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SUSTAINABLE AGRICULTURAL LAND MANAGEMENT BY RECYCLING ALGAL BIOMASS TO LAND

By

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A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

The University of Sheffield

Faculty of Engineering

Department of Chemical and Biological Engineering

September 2018

DECLARATION

I, Emanga Alobwede, declare that this thesis has been composed entirely by myself unless otherwise referenced in the text. I confirm that this thesis has not been submitted for any other degree.

Acknowledgements

I would like to thank the Grantham Centre for Sustainable Futures for funding my research, my supervisors Dr. Jags Pandhal and Prof. Jonathan Leake, for their support, guidance and valuable feedback throughout my PhD thesis.

To my friends and colleagues, thank you for listening and sharing advice. I would also like to thank all of those who helped me during my 4 years of study – through giving general guidance and support: Dr Nella Roccuzzo, Dr Anne Cotton, Irene Johnson and Alan Smalley.

Finally, I would like to thank my family for their love and support even from afar, James, the love of my life, for his endless patience, love and steadfast support and lastly my dad, to whom this thesis is dedicated to. I would not have reached this stage if it weren't for your selflessness and dedication to providing the best for me, as well as for the rest of the family.

Abstract

High rates of inorganic fertiliser use in agriculture can lead to increased eutrophication of local water bodies. This can generate ideal conditions for algal blooms, creating anoxic conditions and detrimental ecosystem impacts. A proposed solution is to utilize the algal biomass to replenish soil nutrients, organic matter and structure and reduce the need for inorganic fertilisers that lead to algae blooms. This thesis investigated the extent to which algal biomass can improve soil quality, and the mechanisms by which any such improvements occur. Greenhouse and field experiments were conducted to test the effect of representatives of five algal species with contrasting elemental composition: (*Arthrospira platensis* (*Spirulina*), *Chlorella* sp., *Palmaria palmata, Laminaria digitata* and *Ascophyllum nodosum*), on soil physico-chemical characteristics and crop yield. None of the algae had an impact on soil aggregate stability. *Chlorella* sp., *Spirulina* and *P. palmata* showed evidence of nitrogen mineralization in both greenhouse and field experiments, with only *C. vulgaris* increasing yields of garden peas under greenhouse conditions. A ^{15}N tracer study was subsequently performed, to assess the fate of nitrogen derived from *C. vulgaris*, into soil and wheat nitrogen pools. Results revealed a significant increase in wheat uptake of algal N after 20 days, from 0.02 mg to 0.5 mg ^{15}N. Low ^{15}N tracer recoveries in plants were attributed to immobilization in the soil (binding of NH_4^+ to clay particles) and/or microbial biomass, making it unavailable to plants. The bulk soil retained a higher amount of nitrogen (31%), highlighting the significant role microbes play in organic matter turnover, and thus prompting an in depth look into microbial communities and taxa responsible for the breakdown and cycling of the algal N. 16S rRNA amplicon sequencing revealed an increase in alpha diversity in soils treated with *C. vulgaris* by day 30, most likely spurred by an increase in relative abundance in taxa (Proteobacteria and Bacteroidetes) putatively involved in facilitating the breakdown of organic residues. The thesis findings provide useful insights into the application of algae biomass to soil as part of a nutrient management strategy, whereby careful consideration would be required for specifying the optimum quantity of biomass required as well as the chemical composition of the algae, as these have been shown to have strong links to measurable effects on soil quality.

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Chapter I

Introduction

This chapter provides an overview of the extent of agricultural intensification and its impact on soil degradation in the UK. This includes impacts on water bodies in the form of proliferation of algal biomass. The role of algae as a nutrient source in agriculture is also discussed.

1.1 Agriculture and soil degradation

The way we produce our food in future is crucial in terms of preserving resources and ensuring that the entire process and practice is sustainable and resilient, particularly in the face of climate change. Currently conventional agriculture relies on practices such as continuous arable cropping with annual soil disturbance, often by ploughing; high use of inorganic fertilisers and agro-chemicals; heavy machinery and intensive irrigation systems all of which degrade the soil, causing loss of organic matter, soil compaction and losses of nutrients, chemicals and soil itself to watercourses (Tilman et al., 2011; Ockenden et al., 2014; Gregory et al., 2015). For agriculture to be sustainable, proper soil management is at the crux of delivering food security and the essential environmental goods and services such as clean drinking water and flood risk reduction (Gregory et al., 2015; Squire et al., 2015).

Intensive farming has altered the biotic interactions and physical and chemical characteristics of the soil. The reduction in plant species richness that comes with

agricultural intensification leads to changes in community composition of not only insects and pests, but of the microbial community of the soil (Manning et al., 2015). Increased compaction through the use of heavy machinery has led to a reduction in soil porosity and poor soil aeration as well as a reduction in root growth (Soane and Van Ouwerkerk, 1995; Lipiec et al., 2003). Soil tillage disrupts the organic matter in the topsoil (upper 20 cm of soil) by increasing its rate of decomposition. It also decreases aggregate stability, which negatively impacts the soil's infiltration and water retention capacity (White, 2006). The destabilization of soil particles in the topsoil, leads to the loss of nutrients and organic matter through leaching and loss of important soil functions (Wood et al. 2016). This results in a loss or reduction of soil physical (e.g. water holding capacity and structure) and biological properties, all of which are important for enhancing soil fertility (Obalum et al., 2017). Furthermore, the application of inorganic fertilisers has led to significant nutrient runoff and an accumulation of these nutrients in waterways (Camargo and Alonso, 2006). Adoption of best practices by farmers and growers has led to the employment of methods such as zero till cultivation, less use of heavy diesel-powered machinery as well as precision timing in the application of fertiliser. However, there is continued heavy reliance of inorganic fertilisers and their impact on the environment such as groundwater and surface water pollution remain a problem even with increasing restrictions on fertilizer use in nitrate-vulnerable zones in the UK (Szoege et al., 1996; Withers and Lord, 2002; Kay et al., 2012).

Nitrogen fertiliser applications on crops and grassland across the UK have changed markedly over the past 65 years. Following a rapid rise in fertilizer use from the 1960s to the mid-1980s (Robinson & Sutherland, 2002), grassland and crop nitrogen fertilizer rates have stabilized at 130-150 kg ha^{-1} since 2010 (AIC, 2017). Application rates on

crops and grassland decreased from 2015 to 2016 by 4 kg ha^{-1} due to a reduction of 5 kg ha^{-1} in the overall application rate on tillage crops (DEFRA, 2017). Despite the recent steady trends in nitrogen application rates, the past over use of fertiliser from 1945 to mid-1980s has left a legacy of increased background nitrogen in the soil and sediments, and therefore resultant leakage into inland waters (Withers et al. 2014). An estimated 70-80% of nitrate in rivers in England reportedly originates from agricultural sources (Kay et al., 2012). NO_3^-, as a result of being a negatively charged ion does not bind