, the top 50 had their sequences downloaded. Occasionally less than 50 matches were found, and all matched sequences were downloaded. The wider NCBI database is not curated and contains many partial and unknown sequences that match target sample sequences.

The wider nucleotide database results were supplemented by a secondary round of searches. For the 16S group, this was against the 16S bacteria database in NCBI. For both ITS groups, this secondary search was against the ITS fungi database. Full sequences of the top 50 matches were downloaded. For the 16S group, the BLAST matches were supplemented by a pre-curated set of cyanobacteria and chloroplast sequences from NCBI spanning most groups (Boden *et al.*, 2021).

As later trials went on, local computing capacity was exceeded for both alignment and tree building, with the number of sequences needing to be reduced. This was done by retaining the

sequence which best matched each sample, and 10-20 sequences with confirmed species or genus names given. This removed most unknown species in the BLAST matches. BLAST sequences of lengths comparable to samples were prioritised. The enlarged full genomes as compared to the shorter (~450 bp) sample sequence would cause alignment inaccuracy, so matches with full genomes were removed, which also aided alignment processing.

The top BLAST search matches for each sequence were manually checked. The best match or matches that had a defined species were used to assign identities to the sample sequences. Where matches had multiple sequences at the same identity percentage, the most common grouping (Order, Family etc.) was used as the identity. The percentage identities of more than 97%, 90%, 85% or 80% was used to determine species, genus, family or order respectively, as used by Tedersoo *et al.*(2015; 2018) on ITS2 data.

3.5.5. *Alignment of samples and reference sequences*

Sequences were aligned using MAFFT version 7.475 (Katoh *et al.*, 2002). After alignment, they were manually checked using an alignment viewer (Bianchini, 2018) . In each group of samples and sequences, aligned sequences that were markedly different and caused long gaps in the middle or end of other alignments were removed, and the MAFFT alignment re-run on the reduced number of sequences.

3.5.6. *Unrooted phylogenetic tree building*

After alignment checking and removing large gaps, trees were built for each sample group. This was done using FastTree version 2.1.10 (Price, Dehal and Arkin, 2009). More accurate programs exist, such as IQ-TREE (Nguyen *et al.*, 2015) , but local computing could not process the more intensive tree-building used by these programs, even with a reduced comparison database (IQ-TREE v 2.0.3).

Trees were input for visualisation into FigTree version 1.4.4 (Rambaut *et al.*, 2018). Due to the wide spread of species, these trees are detailed and complex, with viewing in a larger format needed to read all sample names. To aid visualisation and understanding, notable groups are marked on each tree, with some branches compressed. Trees are not rooted using an outgroup, but are displayed using the midpoint and arranged outward for ease of viewing. From the tree output

and tables with the highest known matches, identities can then be assigned to each of the unique sequences in the samples.

3.6. Results

The results of the ITS sample analysis can be seen in table 3.8 and table 3.9. These show the main algal species, associated fungi and sequences similar to plant chloroplasts that were amplified using the ITS2 primer. The 16S results are shown in table 3.10 for all six samples, though it is restricted to the most common 36 sequences. The full 16S table is shown in Appendix 3 and its results are included in this discussion. The number of reads of each unique sequence are shown, as this can assist the interpretation of the results and shows that many reads were unique to different samples. This highlights that isolates had separate communities of bacteria and algae, though derived from the same samples initially. Sample identification compared to the NCBI database assigned species, genus and family according to percentage sequence identity of above 97%, 90% and 85% respectively (Tedersoo *et al.*, 2015; Tedersoo, Tooming-Klunderud and Anslan, 2018).

3.6.1. *Nant y Brwyn sample 1 – Chlorella vulgaris*

The Nant y Brwyn sample 1 produced ITS reads that were largely identified as a *Chlorella* species within the tree, having NCBI matches of 100% to *Chlorella vulgaris* strains from UTEX 265, UTEX 26 and SAG 211-11t (Table 3.8). The ITS tree also shows that this groups closely with other *Chlorella sp.*, and specifically nests within the *Chlorella vulgaris* species (Figure 3.11). This ITS species designation is supported by the 16S data that was mostly comprised of *Chlorella sp.* chloroplast reads (>66% total reads). The morphology of this species can be seen in figure 3.5.

This isolate appears to be free of most fungal/yeast species detected. The most abundant fungal sequence comprised <0.007% total reads and was identified as *Alternaria tenuissima*. This is a cosmopolitan, saprophytic species of fungi, normally a plant pathogen. This was not seen microscopically in the culture or on agar plates, suggesting that perhaps spores of this species were present with very little active material affecting cultures.

The remaining 16S results showed a low diversity of bacterial species. These were mostly *Roseomonas sp.*, a gram-negative alpha proteobacteria in the order Rhodospirillales, and *Methylophilus sp.*, a gram-negative beta proteobacteria in the order Methylophilales. There were

also sequences of bacteria associated with gut microbiomes (Faecalibacterium, *Ruminococcus* sp. etc.) and environmental samples.

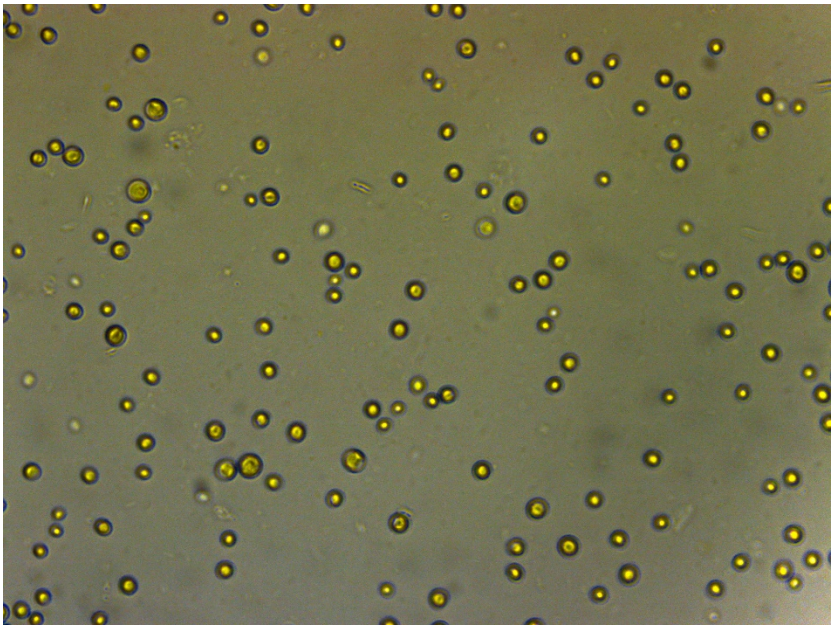


Figure 3.5 - Image of the sample identified as Chlorella vulgaris, using light microscopy at x 400 magnification.

Table 3.8. Identities of ITS sequences present in five of the six samples sequenced.

Sequence name	Species Type	BLAST Match ID		Tree grouping ID	Sample read counts				
		BLAST ID	Similarity (%)		NantYBrwyn sample 1	NantYBrwyn sample 3	PriorsFarm sample 1	PriorsFarm sample 2	PriorsFarm sample 3
ITS5_seq_01	Algae	Lobochlamys segnis	98.84	*Chlamydomonas spp*	0	0	1	42064	0
ITS5_seq_02	Algae	Chlorellaceae sp.	98.96	*Chlorella spp. / Micractinium spp.*	38	2294	0	18	39924
ITS5_seq_03	Algae	Chlorella vulgaris	95.48	*Chlorella spp. / Dictyosphaerium spp.*	41615	0	0	0	0
ITS5_seq_04	Algae	Chlorella vulgaris	100	*Chlorella spp. / Dictyosphaerium spp.*	0	0	38225	1	0
ITS5_seq_05	Amoeba	Naegleria sp.	94.5	*Naegleria sp.*	0	6198	0	1	0
ITS5_seq_06	Fungus / Yeast	Didymellaceae sp.	100	*Family Didymellaceae*	0	899	0	0	0
ITS5_seq_07	Plant	Strelitziana albiziae	99.69	*Strelitziania spp. Chloroplast*	0	815	0	0	0
ITS5_seq_08	Fungus / Yeast	Parapyrenochaeta sp.	98.05	*Pyrenochaeta spp.*	0	539	0	0	0
ITS5_seq_09	Fungus / Yeast	Cladosporium sp.	100	*Cladosporium spp.*	0	461	0	0	0
ITS5_seq_10	Fungus / Yeast	Wallemia sebi	100	*Wallemia c.f. sebi*	0	397	0	0	0
ITS5_seq_11	Fungus / Yeast	Penicillium sp.	100	*Aspergillus spp*	0	458	0	1	0
ITS5_seq_12	Fungus / Yeast	Scopuloides hydnoides	100	*Scopuloides spp*	0	295	0	0	0
ITS5_seq_13	Fungus / Yeast	Aspergillus sp.	100	*Aspergillus spp*	0	289	0	0	0
ITS5_seq_14	Fungus / Yeast	Talaromyces sp. & Penicillium sp.	100	*Talaromyces spp.*	0	265	0	0	0
ITS5_seq_15	Plant (Chloroplast)	Helianthus annuus & Magnoliophyta sp.,	99.73	*Helianthus spp.*	0	624	0	0	0
ITS5_seq_16	Plant (Chloroplast)	Aspergillus flavus & Brassica rapa & Brassica napus & Brassica juncea	100	*Brassica rapa*	0	119	0	0	0

Table 3.8 continued. Identities of ITS sequences in five isolates with paired reads.

Sequence name	Species Type	BLAST ID	Similarity (%)	Tree grouping ID	NantYBrwyn sample 1	NantYBrwyn sample 3	PriorsFarm sample 1	PriorsFarm sample 2	PriorsFarm sample 3
ITS5_seq_17	Fungus / Yeast	Saccosoma farinaceum	77	*Atractiellales order, c.f. Atractiella spp.*	0	296	0	0	0
ITS5_seq_18	Fungus / Yeast	Malassezia restricta & Malassezia arunalokei	99.53	*Malassezia spp.*	0	108	0	0	0
ITS5_seq_19	Fungus / Yeast	uncultured Basidiomycota	99.3	*Malassezia spp.*	0	99	0	0	0
ITS5_seq_20	Fungus / Yeast	Parapyrenochaeta sp.	97.72	*Pyrenochaeta spp.*	0	75	0	0	0
ITS5_seq_21	Fungus / Yeast	Lophiostoma sp. & Dothideomycetes sp.	91.59	*Class Dothideomycetes*	0	173	0	0	0
ITS5_seq_22	Fungus / Yeast	Acremonium charticola	95.61	*Acremonium spp*	0	136	0	0	0
ITS5_seq_23	Fungus / Yeast	Shanorella spirotricha & Thermoascus verrucosus	83.64	*Order Onygenales*	0	145	0	0	0
ITS5_seq_24	Fungus / Yeast	Microascus murinus & Scopulariopsis sp.	100	*Fungal spp*	0	467	0	0	0
ITS5_seq_25	Outgroup, unknown	Paenibacillus sp.	91.94	*Unknown*	0	24	0	0	0
ITS5_seq_26	Fungus / Yeast	Alternaria alternata & A. tenuissima		*Alternaria tenuissima*	3	0	0	0	0

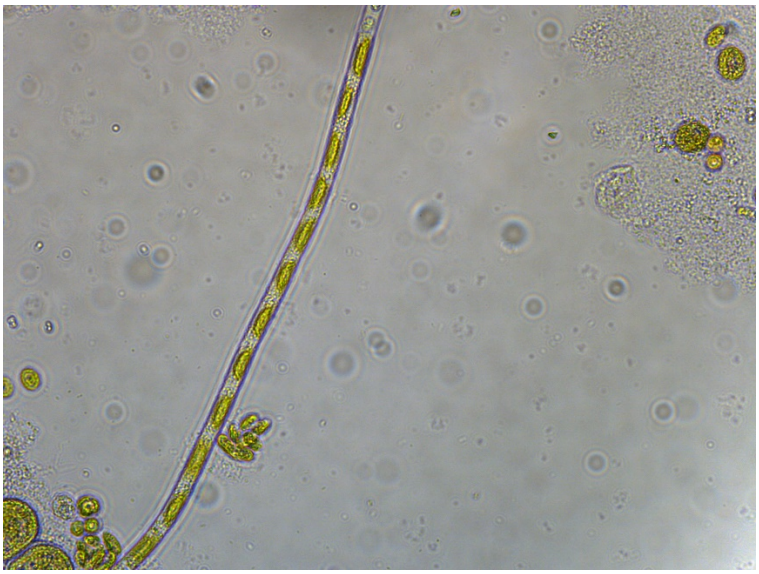
3.6.2. *Nant y Brwyn sample 2 – Klebsormidium sp.*

The Nant y Brwyn sample 2 was processed separately to the other ITS sample for reasons described above. It produced ITS reads (table 3.9) that were mostly determined as a *Klebsormidium sp.* from the phylogenetic tree (Figure 3.12) and NCBI matches. Sequences matched in NCBI with 100% similarity to strains of *Klebsormidium nitens* and *K. fluitans*. As the species could not be definitively assigned, it is referred to by genus only in this thesis - *Klebsormidium sp*. Some ITS reads were found of other algal species, including *Chlorella sp.* (<1.8% total reads), an amoeba (<0.07% total reads) and some fungal species (<0.11% total reads). This is supported by the microscopy and chloroplast arrangement of the filamentous green alga, with very low numbers of any additional species seen in culturing conditions. Species morphology can be seen in Figure 3.6.

This species designation is supported by the 16S data (table 3.10) that was mostly comprised of *Klebsormidium sp.* chloroplast reads (>53% total reads). Other 16S read sequences showed a variety of bacteria present alongside *Klebsormidium sp.*, with a very small number of *Nitzschia sp.* chloroplast reads also present. This diversity is much greater than the ITS2 diversity seen in this sample. The 16S non-algal sequences were largely gram negative proteobacteria; *Bosea* sp. (Order Hyphomicrobiales), and *Brevundimonas sp*. (Order Caulobacterales), both alpha proteobacteria, alongside a beta proteobacteria, *Hydrogenophaga sp*. (Order Burkholderiales).

a)

b)



c)

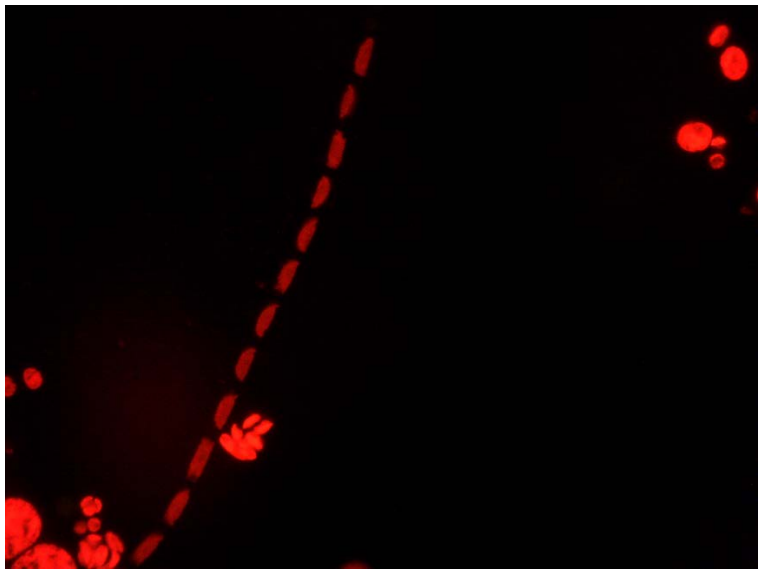


Figure 3.6 – Images of Klebsormidium sp. under light microscopy at x100 (a), and x400 (b), as well as chlorophyll fluorescence microscopy at x400 magnification (c). Images were taken before full isolation was completed.

Table 3.9 Identities of ITS sequences in Nant y Brwyn sample 2 group

Sequence name	BLAST Match ID BLAST ID	Identity Similarity (%)	Tree grouping ID	NB2 sample reads
ITSNB2_seq_01	Klebsormidium sp. (K nitens and K. fluitans)	100	*Klebsormidium sp.*	45742
ITSNB2_seq_02	Klebsormidium sp.	100	*Klebsormidium sp.*	8366
ITSNB2_seq_03	Micractinium sp.	100	*Chlorella sp. UTEX 2069, micractinium sp.*	951
ITSNB2_seq_04	Pezizomycotina sp	99.56	*Uncultured fungus*	55
ITSNB2_seq_05	No matches	0	*Unknown, naegleria relative*	25
ITSNB2_seq_06	Brassica sp.	100	*Brassica spp chloroplast*	12
ITSNB2_seq_07	Bosea sp.	89.59	*Naegleria spp., unknown*	10
ITSNB2_seq_10	Naegleria sp.	94.5	*Naegleria spp.*	3
ITSNB2_seq_09	Cylindrotheca sp. and Nitzschia sp.	99.16	*Nitzschia spp., cylindrotheca spp*	2
ITSNB2_seq_08	Chlorella vulgaris	95.54	*Uncultured fungus, or chlorella spp.*	3
ITSNB2_seq_11	Family Didymellaceae	100	*Didymella glomerata, Didymella spp.*	2

3.6.3. *Nant y Brwyn sample 3 – Nitzschia palea & amoeba (Naegleria sp.)*

The Nant y Brwyn sample 3 was known to be problematic to extract good quality DNA from (*pers. Comm*.). Microscopy clearly showed a diatom and cyanobacteria species present in the isolate. However, most ITS reads were identified as an amoeba, *Naegleria sp*., via the phylogenetic tree. With NCBI matches of ~94% identity to different *Naegleria* species it could only be reliably identified as genus *Naegleria* without species assignation. This is unexpected from the sample morphology. The next most numerous ITS reads were to a *Micractinium* species within the tree, and the NCBI nBLAST search showed a 100% identity match to Family Chlorellaceae samples (>98% identity) and likely *Micractinium* (99.48% identity) genus within that group.

The diatom seen under microscopy was *Nitzschia palea*, according to the tree and NCBI matches of 100% identity and 100% coverage (NCBI accession MH113811.1, AP018511.1), morphology can be seen in figure 3.7. This is a freshwater diatom (Sims, 1996; Crowell, Nienow and Cahoon, 2019), and it is likely that this diatom was the main algal species, along with the cyanobacteria, though there are a low number of reads for this species. The cyanobacteria in culture

was not present in the sequences analysed, and is mentioned in the discussion. A wide range of yeast species can also be seen in this sample (Table 3.8).

The *Nitzschia* sp. identification was supported by the 16S analysis, which found many reads of a *Nitzschia* sp. chloroplast. Bacterial diversity was high amongst these isolates, as seen in Table 3.10. Most single bacterial sequences reads were of genus *Pseudomonas* sequences, a gram negative 91ammaproteobacterial with various animal and plant pathogen species, with anaerobic potential and able to live within a pathogenic biofilm (Hassett *et al.*, 2002).

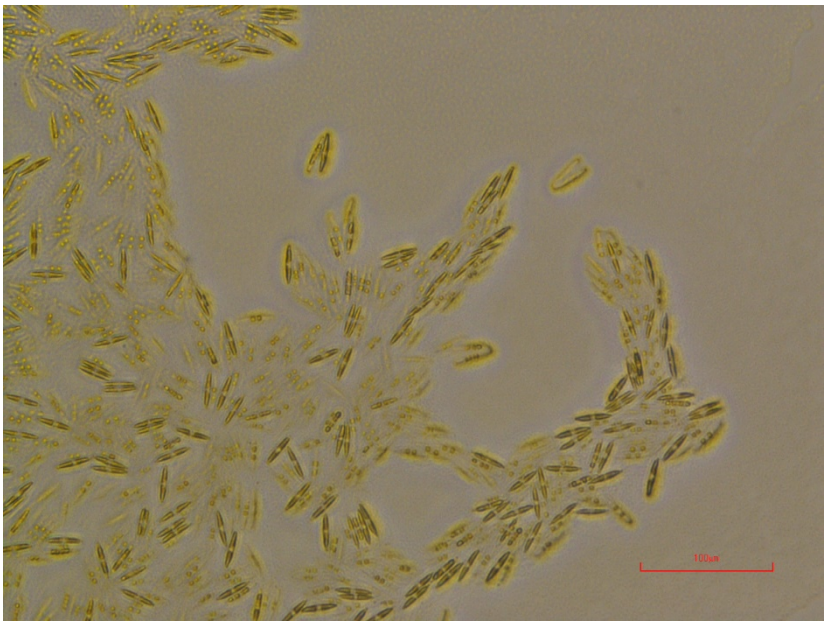


Figure 3.7 – Light microscopy image of Nitzschia sp. diatom from Nant-Y-Brwyn sample 3 at x200 magnification.

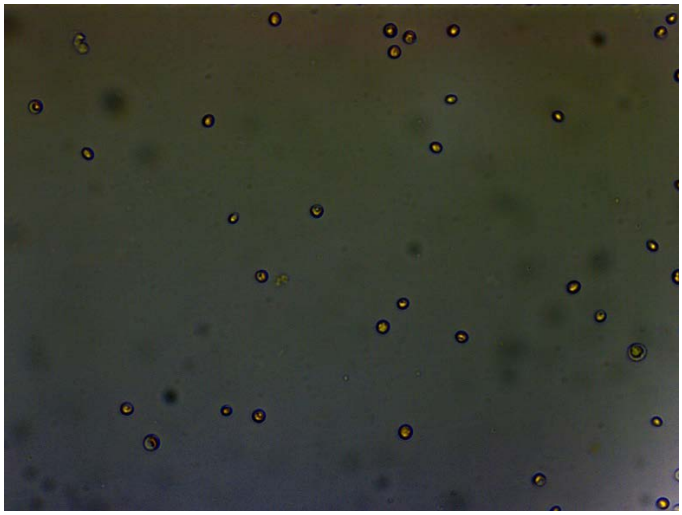
3.6.4. *Priors Farm 1 – Chlorella vulgaris*

The Priors Farm sample 1 ITS sequences were almost exclusively (>99.99%) a single sequence identified as *Chlorella vulgaris*, with no fungal sequences found (Table 3.8). *Chlorella vulgaris* is a highly variable, spherical green algae that is found in a variety of locations worldwide.

The 16S sequencing supported the algal identification, with >55% of sequences identified as a *Chlorella vulgaris* chloroplast. Only four bacteria species were identified in the isolate, of which one species had a single read found (*Faecalibacterium* sp.). The other three were *Phreatobacter sp*., a Family Comamonadaceae species and a Family Microbacteriaceae species. All of these were supported by both NCBI searches and the tree, though not specific enough to determine genus for

two of the bacteria. They matched multiple bacteria species in the respective families with 100% similarity covering 100% of the sample sequences, with the tree not providing enough detail to determine which species each sequence was. Some morphology of this strain can be seen in figure 3.8.

a)



b)

c)

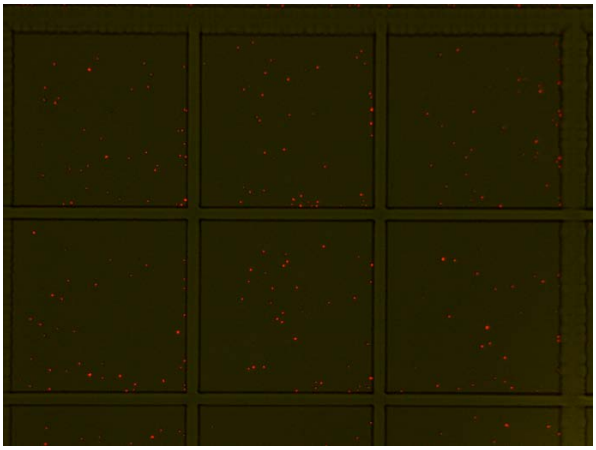


Figure 3.8 – Images of Chlorella vulgaris from Priors Farm sample 1 with light microscopy at (a) x400 magnification and (b) x100 magnification, with (c) chlorophyll fluorescence at x100.

3.6.5. *Priors Farm 2 – Lobochlamys sp.*

The Priors Farm sample 2 algal species was identified through ITS barcoding as Family Chlamydomonadaceae c.f. *Lobochlamys* sp.. A small number of ITS sequences were identified as Family Chlorellaceae, which are related to the Family Chlamydomonadaceae.

Identification of *Lobochalmys sp.* was supported by the 16S sequencing, that showed some sequences of chloroplasts from *Lobochlamys culleus* (>97% match identity). This isolate had a

diverse range of bacteria present. The 16S sequencing found mostly *Rhizobium* sp., followed by a Family Sphingomonadaceae species, Family Microbacteriaceae species, Family Acetobacteraceae species, an Order Hyphomicrobiales species and *Kaistia* sp. with few sequences of other species. Morphology of this strain can be seen in figure 3.9.

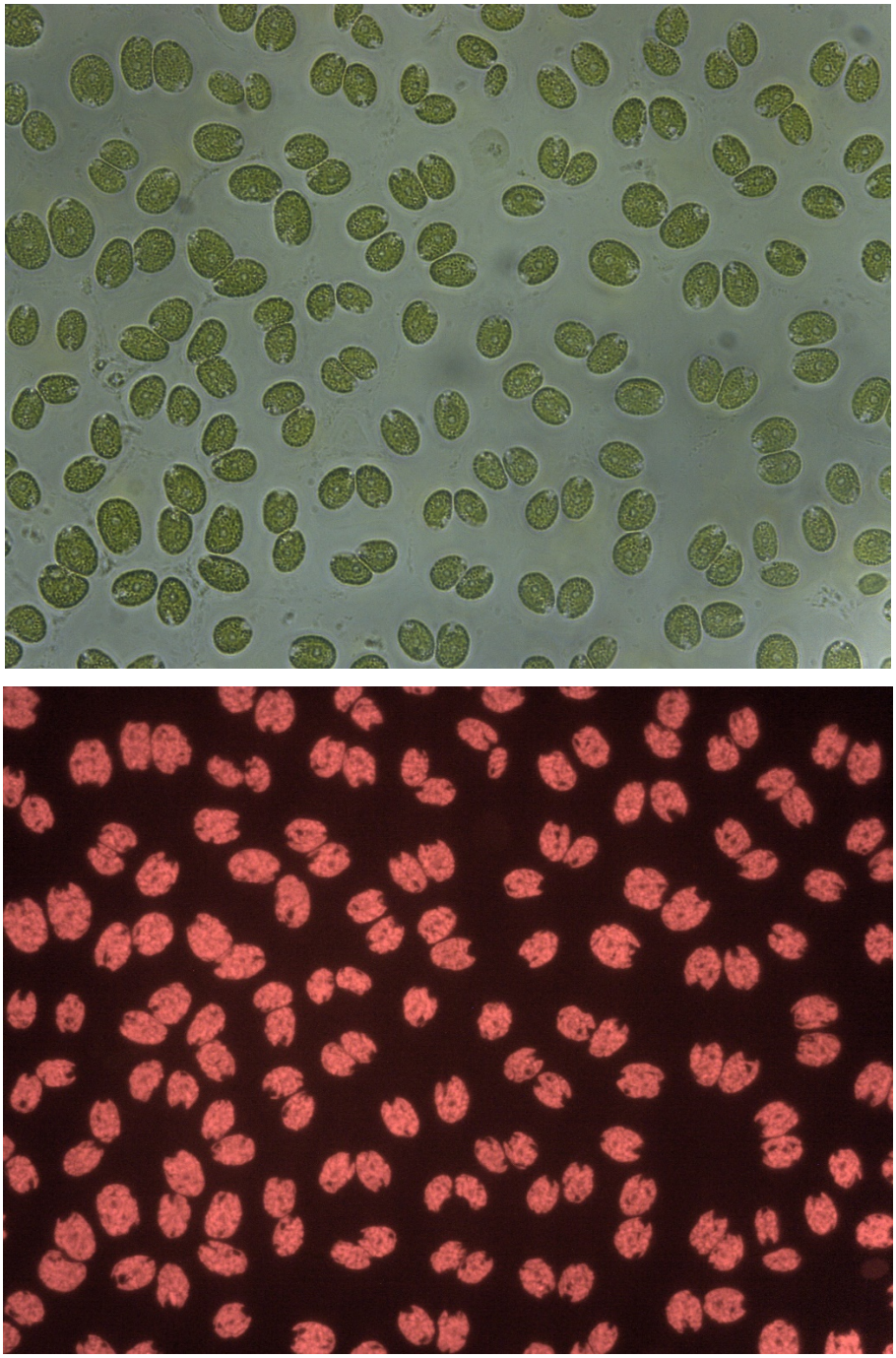


Figure 3.9 – Images of Lobochlamys sp. under (a) light microscopy at x 400 magnification and (b) chlorophyll fluorescence microscopy at x400 magnification.

Table 3.10 Identities of 16s sequences from all samples, showing first 36 unique sequences.

Sequence name	Tree grouping ID	Nant y Brwyn sample 1	Nant y Brwyn Sample 2	Nant y Brwyn sample 3	Priors Farm sample 1	Priors Farm sample 2	Priors Farm sample 3
16s_seq_001	*Chlorella sp. Chloroplast*	0	0	1	0	0	32223
16s_seq_002	*Chlorella sp. Chloroplast*	25107	0	0	0	0	0
16s_seq_003	*Chlorella vulgaris Chloroplast*	0	0	0	22021	0	0
16s_seq_004	*Klebsormidium sp chloroplast*	0	17858	0	0	7	7
16s_seq_005	*Rhizobium sp.*	0	0	0	0	16147	0
16s_seq_006	*Roseomonas sp.*	10525	452	0	0	0	2
16s_seq_007	*Pseudomonas sp.*	0	7	8208	0	1	0
16s_seq_008	*Phreatobacter sp. / Family Rhizobiales*	0	187	0	7539	0	0
16s_seq_009	*Unknown bacteria f*	0	0	0	6834	0	0
16s_seq_010	*Nitzschia sp. Chloroplast*	0	6	3403	0	1	0
16s_seq_011	*Pseudomonas sp.*	0	6	3275	0	2	0
16s_seq_012	*Bosea sp.*	0	3381	0	0	2	1
16s_seq_013	*Family Sphingomonadaceae, c.f. Novosphingobium sp.*	0	1	0	0	3416	3
16s_seq_014	*Hydrogenophaga sp.*	0	3255	0	0	2	3
16s_seq_015	*Methylophilus sp.*	2187	0	0	0	0	0
16s_seq_016	*Brevundimonas sp.*	0	2121	0	0	0	2
16s_seq_017	*Pedobacer sp.*	0	1	2017	0	0	0
16s_seq_018	*Unknown bacterium a*	0	2	1338	0	0	0
16s_seq_019	*Asticcacaulis sp.*	0	2	1338	0	0	0
16s_seq_020	*Roseomonas sp.*	0	0	0	0	1343	0
16s_seq_021	*Rhizobium sp. / Ensifer collicola*	0	1397	411	0	0	2
16s_seq_022	*Bosea sp.*	0	45	0	0	1221	0
16s_seq_023	*Unknown bacterium g*	0	1069	0	0	1	0
16s_seq_024	*Pelomonas sp.*	0	1	1009	0	0	0
16s_seq_025	*Mesorhizobium sp.*	0	926	0	0	1	1
16s_seq_026	*Chlamydomonadales sp. Chloroplast*	0	0	0	0	728	0
16s_seq_027	*Kaistia sp. / Family Hyphomicrobiales*	0	464	0	0	589	1
16s_seq_028	*Unknown bacterium b*	0	0	0	0	0	511
16s_seq_029	*Pedobacter sp.*	0	2	636	0	0	0
16s_seq_032	*Reyanella sp.*	0	282	0	0	0	0
16s_seq_033	*Leifsonia sp.*	0	0	0	3050	1509	0
16s_seq_034	*Rhodopsudomonas*	0	340	0	0	0	0
16s_seq_035	*Methylobacterium sp.*	0	297	0	0	0	0
16s_seq_036	*Methylophilus sp.*	0	2	268	0	0	0

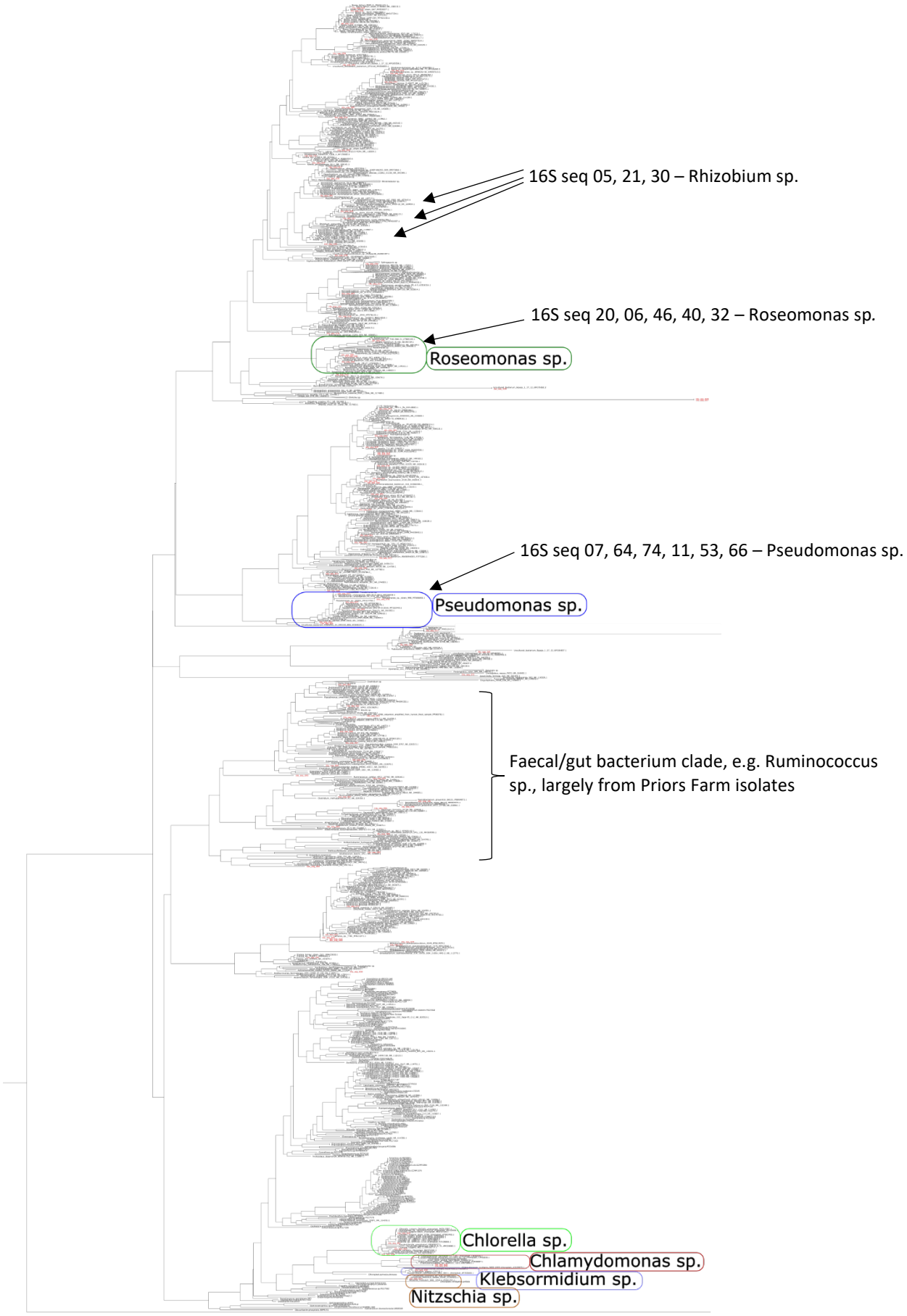


Figure 3.10. Tree of 16s samples alongside a curated comparison database how they align and group within an unrooted tree, sorted by midpoint. Sample sequences are highlighted in red text, with some groups circled.

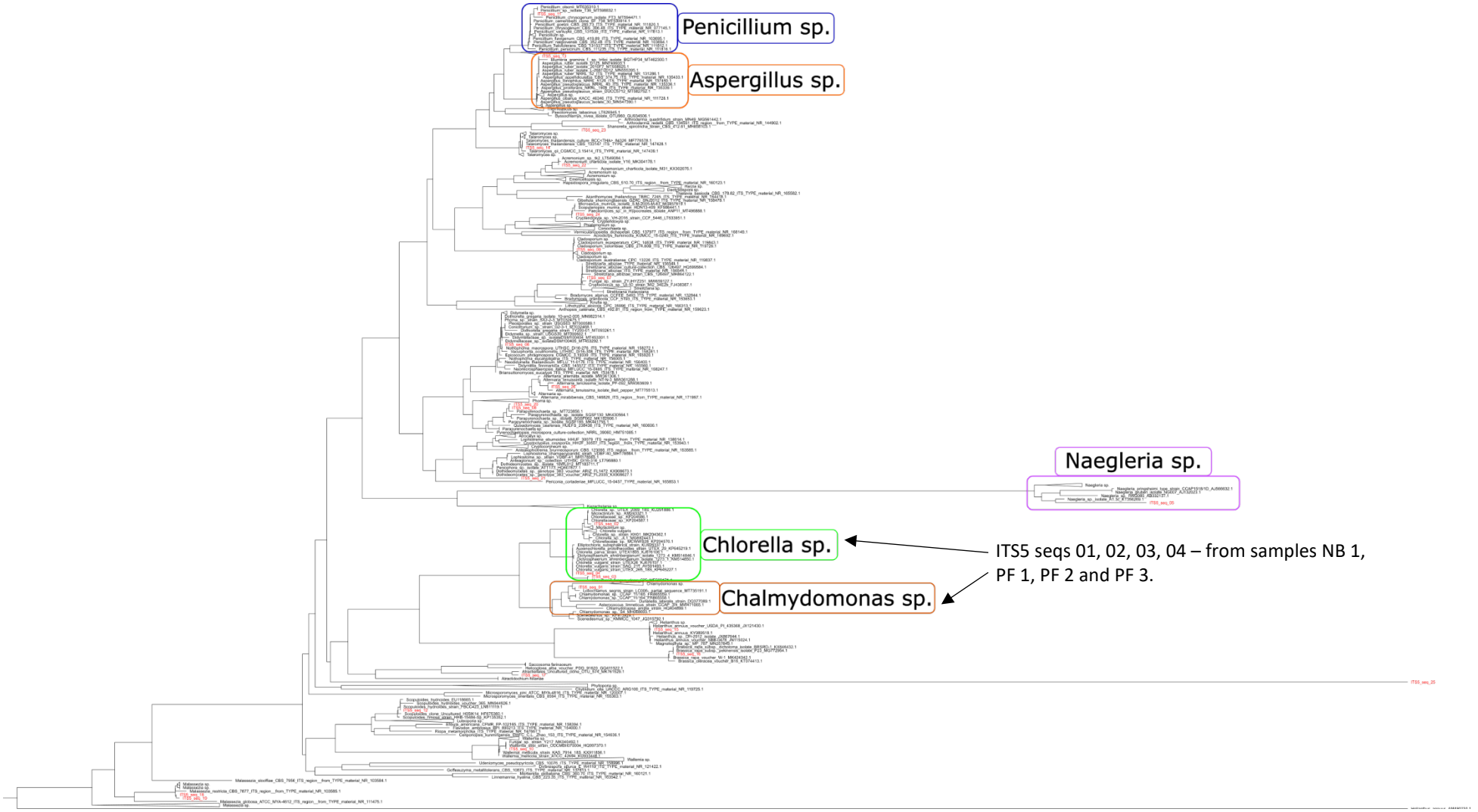


Figure 3.11. Tree of ITS5 sample group alongside similar sequences database, showing how they align and group within an unrooted tree, sorted from the midpoint. Sample sequences are highlighted in red text, with some groups circled.

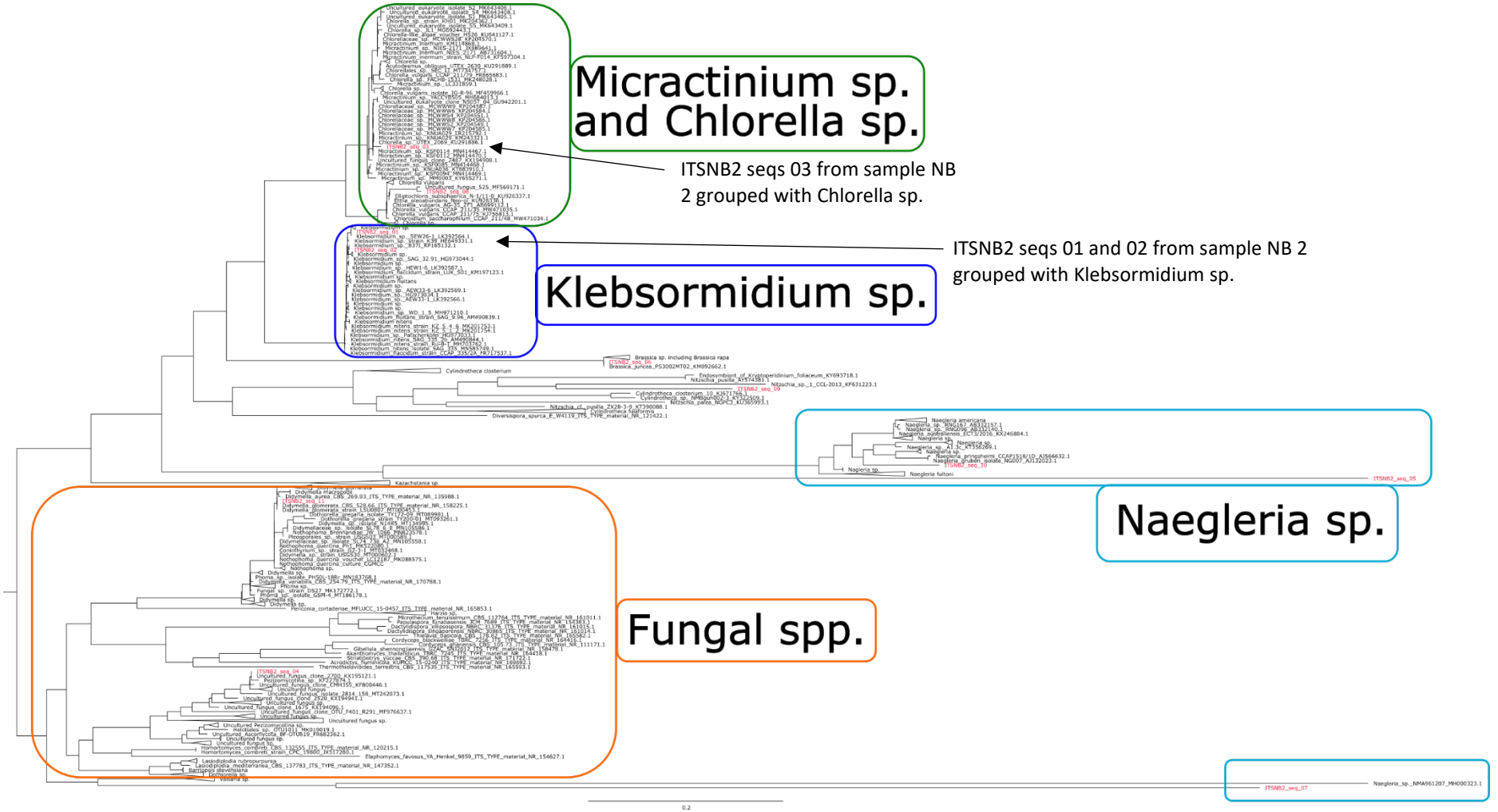


Figure 3.12. Tree of ITSNB2 sample group alongside similar sequences database, showing how they align and group within an unrooted tree, sorted from the midpoint. Sample sequences are highlighted in red text, and some groups are circled.

3.6.6. *Priors Farm 3 – Family Chlorellaceae sp., Order Oscillatoriales sp. and unknown diatom.*

The Priors Farm sample 3 ITS analysis found only one unique sequence. The NCBI BLAST matches identified it as of either an unidentified Family Chlorellaceae sp. (98.96% identity match, 100% sequence cover), Micractinium sp. (98.7% identity match, 95% sequence cover) or Chlorella sp. (96.63% identity match, 100% sequence cover). The ITS tree (Figure 3.6) grouped the related Chlorella sp. with a few Micractinium sp., making definitive sequence identity difficult though the Family identity is more defined. Therefore, this algal species has family definition but not genus or species.

The 16S sequencing found support for the Family identity, with both Micractinium sp. and Chlorella sp. mitochondrion found, with 99.26% identity for both, as well as Micractinium and Chlorella chloroplasts. The 16S also found sequences from a cyanobacteria in the order Oscillatoriales, and a few Klebsormidium sp. chloroplast sequences. Around 12 bacteria species were identified, with very low number of sequences for each. These included *Phyllobacterium* sp., *Sphingomonas* sp., *Roseomonas* sp. and *Mesorhizobium* sp..

3.7. Discussion

Through sequencing using 16S and ITS primers, species were identified in each isolate culture. Identification using NCBI matches alone can be problematic, especially if shorter sequences of 16S and ITS were involved (Edgar, 2018). Phylogenetic analyses further confirmed species identification and helped rectify any issues derived due to low percentage match or multiple species assignation. Tree support was found for all algal species identified through NCBI BLAST searches, though many bacteria could not be identified past family or order level.

The cosmopolitan and highly variable *Chlorella vulgaris* species was found in isolates from both locations. The Nant y Brwyn sample 1 and Priors Farm sample 1 were found to be cultures of a single species of *Chlorella vulgaris*, though both were likely a different strain than those currently registered in the NCBI database. They contained little to no fungal presence, and low bacterial diversity (Table 3.11). Most sequences were based on *Chlorella vulgaris* nuclear or chloroplast DNA. *Chlorella vulgaris* is a generalist algal species, with a wide range of phenotypic plasticity possible (Hollis, 2014; Hollis and Hüner, 2017) and found in a variety of habitats (Aigner *et al.*, 2020). It may

be that the plasticity of the species means it is less dependent on its microbiome, that it grew sufficiently fast to be isolated from local bacteria, or that this is coincidental. A wider study would be needed to ascertain this, which is separate to the aims of this thesis but may be worth investigating. Bacteria present or absent in close proximity to algal species will greatly impact on the bioavailability of DON to the stream as a whole. Other species from the same family, Chlorellaceae, were found in other isolate samples.

Other algal species identified included a filamentous green algae, *Klebsormidium* sp. from the Nant y Brwyn sample 2, with low levels of other algal species found and few fungal matches. This is also a cosmopolitan algal genus, found in various freshwater and terrestrial habitats, including arid soils (Rindi *et al.*, 2011) and urban environments (Rindi, Guiry and López-Bautista, 2008), though it tends to largely be found in moist soils or aquatic environments. The bacterial diversity (via 16S) was much higher in this sample than the fungal and algal species diversity (via ITS) and is the most diverse isolate culture analysed (Table 3.11). This diversity may be due to the filamentous nature of the algae, as it gives a large 3D structure for bacteria to cling to or be encapsulated within when cultures are transferred to new agar plates during sub-culturing. This diversity of community is likely to allow more pathways for DON use, as well as more complexities in handling the filamentous algae.

Cultures isolated from the upland peat location (Nant y Brwyn) tended to be more diverse in terms of total species number than those from the upland agricultural catchment (Priors Farm) (Table 3.11). This may not necessarily reflect initial diversity differences, but does mean that cultures appear to retain more associated microbiome from the upland peat site than the farmland location.

Table 3.11. Comparison of sample diversity (number of unique sequences) from 16S and ITS2 analysis.

Analysis	Sample diversity (number of unique sequences)					
	Nant y Brwyn sample 1	Nant y Brwyn Sample 2	Nant y Brwyn sample 3	Priors Farm sample 1	Priors Farm sample 2	Priors Farm sample 3
16S (bacteria species and algal chloroplasts)	17	48	24	5	23	17
ITS2 (algae & fungus species)	3	11	22	2	5	1

Some samples showed the presence of organisms that may impede growth, such an amoeba (*Naegleria sp.*) and various fungal species. There were also bacteria that may improve their ability to process DON present. The Priors farm *Chlorella vulgaris* (sample 1) was the least diverse culture, and Nant y Brwyn *Klebsormidium* sp. (sample 2) the most diverse. It is worth noting that some *Klebsormidium sp.* and strains can have a superficial sheath layer to the filament (Rindi, Guiry and López-Bautista, 2008), which was seen in this strain under microscopy in liquid culture. This provides some separation from bacteria on the surface of the algal cells, as well as a substrate that can sustain and transfer multiple microbiome species along with the algal cells during sub-culturing.

The Nant y Brwyn sample 1, *Chlorella vulgaris*, had low bacterial diversity compared to other samples, though identified microbial components included some bacteria associated with soil and faeces. This site is largely upland peat that is lightly grazed by sheep, with sheep faeces and bacteria washing in the river, likely where these have originated. It is interesting that these are still present in the samples after a long period of sub-culturing, suggesting they are persisting on agar and in liquid medium, though their contribution and abundance in the isolate culture is unknown.

There was difficulty in extracting DNA from the *Nitzschia palea* diatom of Nant y Brwyn sample 3 via the ITS primer. This was perhaps due to the generalised extraction used and resistance of diatom frustules, though they were sequenced and can generally be sequenced using commercial test kits (Vasselon *et al.*, 2017), though a diatom specific primer could have improved extraction (Mortágua *et al.*, 2019). This isolate showed considerable contamination with an amoeba, various fungi and a range of bacteria present (ITS and 16S data). This sample contained the most diverse range of species, some of which have negative impacts on the growth of photosynthetic algal species. The diatom's slower growth rate means it is more easily outcompeted by fungi or bacteria for the nutrients in the isolation agar or the liquid media, perhaps causing it to rely on interactions with the cyanobacteria, which will compete more favourably with other bacteria. There may be competition between the two photosynthetic organisms as well. The much lower read number of *Nitzschia palea* could be for various reasons including; poor primer performance, poor PCR amplification of these sequences, problems in DNA extraction from the diatom relative to the bacteria and fungi present. The sampling company noted this sample as being problematic to obtain high quality DNA/RNA from (*pers. comm.*). It is also possible that this sample was not representative enough of the isolate culture, perhaps past the exponential stage of growth and in the declining stage, given the higher bacterial and fungal read numbers compared to other samples sent for

analysis. The ITS region has also been shown to provide more divergent taxonomic results for diatoms compared to other algae (Guo *et al.*, 2015).

There was also difficulty in extracting DNA from the Priors Farm sample 3. Under microscopy, a mixture of cyanobacteria and diatom cells were seen. The Osciallatoriales cyanobacteria seen with low read counts, perhaps suggesting difficulty in extraction. It is possible that the cyanobacteria was in very low density or hard to RNA from in this sample, similar to the *Nizschia palea* issues in Nant y Brwyn sample 3 mentioned above. Cyanobacteria often produce exo-polysaccharides (EPS) that can make DNA extraction much more difficult (Billi *et al.*, 1998; Morin *et al.*, 2010). In cultivation this isolate varied between diatom and cyanobacteria dominance, which may have had low cyanobacterial numbers present when sent for genetic sampling. The differences in morphology between cyanobacteria features and a typical *Micractinium* sp. makes it highly unlikely that the two were conflated on visual examination of the cultures. Some *Micractinium* species do have spindle extrusions (Chae *et al.*, 2019) , but these look different to diatom frustules, and it is possible the green algae is Chlorella species instead. The isolate likely contains three algal species not fully identified – Family Chlorellaceae sp., Order Oscillatoriales sp. and an unknown diatom species, alongside the fungal and bacterial species identified.

DNA barcoding is not a new technique for identifying species. It does rely on the reference library covering a wide and deep enough range of species to enable useful comparisons. A number of unique sequences had difficulty in reliably being identified to species level. This is not a novel issue in algal and bacterial identification, though it varies between algal groups (Mortágua *et al.*, 2019). As research progresses in future and more verified species undergo genetic analysis, the coverage and depth of genetic reference libraries will increase, and re-analysis of previous identifications could improve field understanding. Some species also proved more difficult to extract (Cyanobacteria, diatoms) which may necessitate more targeted extraction techniques. As shown by Priors Farm sample 3, genetic analysis benefits from being combined with traditional microscopy and taxonomic skills to identify issues with sample processing or primer adhesion. It also highlights that though this chapter can define what species were definitely present in each sample, there may also be undetected species not represented due to very low abundance or cells resistant to DNA extraction.

3.8. Conclusions

A range of algal species were isolated, including similar species at both locations (Chlorella sp.). The associated microbiome varied distinctly between samples, both in terms of fungi and

bacteria present. This is likely to impact on the algal ability to utilise DON, and potential detrimental effects of competition for similar resources from fungal species, or predation by amoeba. More detailed genome analysis of the species found, or close relatives, could help in understanding what potential pathways are present for DON processing and bioavailabilty in the freshwater habitat isolates originated from.

Chapter 4 – Can dissolved organic nitrogen support algal growth in streams draining from a heavily farmland impacted area?

4.1. Aims

This section explores whether dissolved organic nitrogen can be used as a nutrient resource by algae, through experimental testing. These tested biomass responses in vitro, to a series of different DON and inorganic nitrogen compounds under replicated, controlled laboratory conditions. Isolated species from an upland, farmland catchment were used to experimentally compare growth on inorganic and organic nitrogen compounds. This is in an agricultural dominated catchment. In the River Sem near Priors Farm, mean annual DON concentration over the three year period 2015-2018 was 1.25 mg N L^{-1} and 33% of the TDN pool instream (Yates and Johnes, 2013).

4.2. Introduction and context

Algal production in rivers varies according to a range of conditions, between rivers of differing environmental character, and even between different reaches of the same river, depending on local light conditions and whether there is overhanging riparian vegetation, riverine hydro-morphology, and the species composition of the reach. To determine the specific response of algal species to DON chemistry relative to inorganic N chemistry, a series of experiments were designed using algal species isolated from just one site location, removing this set of environmental controls from the resulting response of the algal isolates. This allowed investigation of whether isolated species are able to use DON as a nutrient resource, whether that ability varies according to the DON chemistry presented, by species, and in comparison to algal growth responses when presented with comparable N dosing in an inorganic form (nitrate, ammonium). The specific hypotheses tested are:

Hypothesis 1 – Isolated algal species can utilise both inorganic and organic nitrogen sources for growth.

Hypothesis 2 – Isolated algal species have different species-specific and compound-specific preferences for growth on inorganic and organic nitrogen.

4.2.1. *Experimental approach and methods*

This chapter used a bioassay approach including replicate testing of algal growth responses to algal cultures isolated from the River Sem in the Hampshire Avon catchment which is intensively farmed for dairy production, as described in Lloyd *et al.* (2019) and Yates *et al.* (2019). Isolate collection and cultivation is described in Chapter 3, where the methods used for analysis of all algal samples are also described in detail. A broad experimental overview is shown in Figure 4.1.

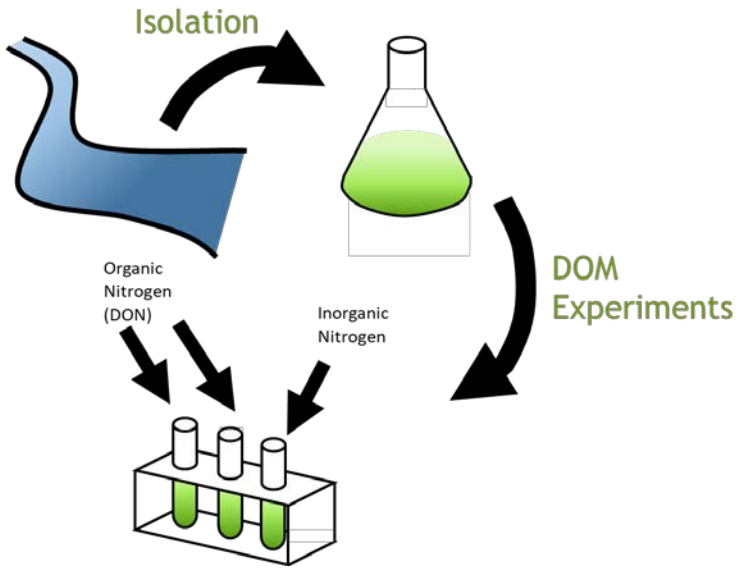


Figure 4.1. Overview of sampling and experimental process

4.3. Results and discussion

Using the results from the pilot experiments, the main experiments were designed to test the hypotheses outlined in 4.1. These comprised a series of 3 replicate experiments each with a different species isolated and cultured from samples collected in the River Sem at Priors Farm. The experimental design and methodology was as described in Chapter 2, unless otherwise stated. Results are presented with mean cell counts or concentrations ± standard error of the mean.

4.3.1. *Experiment 1 - Chlorella vulgaris*

This experiment used an isolate of *Chlorella vulgaris*, isolated alongside some bacteria but with no other algal species found in culture. It started on 27 Sept 2018, running for 14 days. This initial experiment differed from the main methodology, as no B vitamins were added and it was not

buffered with $NaHCO_3$ (though all media started at pH 7 and ended at pH 6.5-7.5). This experiment took place between pilot 6 and 7, before the need for buffering and additional vitamins were seen later in other isolates.

Cell growth above initial levels was observed in the nitrate and urea treatments after two weeks. The nitrate treatment increased cell counts to 1.5 ± 0.1 million cells mL^{-1}, while the urea treatment resulted in an increase to 2.8 ± 0.2 million cells mL^{-1}. The increase in cell numbers in the nitrate and urea treatments is supported by the increase in particulate carbon and nitrogen concentrations in samples collected at the end of the experiment (Figure 4.2). By contrast, this species did not grow well on the ammonium (0.027 ± 0.001 million cells mL^{-1}) showing no statistically significant response relative to the control treatment (control 0.027 ± 0.002 million cells mL^{-1}, $t_{(3.6)}$ = 0.3, p=0.77). The glutamate treatment cell count of 0.017 ± 0.001 million cells mL^{-1} was significantly less than the control ($t_{(3.0)}$ = 4.5, p=0.02).

The nitrate and urea treatments not only showed increased cell growth but also showed a decrease in the N fraction concentration provided to them at the start of the experiment, as well as decreased media concentrations of TDP and PO_4-P (Figure 4.2). Media nutrient concentrations in the ammonium, glutamate and control treatments remained unchanged over the course of the experiment. This suggests that the media nitrogen and phosphorus were removed by the *Chlorella* spp. in the treatments showing cell growth, but not in treatments where cell growth did not occur.

Dissolved organic carbon (DOC) increased in all treatments except in the glutamate treatment (Figure 4.2). The glutamate treatment started at a higher concentration (4.9 ± 0.02 mg C L^{-1}) and remained similar at the end point (5.1 ± 0.3 mg C L^{-1}, $t_{(4)}$ = -0.6, p= 0.57). This increase in the non-glutamate treatments is potentially due to the release of an exudate from the algal cells over the course of the experiment, as all treatments other than the glutamate treatment showed DOC in the media at the end of the experiment. Both organic nitrogen sources contained carbon, hence the higher initial DOC concentration in all DON treatments at the start of each experiment, compared to the inorganic N treatments.

Particulate carbon concentrations increased in both the nitrate and urea treatments (Figure 4.2), as would be expected in a rapidly growing culture. This was alongside an increase in particulate nitrogen, that increased from 0.21 ± 0.019 µg N L^{-1} starting concentrations to 0.94 ± 0.043 µg N L^{-1} in the nitrate treatment ($t_{(9)}$ = -2.2, p= 0.053) that bordered on statistical significance at the 95% level, and 0.78 ± 0.007 µg N L^{-1} in the urea treatment ($t_{(11)}$ = -1.8, p= 0.096) that was not statistically significant. The incorporation of additional nitrogen into the biomass of the algae over the course of the two-week experiment and a reduction in media nitrogen concentrations supports the

conclusion that the media nitrogen was being used for cellular processes. The treatments that did not increase in cell numbers, ammonium and glutamate, decreased in particulate carbon during the experiment, with a small decrease in particulate nitrogen concentrations over the same time.

All treatments decreased in their carbon to nitrogen ratio. For the actively growing nitrate and urea treatments the increased rate of particulate nitrogen accumulation over particulate carbon caused this effect. The ammonium and glutamate treatments that did not exhibit a cell growth response decreased in their carbon content to a greater extent than for their nitrogen content, causing the C:N ratio to decrease but for very different fundamental reasons.

These data show that *Chlorella vulgaris* can grow when provided with nitrate or urea in the surrounding media, but not when provided with ammonium or glutamate as nitrogen sources. As this was one of the earlier experiments, however, the media was not buffered by $NaHCO_3$ and did not have additional B vitamins added. This may have impacted on this species ability to use the ammonium and glutamate provided, though they were able to make extensive use of the nitrate and urea provided under the same conditions.

The lack of response to ammonium dosing contrasts with what would be expected from literature which suggest that ammonium is an important source of nitrogen for algal species in freshwaters (Glibert and McCarthy, 1984; Ganuza, Anderson and Ratledge, 2008; Flynn, Fasham and Hipkin, 1997; Arango *et al.*, 2008). It may be that this species depends on the presence of bacteria, other algae or micro-invertebrates acting as consortia to access these compounds, while it is inherently able to utilise nitrate and urea, perhaps due to the presence of nitrate and urea transporter genes and absence of direct ammonium or glutamate transporter genes (Hildebrand and Dahlin, 2000; Calatrava *et al.*, 2019; Terrado *et al.*, 2015). Another possibility is that the pH or light levels restricted the ability to utilise the ammonium present. There is also some literature using stream biofilms that found reduced nitrate uptake when presented with ammonium, and the inverse may also be impacting these results (Ribot *et al.*, 2015). These require further investigation to properly attribute this response to the correct causal factors.

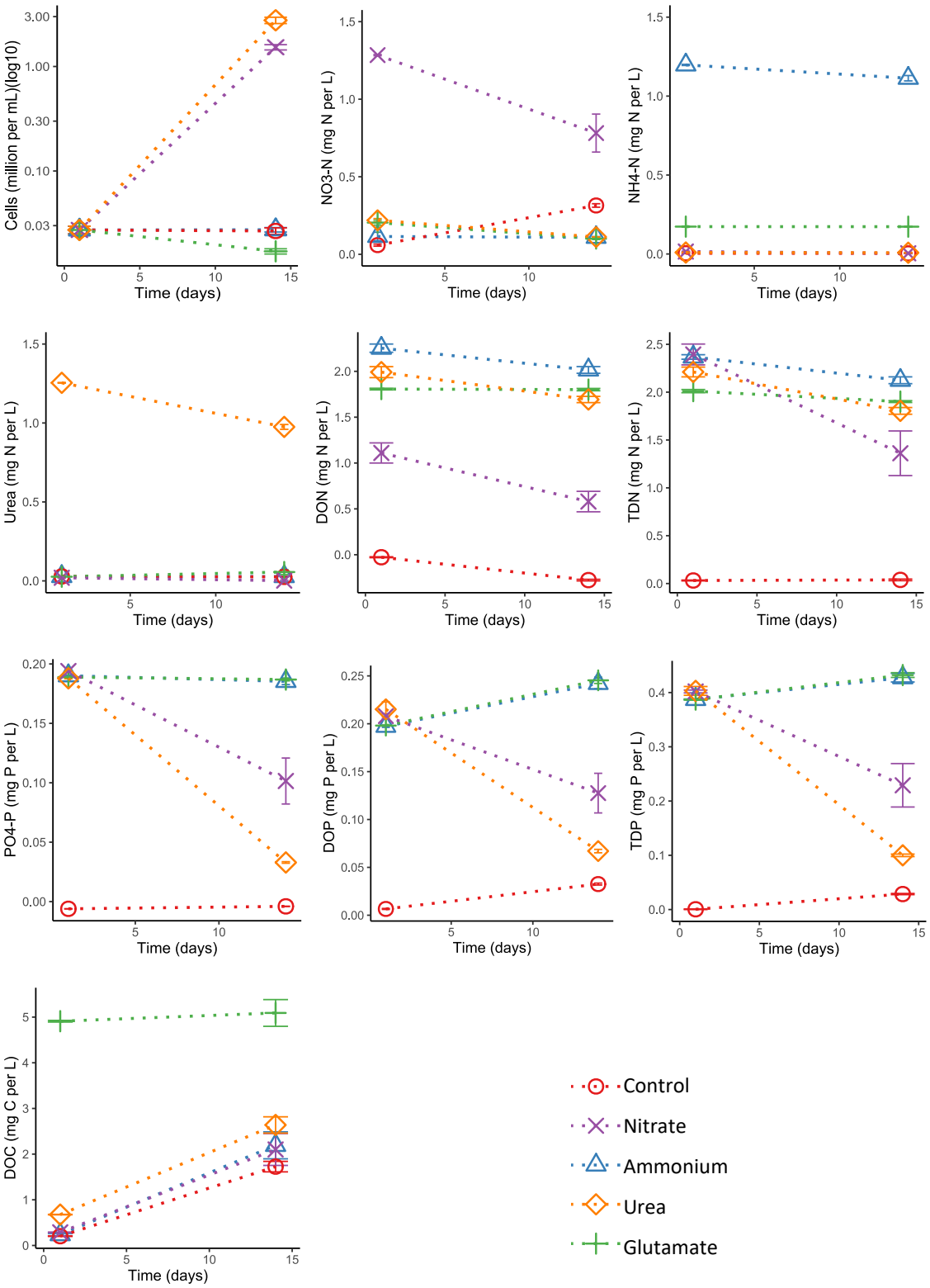


Figure 4.2. Analyses of media and particulate compounds from Experiment 1, Chlorella vulgaris., showing cell number, NO_3-N, NH_4-N, Urea, DON, TDN, PO_4-P, DOP, TDP, DOC, particulate C, particulate N, and particulate C:N ratio. All points show mean ± standard error.

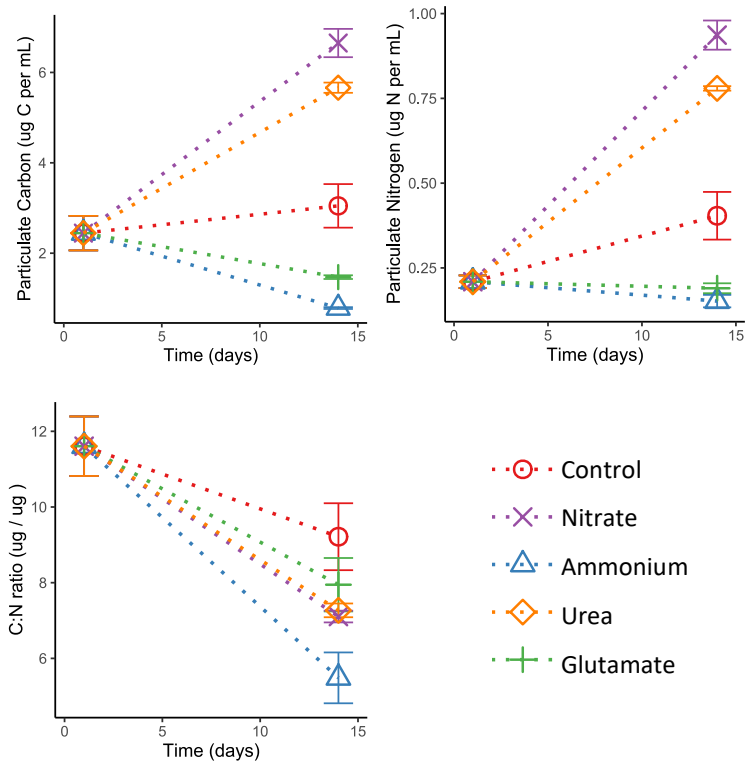


Figure 4.2 Continued. Analyses of media and particulate compounds from Experiment 1, Chlorella vulgaris, showing cell number, NO_3-N, NH_4-N, Urea, DON, TDN, PO_4-P, DOP, TDP, DOC, particulate C, particulate N, and particulate C:N ratio. All points show mean ± standard error.

4.3.2. *Experiment 2 - Family Chlamydomonadaceae c.f. Lobochlamys sp.*

This experiment involved an isolate of c.f. *Lobochlamys sp.* cultured from the same site as in Experiment 1, as described in the methodology (Chapter 2) and identified in Chapter 3. The species is a spherical, flagellated green algae, and was often seen in its dividing stages during the experiment (multiple individual cells grouped together and suspended in gelatinous mucus). The experiment started on 26 Nov 2018 and ran for 18 days. This initial experiment differed from the main methodology, as no B vitamins were added, but unlike in Experiment 1, it was buffered with $NaHCO_3$.

In marked contrast to Experiment 1, all nitrogen addition treatments saw an increase in cell numbers over the course of the experiment. The largest increase from starting cell numbers (57 ± 6 thousand mL^{-1}) was seen in the urea treatment (128 ± 7 thousand mL^{-1}, $t_{(14)} = -7.7$, $p < 0.001$), then nitrate (108 ± 6 thousand mL^{-1}, $t_{(17)} = -5.7$, $p < 0.001$), ammonium (93 ± 4 thousand mL^{-1}, $t_{(17)} = 5.0$, $p < 0.001$) and glutamate (84 ± 5 thousand mL^{-1}, $t_{(18)} = -3.2$, $p = 0.0045$). The magnitude of this increase compared to starting cell numbers, while relatively consistent across nitrogen compound

types, was much lower than that seen in the responses of *Chlorella vulgaris* mentioned above to the same conditions.

TDN concentrations decreased in all nitrogen addition treatments by the end of the experiment, consistent with nitrogen uptake by the algal cells, with the largest decrease in the urea treatment that showed the highest cell growth ($t_{(1.7)} = -54.8$, $p = 0.001$). The other three treatments showed a similar final TDN concentration between 1.7-1.9 mg N L^{-1}, which was nevertheless still greater than the control concentration of 0.1 ± 0.04 mg N L^{-1}.

Each type of nitrogen addition showed a decrease in the concentration of the supplemented form of nitrogen (Figure 4.3). This suggests the use of the nitrogen provided to support algal growth, though no treatment depleted the media nitrogen fully. The pattern of media nitrogen use differs compared to that observed in the first experiment, as all treatments showed some media N depletion in association with cell growth response to dosing.

Phosphorus is mostly present at the start of the experiment as PO_4-P in the concentration range 0.39 ± 0.003 mg P L^{-1}, with concentrations decreasing in each nitrogen addition treatment. There were two groups of end point PO_4-P concentration from similar starting values, ranging from 0.07-0.08 mg P L^{-1} in the organic N treatments (urea $t_{(4.8)} = -84.6$, $p < 0.001$, glutamate $t_{(3.3)} = -77.6$, $p < 0.001$), and 0.14-0.18 mg P L^{-1} in the inorganic N treatments (nitrate $t_{(4)} = -67.3$, $p < 0.001$, ammonium $t_{(2.4)} = -95.9$, $p < 0.001$). The increased reduction in available phosphorus by the organic N treatments is not reflected in a higher growth rate than in the inorganic N treatments: although both removed a similar amount of PO_4-P, only urea showed a higher rate of cell growth. The control increased in DOC over the course of the experiment but was not statistically significant ($t_{(2.1)} = 3.6$, $p = 0.062$), and all other treatments did not show any significant changes over time.

The highest growth response in Experiment 2 was to the urea treatment, which was higher than that for both inorganic N treatments. The magnitude of all cell number increases were lower here, however, compared to Experiment 1. It may be that the production of mucus or differences in growth speed may be the reason. It may also be that the *Lobochalmys sp.* culture found other conditions in the growth cabinet to be more challenging to their growth. For example it may have needed access to higher light intensity, or to grow within a consortium of algal and microbial cells to maximise its growth response to nutrient dosing.

Samples were collected in this experiment for particulate carbon and nitrogen analysis, as in Experiment 1, but although samples were prepared for analysis and combusted, a technical malfunction meant they were not analysed. Due to this, no particulate C and N data are available in this experiment.

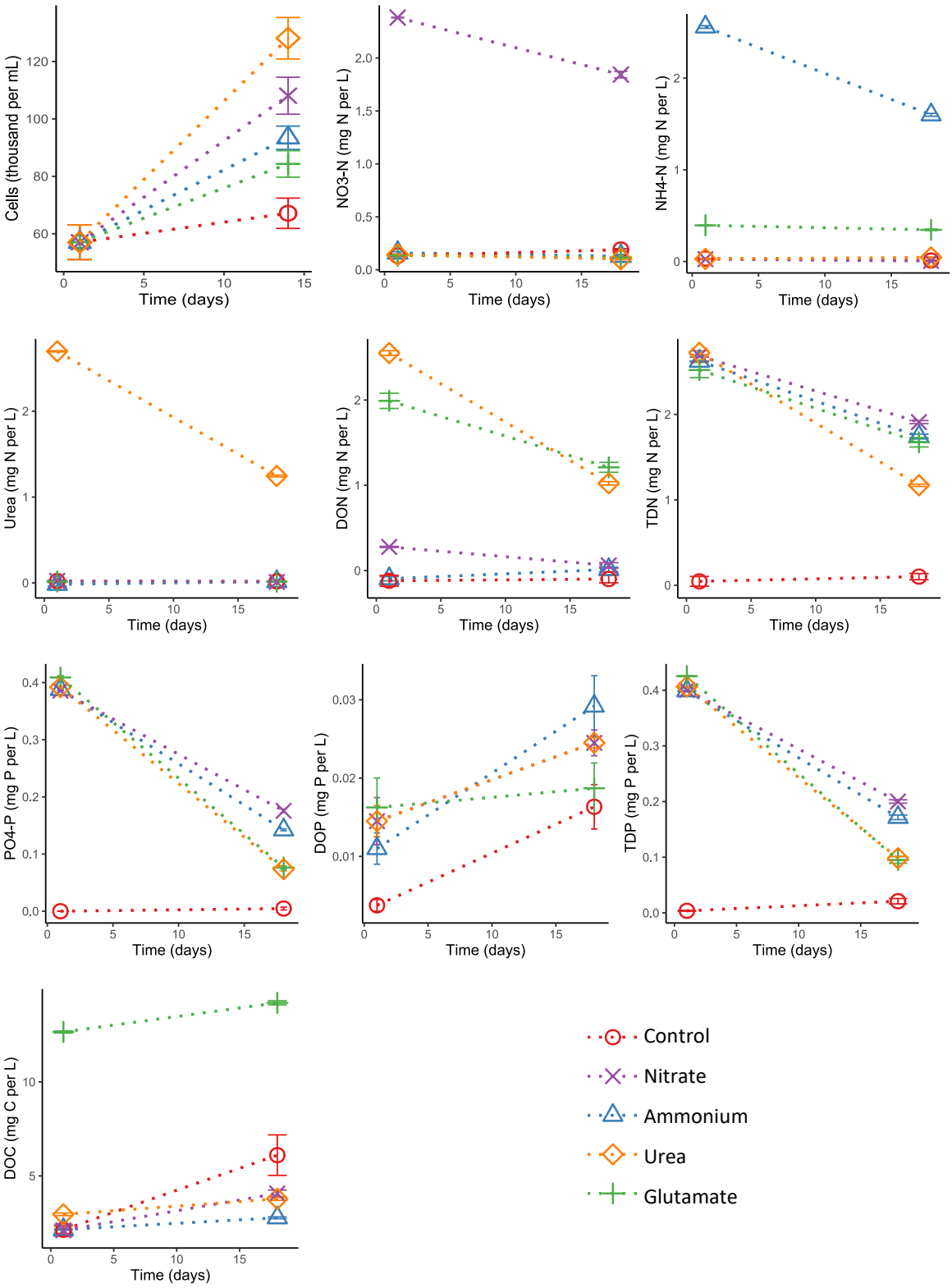


Figure 4.3. Analyses of media and particulate compounds from Experiment 2, Lobochalmys sp., showing cell number, NO_3-N, NH_4-N, Urea, DON, TDN, PO_4-P, DOP, TDP, and DOC. All points show mean ± standard error.

4.3.3. *Experiment 3 - Family Chlorellaceae sp., Order Oscillatoriales sp. and unknown diatom*

This experiment used a unicellular diatom isolate (*unknown species*) in co-culture with a cyanobacteria species (Order Oscillatoriales, see Chapter 3) and a (*Chlorella* sp. or *Micractinium* sp.) of the Family Chlorellaceae. These were from the same geographical site as in Experiments 1 and 2 (see Chapter 2). The experiment started on 05 February 2019 and ran for 14 days. It ran as per the main methodology described in Chapter 2, using $NaHCO_3$ buffering to adjust each treatment to pH 7, with three additional B vitamins added to each treatment to meet cell micronutrient needs. This species was initially cultured in experimental conditions without vitamins, and was shown to require them for any growth to occur.

As many stream locations have diatoms adhered to rocks and benthic debris, the response of a diatom species was thought important to understanding how many river reaches would respond to changes in organic nitrogen loading from catchment sources. It was very difficult to isolate the diatom without the cyanobacteria associated with it, though the cyanobacteria could be grown without the diatom for stretches at a time. Maintenance of culture plates during their lab cultivation has shown this species to fluctuate between cyanobacteria and diatom dominance on a plate. Repeated attempts were made to keep the two species separate but without success. The cyanobacteria was able to grow in an almost mono-culture state on an agar plate for >3months before diatoms visibly grew. The diatoms have been very difficult to grow successfully on the agar plates without the cyanobacteria. The few cases where cyanobacteria have been successfully excluded from the plate, the diatoms have not grown well and began to lose their pigmentation after a few weeks. It is likely that the cyanobacteria is providing some trace nutrient or compound to the diatoms which is not present in the isolation or experimental media. Therefore, a decision was taken to run this culture using both species.

The results shown here have more complex interactions than those previously shown in Experiments 1 and 2, with two species present in the culture concurrently. This experiment was attempted twice, as the first time the culture did not grow (over a 2-week time period) until the isolation media vitamin solution was added at the same concentration as the isolation media. To prevent this in future, all experiments from this point on had vitamins B1, B7 and B12 added to them. A variety of algae from different taxonomic groups have been shown to require some (or all) these B vitamins to grow (Croft *et al.*, 2005; Sañudo-Wilhelmy *et al.*, 2014).

A starting culture with a diatom dense culture and very few cyanobacteria visible under a microscope (x400) was used as the inoculation of this experiment. Both types of cells were counted

separately. Few cyanobacteria cells and almost no filaments were seen in counting chambers at the start of the experiment. Cyanobacteria were much more visible when counting towards the end of the experiment, increasing accuracy of counts at the experiment end point. Figure 4.4 shows two cell count graphs, one for the diatom species, the other for the cyanobacteria.

The diatom cells showed the greatest increase in cell numbers compared to the control in the urea (56.0 ± 11.8 thousand cells, $t_{(7.4)} = -1.1$, $p = 0.31$) and glutamate (60.0 ± 14.9 thousand cells, $t_{(6)} = -1.2$, $p = 0.29$) treatments, though this was not significantly different to the control. There was a wide range spread of cell counts and a relatively large standard error range (Figure 4.4 a). The nitrate (43.5 ± 3.0 thousand cells, $t_{(8.1)} = -0.44$, $p = 0.67$) and ammonium (36.3 ± 4.5 thousand cells, $t_{(9.5)} = -0.22$, $p = 0.83$) treatments had growth similar to the control (38.8 ± 10.4 thousand cells), all of which were above starting cell numbers (22.8 ± 1.5 thousand cells). The cell numbers of both species increased in the control compared to the start ($t_{(7.3)} = 1.5$, $p = 0.17$), possibly showing that there was a movement of essential nutrients between the two species and their initial, internal nutrient pools were enough to support some small growth, or that the cyanobacteria were fixing N actively during the experiment under the lower N concentrations in the control.

The Oscillatoriales species had low initial cell counts (1.7 ± 0.4 thousand cells mL^{-1}), and increased the most after two weeks in the ammonium treatment (34.0 ± 7.8 thousand cells mL^{-1}, $t_{(5)} = 4.1$, $p = 0.001$), with the next largest increase in the nitrate treatment (23.5 ± 5.0 thousand cells mL^{-1}, $t_{(5)} = 4.4$, $p = 0.007$). The glutamate and urea treatments showed some cell growth at 21.3 ± 11.8 thousand cells ($t_{(3)} = 1.7$, $p = 0.20$) and 18.1 ± 4.4 thousand cells ($t_{(3)} = 3.7$, $p = 0.033$) respectively, but unlike in the diatom cell counts, these were much closer to the control cell numbers of 10.6 ± 1.5 thousand cells.

Dissolved organic carbon concentrations had decreased by the end point measurement in the glutamate treatment. This differs from the trends seen in the previous two experiments, where the DOC in the glutamate treatment remained close to starting concentrations. DOC remained largely the same as the starting levels in the ammonium, nitrate and urea treatments, though they increased in the control treatment ($t_{(11)} = 19.5$, $p < 0.001$). DON compounds as a source of nitrogen are the focus of the experimental work, but it may also be possible that the carbon within the compound is of use to algal species.

All treatments showed a decrease in the concentration of the dosed media N sources, as observed for *Lobochalmys sp.* in Experiment 2. Both urea and glutamate treatments showed an increase in media ammonium concentrations at the end of the experiment. This was not seen in the first two experiments using green algae species. This may indicate that the organic N treatments

were converted, by either the diatom or more likely the Oscillatoriales species, into other N compounds before use. This transformation of N between different N compounds is highly likely to occur in a river location as there are multiple species capable of a wider range of biochemical processes, making organic matter more bioavailable (Stevens and Quinton, 2009). The concept of nutrient spiralling in river systems is linked to this (Durand *et al.*, 2011; Newbold *et al.*, 1981; Dodds *et al.*, 2004; O'Brien *et al.*, 2007). The first two experiments using green algae showed that species can apparently use urea and glutamate directly, but the evidence in Experiment 3 suggests there may be an intermediate step where these are broken down to release inorganic N compounds prior to uptake for other species. Making use of N compounds as they alternate in chemical forms after processing by various species would be a valid survival strategy in a complex, biodiverse river location.

Many cyanobacteria can fix atmospheric N, however this experiment did not see an increase in the particulate N concentration in the control treatment, even though C fixation and some cell growth occurred during the two-week period. It is therefore unlikely that the cyanobacteria provided any significant quantity of N above that already present.

The total dissolved nitrogen concentrations decreased in all treatments. Regardless of which cells increased in number, all treatments showed a similar decrease to each other with the control remaining around zero. The evidence collected suggests that regardless of the pathway, media N is being used to support cell growth in addition to the dosed N form. It is likely in each treatment that different uptake pathways are being used, favouring each of the two species in different ways. Further investigation would be required to determine how the N is being used by the cells, and the dynamics between the species. The same trend was also seen in PO_4-P concentrations (Figure 4.4), where all treatments used similar amounts of P. The decrease in media N and P concentrations is likely due to its use to support the algal cell growth for both species.

All forms of N were used in this experiment, though each species utilised different N compounds. This may be due to preferences in uptake ability, different internal pathways for use or different uptake speeds that caused species dominance to be established early in the experiment that was then sustained. Variations in the presence of N compound transporters may also be important in controlling which N forms each species can use. Where this is lacking, the extracellular breakdown of more complex molecules may then allow the algal cell to access the N within these organic molecules. Investigation of multi-species utilisation of organic N would be better understood when using a time course experiment with mixed consortia to track dynamics between species. Particulate C concentrations increased in all treatments, including the control. The increase

in control particulate C amounts reflects the increase in cell numbers seen in the control treatment. The control treatment remained at initial particulate N concentrations. All N addition treatments increased in particulate N.

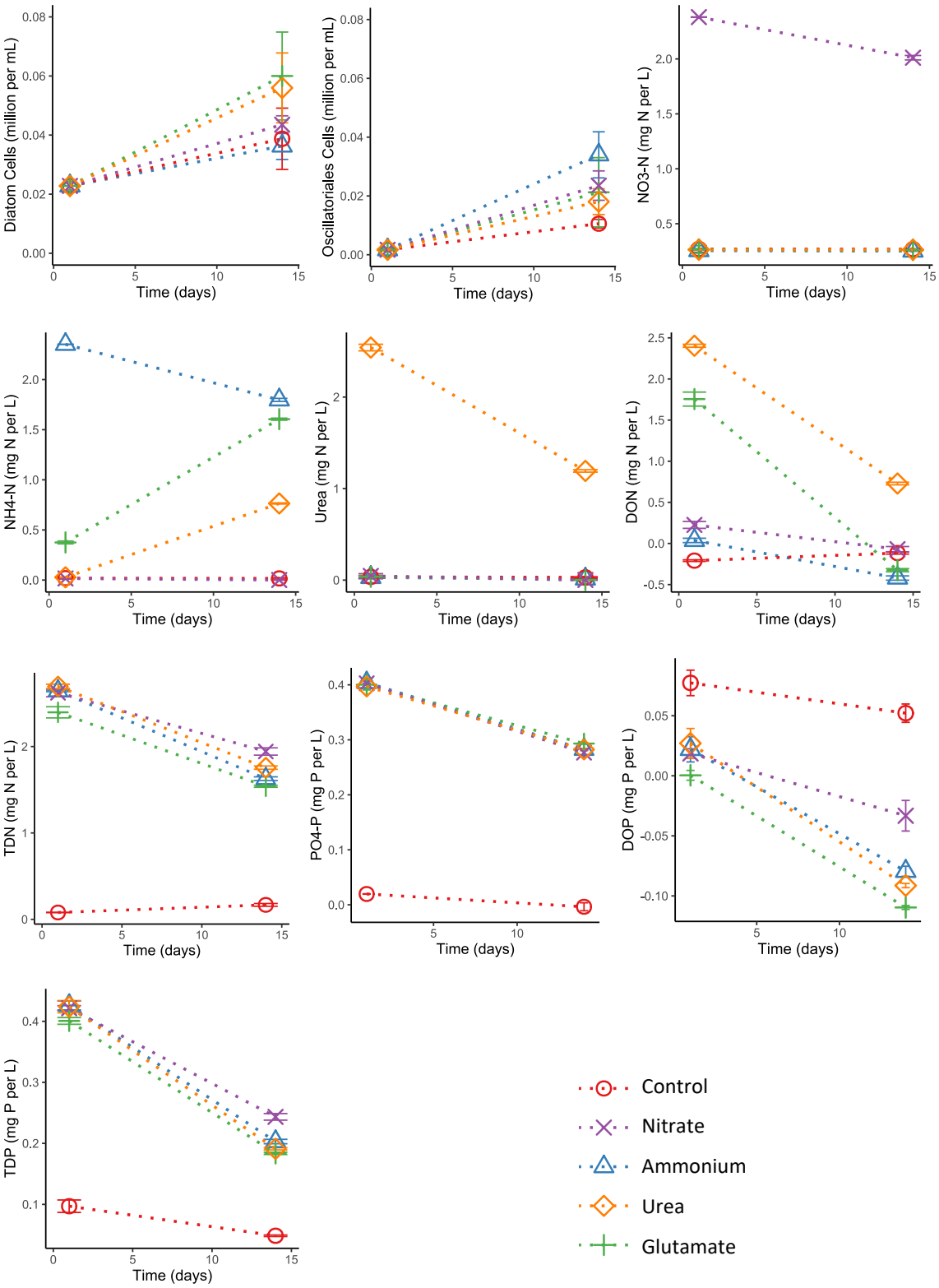


Figure 4.4. Analyses of media and particulate compounds from Experiment 3, mixed species including Family Chlorellaceae sp., Order Oscillatoriales sp. and unknown diatom, showing diatom cell numbers, Oscillatoriales cell number, NO_3-N, NH_4-N, Urea, DON, TDN, PO_4-P, DOP, TDP, DOC, particulate C, particulate N and particulate C:N ratio. All points show mean ± standard error.

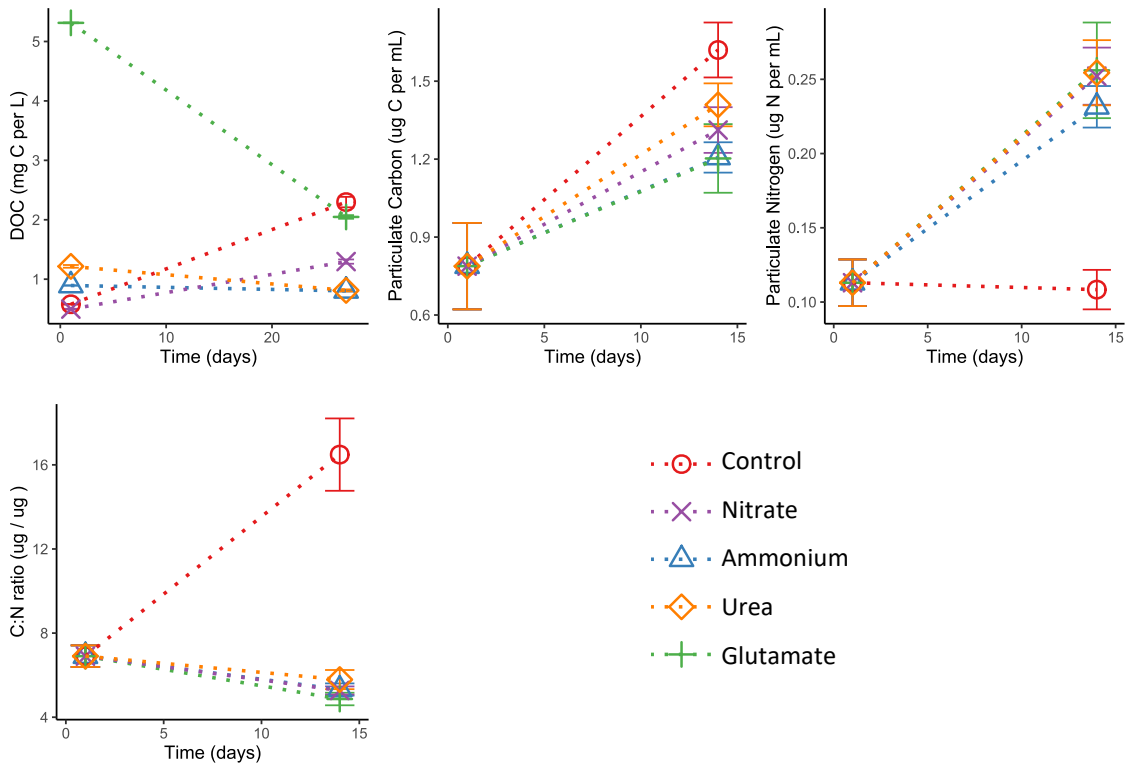


Figure 4.4. Continued. Analyses of media and particulate compounds from Experiment 3, mixed species including Family Chlorellaceae sp., Order Oscillatoriales sp. and unknown diatom, showing cell number of diatom, cell number of Oscillatoriales, NO_3-N, NH_4-N, Urea, DON, TDN, PO_4-P, DOP, TDP, DOC, particulate C, particulate N and particulate C:N ratio. All points show mean ± standard error.

4.3.4. *Comparison of species*

The three experiments examined how different species of algae isolated and cultured from the same initial N-rich stream varied in the magnitude of their response to different N compounds, and whether there was a species-specific and compound-specific response evident in the cell growth data. *Chlorella vulgaris* showed a positive cell growth response to nitrate and urea, but no significant response to ammonium or glutamate dosing. *Lobochalmys sp.* showed a lower magnitude response in cell growth than Chlorella, but could use all four N compounds, favouring urea and nitrate over ammonium and glutamate, though all had a positive response compared to the control. The Oscillatoriales/diatom culture responded to both urea and glutamate in preference to nitrate and ammonium, while the cyanobacteria alone responded to ammonium in preference to all of the other N compounds. An overview of this is present in Table 4.1.

Table 4.1. Overview of species responses to DON and inorganic treatments isolated from a clay catchment in agriculturally dominated location.

	Growth seen compared to starting point (positive or negative) per treatment				
Species	Control	Nitrate	Ammonium	Urea	Glutamate
Chlorella vulgaris	-	**+**	-	**+**	-
Lobochlamys spp.	-	**+**	+	**+**	+
Mixed species - Family Chlorellaceae spp., Order Oscillatoriales spp., Unknown diatom spp.	-	**+**	**+**	**+**	**+**

Nitrogen transformations of the dosed compounds was not evident in the single species experiments at the end point of the experiments. It is possible that nutrients were cycled through other chemical forms in the media during the experiment that were not captured by the end point sampling. However, this contrasts with the cycling apparent in the mixed species experiment where cyanobacterium spp. was present and showed a preference of ammonium over the other N compounds. In this third experiment, ammonium concentrations were clearly increased in the media of the organic N treatments at the end of the dosing period. There will be a complex interplay in real world stream locations between different sections of any biofilm or suspended algal community, and cycling of N through different chemical forms is more likely to occur in mixed consortia under ambient environmental conditions (Amon and Benner, 1994; Carlsson, Graneli and Segatto, 1999; Dodds *et al.*, 2004; O'Brien *et al.*, 2007). It is notable that green algae, the subject of the first two experiments, are clearly able to grow in response to both inorganic and organic N compounds, apparently without much intermediate transformation of more complex organic N molecules to inorganic N compounds occurring in the media via the microbiome.

The differences in processing organic N, as well as inorganic N support the idea that river reach processing of DON is species dependent, and that there is a compound-specific preference that varies between species (McCarthy, Lehman and Kudela, 2013; Evershed *et al.*, 2017; Reay *et al.*, 2019a). Greater diversity of species within stream microbial consortia is therefore likely to lead to utilisation of more N across a range of different compound classes (Zimmerman and Cardinale, 2014; Baker, de Guzman and Ostermiller, 2009). This has implications for river N processing

capabilities after local disturbance to or reduction of the stream algal biodiversity. Downstream N transport, both in terms of its quantity and molecular composition is likely also affected if there are major or lasting changes to abundance or species composition of the algal community, in addition to any changes in the bacteria present.

To explore this further, the mixed species experiment would need to be repeated with isolates, rather than as a multi-species mix, but this might not be possible without alterations to the media, to provide the essential compounds shared between them in co-culture. Overall, DON compounds were used by each species for growth. Though each N treatment differed in which species was making use of specific N compounds, overall DON was incorporated into algal biomass.

4.4. Conclusion

Riverine algal species isolated from a stream draining through intensively farmed land, have been shown to exhibit cell growth and an increase in their C and N content when presented with both organic and inorganic N sources. Species differ in their apparent ability to utilise forms of N, with some able to use nitrate and urea, but not ammonium and glutamate, while others appear to favour ammonium and glutamate. The magnitude of the response differed between each species tested. A combined species experiment incorporating an N-fixing cyanobacterial species showed depletion of TDN over the course of the experiment and a transformation in N speciation in the water column by the end of the experiment. Though each of the species in the combined vessel appeared to utilise separate N compounds to increase in cell number, and all N forms were used by both species in consortium, there was evidence of apparent transformation of organic N to inorganic N forms in the cyanobacteria-only data. The findings from this suite of experiments suggest that there are both species-specific and compound-specific responses to N compounds in riverine algal communities, and that both organic and inorganic N compounds are bioavailable nutrient resources to the freshwater algae.

Chapter 5 – Does catchment character and nutrient enrichment status affect freshwater algal utilisation of low molecular weight DON?

5.1. Aims

The work reported in Chapter 4 was based on algal species isolated from a single, nutrient rich stream draining through a landscape used for intensive dairy cattle production (Lloyd *et al.,* 2019). It could be argued that species in this stream would be acclimatised to a nutrient pool rich in lower molecular weight DON compounds such as urea and glutamate, both of which would be abundant in cattle slurries, urine and manures (Yates *et al.*, 2019). In the River Sem near Priors Farm, mean annual DON concentration over the three year period, 2015-2018, was 1.25 mg N L^{-1} and 33% of the TDN pool instream. This chapter established whether the same ability to utilise DON compounds as a nitrogen resource to support cell growth is present in upland, oligotrophic waters. Algal species were therefore isolated and cultured from another well-researched stream where DON concentrations are much lower than in the River Sem at Priors Farm, but where DON is also the dominant nitrogen form: Nant-y-Brwyn in the upper Conwy catchment in North Wales where mean annual DON concentration over the 2015-2018 period was less than half of that at the River Sem site, 0.452 mg N L^{-1} but it comprised 87% of the TDN pool available to the biota (Yates *et al.*, 2019). This allowed testing of the following hypotheses:

Hypothesis 2 – Isolated algal species have different species-specific and compound-specific preferences for growth on inorganic and organic nitrogen.

Hypothesis 3 – Similar species isolated from differing headwater catchments show the same growth response to DON chemistry.

5.2. Results

5.2.1. *Experiment 4 – Chlorella vulgaris*

This experiment was based on a spherical, single celled green algal species, *Chlorella vulgaris*. This is the same species as isolated and used in experiment 1, though a different strain. Methodology was as described in Chapter 2. The experiment took place over 14 days from 17 April 2019. Positive cell growth above the initial level (49 ± 3 thousand cells per mL) was seen in the glutamate treatment (248 ± 17 thousand cells per mL, $t_{(4)} = 11.7$, $p < 0.001$), and to a lower amount

in the ammonium treatment (94 ± 5 thousand cells per mL, $t_{(4)} = 9.6$, $p < 0.001$). Other treatments did not show growth above the control (Figure 5.1).

Though both ammonium and glutamate showed cell growth, only glutamate decreased in TDN and the provided DON (Figure 5.1, TDN - $t_{(1.4)} = 26$, $p = 0.007$, DON - $t_{(1.2)} = 17.7$, $p = 0.021$). The glutamate treatment largely had nitrogen in dissolved organic form, with some consistently present as ammonium at the start and end point. None of the other nitrogen treatments decreased in their provided nitrogen compound, nor were they transformed into other compounds during the experiment. TDN decreased in the glutamate treatment, but remained constant in all other treatments. This contrasts with the expectation of a decrease in media nitrogen in treatments that showed growth, as only a slight decrease in TDN or ammonium fraction was seen in the ammonium treatment. A larger decrease in TDN was seen in the glutamate treatment. The ammonium treatment may have used internal stores of nitrogen, but if so then growth should be seen in all other treatments as the internal nitrogen stores were similar. As it is not, there may be other limiting factors to growth. Regardless, *Chlorella vulgaris* seems able to grow in the glutamate treatment better than when provided with the other forms of nitrogen.

All treatments decreased slightly in TDP, though PO_4-P did not change for any treatment over the course of the experiment. The growth in the glutamate treatment seems fuelled by the unreactive media phosphorus and/or internal stores of phosphorus rather than the PO_4-P provided in the media.

Samples on glass filters taken for particulate nitrogen and carbon measures were analysed, but were in the same problematic sample batch as mentioned in the previous chapter. Sample analysis was conducted in bulk after completion of all experiments. Samples were destructively combusted, but an error with the analytical machine meant they were not analysed.

Samples of unfiltered media were also taken at the end point of this experiment, with the original intention of comparing with the filtered nitrogen measures. Analysis of total nitrogen and phosphorus in the aqueous, unfiltered samples gives the total nitrogen and total phosphorus in the cultures. Dissolved nitrogen and phosphorus were removed from these total values to give particulate nitrogen and phosphorus respectively. Particulate nitrogen was highest in the glutamate treatment, with all other treatments similar to the control. Particulate phosphorus suggest the analysis of the TP was not complete, as the TP measures were lower than the TDP and resulted in slightly negative values for particulate phosphorus for the glutamate treatment, though standard error values generally still encompassed zero.

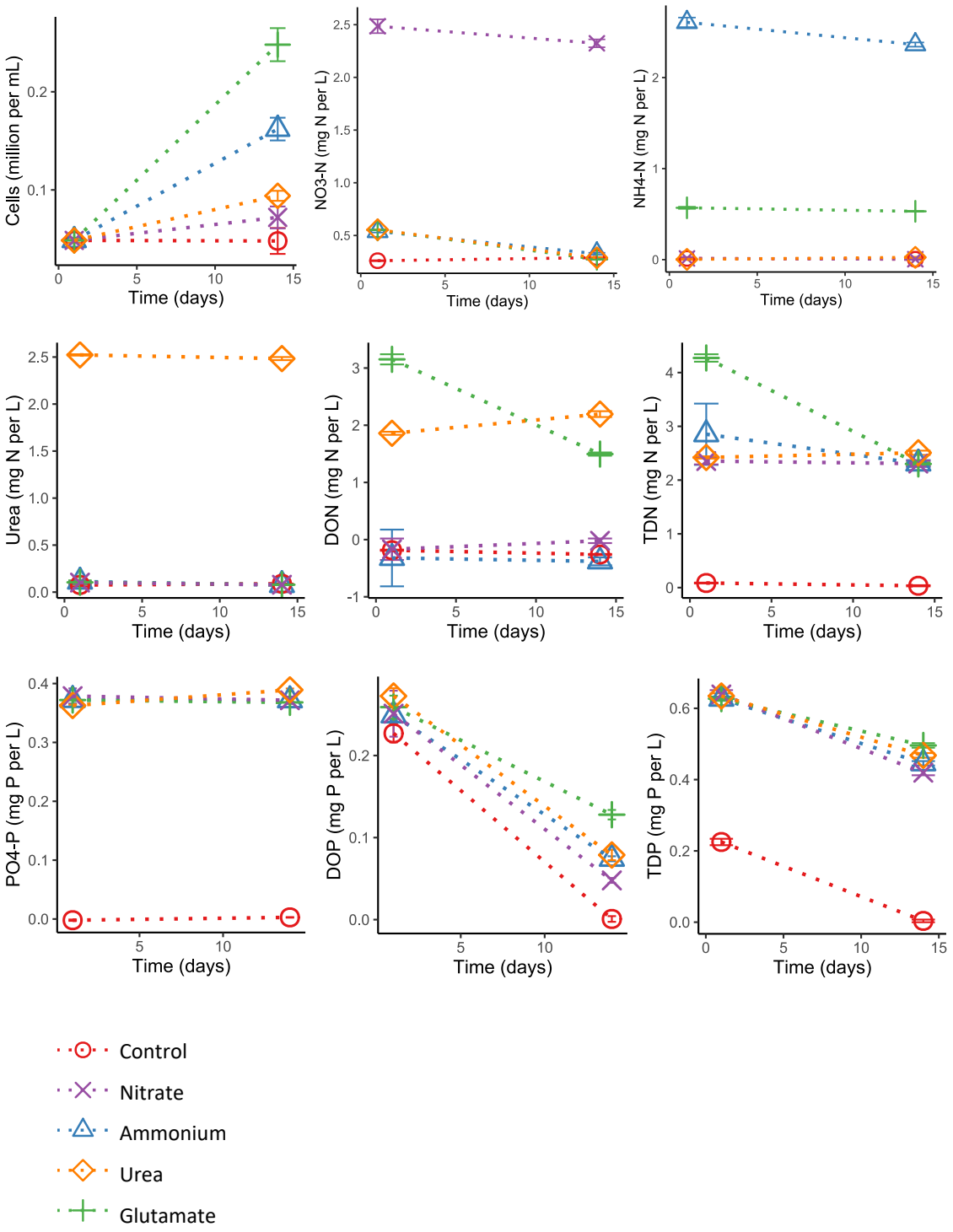


Figure 5.1. Analyses of media and particulate compounds from Experiment 4, Chlorella vulgaris., showing cell number, NO_3-N, NH_4-N, Urea, DON, TDN, PO_4-P, DOP, TDP, TN, TP, particulate C and particulate N. Starting samples for particulate and TN/TP measures were lost due to machine malfunction. All points show mean ± standard error.

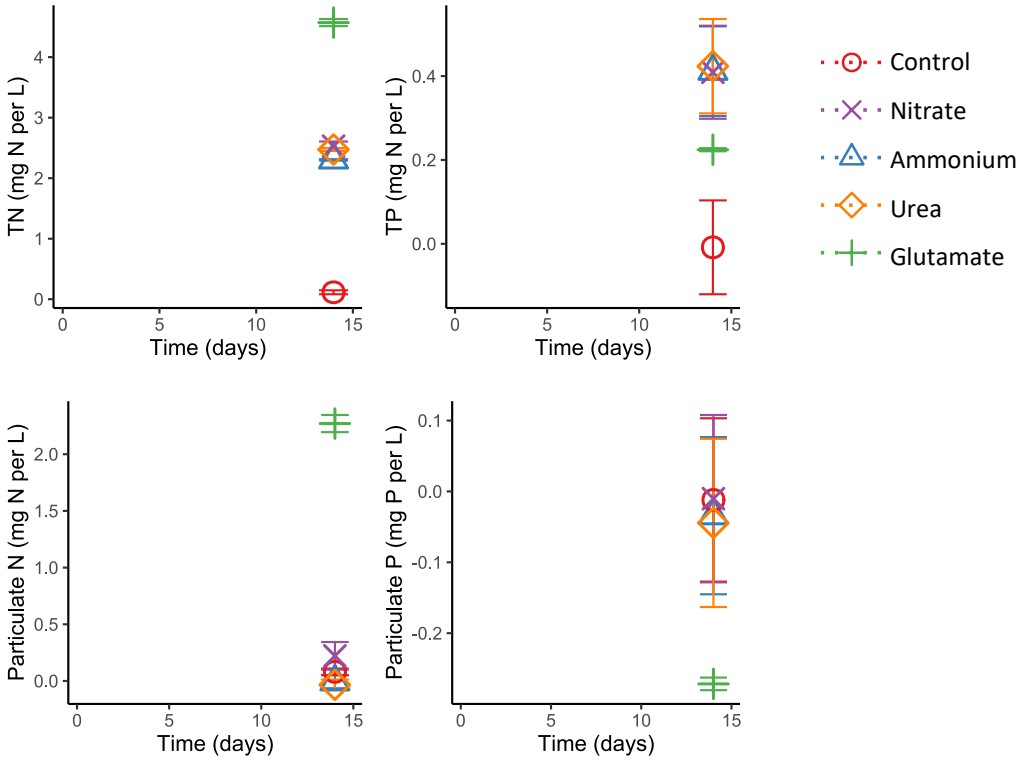


Figure 5.1. Analyses of media and particulate compounds from Experiment 4, Chlorella vulgaris., showing cell number, NO_3-N, NH_4-N, Urea, DON, TDN, PO_4-P, DOP, TDP, TN, TP, particulate C and particulate N. Starting samples for particulate and TN/TP measures were lost due to machine malfunction. All points show mean ± standard error.

5.2.2. *Experiment 5 – Klebsormidium sp.*

This experiment used an isolate of a filamentous green algae with a thin superficial cellular sheath, *Klebsormidium sp*. The experiment started on 17th of June 2019 for 14 days. The highly adhesive filaments caused some loss of biomass when preparing cell count samples. The end point counts will not be accurate in absolute terms, nor directly comparable to the starting point samples. They were all handled identically, so losses should be very similar between all treatments, with the end point cell counts being relatively accurate to others at the same time point. Urea and nitrate treatments ended with the highest cell numbers at the end of the experiment compared to the control ($t_{(3)} = -2.4$, $p = 0.09$ and $t_{(3)} = -5.4$, $p = 0.01$ respectively). The glutamate treatment showed some growth above the control ($t_{(3.7)} = -4.8$, $p = 0.011$), with the ammonium treatment cell numbers being similar to the control ($t_{(3.9)} = 1.2$, $p = 0.31$).

As would be expected, glutamate started with the highest DOC. Glutamate then decreased in DOC during the experiment ($t_{(4.7)} = 37.8$, $p < 0.001$). Other treatments increased slightly from starting concentrations, with the control treatment increasing about 7 times from starting

concentrations ($t_{(4.4)}$ = -11.7, $p < 0.001$). This appears to show that the cells in the glutamate treatment used the DOC present, and the other treatments produced DOC during the experiment. This shows an alternate interaction in the use of DON, where the carbon may be more 'valuable' to the cells than the nitrogen it contains.

The relevant nitrogen addition decreased in all treatments, with the control concentration remaining near zero for all forms of nitrogen. TDN was largely depleted from starting levels in the glutamate treatment ($t_{(3.6)} = 47.5$, $p < 0.001$), although it was not the highest cell number treatment. TDN decreased in all other treatments but was not depleted, reaching about half of initial concentrations.

TDP was largely PO_4-P in all nitrogen addition treatments and both followed the same pattern (Figure 5.2). All treatments decreased in TDP and PO_4-P to about half the initial concentrations of each respectively. The glutamate addition treatment had largely depleted TDP ($t_{(4)} = 93.9$, $p < 0.001$) and PO_4-P ($t_{(4)} = 73$, $p < 0.001$) by the end of the experiment. Soluble unreactive phosphorus was a small percentage of total phosphorus in all treatments and the control at all time points.

The glutamate treatment removed most nitrogen and phosphorus from the media, though it was not the highest in cell numbers. Previous experimental treatments in this thesis that showed a lot of cell growth tended to also remove a majority of nitrogen and phosphorus, ostensibly to support population growth. The adhesive nature of the filaments and losses during handling should have been equal amongst treatments as handling was identical. Though lower than the actual cell counts, they would be expected to be relatively correct between treatments at the end point. There may be a reason for larger losses from the glutamate treatment, or it may be that cell size increased slightly rather than cell number as this was not measured. Alternatively, it may be that the media nitrogen and phosphorus in the glutamate treatment were taken up by the cells but cell growth did not actually occur. It could have potentially been stored internally or adhered to the cells rather than used for growth. The latter is less likely as it had lower DOC than other treatments, with a lower amount of exo-polysaccharides (mucus) to trap molecules outside the cells.

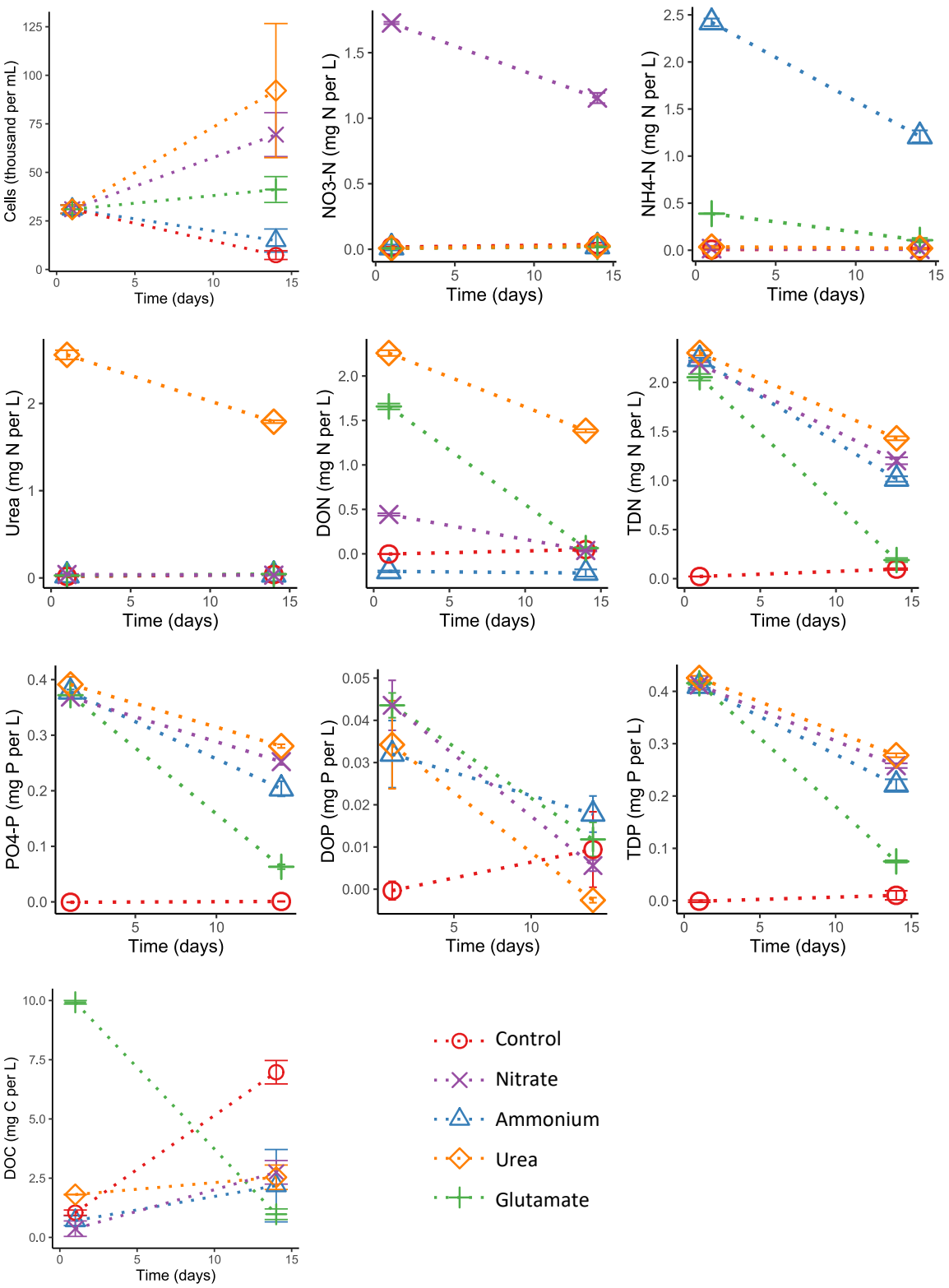


Figure 5.2. Analyses of media and particulate compounds from Experiment 5, Klebsormidium sp., showing cell number, NO_3-N, NH_4-N, Urea, DON, TDN, PO_4-P, DOP, TDP, particulate C, particulate N and particulate C:N ratio.

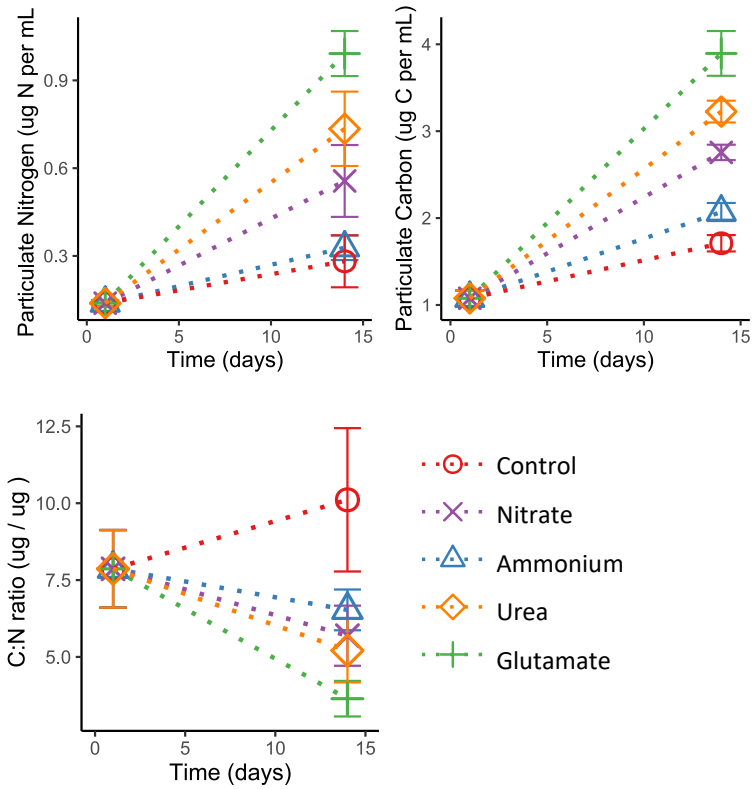


Figure 5.2. Continued. Analyses of media and particulate compounds from Experiment 5, Klebsormidium sp., showing cell number, NO_3-N, NH_4-N, Urea, DON, TDN, PO_4-P, DOP, TDP, particulate C, particulate N and particulate C:N ratio.

Particulate nitrogen and carbon show a different pattern to the cell numbers in terms of highest growth. Glutamate accumulated the highest concentration of particulate nitrogen and carbon as captured on the 0.7 µm filters analysed compared to the control ($t_{(9)} = -6.0$, $p < 0.001$ and $t_{(11)} = -7.9$, $p < 0.001$ respectively). The next highest in terms of particulate nitrogen and carbon was the urea addition treatment, then the nitrate. The ammonium treatment remained similar to the control in nitrogen ($t_{(7.1)} = 0.47$, $p = 0.65$) though the carbon differed to the end point control ($t_{(17.8)} = 2.6$, $p = 0.019$). As in the previous experiments in this thesis, the control increased in carbon relative to nitrogen during the course of the experiment, increasing the control treatment C:N ratio though this was highly variable and not statistically significant ($t_{(5.8)} = -0.84$, $p = 0.43$). All nitrogen addition treatments increased in nitrogen relative to carbon, with the largest C:N ratio decrease in the glutamate treatment ($t_{(1.4)} = 3.0$, $p = 0.14$), followed by the urea ($t_{(1.6)} = 0.9$, $p = 0.47$) and nitrate ($t_{(2.4)} = 1.4$, $p = 0.29$). This is the same pattern shown in other analyses here, suggesting glutamate uptake and cellular incorporation of media nitrogen, as well as uptake and utilisation in urea and nitrate treatments, though measurements are variable between replicates and statistical significance is low.

All treatments increased to some degree in particulate nitrogen and carbon. As internal nitrogen stores at the start of the experiment would be measured in this method, the extra nitrogen may have been removed from the media or captured in the mucus sheath as the samples were rinsed with deionised water to prevent adhesion. As the mucus sheath composition may vary between treatments this may not have been adequate for all treatments, and DOC exudate composition may change under stress conditions.

Although a cursory inspection of cell numbers appears to show the largest response in the urea treatment, followed by the nitrate and glutamate treatments, other analyses suggested the glutamate treatment responded more positively. There is some uncertainty in the order of which treatment showed the largest response, but both DON treatments and the nitrate treatments saw positive growth responses and uptake of nitrogen into particulate (largely algal) biomass. The ammonium addition did not result in increased growth compared to the control. Again, this contradicts literature where ammonium is considered bioavailable. While likely true for bacterial riverine organisms, it does not seem to be true for the algal isolates described here and the co-isolated microbiome.

5.2.3. *Experiment 6 – Nitzschia palea & amoeba (Naegleria sp.)*

To contrast with Chapter 4 and investigate a different type of algae, a diatom (*Nitzschia palea*) isolated from the Conwy location was chosen. This experiment started on 25th of July 2019 and ran for 14 days, with a single celled, non-colonial diatom species.

Cell numbers increased in the nitrate addition treatment compared to starting numbers ($t_{(5.5)}$ = 4.9, $p < 0.001$), stayed the same in the urea treatment ($t_{(6.4)}$ = 0.75, $p = 0.48$) and decreased in the control ($t_{(7.1)}$ = -4.1, $p = 0.004$), glutamate ($t_{(6.6)}$ = -5.0, $p = 0.002$) and ammonium ($t_{(7.7)}$ = -4.2, $p = 0.003$) treatments. The nitrate media concentrations decreased in the nitrate treatment, and remained low in all other treatments. Media ammonium concentrations stayed constant in the ammonium treatment ($t_{(5.4)}$ = 0.64, $p = 0.55$) and increased in the glutamate treatment ($t_{(4)}$ = -93, $p < 0.001$). DON decreased in the glutamate treatment, but some of this will be due to the conversion to ammonium instead of uptake of the glutamate. Urea stayed constant in the urea addition treatment. The majority of DON in each treatment was the compound provided in the starting media. TDN decreased slightly in the glutamate treatment ($t_{(4.5)}$ = 13, $p < 0.001$). No treatment utilised the majority of nitrogen provided, regardless of compound form.

DOC started higher in the glutamate treatment, and started slightly higher than control in the urea treatment. DOC decreased to near 0 mg C mL^{-1} in the glutamate addition treatment from initial concentrations ($t_{(4)} = -22.8$, $p < 0.001$). All other nitrogen addition treatments and the control increased in DOC content by the end of the two week experiment. All nitrogen addition treatments removed about a third of the TDP in the media by the end of the experiment, irrespective of whether cell growth was seen. The PO_4-P followed a similar trend to the TDP.

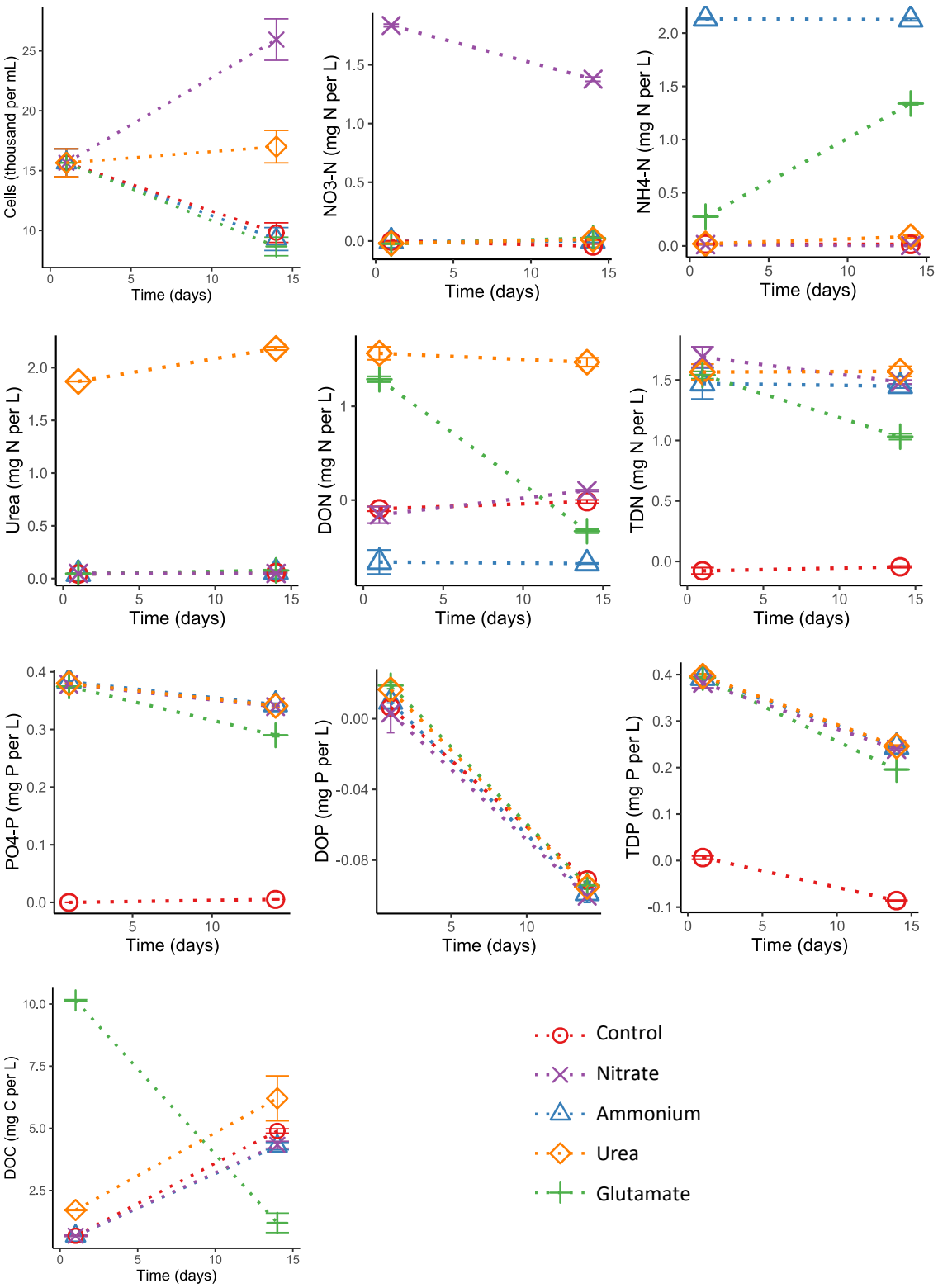


Figure 5.3. Analyses of media and particulate compounds from Experiment 6, Nitzschia palea, showing cell number, NO_3-N, NH_4-N, Urea, DON, TDN, PO_4-P, DOP, TDP, particulate C, particulate N and particulate C:N ratio.

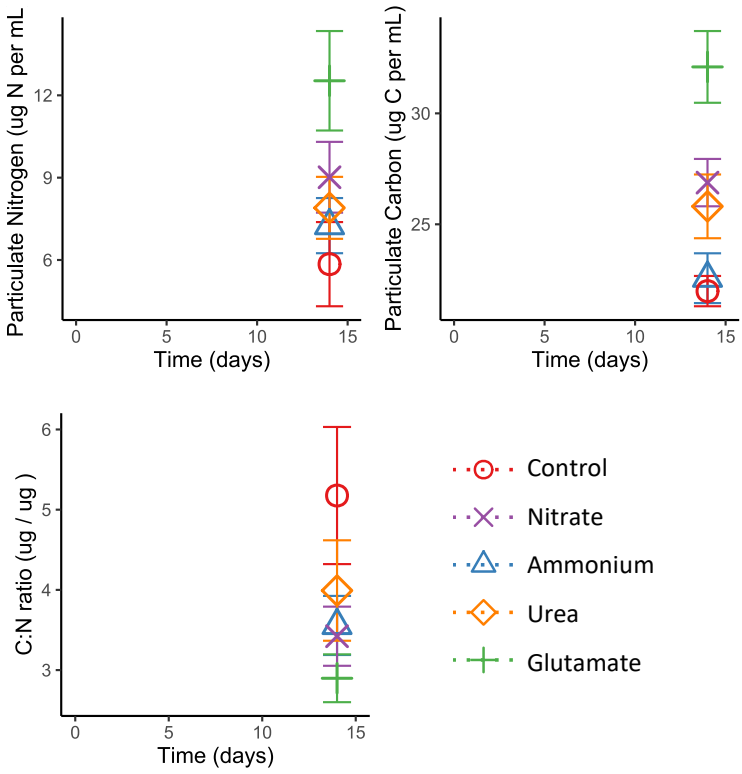


Figure 5.3. Analyses of media and particulate compounds from Experiment 6, Nitzschia palea, showing cell number, NO_3-N, NH_4-N, Urea, DON, TDN, PO_4-P, DOP, TDP, particulate C, particulate N and particulate C:N ratio. Starting samples for particulate measures were lost due to machine malfunction.

Cells number samples at the end of the two weeks experiment saw large, thick walled cells apparent that were not in the initial cell number samples. These may have affected the cell growth and decreases in cell number seen compared to the initial numbers. Given the DNA barcoding identified the presence of *Naegleria sp.*, it is likely that an increase in this amoeba was seen, though the cells noticed were of a cyst stage rather than of a growing stage. It is not photosynthetic, and is a free-living single celled protist that tends to live by phagocytosing bacteria in aquatic or soil habitats (Jonckheere, 2002), though it does have a human pathogenic species (Carter, 1970). It moves between flagellated and cyst stages, and microscopy only saw cyst stages during the experiment. *Naelgeria sp.* cyst numbers increased gradually over time though they remained very infrequent, so flagellated stages could have been present but not encountered. The *Naegleria sp.* is unlikely to have competed directly for nutrients, though it would have been accumulating bacteria that may have incorporated nutrients, locking them in its' cells and leaving less available for the algae. No *Naegleria sp.* was seen containing diatom cells or chloroplasts. It is possible this amoeba

contributed to the increase in DOC content seen in most treatments and the control in this experiment.

Samples at time zero for particulate measures were subject to the analytical issue previously mentioned, and were destructively combusted without the resulting sample being analysed. End point particulate carbon trends follow the cell number trends, with glutamate the highest carbon content, followed by nitrate and urea. The ammonium and control treatments were similar with the lowest concentration of particulate carbon. Particulate nitrogen had less clearly grouped treatments, with the highest end point concentration in the glutamate treatment, then the nitrate, urea, ammonium and control. The C:N ratio followed previous experiment trends with the lowest C:N ratio associated with the largest uptake of nitrogen, and the control having the highest C:N ratio.

5.3. Discussion and comparison of species

Of the species isolated in the headland catchment of the River Conwy, all showed a positive response to nitrate (inorganic) addition and generally positive responses to urea (organic) addition (Table 5.1). Two showed some positive response to glutamate (organics), and one showed a positive response to ammonium (inorganic). This compares with the species isolated from the farmland, clay catchment on the River Sem with all species able to utilise nitrate (inorganic) and urea (organic), and two species also able to use ammonium and glutamate to some degree.

Chlorella vulgaris showed positive responses to the widest range of compounds, inorganic and organic. Recent research has shown *Chlorella vulgaris* culture collection species (UTEX, 2021) are capable of utilising bulk wastewater DON (Sun *et al.*, 2017; Sun and Simsek, 2017). It has also been isolated from wastewater locations (Chinnasamy *et al.*, 2009), with literature appearing more focused on potential wastewater treatment (remediation) or biomass production (for biofuel) than the impacts of DON bioavailability in natural waters. The strains here, recently isolated from two contrasting locations, showed different responses to DON compounds compared to each other. *Chlorella vulgaris* is a highly plastic species (Aigner *et al.*, 2020; Hollis, 2014), with different strains specialised to a variety of environments. The differences seen between the two strains isolated here may be due to the species ability to acclimate to a variety of habitats and nitrogen sources, as well as the differences in associated microbiome of these non-axenic cultures. This is a widespread species, and these experiments show it can use urea and glutamate for growth, and possibly other DON compounds as well.

*Table 5.1. Comparison of species in this chapter in growth responses to treatments as determined by nitrogen uptake, cell growth and an increase in particulate nitrogen. *All Klebsormidum species samples had handling issues due to adhesive filaments affecting cell counts.*

Isolation Location	Culture Species	Growth seen compared to starting point (positive or negative)				
		Control	Nitrate (inorganic)	Ammonium (inorganic)	Urea (DON)	Glutamate (DON)
Priors Farm, Upper Nadder catchment, South England	*Chlorella vulgaris*	-	**+**	-	**+**	-
	Lobochlamys spp.	-	**+**	+	**+**	+
	Mixed species - Family Chlorellaceae spp., Order Oscillatoriales spp., Unknown diatom spp.	+	**+**	**+**	**+**	**+**
Nant y Brwyn peatland, Upper Conwy catchment, North Wales	*Chlorella* spp.	-	**+**	+	+	**+**
	**Klebsormidium* spp.	**-**	**+**	-	**+**	+
	Nitzschia palea	**-**	**+**	-	+	**-**

The filamentous *Klebsormidium* sp. mainly responded to the urea and nitrate additions, and to a lesser degree the glutamate. This is also a widely found species, occurring in aquatic, soil and urban environments (Rindi, Guiry and López-Bautista, 2008). As this is a filamentous algae with an occasional mucus sheath, it is likely to be a key component of freshwater biofilm formation, creating niches for a wider range of other algal and bacterial species to grow. Literature has investigated *Klebsormidium sp.* growth rates on inorganic media (Ryšánek, Holzinger and Škaloud, 2016), as well as potential for wastewater phosphorus removal (Liu and Vyverman, 2015; Valchev *et al.*, 2021), but it is difficult to find literature information on *Klebsormidium sp*. and DON bioavailability. This is the only green filamentous alga used in this thesis, and does not show notable nitrogen preferences compared to the other species.

The mixed culture diatom species and the *Nitzschia palea* culture showed differences in responses. They both responded positively to the nitrate (inorganic) addition, and to a small degree to the urea (organic) addition. They differed in response to ammonium (inorganic) and glutamate (organic), with the single algal species not responding, and the mixed culture showing positive

responses to all nitrogen addition treatments. The peatland culture of *Nitzschia palea* had some complications from the presence of an amoeba, *Naegleria sp.,* that seemed to suppress diatom growth. The positive response shown here is compared to the end-point control that had greatly declined in number over time, likely due to cell death (perhaps increased due to the amoeba) overtaking any cell growth possible on the nutrients present. The mixed species culture from the farmland location also has DON use possible through a cyanobacteria (Family Oscillatoriales) and green algal (Family Chlorellaceae) that were also identified within the culture. This culture showed the widest range of compounds causing positive growth responses, including ammonium.

Some nutrient cycling was seen in the media of these isolates. Conversion of the glutamate addition to ammonium occurred in the *Chlorella vulgaris* and *Nitzschia sp*. isolated from the River Conwy. This suggests some cycling of nitrogen is occurring, rather than more direct uptake and use. The *Chlorella vulgaris* glutamate treatment showed a strong positive response alongside this nitrogen cycling, while the *Klebsormidium sp.* did not appear to have such cycling and showed some positive response to the glutamate treatment.

Most treatments did not show growth on inorganic ammonium, which is it often regarded as an easily processed compound and is used in nutrient addition treatments (Shilova *et al.*, 2017; Arango *et al.*, 2008; Lachmann *et al.*, 2019). Monitoring and legislation often involve ammonium targets (Markogianni *et al.*, 2018; Romero *et al.*, 2016; Schuurkes and Mosello, 1988). Ammonium in aquatic environments often needs to be transformed into nitrate/nitrite first by microbes before algae and plants can utilise it, with algal response saturated at higher concentrations (O'Brien and Dodds, 2008). Pure ammonium ions are known to be toxic to plants (De Rijck and Schrevens, 1997) and impact freshwater macroinvertebrates (Berenzen, Schulz and Liess, 2001) with some investigations with algae finding high concentrations inhibit algal growth (Li *et al.*, 2016). This may be due to the pH dependent forms of ammonium. The equilibrium between NH_4+ and NH_3 in solution can be found using equations originally derived by Emerson *et al.* (1975), and is shown in Figure 5.4 for the experimental temperature of 15˚C across a range of pH concentrations.

The ammonium was provided as $NaNO_3$, but would have dissociated in solution with the buffer keeping it around pH 7. This may be part of the reason why ammonium alone was not a preferred source of nitrogen for growth, in contrast to some literature. Where ammonium has been processed by other biota it will no longer be in NH_4^+ or NH_3^- form and may be used more by algal species. Processed ammonia is likely to have transformed into a DON compound, and in doing so may be utilised more completely than in the original inorganic form. The pH in the natural

environment can be highly varied across a river reach, both in water and at the sediment boundary. The pH impacts not only ammonium form (Figure 5.4) but also nitrate forms, altering the bioavailability of these compounds. As experiments in this thesis were done around pH 7, there may be different response in real-world situations that are more acid or more alkaline due to the different nitrogen ions present, and the associated different pathways for assimilation.

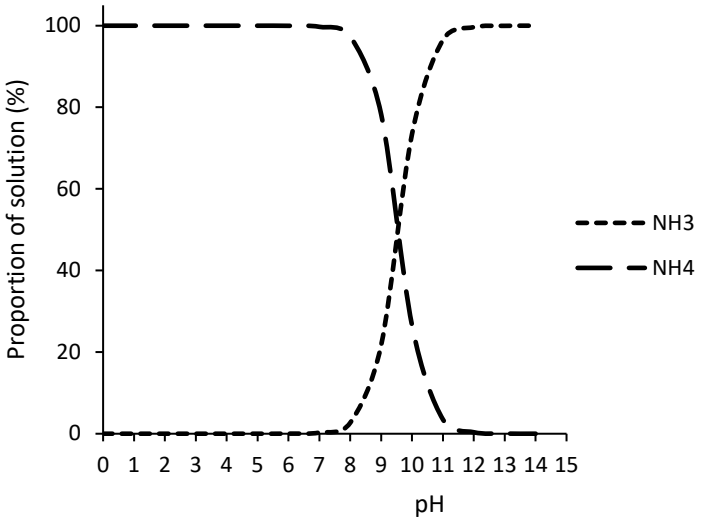


Figure 5.4. Proportion of NH_4^+ and NH_3 in solution at 15 ˚C.

DOC tended to decrease in the glutamate treatment, while increasing in all other treatments and the control. This suggests that the carbon in the more carbon rich glutamate could be a desired resource. Stress environments have been shown to increase algal carbon exudates (Stuart *et al.*, 2016b; Stuart *et al.*, 2016a). The control C:N ratio increased towards the end of all experiments. Though the control is not provided with dissolved carbon, nitrogen or phosphorus and there is no increase in cellular nitrogen, the cellular carbon tended to increase. This may be a stress response to the environment, as the cells metabolise internal stores of nitrogen and break down non-essential components to survive the starvation conditions. As mentioned in literature (Mayers, Flynn and Shields, 2014; Mineau *et al.*, 2013; Yvon-Durocher *et al.*, 2015), the C:N ratio though often used in carbon modelling varies widely with algal stress and nutrient status. Care is needed when applying blanket values to a very diverse group of organisms that will fluctuate between nutrient replete and deplete conditions in rivers, as well as light and temperature stresses to determine freshwater DON and DOC fluxes.

Riverine algal species consist of various groups of distantly related species. As shown here, they respond in a variety of ways to the same nitrogen compounds and environment, with both urea and glutamate being bioavailable DON compounds. The plasticity in response to nitrogen compounds is favourable for the community structure in which algae exist in the wild. Specialising in different compounds allows species to target different niches in the physical location, while still competing for light and nutrients with other photoautotrophs.

This chapter has shown hypothesis 2 to be correct – isolated algal species have different species-specific and compound-specific preferences for growth on inorganic and organic nitrogen. Hypothesis 3 has not been shown to be correct, as similar (or identical) species isolated from differing headwater catchments did not show the same growth response to DON. This is exemplified by the response of Chlorella vulgaris, isolated from both locations.

5.4. Conclusion

The algal species isolated from the headland section of the river Conwy have shown different responses to those isolated from the chalk river, farmland location on the river Sem. All species responded positively to nitrate but varied in their responses to other compounds. Ideally, experimenting with more species would help to build a better picture of nitrogen and DON use in the algal community in stream. As with the previous chapter, there was a lack of positive response to ammonium in all species tested here. This contrasts with general literature, where ammonium is considered a fairly bioavailable compound due to its chemical form and the metabolism pathways of ammonium and nitrate within stream algae and associated bacteria.

Chapter 6 – Investigating mechanisms of algal DON use through stable isotope tracer uptake

6.1. Aims

This chapter aimed to investigate how DON and inorganic N is assimilated into algal cells by presenting stable isotope-labelled substrates to natural isolates in laboratory cultures. The *Chlorella* spp. was isolated from an upland farmland catchment and allowed to grow on dual ^{15}N and (and ^{13}C) -labelled analogues of the identical substrates used in Chapter 5. The cultures were then sampled in a time course and EA-IRMS used to determine the uptake of the substrate into algal biomass to assess differences in the utilisation of DON and inorganic N by the algae, thereby allowing the implications for water quality impacts of different N sources to be compared.

6.2. Introduction

The use of common element stable isotopes originated in probing unculturable soil methylotrophic bacteria (Radajewski *et al.*, 2000; Bull *et al.*, 2000). Stable isotope probing (SIP) has since been used in various investigations (Hungate *et al.*, 2015; Chen and Murrell, 2010; Mader *et al.*, 2017), often into difficult to culture organisms or to track biological compounds within or between cells (Haichar *et al.*, 2016; Kreuzer-Martin, 2007; Lerche *et al.*, 2018). The basic approach is to use heavier isotopes of an element to track how a compound given to organisms is processed, often in an incubation experiment. This is either through analysing the specific compounds present within the biomass or exudates or present in the DNA of the organisms involved which will also contain the heavier isotope. These approaches generally investigate the 'what' of a substrate metabolism through compound analysis versus the 'who' via DNA methods (Evershed *et al.*, 2017). Commonly carbon, nitrogen and oxygen stable isotopes are used, being common elements utilised by all living organisms.

Previous experiments in this thesis have shown algae species can make use of urea and glutamate as DON sources for growth (Chapter 4 and 5). The exact mechanism for this is unknown and is likely to differ amongst species (Mandal *et al.*, 2018; Killberg-Thoreson *et al.*, 2021; Halterman and Toetz, 1984). As bulk determinations of algal biomass N were performed for the latter experiments, it remains to be determined whether the N-containing substrates were truly incorporated into cellular biochemicals or were adhering on to the cell mass in their applied form

and, thus, not truly assimilated. The objective of the experiments described in this chapter was to investigate the pathways of use of the applied substrates to enable tracing of N (and C where present in DON) from dissolved organic and inorganic substrates via biochemical assimilation into the cell biomass using SIP.

As an overview, stable isotope is used here to describe a heavier, non-radioactive isotope compared to the average, or most common isotope of that element, with the numbers preceding the element refer to the atomic mass (neutrons plus protons) of that isotope. Stable isotopes are found naturally, but those referred to as stable isotope are usually not the most abundant isotope of that element. An above average proportion of the heavier isotope can be used to track elements through various compounds, with the heavier compounds 'enriching' the normal values. The average isotopic value is that used in the periodic table of elements. Here, ^{15}N (and ^{13}C where present) were used to label the substrates. Then stable isotope ratio mass spectrometry (IRMS) was used to follow the uptake of the labels into the algae qualitatively and quantitatively. Any enrichments in the algal biomass compared to control cultures is used to confirm that algae had assimilated and grown on the labelled substrates. The isotopically labelled compounds used in the experiments are shown in figure 6.1. The treatment of the cultures with highly isotopically labelled substrates was avoided as these this has been shown to affect biological uptake and processing resulting isotope effects which can affect quantification (Andriukonis and Gorokhova, 2017). A 5 atom % enrichment in ^{15}N was used for all substrates, which led to a corresponding ^{13}C enrichment of 2.5 atom % in the urea treatment and 5 atom % in the glutamate treatment.

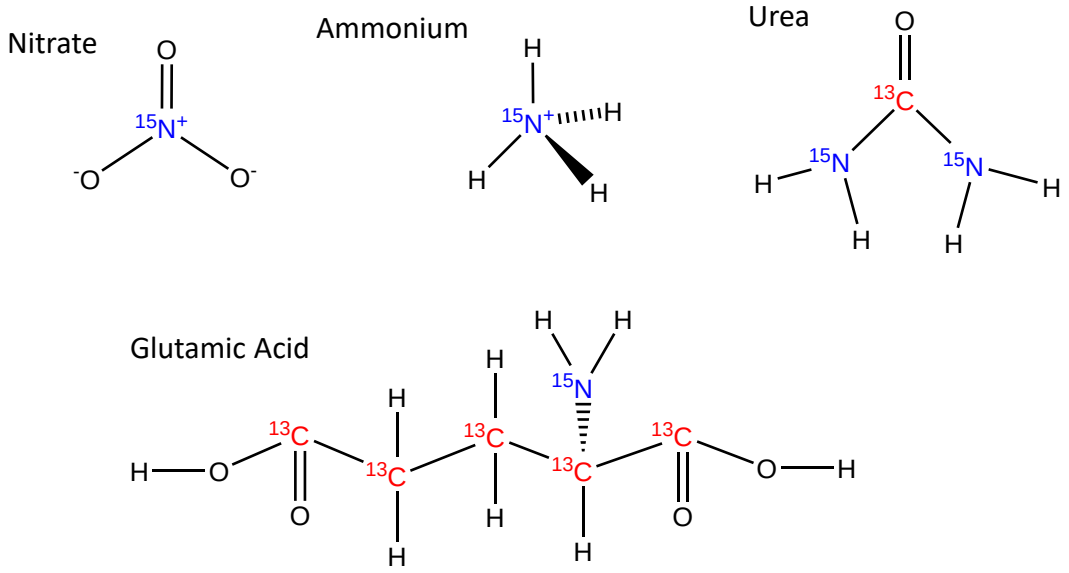


Figure 6.1 Isotope labelled version of all treatment compounds, with the heavier isotopes of carbon ($\delta^{13}C$) highlighted in red and nitrogen ($\delta^{15}N$) highlighted in blue.

Figure 6.1 shows the isotopically labelled substrates used in the tracer experiment. As can be seen from the structures, the compounds are universally labelled in N and C where present. The largest naturally occurring isotope of nitrogen is ^{14}N, with ^{15}N used here as the stable isotope label in all compounds. The major naturally occurring isotope of carbon is ^{12}C, with ^{13}C used here as the stable tracer isotope within organic treatment compounds of urea and glutamate. The ammonium and nitrate substrates contain a single ^{15}N labelled N-atom. In urea and glutamate the labelling is more complex because of the presence of both ^{15}N and ^{13}C in the same compound. Urea contains two labelled N-atoms, while glutamate contains only one. Urea contains a single labelled carbon atom, while glutamate contains 5 labelled carbon atoms. As noted above the commercially acquired 99 atom % ^{15}N and ^{13}C-labelled substrates were diluted with natural abundance material to avoid undesirable isotope effects.

6.3. Methods overview

The experiment set up as described in Chapter 2. Briefly, this consisted of a temperature-controlled cabinet 15˚C, with 16 h light, 8 h dark with light at 50 µmol m^{-2} s^{-1}. Media treatments were a negative control, with treatments being separate additions of nitrate, ammonium, urea and glutamic acid. The media is the same as in Chapter 4 and 5, with 5 atom % ^{15}N labelled concentrations of sodium nitrate, ammonium chloride, urea, glutamic acid. As mentioned in Chapter 2 (Section 2.4.6.2), isotope enrichment using the delta notation is reported relative to atmospheric nitrogen for $\delta^{15}N$ values, and relative to δ_{VPDB} (the Vienna Pee Dee Belemnite fossil) for $\delta^{13}C$ values (Bay et al. 2015).

The same algal species as in Experiment 1 (Chapter 4) was used; namely a single celled green algae, *Chlorella vulgaris* isolate. Samples were taken throughout the time course of the experiment. Practicalities in sampling prevented all samples being taken concurrently. Samples within the first 8 h were taken as soon as practically possible. Results are presented as mean ± standard error of the mean (± s.e.). Samples were taken throughout the experiment, with timings and analytical methods summarised in Chapter 2. Treatments replicates (n=3) were each sampled once for cell number, and twice each for TDN, TDP, bulk particulate C & N, and enriched C & N.

6.4. Results

6.4.1. *Cell numbers and media nutrient results*

Figure 6.2 (a) shows the cell numbers, TDN and TDP over the seven day experiment. After 7 days, cell numebrs increased most significantly in the nitrate treatment (1.11 ± 0.24 million cells mL^{-1}) compared to the control (0.13 ± 0.01 million cells mL^{-1}), although the standard error is much larger for the nitrate treatment than the other treatments at the final time point. The cell counts remained closer to the control for the other 3 treatments, i.e. glutamate (0.34 ± 0.01 million cells mL^{-1}), urea (0.29 ± 0.03 million cells mL^{-1}) and ammonium (0.12 ± 0.02 million cells mL^{-1}).

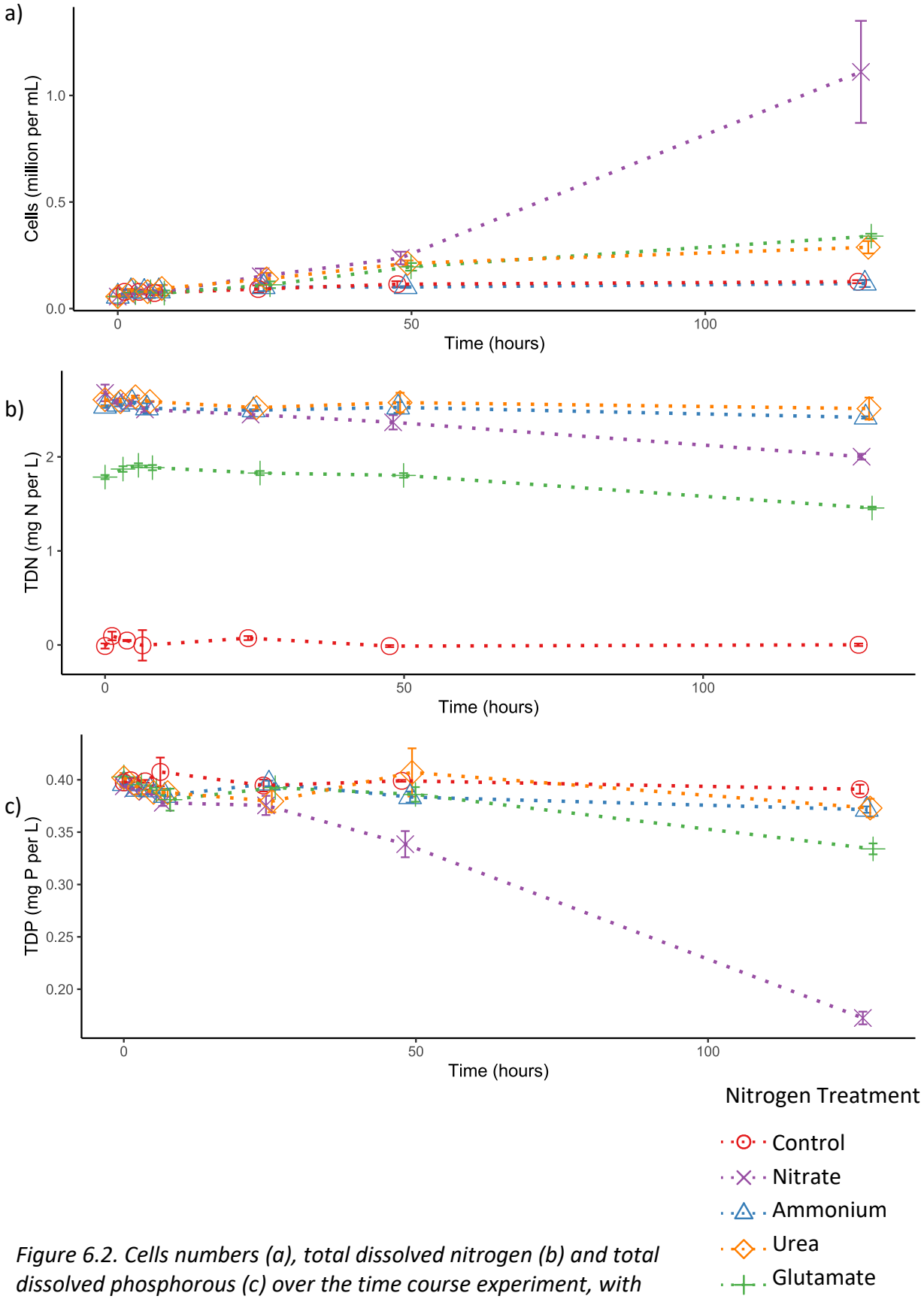


Figure 6.2. Cells numbers (a), total dissolved nitrogen (b) and total dissolved phosphorous (c) over the time course experiment, with error bars ± s.e.

Figure 6.2b showed total dissolved nitrogen (TDN) decreased slightly from t_0 concentrations in the nitrate and glutamate treatments, but not in the control, urea or ammonium treatments. Figure 6.2c showed total dissolved phosphorus (TDP) to exhibit greater differences between the start and end of the experiment. At the last sampling point, media TDP was halved in the nitrate treatment (0.173 ± 0.006 mg P L^{-1}) as compared to the control (0.391 ± 0.004 mg P L^{-1}). Both nitrate ($t_{(3.6)}$ = -59.8, p<0.001) and glutamate ($t_{(3.8)}$ = -74.3, p <0.001) TDP concentrations were significantly lower than the end point of the control, as well as being different to one another ($t_{(3.9)}$ = -16, p<0.001).

Cell growth and removal of TDN and TDP from the media show a lag phase at the start of the experiment. Very little changed for the first few days, though by the end of seven days changes in these nutrients and cell numbers became more apparent between treatments. These data indicate that the nitrate treatment stimulated the highest growth and removed the most media TDP, with some utilisation of the provided inorganic N occurring over the seven day of the experiment. The glutamate treatment also saw some algal growth concomittant with removal of TDP from the media, but with relatively minor utilisation of organic nitrgoen over the same time.

6.4.2. *Bulk particulate carbon and nitrogen results*

Bulk particulate nitrogen was highly variable (Figure 6.3, a), with only the nitrate treatment (0.755 ± 0.078 ug N mL^{-1}) being significantly different to the ammonium (0.355 ± 0.104 ug N mL^{-1}, $t_{(7.8)}$ = -3.1, p=0.016) and urea (0.297 ± 0.066 ug N mL^{-1}, $t_{(9.0)}$ = 4.5, p=0.015) at the end point of the experiment, although nitrate was not significantly different in concentration from the control ($t_{(3.7)}$ = , p=0.56).

Bulk particulate carbon concentrations were more constrained with treatments showing clearer differences (Figure 6.3, b). After seven days the nitrate treatment achieved 3.73 ± 0.07 ug C per mL culture, which was significantly higher than all other treatments (glutamate $t_{(8.8)}$ = -11.7, p < 0.001; urea $t_{(8.7)}$ = 22.6, p < 0.001 ; ammonium $t_{(7.7)}$ = -22.6, p < 0.001) and the control ($t_{(4.5)}$ = -11.1, p < 0.001). For the glutamate treatment at 2.51 ± 0.08 ug C per mL the bulk particulate carbon was significantly higher than the control treatment after seven days ($t_{(4.6)}$ = -3.6, p = 0.017). Carbon-to-nitrogen ratio remained similar between all treatments (Figure 6.3, c).

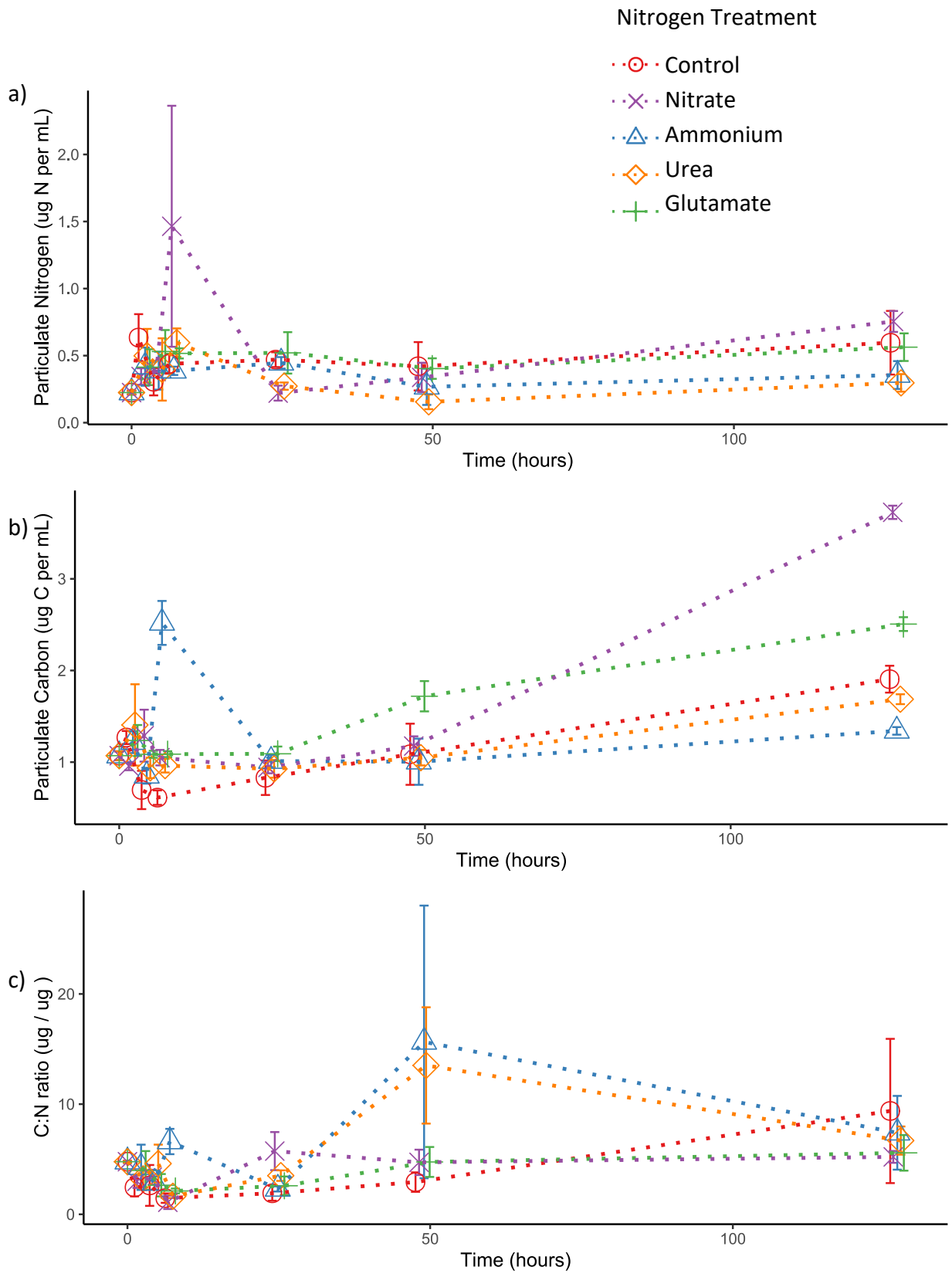


Figure 6.3. Bulk particulate nitrogen (a), bulk particulate carbon (b) and bulk carbon to nitrogen ratio (c) over time, with error bars ± s.e.

6.4.3. *Bulk stable isotope results*

During data processing, it was noted that sample variance greatly increased as the detector peak height approached zero. No set detection limit could be obtained from the manufacturer, the variance across detector peak heights was used to determine the lowest reliable detection limit. This was 0.5 µA peak height as measured by the detector, and samples with values below this were removed from the dataset. Notably low samples were doubled during the run once this was noted. This meant that the ammonium and urea treatment samples had n=1 for $\delta^{13}C$ and $\delta^{15}N$ enrichment, and as such statistical testing or standard error calculation was not possible for these treatments.

As shown in Figure 6.4a enrichment in ^{15}N was seen in all nitrogen treatments (Figure 6.4 a, log scale). The ^{15}N-isotope enrichment increased immediately at the start of the experiment for all treatments. This rate slowed over the seven days of the experiment for the ammonium and urea treatments that reached 2900 ‰ $\delta^{15}N_{(air)}$ and 2400 ‰ $\delta^{15}N_{(air)}$ respectively at the end of seven days. The highest enrichment was seen in the nitrate and glutamate treatments, which also showed increased cell counts and use of media TDP. The nitrate and glutamate treatments enrichment rate increased markedly between the first two sampling points and slowed but remained at a high abundance until day seven. At the end of seven days, the nitrate treatment reached 31200 ± 7800 ‰ $\delta^{15}N_{(air)}$, which was statistically similar to the glutamate treatment (78000 ± 54000 ‰ $\delta^{15}N_{(air)}$, $t_{(4.2)} = 0.9$, $p = 0.44$). The ^{15}N-enrichment of the cell mass from the nitrate treatment was statistically different to the control (30 ± 9 ‰ $\delta^{15}N_{(air)}$, $t_{(3)} = -4.0$, $p = 0.028$).

Only the urea and glutamate treatments were provided with ^{13}C-label, both being DON compounds. Glutamate showing a small and stable enrichment of $\delta^{13}C$ during the first three days that increased at seven days. Glutamate reached 1678 ± 83 ‰ $\delta^{13}C_{(VPDB)}$ after seven days, significantly different to the control (-21.7 ± 0.6 ‰ $\delta^{13}C_{(VPDB)}$, $t_{(4)} = -20$, $p < 0.001$). This correlates temporally with the increase in cell numbers and decrease in media TDP after seven days for the glutamate treatment. Urea showed no difference in $\delta^{13}C$ compared to the control after seven days (Figure 6.4 b).

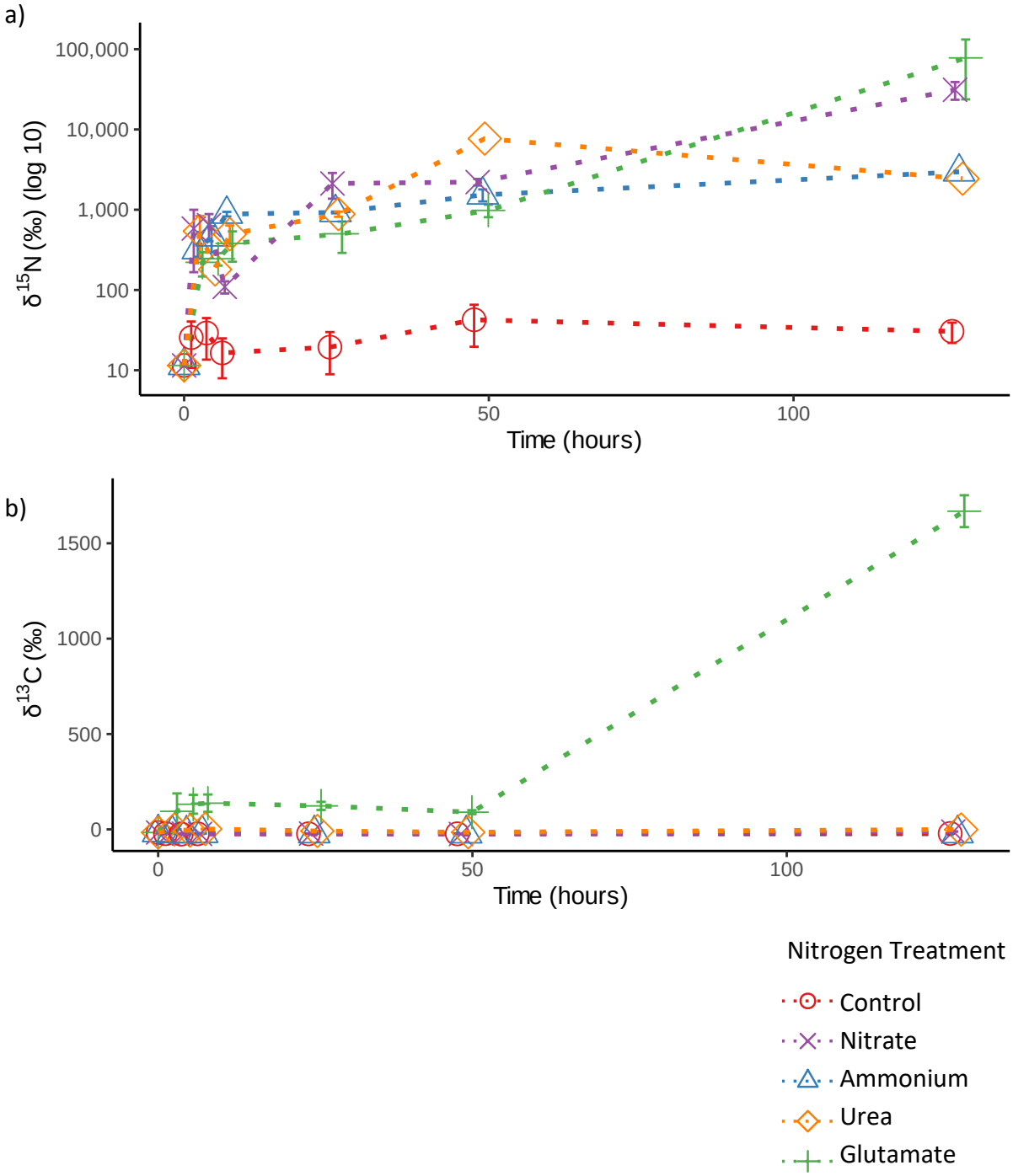


Figure 6.4. Isotope enrichment values for nitrogen (a) and carbon (b), with error bars ± s.e.

6.5. Discussion

The results shown here support the use of DON (glutamate) by an isolated strain of *Chlorella vulgaris.* The algae removed both nitrogen and carbon present in glutamate, with a longer lag phase than that for an identical concentration nitrate substrate. No response was seen to ammonium or urea, highlighting the species-specific responses that combine to form environment responses to nitrogen compounds.

Varied responses were seen to the different inorganic and organic nitrogen treatments, as seen in previous experiments (Chapter 4 & 5). An overview of the statistical differences between treatments is shown in table 6.2. The initial *Chlorella vulgaris* inoculum was not phosphorus limited, as seen by the control treatment not increasing in cell numbers and no depletion of TDP occurring in the control treatment after seven days. Nitrogen is therefore one of the likely limiting nutrients for growth in this experiment, as light and pH are the same as in previous experiments.

The nitrate (inorganic) treatment and to a lesser degree, the glutamate (DON) treatment, saw a positive response, as evidenced by increased cell numbers, and not unexpectedly the use of some TDP to support growth. The ammonium (inorganic) and urea (organic) treatments showed no evidence of growth, with cell number being similar or lower than the control at most time points. This may be due to the concentration used, as mixed riverine assemblages have been shown by Mackay *et al.* (2020) to respond better to some DON compounds when they are presented at very low concentrations.

Table 6.2. Overview of statistical differences between treatments after seven days, for various analyses. Vertical groups with the same letter are not statistically different at the 95% level. Statistical analysis was not possible for ammonium and urea as the sample signal was above the detection limit for only one sampling replicate in each treatment.

Treatment	Cell Number	Bulk Nitrogen	Bulk Carbon	TDN	TDP	$\delta^{15}N$ Enrichment	$\delta^{13}C$ Enrichment
Control	A	a, b	a	a	a	a	a
Nitrate	a, b	b	b	b	b	b	b
Ammonium	a, b	a	c	c	c	-	-
Urea	a, b	a	a	c	a, c	-	-
Glutamate	b	a	d	d	d	a, b	c

The experiments reported in this Chapter were carried out over a shorter (7 days) period than the experiments discussed in Chapters 4 and 5 (≥ 14 days). Perhaps due to this, lower maximum cells counts were observed, as well as no complete depletion of TDN or TDP. Growth as measured by particulate carbon and cell counts were seen in the nitrate treatment, as well as a lower response seen in the glutamate treatment. The urea and ammonium treatments were not significantly different from the control for most analyses. A lower uptake of TDN and TDP was seen after seven days, although some removal was seen in the nitrate and glutamate treatments.

^{15}N-label was found in the cell mass recovered from all the nitrogen addition treatments, even if other analyses did not indicate cellular growth had occurred or utilisation P and/or N. From this it seems that the incorporation of some labelled compound may be separate to growth on those compounds. Since the cell mass from all treatments became enriched in ^{15}N it appears that small amounts of nitrogen may be taken up by algal cells before large scale population growth is seen. The cell growth leading to this incorporation of isotope could be due to cells, or likely the cell death rates were balanced by small scale cell growth, leaving the overall cell measures looking stationary and stable. This lag in growth in the glutamate treatment is not unexpected, as the presence or absence of different nitrogen compounds would be a logical trigger for changes to cellular mechanisms, so the stimulation of nitrogen uptake pathways may take time to respond to a potentially bioavailable compound (Duarte, 1990; Cerón García *et al.*, 2005).

The particulate carbon showed similar trends to the cell counts, while the bulk particulate nitrogen was variable and more difficult to see differences or clear uptake of nitrogen. The reason for this difference in variability is unknown. Particulate carbon is a larger percentage of algal biomass, and so is easier to detect at the small concentration of biomass this experiment involved. It is possible there were different cell groups in cultures at any one time (dividing or resting, motile or stationary etc.), and that these had more divergent nitrogen processing than carbon, contributing to the variability seen.

The nitrate treatment showed the strongest growth response, but a response was also seen in the glutamate treatments. The bulk particulate carbon increase, as well as other measures such as TDP use, suggest that the glutamate treatment had a longer lag phase and was beginning to grow after seven days. There is a temporal aspect to use of dissolved organic nitrogen and associated carbon compounds within the treatments used here, and algal lag phases are known in literature (Tilman, Kilham and Kilham, 1982; Fogg and Thake, 1987). Algal lag phases have also been shown to be longer with additional ^{15}N enrichment (Andriukonis and Gorokhova, 2017), and a longer

experimental period may be needed to counteract this initial lag phase to see the longer term uptake rates.

Glutamate is an important precursor molecule to amino acid creation. Laboratory studies with soil bacterial cultures have shown glutamate is essential to mineralisation and assimilation of nitrogen (Miller and Stadtman, 1972; Yuan *et al.*, 2006; Knowles *et al.*, 2010). The $\delta^{13}C$ ‰ seen in this experiment showed ^{13}C was incorporated into biomass and used alongside the nitrogen that entered the algal cells (Figure 6.4 b).

This incorporation of carbon in the glutamate treatment, as well as the nitrogen provided, is interesting as it means that DON compounds may be used for their carbon content, not just the nitrogen present (Mineau *et al.*, 2016; Bechtold *et al.*, 2012). The signal of ^{13}C in the biomass shows it has been incorporated, and not lost as CO_2 or dissolved carbon fractions. This pattern was not seen in the urea treatment, perhaps because it is a much smaller compound and the carbon may have been lost as CO_2 if the urea compound was split. For urea, the urease enzyme splits it into CO_2 and ammonia, and this may happen outside or inside of the algal cells. Most species isolated in this thesis have not grown in response to ammonium addition (see Chapter 5), so it is unsurprising that there is less increase in cell numbers in the urea and ammonium treatment here. Ammonium is usually thought of as being processed into nitrate before use by algae cells (O'Brien and Dodds, 2008; Arango *et al.*, 2008; Lachmann *et al.*, 2019). It looks like the bacteria present in this isolate either could not do so, or the rate was not high enough to sustain algal growth. It is interesting to note the similar response between urea and ammonium treatments though, as this hints to similar pathways being used in the uptake and assimilation of these compounds.

Glutamate can be cleaved to provide an amino group for the creation of other amino acid molecules (Miller and Stadtman, 1972; Nelson and Cox, 2000; Knowles *et al.*, 2010). The remaining carbon-based molecule (α-keto-glutatarate) could then be further used (Gelfand and Steinberg, 1977). Other C*hlorella sp.* are known to have glutamate synthase pathways, use to convert ammonium into glutamine, then into glutamate for use inside the cell (Lea and Miflin, 1975), so the components of glutamate are well used in algal metabolic pathways. Glutamate is converted into aspartate in cyanobacteria (Strasser and Falkner, 1986), supporting the production of other amino acids and proteins. *Chlorella vulgaris* is known to produce a variety of amino acids and omega-3 polyunsaturated fatty acids for human metabolism (Panahi *et al.*, 2016; Schieler, McClure and Dunn, 1953). *Chlorella* species have the potential for heterotrophic growth, skipping the fixation of carbon through photosynthesis by using glucose (Chen and Johns, 1991; Endo *et al.*, 1974) or other compounds (Heredia-Arroyo *et al.*, 2011; Liang, Sarkany and Cui, 2009; Shi, Zhang and Chen, 2000).

The mechanisms that this takes place through should be the same that DON could be utilised through. Knowledge of the amino acid pool changes in this thesis experimental treatment would help to elucidate these pathways more clearly.

DON, in this case specifically glutamate, is bioavailable to green algae. This has implications due to the increased nitrogen inputs into freshwaters globally. There are often limits on nitrate and ammonium (DIN) release to freshwater, but wastewater treatment plant often adhere to these through converting inorganic compounds to DON. Though DON may initially take a little longer to be utilised by local biota, many components will still be bioavailable. Processing of nitrogen and amino acid composition of algae has been shown to differ between eukaryotic and cyanobacterial groups (McCarthy, Lehman and Kudela, 2013). Cyanobacteria are also often HAB forming species, alongside green algae, but care should be taken in extrapolating these results outside of green, ideally verified by similar experimental work.

As part of this section of work, analysis of the isotope enrichment within extracted amino acids of the algal cells would be undertaken following the methodology of Reay *et al.* (2019a) and recently used in literature for bacteria (Knowles *et al.*, 2010; Charteris *et al.*, 2016). This would enable the pathway through to internal algal nitrogen stores to be clarified, hopefully showing where glutamate ^{15}N and ^{13}C appear first in the internal amino acid pools of algal cells, as well as how it flows through other amino acid groups. This approach would clarify the pathways used in DON processing. Due to COVID-19 related time restraints and restrictions on research activities, this has not been possible. Samples were taken for this analysis and will be processed post PhD submission. More detailed analysis of the internal amino acid pools would help define the pathways and timings of incorporation into the cells, and ultimately help gauge the impact of N_r on freshwater ecosystems.

6.6. Conclusions

The use of glutamate (DON) alongside nitrate as a substrate for growth of a green algae, *Chlorella vulgaris*, has been shown here. Urea and ammonium substrates resulted in a lower level of growth and were similar to each other. Glutamate and likely other carbon skeleton DON compounds can be used by a widespread green algae for growth and assimilation of carbon as well as nitrogen, showing bioavailability of these compounds. Care should be taken in extrapolating to other species, as shown earlier in this thesis, algal species differ in their responses to DON. Variable

bioavailability of these compounds means more focus is needed determining varied species responses, as well as monitoring and reducing human inputs of DON to freshwater ecosystems.

Chapter 7 – DON impact and interactions with freshwater algal species: overview, future work and concluding remarks

7.1. Introduction

Algae form an important part of the food web in freshwater ecosystems (Masclaux *et al.*, 2015; Woodward and Hildrew, 2002; Weitere *et al.*, 2018). Environmental stressors which impact on algae therefore have consequences for higher tropic level species and wider ecosystem functions (Deininger *et al.*, 2017). While their responses collectively and as individual species to inorganic nutrient stressors has been extensively researched, their responses to the more complex DON pool and the many compounds this comprises are not well understood in freshwater ecosystems. Recent work on DON use by freshwater algae has largely investigated mixed communities (Mackay *et al.*, 2020) or some single species culture collections (Fiedler *et al.*, 2015). In these experiments, DON use is often inferred from algal growth rather than by directly measuring nitrogen or carbon uptake and metabolism within the algal cells. Contested theories therefore exist concerning DON uptake by freshwater algae, varying from uptake of whole molecules, to extracellular breakdown and uptake of inorganic N fractions only from DON compounds.

Knowledge of the interactions between DON and freshwater algal species, whether phytoplankton or benthic, is important to improve understanding of how freshwater ecosystems will change in future in response to changing nutrient enrichment, or changing composition of the N pool in freshwaters. Tracking the algal response to specific DON chemistries is also important, as these may be taken up directly or split into simpler molecules which are then taken up by the algae. Recent advances in analytical methods to determine the biological flow, or metabolism of nitrogen in DON, including stable isotope probing of DON into amino acid synthesis has been demonstrated recently in soil bacterial communities (Charteris *et al.*, 2016; Reay *et al.*, 2019a), and has offered the opportunity in this thesis to trace those pathways in the freshwater algae for the first time.

Knowing what impacts DON compounds have on algal production is important, as there is increasing pressure on freshwater ecosystems from N enrichment globally and climate change. Changing climate is impacting freshwater ecosystems though changes in rainfall amount and patterns, extreme heat waves (Woolway *et al.*, 2021), as well as increasing average temperatures (Kernan, Battarbee and Moss, 2010), which in turn have changed the rates and timing of nutrient flux to freshwaters and rates of cycling in freshwaters (Ockenden *et al.*, 2017). Changes to the range of temperatures within freshwater environments will negatively impact fish, mollusc and plants

species (Kärcher *et al.*, 2019) as well as algae which underpin the food web. Rapid warming is likely to lead to the dispersal, death or adjustment of food web structures and rates of nutrient spiralling within waters as thermal regimes adapt (Woolway and Maberly, 2020), including in tropical freshwater environments (Gallardo *et al.*, 2018). All of these will change local vegetation and DON inputs to river systems, community structure and potentially the chemical composition of the N pool and the rates of DON uptake by freshwater biota. Recent climate change is increasingly human driven, but there are other anthropogenic impacts on freshwater ecosystems (Crutzen, 2006; Steffen, Crutzen and McNeill, 2007) such as changing of wastewater treatment and its discharge to waters (Yates, Johnes and Spencer, 2019), and changing land use and management (Yates *et al.*, 2019; Lloyd *et al.*, 2019), that will also alter both the rates and chemical composition of DON input to freshwaters and potentially its bioavailability to the freshwater algae. Catchments supplying freshwater environments are subject to increasing urbanisation and agricultural pressure, including changes to runoff pathways, soil microbial function, the drainage of wetlands, livestock densities, and water abstraction or flow diversions (Sabater, Elosegi and Ludwig, 2018). These will all affect both the rates and composition of nutrient inputs to freshwater systems, including DON, and provide multiple concurrent stressors to freshwater organisms.

Freshwater as a resource is also under pressure, increasingly extracted or diverted from rivers or lakes (Broadley *et al.*, 2020). Catchment based approaches to freshwater management have been introduced in some countries, for example under the EU Water Framework Directive (Directive 2000/60/EC) to varying effect (Hering *et al.*, 2010; Moss, 2008). Human use of water resources is still politicised (Huitema and Meijerink, 2017), though increasing public participation could improve freshwater conservation (Cañedo-Argüelles *et al.*, 2019; Gibbs, 2014), helping to support freshwater ecosystem services. Knowing which DON compounds to target through mitigation efforts, which are those most likely to stimulate freshwater biotic uptake, would help provide data to start these conversations positively.

Appropriate monitoring is needed to understand how changes in water quality impact river biology (White *et al.*, 2019; Chen and Olden, 2018), and more understanding is needed globally to understand and predict freshwater flows and how they might change in response to management interventions within catchments or climate change (Döll *et al.*, 2016). This could be aided by better understanding the historic ecology of rivers (Haidvogl *et al.*, 2015), along with increased monitoring at higher frequencies and for a wider range of the stressors the drive ecosystem responses in freshwaters (Lloyd *et al.*, 2019). These multiple stresses in river ecosystems make it more difficult to predict how changes will affect freshwater ecology (Sabater, Elosegi and Ludwig, 2018). A better

understanding of DON processing is also needed to the likely combined effects of changes in flow, thermal regime and the change of other stressors to freshwaters on the ecology of freshwaters. Freshwater bacterial interactions with DON are also not well understood, but more recent work is investigating the microbiological response to dissolved organic matter more generally and DON in particular (Brailsford *et al.*, 2017; Brailsford *et al.*, 2019; Ghosh and Leff, 2013; Brailsford *et al.*, 2021), and more work on marine interactions has been conducted as part of global carbon cycle research (Cherrier, Bauer and Druffel, 1996; Carlsson, Graneli and Segatto, 1999; Somes and Oschlies, 2015a) and in relation to DOM uptake by estuarine and coastal algae (Glibert *et al.*, 2021; Glibert *et al.*, 2006a).

The work presented in this thesis has addressed this knowledge gap, generating species-specific understanding of algae-DON interactions in freshwater ecosystems. Using a set of algal isolates from headwater catchments in catchments with contrasting environmental character, and DOM composition instream, a suite of incubation experiments, including with stable isotope substrates has revealed a range of species-specific and DON compound-specific responses. This chapter summarises these findings and discusses their implications for wider research and understanding of the role of DON in freshwater ecosystems.

7.2. Key findings

The broad aims of this thesis were to determine the types of DON that different algal species could use for growth in the absence of other forms of nitrogen, if that varied between species or environmental conditions, and DON uptake could be traced into algal cells or cellular components using stable isotope approaches. An overview of the main species results are shown in Table 7.1. The following sections address the thesis objectives in more detail and summarize results.

Table 7.1. Overview of species and catchment related responses to inorganic and two organic DON compounds as well as a negative control.

Isolation Location	Species	Growth seen compared to starting point (positive or negative)				
		Control	Nitrate	Ammonium	Urea	Glutamate
Priors Farm, Upper Nadder catchment, South England	*Chlorella vulgaris*	-	**+**	-	**+**	-
	Lobochlamys spp.	-	**+**	+	**+**	+
	Mixed species - Family Chlorellaceae spp., Order Oscillatoriales spp., Unknown diatom spp.	+	**+**	**+**	**+**	**+**
Nant y Brwyn peatland, Upper Conwy catchment, North Wales	*Chlorella* spp.	-	**+**	+	+	**+**
	Klebsormidium spp.	**-**	**+**	-	**+**	+
	Nitzschia spp.	**-**	**+**	-	+	**-**

Objective 1: To determine whether riverine algae can use DON as a nutrient resource

This was investigated using experimental incubations of algae isolated from UK rivers in the presence of a selection of commonly occurring DON compounds (Chapter 4 and 5). This demonstrated that DON (urea or glutamate) was used as a nutrient resource for growth by all species investigated. All treatments were set up to ensure nitrogen, rather than phosphorus limitation and so drive N uptake by the autotrophs. Uptake of DON was evidenced through a decrease in DON concentration in the media in association with a parallel decrease in P concentrations as algal biomass (cell counts and particulate carbon) increased.

Hypothesis 1 – Isolated algal species can utilise both inorganic and organic nitrogen sources for growth.

Outcome – Yes, the DON compounds tested can be used by algal isolate cultures as source of nitrogen, generally sustaining biomass production at rates comparable to those for inorganic N compounds and above a negative control.

Objective 2: To investigate whether there is a preference for different DON compounds, and whether this is consistent between species groups.

Species identification was obtained through DNA barcoding showed a range of algal isolates present (Chapter 3). These included various spherical *Chlorella* species, a filamentous *Klebsormidium* species and *Lobochlalmys* species, all of which were chlorophytes or green algae, along with a Nitzschia species diatom. Other strains were identified within some samples, highlighting some species having a residual mixed algal community present despite efforts being made to isolate axenic cultures. Algal exudates are known to support aquatic bacterial communities (Azam *et al.*, 1983), including in riverine biofilms (Weitere *et al.*, 2018). A range of associated bacteria, and in one isolate an amoeba, were also identified by DNA barcoding, providing a supporting microbiome in each algal isolate culture.

Hypothesis 2 – Isolated algal species have different species-specific and compound-specific preferences for growth on inorganic and organic nitrogen.

Outcome – Yes, different species usually showed a preference for urea or glutamate, usually not both, with preferences either for nitrate plus urea or ammonium plus glutamate generally observed over six species.

Objective 3: To determine whether DON uptake varies between species isolated from streams with differing catchment characteristics

Algal responses to DON and inorganic N dosing were reported for stream of contrasting environmental character in Chapter 5: a headwater peatland stream at Nant y Brwyn in the Upper Conwy catchment, and a headwater farmland stream at Priors Farm which drained through an intensive dairy catchment in the Upper Nadder catchment. The results did not reveal consistent differences between the two catchments. Most species showed a positive growth response to urea (DON) and nitrate (inorganic) compounds, irrespective of isolation location. Glutamate caused a strong positive response in one species from each location (*Chlorella* spp. single culture, and a mixed culture of Family Chlorellaceae spp., Order Oscillatoriales spp., Unknown diatom spp), and a weaker response in a different species from each location (*Lobochlamys* spp and *Klebsormidium* spp.). Urea saw more widespread positive impact on algal biomass, all species isolated from the farmland

catchment showed strongly positive responses, with some growth above initial biomass shown by all peatland isolates.

Very similar farmland (Priors Farm) *Chlorella vulgaris* and peatland (Nant y Brwyn) *Chlorella* spp. showed different responses to the urea and glutamate, though both showed growth in the nitrate treatment. The ability to utilise glutamate seemed to coincide with utilisation of ammonia. From the six isolates used here, species/isolate appears a stronger impact on the ability to process particular compounds DON than the character of the catchment from which the isolate was collected. However, the species present and isolates from each catchment were different, so the response of each catchment may differ due to algal biodiversity, which is indirectly affected by catchment character.

Hypothesis 3 – Similar species isolated from differing headwater catchments show the same growth response to DON chemistry.

Outcome – No, very similar species showed different responses to the same DON chemistry, while different species showed similar responses to individual DON compounds.

Objective 4: Investigating evidence of DON uptake into protein synthesis pathways in algal cells.

In a final suite of experiments reported in Chapter 6, dual-labelled DON compounds and single-labelled inorganic N compounds were used to investigate the uptake and metabolism of N by *Chlorella vulgaris*, isolated from an agriculturally impacted clay stream (Priors Farm). Glutamate generated enrichment of both ^{15}N and ^{13}C within the algal biomass, though the rate of biomass production was slower than on nitrate. All nitrogen substrates, generated ^{15}N enrichment in the algal biomass though not all saw an increase in cell numbers. This may be due to the different metabolic pathways involved in processing the various substrates. The lag in cell growth, even though cells became isotopically enriched, also suggests that a small amount of uptake occurs before cells are fully able to utilise the substrate for population growth, perhaps due to the effort required to switch metabolic processes to alternate substrates.

Hypothesis 4 – Algae show isotopic enrichment in bulk biomass after growth on isotopically labelled inorganic and organic nitrogen compounds.

Outcome – Yes, algae showed isotopic enrichment of nitrogen in the bulk biomass measures. The DON substrates showed varied responses, consistent with the findings for this particular algal species reported earlier in Chapter 4, with only glutamate showing an increase in bulk ^{13}C.

Hypothesis 5 – Algal amino acid pools will show enrichment after growth on isotopically labelled inorganic and organic nitrogen compounds.

Outcome – Though this was originally an aim of the thesis, time constraints imposed by Covid lockdowns meant these samples could not be analysed and reported in this thesis. See Covid statement for details.

7.3. Wider implications for freshwater ecosystems

DON is a bioavailable nutrient resource for freshwater algae, sometimes used in preference to inorganic nitrogen compounds, with both species-specific and compound-specific responses evident in the results from this thesis. Changes in environmental function due to climate change, changes in land use and management or wastewater discharge rates or composition will likely change the nitrogen chemistry of stream N_r pools, and then affect changes in algal biomass, but also potentially species composition of algae and algal blooms. Not only does this indicate the need to control both inorganic and organic nitrogen inputs to waters in any catchment mitigation efforts, but also control on currently increasing DON changes to waters from locations including wastewater discharges or livestock farming, or switches from inorganic to organic fertiliser use in farming systems, could lead to increased negative effects in freshwater ecosystems, via eutrophication and/or toxin production (Jos and Cameán, 2020). Other changes and pressures are also occurring in freshwater ecosystems, alongside nutrient and DON composition changes. Depending on how these interact, not all consequences may be negative. If algal growth is balanced with increases in algae-grazing organisms and higher trophic organisms, changes to the nutrient composition of streams could still sustain a fully functioning ecosystem. Even if the species biodiversity is lost or altered, if the functional diversity between species is retained then freshwater ecosystems will have fewer negative impacts (Dunck *et al.*, 2016; Cibils *et al.*, 2015; Sandin and Solimini, 2009). This may require more concerted management of freshwater ecosystems, and require better monitoring, including of DON (Willett *et al.*, 2004; Heathwaite and Johnes, 1996).

Most freshwater monitoring covers nitrate and ammonium, but often fails to determine either Total N or DON as a bulk fraction (Durand *et al.*, 2011; Yates, Johnes and Spencer, 2019). It also fails to include individual DON compounds, which as DON is a massive pool of potential compounds, and only more recent, untargeted analytical techniques are able to cover the full range of compounds present (Pemberton *et al.*, 2019). Measuring freshwater nitrogen pools, however, not only benefits understanding of the pollutant loading in freshwater ecosystems, but also helps

to identify where and what DON fluxes occur to streams and the synthesis of new compounds instream through the cycling of nitrgoen through biotic loops within the stream ecosystem (Andersen *et al.*, 2014).

The view of which measure of N loading is most important for predicting algal responses is not a one-size-fits-all, and needs to be tailored to the species present at each location to best understand and predict the ecosystem responses. As shown here, even taxonomically similar *Chlorella* species can respond in different ways to the same substrates and environmental conditions (Chapter 4 and 5). Nevertheless, what this thesis has shown is that DON is an important N resource for freshwater autotrophs. Common and widespread *Chlorella* and *Klebsormidium* species tested in this suite of experiments demonstrated strong responses to both urea and glutamate dosing, highlighting the importance of DON as a bioavailable nutrient resource to both biofilm and planktonic algae. DON is this likely to be more bioavailable than previously thought, contributing to the overall dissolved and bioavailable nitrogen pool that supports algal growth in freshwaters (Gillor *et al.*, 2010). This mirrors the responses to DON previously reported and well-established in the literature for both coastal and estuarine ecosystems (Glibert *et al.*, 2006b; Glibert *et al.*, 2020), suggesting that the general principle of DON bioavailability to photoautotrophs is common across aquatic ecosystems rather than particular to estuarine and coastal waters alone.

7.3.1. *Potential pathways for freshwater DON uptake*

Different substrates have different potential uptake pathways into algal cells, likely mediated by the bacteria and fungi present in the environment. Fungi and bacteria are an integral part of freshwater ecosystems (Moss, 1998), and most isolates used here had co-associated microbiomes that will play a role in the uptake of DON into cells. These interactions require further study, as there are complex multi-species systems within the freshwater environment.

Nitrate (inorganic N) metabolism by algae in marine and freshwater locations has been more closely studied than DON interactions (Lomas and Glibert, 2000; Flynn, Fasham and Hipkin, 1997; Glibert *et al.*, 2015; Hildebrand and Dahlin, 2000). Certainly, all species studied in this thesis showed positive growth on nitrate substrate. However, most treatments did not show a growth response to ammonium (inorganic N), which is not what would be expected from most literature as ammonium has a lower energy requirement for use than nitrate (Lachmann *et al.*, 2019; LeKieffre *et al.*, 2020). However other research has found ammonium to inhibit growth at above optimum concentrations (Glibert *et al.*, 2016), with responses usually being species-dependent (Domingues *et al.*, 2011), and

potentially light-dependent (Sinclair, Kamykowski and Glibert, 2009). This is interesting, as microbial degradation of organic matter produces ammonium (Figure 7.1).

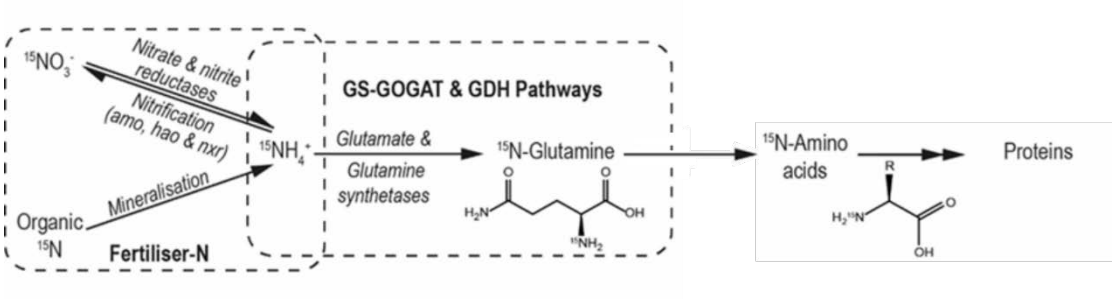


Figure 7.1. Pathways for assimilating ammonia, from inorganic or organic sources, into glutamate within bacteria and plant cells, showing probing by ^{15}N labelled compounds.

The pathways for mineralised organic nitrogen uptake shown in figure 7.1 include glutamine dehydrogenase (GDH) and glutamine synthase (GS) alongside NADPH dependent glutamine-2-oxoglutarate aminotransferase (GOGAT). GDH and GS-GOGAT are known pathways in plants (Masclaux-Daubresse *et al.*, 2006; Dubois *et al.*, 2003), bacteria (van Heeswijk, Westerhoff and Boogerd, 2013; Mörsdorf and Kaltwasser, 1989; Yan, 2007; Vairinhos, Bhandari and Nicholas, 1983) and yeasts (Liu, Sutter and Tu, 2021; Mara *et al.*, 2018). It is not unreasonable to suggest that these pathways may be active in some freshwater algal species, alongside the potential to use organic compound through other pathways.

The bioavailability of DON may be related to the carbon it contains, not just the nitrogen. A *Chlmydomonas* spp., related to the *Lobochalmys* spp. isolated here, shows carbon concentrating mechanisms to aid growth (Spalding, 2008). As DON contains carbon this may be an important resource for algae, alongside the nitrogen present. If carbon is liberated from the compound first, this will increase the bioavailability of the nitrogen present. Bacteria could liberate some of the carbon in DON for algae to use, resulting in the same effect. Some algae can also be grown heterotrophically, such as *Chlorella vulgaris*, also isolated here (Heredia-Arroyo *et al.*, 2011; Jia *et al.*, 2011). This may help to explain the enrichment of ^{13}C in the Chlorella vulgaris used in Chapter 6. Although the population (cell numbers) did not increase for that treatment the use of carbon may have been de-coupled from the nitrogen use. Dual-labelled isotope dosing coupled with compound specific stable isotope probing would have (if time had permitted) revealed whether this cleaving of molecules and the uptake of their constituent C and N components was the common or dominant uptake pathway, or whether there was whole molecule uptake into protein synthesis within the algal cells.

7.4. Limitations and further work

This work was limited by the lack of time available to complete the amino acid specific analysis on the enriched algal biomass. This would have allowed tracing of uptake of DON and inorganic N into the algal amino acid pools, and determination of whether this involved the extracellular splitting of molecules or their uptake as whole molecules into the algal cells. COVID-19, unfortunately, substantially reduced the amount of laboratory work that could be completed during 2020, not only while the laboratories were closed but also due to the slower pace of laboratory work and faulty instruments available once labs re-opened under COVID rules. Nevertheless, the samples have been prepared and stored, and will be analysed in the near future. Had these circumstances not arisen, compound-specific stable isotope analyses would have been completed to unequivocally determine the pathways by which DON was incorporated into the algal amino acid pool and algal biomass.

Another limitation to this programme of experiments was that the isolates used here were not axenic, so the effect of bacteria was not separated from the algal ability to utilise DON compounds. Analysing axenic responses compared to the mixed algal-bacterial responses would help to locate which sections of the community are most essential in DON bioavailability, and most important to target for monitoring or conservation efforts. Metagenomic work to probe deeper into the known and potential pathways of the co-associated fungi and microbiome of algae would aid in understanding the pathways controlling the bioavailability of DON and predict how future changes to the composition of the stream N pool might impact on freshwater ecosystems.

Improvements in characterisation of the complex types of DON present in freshwater environments (Pemberton *et al.*, 2019), would allow a wider range of environmentally relevant compounds to be tested in incubation experiments, either in laboratory conditions or in field mesocosm studies. Alongside this, investigation of how changes in algal biodiversity alter the rates of DON uptake in freshwaters would approach what is seen in environmental mixed species communities with complex DON pools. Species differing in nutrient and energy content will affect higher trophic levels, so co-mixed experiments would be useful to investigate dominance shifts in relation to different DON substrates.

There are, therefore, many more avenues of research possible and great potential to better understand DON uptake and synthesis pathways in freshwaters, how much algal uptake is mediated by the local microbiome under natural conditions, and how many compounds can be directly used

by the algae, and what consequences there are for an ecosystem under increasing pressure and stresses.

7.5. Concluding remarks

This thesis has shown the recently isolated algal species including green algae and diatoms can utilise DON for growth. The rate of uptake depends on the species in question and is likely impacted by any associated microbiome. Stable isotope experiments showed uptake of both nitrogen and carbon from DON compounds into algal biomass, with varying levels of growth and lag phases compared to inorganic substrates. DON is thus demonstrated to be bioavailable to freshwater algae, and likely to be impacting algal succession and diversity in freshwater ecosystems. More research is needed to better understand these interactions and to correctly conceptualise and contextualise the role of DON as a bioavailable nutrient resource in freshwater ecosystems.

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Appendix 1 – Supplementary information for Chapter 1

A.1 Chemical reactions of the classic inorganic nitrogen cycle

Reactions shown below cover the inorganic nitrogen cycle. Some need to occur in anoxic conditions, these are red. Others need oxygenated environments; these are light blue.

$$\mathrm{N_2 + 16\,H_2O\ \ [+ 8\,e^- + 8\,H^+ + 16\,ATP\,]} \rightleftharpoons 2\,NH_3 + H_2\ \ [+ 16\,ADP + 16\,P_i\,]$$

N2 Fixation (biological)

$$N_2 + 3\,H_2 \xrightarrow[\substack{>400\,°C \\ >150\ BAR \\ +\ catalyst}]{} 2\,NH_3 + H_2$$

N2 fixation (Haber-Bosch)

$$2\,NH_4^+ + 3\,O_2 \xrightarrow[+\ Enzyme]{} 2\,NO_2^- + 4\,H^+ + 2\,H_2O$$

Nitrification part 1

$$2\,NO_2^- + O_2 \xrightarrow[+\ Enzyme]{} 2\,NO_3^-$$

Nitrification part 2

$$2\,NO_3^- + 10\,e^- + 12\,H^+ \xrightarrow[+\ Enzyme]{} N_2 + 6\,H_2O$$

Denitrification

$$sugar + \frac{nitrate\ or}{nitrite} \xrightarrow[(Respiration)]{+\ Enzyme} NH_4^+ + H_2O + energy\,(ATP) + CO_2$$

Dissimilatory nitrate reduction to ammonium (DNRA)

$$NH_4^+ + NO_2^- \xrightarrow[+\ Enzyme]{} N_2 + 2\,H_2O$$

Anammox

Appendix 2 – Supplementary information for Chapter 2

B.1 - Comparison for Isolation Media

Appendix Table B.1 Comparison of various media and the compounds they contain, used to derive an isolation media covering most requirements of generalist microalgal species.

Compound type	Main element / nutrient	Compound in media (ug L^{-1})	BG11	Walnes Medium	Bold Basal Medium (BBM)	Jaworskis Medium (JM)	Spirulina Medium (SM) (marine)	F/2 (marine)
Nitrogen	Nitrogen	$NaNO_3$	1500000	100000	250000	80000	2500000	75000
Phosphorus	Phosphorus	K_2HPO_4	40000		75000			
	Phosphorus	KH_2PO_4			175000	12400	500000	
	Phosphorus	$NaH_2PO_4.2H_2O$		20000				5650
	Phosphorus	$Na_2HPO_4.12H_2O$				36000		
Metals	Calcium	$CaCl_2.2H_2O$	36000		25000		10000	
	Calcium	$Ca(NO_3)_2.4H_2O$				20000		
	Calcium	$C_{18}H_{32}CaN_2O_{10}$					50	
	Cobalt	$Co(NO_3)_2.6H_2O$	50		500		5	
	Cobalt	$CoCl_2.6H_2O$		20				10
	Copper	$CuSO_4.2H_2O$	80					
	Copper	$CuSO_4.5H_2O$		20	1600		0.25	10
	Iron	$C_6H_8FeNO_7$	6000					
	Iron	$FeCl_3.6H_2O$		1300				3150
	Iron	$FeSO_4.7H_2O$			4980		13500	
	Magnesium	$MgSO_4.7H_2O$	75000		75000	50000	200000	
	Manganese	$MnCl_2.4H_2O$	1800	400	1400	1400		180
	Manganese	$MnSO_4.7H_2O$					10	
	Molybdenum	$Na_2MoO_4.2H_2O$	400				5	6
	Molybdenum	$(NH_4)_6Mo_7O_{24}.4H_2O$		9		1000		
	Molybdenum	MoO_3			700			

Appendix Table B.1 continued. Continuation of table B.1 - Comparison of various media and the compounds they contain, used to derive an isolation media covering most requirements of generalist microalgal species.

Metals	Potassium	KOH			31000			
	Potassium	K_2SO_4					1000000	
	Sodium Chloride	NaCl			25000		1000000	
	Zinc	$ZnSO_4.7H_2O$	200		8800		5	22
	Zinc	$ZnCl_2$		21				
Vitamins	Vitamin	Vitamin B1 (Thiamine HCl)		10		40	250	100
	Vitamin	$C_6H_5NO_2$ (Niacin / Vitamin B3)					50	
	Vitamin	$C_6H_{12}O_6$ (Inostiol / Vitamin B8)					2500	
	Vitamin	Vitamin B12 (Cobalamin)		10		40	10	0.5
	Vitamin	$C_{19}H_{19}N_7O_6$ (Folic acid)					1	
	Vitamin	Vit H (Biotin)		0.2		40	0.5	0.5
Other	Acid	H_2SO_4			1 (µL)			
	Acid	$C_6H_8O_7$	6000					
	Boron	H_3BO_3	2860	33600	11400	2500	50	
	Carbonate	Na_2CO_3	20000				4030000	
	Carbonate	$NaHCO_3$				15900	13610000	
	EDTA FeNa	$C_{10}H_{12}N_2NaFeO_{83}$				2300		
	EDTA Na_2	$C_{10}H_{14}N_2Na_2O_8{\cdot}2H_2O$	1000	45000		2300	84000	4160
	EDTA	$C_{10}H_{16}N_2O_8$			50000			
	Other	$C_5H_6N_2O_2$ (Thymine)					1500	

B.2 - Experimental Media Composition

Media used in experimental phase of research is described in more detail here. It is based on Jaworski's media, using alternative compounds where required and with the addition of Silica for diatoms and B1, B7 and B12 vitamins.

Compounds listed below are weighed, diluted and then usually passed through a 0.2 µm filter (Sartorius, Germany) to sterilise the stock solutions. There are exceptions that are autoclaved instead (calcium carbonate and iron solution). Stock solutions are then added aseptically to autoclaved DI, usually 500mL or 1000mL, via sterile pipetting within a Class II flow hood. The resulting media is stored at 4°C until required or disposed of after 2 months. Stock solutions are kept for up to 6 months at 4°C.

Table B.2. Media used in experimental treatments. Chemicals with the same letter prefix are made up in the same stock vessel. Stock solutions A-D are added to all media treatments, stock solutions E-F are only added to the respective treatment media.

Name	Formula	Media final Concentration (mg L-1)	Stock solution used 1mL L-1 (mg per 50 mL)	Stock solution used 0.1 mL L^{-1} (mg per 25 mL)
(A) Sodium phosphate	Na2HPO4. 12H2O	4.30	215.0	-
(B) Iron citrate	**C6H5FeO7**	**1.18**	**58.9**	-
(C) Boric acid	H3BO3	0.50	24.0	-
(C) Calcium chloride dihydrate	CaCL2. 2H2O	14.70	735.0	-
(C) Magnesium sulphate	MgSO4. 7H2O	9.60	478.0	-
(C) Manganese chloride	MnCl2. 4H2O	0.27	14.0	-
(C) Sodium molybdate VI dihydrate	NaMoO4. 2H2O	1.18	58.8	-
(D) Zinc chloride	**ZnCl2**	**0.004**	**-**	**1**
(D) Cobalt chloride	**CoCl2. 6H2O**	**0.004**	**-**	**1**
(D) Copper sulphate	**CuSO4. 5H2O**	**0.004**	**-**	**1**
(E) Sodium Nitrate	NaNO3	15.30	765.0	-
(F) Ammonium chloride	**NH4Cl**	**9.60**	**481.4**	**-**
(G) Urea	CO(NH2)2	5.40	270.0	-
(H) Glutamic acid monosodium salt	**C5H8NNaO4 ·xH2O**	**30.40**	**1522.0**	**-**

Appendix 3 – Supplementary information for Chapter 3

C.1 - Commands for bioinformatics analysis

Program version used can be found in main text. Code written for use in Python environment, and annotated before each command explaining the action.

#Data import for paired samples, ITS and 16S.
qiime tools import --type 'SampleData[PairedEndSequencesWithQuality]' --input-path [path_name] --input-format CasavaOneEightSingleLanePerSampleDirFmt --output-path [demux_output_paired.qza]

#Data import of ITS forward reads only of sample NMspp2.
qiime tools import --type 'SampleData[SequencesWithQuality]' --input-path [path_name] --input-format CasavaOneEightSingleLanePerSampleDirFmt --output-path [demux_output_single.qza]

#Visualise imported data, done for all import files.
qiime demux summarize --i-data [demux_output.qza] --o-visualization [demux_visualisation.qzv]

#Denoising and pairing reads of ITS and 16S.
#16s and ITS5 denoise and pair reads
qiime dada2 denoise-paired --i-demultiplexed-seqs [demux_output_paired.qza] --p-trim-left-f 0 --p-trim-left-r 0 --p-trunc-len-f 225 --p-trunc-len-r 223 --o-table [output_table.qza] --o-representative-sequences [sequences.qza] --o-denoising-stats [denoising_stats.qza]
#Denoising single, unpaired reads.
qiime dada2 denoise-single --i-demultiplexed-seqs [demux_output_single.qza] --p-trim-left 0 --p-trunc-len 224 --o-table [single_output_table.qza] --o-representative-sequences [sequences_unpaired.qza] --o-denoising-stats [denoising_stats_unpaired.qza]

#Summarizing output of denoising and pairing steps.
#Sequnces summarised
qiime feature-table tabulate-seqs --i-data [sequences.qza] --o-visualization [sequences_visualise.qzv]
#Denoising summarised
qiime metadata tabulate --m-input-file [denoising_stats.qza] --o-visualization [denoising_stats_visualise.qzv]
#Feature table created
qiime feature-table summarize --i-table [output_table.qza] --o-visualization [table_type.qzv] --m-sample-metadata-file [metadata.tsv]

```
#Python used to remove dulpicate chartacter sequences in the sequencs fasta file.
cat ITSNB2_final_sequences_start_id_edit.fasta | sed -n '0~2p' | while read sequence ; do cat
        ITS5_final_sequences_start.fasta | awk '/^>/ {seq=$1} /'${sequence}'/ {print seq,
        "'${sequence}'"}' ;done
#Alternatively the following python script was run
#unique_sequences.py
import sys

sequences = {}

for line in sys.stdin.readlines():
   line = line.rstrip('\n')
   if line.startswith('>'):
      sequence_name = line
      sequences[sequence_name] = []
   else:
      sequences[sequence_name].append(line)

for sequence_name, sequence_lines in sequences.items():
   print(sequence_name)
   for sequence_line in sequence_lines:
      print(sequence_line)


#These were then BLAST searched. Files were manually checked during download, and complete
        genome matches or duplicates were removed where possible to ensure downstream
        processing could take place.
#Each BLAST match file had names shortened or adjusted so that name would be more legible on
        the final tree.
#Original and downloaded sequences were combined, as shown below.
cat [list of fasta files] >> cat16smatches+original.fasta
cat [list of fasta files] >> catITSmatches+original.fasta
cat [list of fasta files] >> catITS_NB2matches+original.fasta

#Spaces present in the filenames of downloaded sequences were replaced with underscores. The
        below commands are shown for 16S only, but all three grouped files shown above followed
        the same process shown from this point onwards.
sed 's/ /_/g' cat16smatches+original.fasta >> cat16smatches+original_nospace.fasta
#Duplicate BLAST matches were also removed, using the previously shown command for removing
        duplicate character sequences.

#Sequences and matches were aligned using mafft.
```

```
mafft --localpair --maxiterate 1000 cat16smatches+original_nospace.fasta >>
        mafftalignout_16s.fasta

#Fasttree was used to create a tree, with trial using IQtree shown to be impossible on my local
        machine.
fasttree -nt mafftalignout_16s.fasta > mafftalignout_16s_tree.tre

#Output tree files were viewed and formatted in Figtree, then adjusted with additional text in
Inkscape and Word.
```

C.2 – Nant y Brwyn sample 3 ITS quality checks

Investigation of ITS sample NM spp. 3 with lower than average read counts (Figure A.1). A visual comparison of the overall read qualities showed the reverse reads of both group to be much lower quality than the forward reads, and reverse reads of Nant Y Brwyn species 3 (Figure X d) were better than the other 5 species grouped together (Figure A.1 b). Visualisation of the imported QIIME artifacts was through export and visualisation on the website visualiser for QIIME artifacts. No major decrease in quality scores is seen before the other five ITS samples. The five ITS sample quality scores decreased below 20 much sooner than would be expected, and other samples were isolated to identify any outliers.

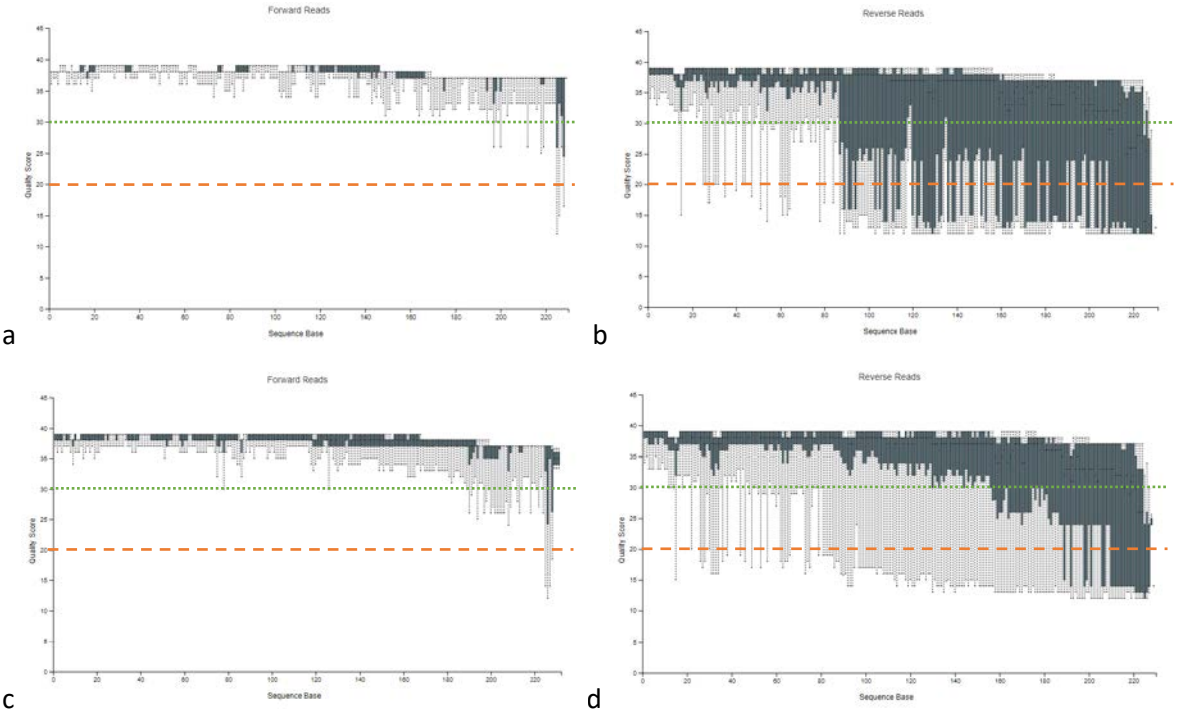

Figure C.1. Quality scores of raw data forward and reverse ITS V3/V4 reads of 5 samples forward (a) and reverse (b) and ITS reads of potential outlier NM spp. 3 forward (c) and reverse (d), with lines at quality scores of 30 (green) and 20 (red). (Visualisation through export from QIIME2 and visualisation on QIIME2 website visualiser (ref)).

Samples were put through a DADA2 pipeline, of which various results are shown in the tables below.

Table C.2. Summary of 16S reads passing through DADA2 pipeline to trim, denoise and merge reads.

16s DADA2 pipeline with trim - sample-id	Input count	Filtered count	% of input passed filter	Denoised count	Merged count	% of input merged	Non-chimeric count	% of initial input non-chimeric
NB34-S1-spp14	41995	38335	91.28	38291	38215	91	36228	86.27
NB34-S2-spp4	42248	36350	86.04	35947	34866	82.53	29534	69.91
NB34-S3-spp8	29232	26379	90.24	26193	25396	86.88	21413	73.25
PF34-S1-spp07	43680	37982	86.96	37915	37796	86.53	36532	83.64
PF34-S2-spp08	31875	25351	79.53	25249	24964	78.32	22044	69.16
PF34-S3-spp10	35223	31065	88.2	31025	30997	88	30962	87.9

Table C.3. Summary of 16S reads passing through DADA2 pipeline without trimming, and with denoising and merging reads. Processes occur left to right.

16S DADA2 pipeline, no trimming - sample-id	Input count	Filtered count	% of input passed filter	Denoised count	Merged count	% of input merged	Non-chimeric count	% of initial input non-chimeric
NB34-S1-spp14	41995	38400	91.44	38337	38238	91.05	36271	86.37
NB34-S2-spp4	42248	36532	86.47	36150	34916	82.65	29674	70.24
NB34-S3-spp8	29232	26363	90.19	26185	25331	86.66	21410	73.24
PF34-S1-spp07	43680	40731	93.25	40692	40514	92.75	39161	89.65
PF34-S2-spp08	31875	29247	91.76	28945	28252	88.63	25259	79.24
PF34-S3-spp10	35223	31119	88.35	31067	31037	88.12	30997	88

Table C.4. Summary of ITS reads passing through DADA2 pipeline without trimming, and with denoising and merging reads. Processes occur left to right, and the anomalous results is highlighted.

ITS DADA2 pipeline (2ee) sample-id Table X-1	Input count	Filtered count	% of input passed filter	Denoised count	Merged count	% of input merged	Non-chimeric count	% of initial input non-chimeric
NB-ITS-sample1	45065	38417	85.25	38414	38410	85.23	38390	85.19
NB-ITS-sample2	59089	1823	3.09	1803	1076	1.82	1076	1.82
NB-ITS-sample3	21517	16142	75.02	16038	12917	60.03	12797	59.47
PF-ITS-sample1	41682	35456	85.06	35450	35450	85.05	35450	85.05
PF-ITS-sample2	45602	40489	88.79	40484	40482	88.77	40482	88.77
PF-ITS-sample3	42632	36801	86.32	36799	36799	86.32	36799	86.32

Table C.5. Summary of ITS reads passing through DADA2 pipeline without trimming, and with denoising and merging reads, with a relaxed error rate (10 ee). Processes occur left to right, and the anomalous result is highlighted.

ITS DADA2 pipeline (10ee) sample-id Table X-2	Input count	Filtered count	% of input passed filter	Denoised count	Merged count	% of input merged	Non-chimeric count	% of initial input non-chimeric
NantYBrwyn1spp14.1	45065	45065	100	45051	40816	90.57	40794	90.52
NantYBrwyn2spp4.2	59089	59081	99.99	59051	5441	9.21	5373	9.09
NantYBrwyn3spp8.3	21517	21508	99.96	21349	15124	70.29	14989	69.66
PriorsFarm1spp07.4	41682	41682	100	41670	36633	87.89	36633	87.89
PriorsFarm2spp08.5	45602	45601	100	45589	41389	90.76	41389	90.76
PriorsFarm3spp10.6	42632	42632	100	42620	39705	93.13	39705	93.13

Table C.6. Summary of ITS forward only reads for Nant y Brwyn sample 2, after passing through DADA2 pipeline without trimming, and with denoising and merging reads and the default error rate. Processes occur left to right.

ITS NB2 DADA2 pipeline, forward reads only	Input count	Filtered count	% of input passed filter	Denoised count	Non-chimeric count	% of initial input non-chimeric
Nant Y Brwyn sample 2	59089	57423	97.18	57412	57344	97.05

C.3 – All 16S matches and identifications in full

Table C.7. All 16S sequence matches for all samples.

Sequence Name	NCBI match	Identity match (%)	Coverage	Tree grouping	Identification	Nant y Brwyn sample 1	Nant y Brwyn sample 2	Nant y Brwyn sample 3	Priors Farm sample 1	Priors Farm sample 2	Priors Farm sample 3
>16s_seq_001	Micractinium sp.	100	100	Chlorella sp. Chloroplast	Chlorella sp. Chloroplast	0	0	1	0	0	32223
>16s_seq_002	Chlorella vulgaris	98.76	100	Chlorella sp. Chloroplast	Chlorella sp. Chloroplast	25107	0	0	0	0	0
>16s_seq_003	Chlorella vulgaris	100	100	Chlorella vulgaris Chloroplast	Chlorella vulgaris Chloroplast	0	0	0	22021	0	0
>16s_seq_004	Klebsormidium sp.	99.76	100	Klebsormidium sp chloroplast	Klebsormidium sp chloroplast	0	17858	0	0	7	7
>16s_seq_005	Rhizobium sp.	100	100	Rhizobium sp.	Rhizobium sp.	0	0	0	0	16147	0
>16s_seq_006	Roseomonas sp.	99.5	100	Roseomonas sp.	Roseomonas sp.	10525	452	0	0	0	2
>16s_seq_007	Pseudomonas sp.	100	100	Pseudomonas sp.	Pseudomonas sp.	0	7	8208	0	1	0
>16s_seq_008	Phreatobacter cathodiphilus	99.25	100	Phreatobacter sp.	Phreatobacter sp.	0	187	0	7539	0	0
>16s_seq_009	Comamonadaceae bacteria	99.3	100	Unknown bacteria f	Comamonadaceae bacteria	0	0	0	6834	0	0
>16s_seq_010	Nitzschia palea, Durinskia baltica, Microcystis sp.	100	100	Nitzschia sp. Chloroplast	Nitzschia sp. Chloroplast	0	6	3403	0	1	0
>16s_seq_011	Pseudomonas sp.	100	100	Pseudomonas sp.	Pseudomonas sp.	0	6	3275	0	2	0
>16s_seq_012	Bosea sp.	100	100	Bosea sp.	Bosea sp.	0	3381	0	0	2	1
>16s_seq_013	Blastomonas sp., Sphingomonas sp.	100	100	Family Sphingomonadaceae	Family Sphingomonadaceae	0	1	0	0	3416	3

Table C.7 continued. All 16S sequence matches for all samples.

Sequence Name	NCBI match	Identity match (%)	Coverage	Tree grouping	Identification	Nant y Brwyn sample 1	Nant y Brwyn sample 2	Nant y Brwyn sample 3	Priors Farm sample 1	Priors Farm sample 2	Priors Farm sample 3
>16s_seq_014	Hydrogenophaga sp.	99.77	100	Hydrogenophaga sp.	Hydrogenophaga sp.	0	3255	0	0	2	3
>16s_seq_015	Methylophilus sp.	100	100	Methylophilus sp.	Methylophilus sp.	2187	0	0	0	0	0
>16s_seq_016	Brevundimonas sp., Mycoplana sp., Caulobacteraceae bacterium	100	100	Brevundimonas sp.	Caulobacteraceae	0	2121	0	0	0	2
>16s_seq_017	Pedobacter sp.	100	100	Pedobacer sp.	Pedobacer sp.	0	1	2017	0	0	0
>16s_seq_018	Ancylobacter sp., Hyphomicrobiaceae bacterium	95.27	100	Unknown bacterium a	Order Hyphomicrobiales	0	2	1338	0	0	0
>16s_seq_019	Asticcacaulis sp.	100	100	Asticcacaulis sp.	Asticcacaulis sp.	0	2	1338	0	0	0
>16s_seq_020	Paracraurococcus sp., Roseomonas stagni, Roseomonas sp.	100	100	Roseomonas sp.	Family Acetobacteraceae	0	0	0	0	1343	0
>16s_seq_021	Rhizobium alvei, Rhizobium rosettiformans, Neorhizobium huautlense, Rhizobium sp.	100	100	Rhizobium sp.	Rhizobium sp.	0	1397	411	0	0	2
>16s_seq_022	Bosea sp., Rhizobiales bacterium, Afipia sp.	100	100	Bosea spp.	Order Hyphomicrobiales	0	45	0	0	1221	0
>16s_seq_023	uncultured bacterium	100	100	Unknown bacterium g	uncultured bacterium	0	1069	0	0	1	0
>16s_seq_024	Pelomonas sp., Pseudomonas sp.	100	100	Pelomonas sp.	Pelomonas sp.	0	1	1009	0	0	0

Table C.7 continued. All 16S sequence matches for all samples.

Sequence Name	NCBI match	Identity match (%)	Coverage	Tree grouping	Identification	Nant y Brwyn sample 1	Nant y Brwyn sample 2	Nant y Brwyn sample 3	Priors Farm sample 1	Priors Farm sample 2	Priors Farm sample 3
>16s_seq_025	Mesorhizobium sp.	99.75	100	Mesorhizobium sp.	Mesorhizobium sp.	0	926	0	0	1	1
>16s_seq_026	Lobochlamys culleus	97.52	100	Chlamydomonadales sp. Chloroplast	Chlamydomonadales sp. Chloroplast	0	0	0	0	728	0
>16s_seq_027	Kaistia sp., Rhizobiales bacterium	99.5	100	Kaistia sp.	Kaistia sp.	0	464	0	0	589	1
>16s_seq_028	Micractinium singularis mitochondrion, Chlorella sp. mitochondrion	99.26	100	Unknown bacterium b	Family Chlorellaceae mitochondrion	0	0	0	0	0	511
>16s_seq_029	Pedobacter cryoconitis, Pedobacter lusitanus	100	100	Pedobacter sp.	Pedobacter sp.	0	2	636	0	0	0
>16s_seq_030	Ensifer collicola, Rhizobium sp., Sinorhizobium sp.	100	100	Ensifer collicola	Family Rhizobiaceae	0	320	41	0	0	0
>16s_seq_031	Rhizobiaceae bacterium, Kaistia sp.,	99.5	100	Rhizobiaceae bacterium	Family Rhizobiaceae	0	294	35	0	0	0
>16s_seq_032	Reyranella sp.	100	100	Reyanella sp.	Reyanella sp.	0	282	0	0	0	0
>16s_seq_033	Microcella sp., Salinibacterium sp., Chryseoglobus sp.	99.51	100	Unknown bacteria t	Family Microbacteriaceae	0	0	0	3050	1509	0
>16s_seq_034	Bosea sp., Rhodopseudomonas sp.	100	100	Rhodopsudomonas	Order Hyphomicrobiales c.f. Rhodopseudomonas	0	340	0	0	0	0
>16s_seq_035	Methylobacterium sp.	100	100	Methylobacterium sp.	Methylobacterium sp.	0	297	0	0	0	0

Table C.7 continued. All 16S sequence matches for all samples.

Sequence Name	NCBI match	Identity match (%)	Coverage	Tree grouping	Identification	Nant y Brwyn sample 1	Nant y Brwyn sample 2	Nant y Brwyn sample 3	Priors Farm sample 1	Priors Farm sample 2	Priors Farm sample 3
>16s_seq_036	Methylophilaceae bacterium	99.77	100	Methylophilus sp.	Methylophilus sp.	0	2	268	0	0	0
>16s_seq_037	Chryseoglobus frigidaquae, Microcella sp., Leifsonia sp., Microcella sp.	100	100	Microbacteriaceae family	Family Microbacteriaceae	0	0	0	201	5	0
>16s_seq_038	Hyphomicrobium sp., Pedomicrobium sp.	99.75	100	Hyphomicrobium sp.	Hyphomicrobium sp.	0	196	0	0	0	0
>16s_seq_039	uncultured bacterium	100	100	Unknown bacterium c	Unknown bacterium	0	229	0	0	0	0
>16s_seq_040	Roseomonas sp.,	99.25	100	Roseomonas sp.	Roseomonas sp.	2	181	0	0	0	0
>16s_seq_041	Bacteroidetes bacterium, Chitinophaga sp., Sphingobacteriales, Bacteroidetes, Flavobacteriia	99.29	100	Unknown bacterium l, sphingobacteriales	Phylum Bacteroidetes	0	147	0	0	0	0
>16s_seq_042	Rhizobiales bacterium, Phreatobacter sp.	99.5	100	Uncultured rhizobiales bacterium	Family Rhizobiaceae	0	150	8	0	0	0
>16s_seq_043	Mesorhizobium albiziae	100	100	Mesorhizobium sp.	Mesorhizobium sp.	0	190	12	0	0	0
>16s_seq_044	uncultured bacterium	100	100	Rhizobacter sp.	Family Rhizobiaceae	0	0	87	0	0	0
>16s_seq_045	Rhodopseudomonas sp. , Bradyrhizobiaceae bacterium,	100	100	Nitrobacteraceae family	Family Nitrobacteraceae	0	0	78	0	0	0
>16s_seq_046	Roseomonas sp.	99.5	100	Roseomonas sp.	Roseomonas sp.	0	0	62	0	0	0

Table C.7 continued. All 16S sequence matches for all samples.

Sequence Name	NCBI match	Identity match (%)	Coverage	Tree grouping	Identification	Nant y Brwyn sample 1	Nant y Brwyn sample 2	Nant y Brwyn sample 3	Priors Farm sample 1	Priors Farm sample 2	Priors Farm sample 3
>16s_seq_047	Limnohabitans sp., Comamonadaceae, Curvibacter sp.,	100	100	Curvibacter sp.	Family Comamonadaceae	0	0	0	58	0	0
>16s_seq_048	Bosea sp., Afipia sp.	100	100	Bosea spp.	Bosea sp.	0	53	0	0	0	0
>16s_seq_049	Tardiphaga sp., Rhodopseudomonas sp.	100	100	Tardiphaga robinae	Tardiphaga sp.	0	0	47	0	0	0
>16s_seq_050	Hydrogenophaga sp.	99.77	100	Hydrogenophaga sp.	Hydrogenophaga sp.	0	51	0	0	0	0
>16s_seq_051	Pelomonas sp., Paucibacter sp., Pseudomonas sp.	99.3	100	Paucibacter sp.	Family Comamonadaceae c.f. Paucibacter sp.	0	47	0	0	0	0
>16s_seq_052	Polaromonas sp., Comamonadaceae bacterium	99.77	100	Polaromonas sp.	Polaromonas sp.	0	41	0	0	0	0
>16s_seq_053	Oxalobacteraceae , Rhodocyclaceae	92.96	100	Unknown bacterium i	Class betaprotobacteria	0	41	0	0	0	0
>16s_seq_054	Piscinibacter defluvii, Piscinibacter aquaticus	98.59	100	Piscinibacter sp.	Piscinibacter sp.	0	43	0	0	0	0
>16s_seq_055	Chryseoglobus frigidaquae, Diaminobutyricimonas aerilata, Cryobacterium soli	99.51	100	Unknown bacteria s	Family Microbacteriaceae	0	0	0	31	4	0
>16s_seq_056	Sphingopyxis sp.	100	100	Sphingopyxis alaskensis	Sphingopyxis sp.	0	28	0	0	1	0
>16s_seq_057	No matches	0	0	Unknown bacterium b		0	0	0	0	0	28
>16s_seq_058	Actinomarinicola tropica	92.29	99	Family Lamiaceae	Class Acidimicrobiia	0	32	0	0	0	0

Table C.7 continued. All 16S sequence matches for all samples.

Sequence Name	NCBI match	Identity match (%)	Coverage	Tree grouping	Identification	Nant y Brwyn sample 1	Nant y Brwyn sample 2	Nant y Brwyn sample 3	Priors Farm sample 1	Priors Farm sample 2	Priors Farm sample 3
>16s_seq_059	Order Oscillatoriales	85.27	98	Chlorella sp. Chloroplast	Order Oscillatoriales	0	0	0	0	0	25
>16s_seq_060	Paucibacter toxinivorans	98.12	99	Unknown bacteria f		0	24	0	0	0	0
>16s_seq_061	Variovorax ureilyticus	98.83	100	Unknown bacterium e		0	0	24	0	0	0
>16s_seq_062	Bifidobacterium catenulatum, Bifidobacterium pseudocatenulatum	100	100	Bifidobacterium pseudocatenulatum		6	5	0	0	0	0
>16s_seq_063	Blautia wexlerae	100	100	Blautia sp.		11	0	0	0	0	0
>16s_seq_064	Pseudomonas vancouverensis	98.36	100	Pseudomonas vancouversensis		0	0	13	0	0	0
>16s_seq_065	Herbaspirillum autotrophicum	100	100	Herbaspirillum autotrophicum		0	0	19	0	0	0
>16s_seq_066	Cupriavidus necator	93.01	100	Unknown bacterium i		0	0	29	0	0	0
>16s_seq_067	Roseburia faecis	98.26	100	Unknown bacterium o, faecal bacterium		8	0	0	0	1	0
>16s_seq_068	Aminipila butyrica	91.36	100	Order Clostridales		8	0	0	0	0	0
>16s_seq_069	Labrys wisconsinensis	99.25	100	Labrys sp.		0	14	0	0	0	0
>16s_seq_070	Cryobacterium roopkundense, Cryobacterium levicorallinum	91.82	100	Unknown bacteria t.2		0	26	0	0	0	0
>16s_seq_071	Fimbriiglobus ruber	91.46	100	Family Planctomycetaceae		0	15	0	0	0	0
>16s_seq_072	Variovorax boronicumulans	99.3	100	Varivorax sp.		0	0	11	0	0	0

Table C.7 continued. All 16S sequence matches for all samples.

Sequence Name	NCBI match	Identity match (%)	Coverage	Tree grouping	Nant y Brwyn sample 1	Nant y Brwyn sample 2	Nant y Brwyn sample 3	Priors Farm sample 1	Priors Farm sample 2	Priors Farm sample 3
>16s_seq_073	Salinibacterium xinjiangense, Lysinimonas soli	99.51	100	Herbiconiux sp.	0	108	0	0	0	0
>16s_seq_074	Pseudomonas kilonensis, Pseudomonas corrugata	99.53	100	Pseudomonas sp.	0	0	11	0	0	0
>16s_seq_075	Ruminococcus callidus	93.83	100	Ruminococcus sp.	6	0	0	0	0	0
>16s_seq_076	Fusicatenibacter saccharivorans	97.76	100	Unknown bacteria n	0	6	0	0	0	0
>16s_seq_077	Methylibium petroleiphilum	98.36	100	Methylibium sp.	0	12	0	0	0	0
>16s_seq_078	Faecalibacterium prausnitzii	99.5	100	Faecalibacterium sp.	4	0	0	0	0	0
>16s_seq_079	Coprococcus eutactus	97.76	100	Family Lachnospiraceae	0	7	0	0	0	0
>16s_seq_080	Gracilibacter thermotolerans	88.48	100	Unknown bacterium r	5	0	0	0	0	0
>16s_seq_081	Dorea longicatena	99.75	100	Dorea longicatena	0	0	0	0	0	2
>16s_seq_082	No matches	0	0	Unknown bacterium k	0	0	0	0	0	0
>16s_seq_083	Anaerostipes hadrus	92.56	100	Order Clostridiales	5	0	0	0	0	0
>16s_seq_084	Methyloversatilis universalis, Methyloversatilis discipulorum	92.34	100	Unknown bacterium h	0	6	0	0	0	0
>16s_seq_085	Tardiphaga robiniae	97.26	100	Unknown bacterium d	0	0	1	0	0	0

Table C.7 continued. All 16S sequence matches for all samples.

Sequence Name	NCBI match	Identity match (%)	Coverage	Tree grouping	Nant y Brwyn sample 1	Nant y Brwyn sample 2	Nant y Brwyn sample 3	Priors Farm sample 1	Priors Farm sample 2	Priors Farm sample 3
>16s_seq_086	Phyllobacterium myrsinacearum, Phyllobacterium brassicacearum	100	100	Phyllobacterium sp.	1	0	0	0	0	4
>16s_seq_087	Ruminococcus bromii	99.5	100	Ruminococcus sp.	0	0	0	0	4	0
>16s_seq_088	Roseburia hominis	99.5	100	Roseburia hominis	0	0	0	0	2	0
>16s_seq_089	Christensenella massiliensis, Christensenella minuta	91.07	99	Order Clostridia	0	0	0	0	2	0
>16s_seq_090	Eubacterium xylanophilum, Anaerotaenia torta, [Clostridium] populeti	97.26	100	Order Clostridiales	0	4	0	0	0	0
>16s_seq_091	Frankia alni	98.46	100	Frankia sp.	3	0	0	0	0	0
>16s_seq_092	Herbaspirillum autotrophicum	98.37	100	Herbaspirillum sp.	0	0	7	0	0	0
>16s_seq_093	Kineothrix alysoides	97.01	100	Unknown bacteria m	0	5	0	0	0	2
>16s_seq_094	Faecalibacterium prausnitzii, Gemmiger formicilis	96.02	100	Unknown bacteria q	4	0	0	0	0	0
>16s_seq_095	Kineothrix alysoides	90.32	99	Unknown bacteria p	0	2	0	0	0	0
>16s_seq_096	Limnoraphis robusta	85.82	99	Chlamydomonadales sp. Chloroplast	0	0	0	0	3	0
>16s_seq_097	Novosphingobium rosa	100	100	Novosphingobium sp.	0	1	0	0	0	0
>16s_seq_098	No matches	0	0	Unknown bacterium j	0	1	2	0	0	0

Table C.7 continued. All 16S sequence matches for all samples.

Sequence Name	NCBI match	Identity match (%)	Coverage	Tree grouping	Nant y Brwyn sample 1	Nant y Brwyn sample 2	Nant y Brwyn sample 3	Priors Farm sample 1	Priors Farm sample 2	Priors Farm sample 3
>16s_seq_099	Naasia aerilata, Yonghaparkia alkaliphila, Microcella putealis	98.85	100	Unknown bacteria t	0	0	0	0	2	0
>16s_seq_100	Sphingomonas kyeonggiensis, Sphingomonas leidyi	100	100	Sphingomonas sp.	2	0	0	0	0	3
>16s_seq_101	Faecalibacterium prausnitzii	98.26	100	Faecalibacterium prausnitzii	1	0	0	1	2	0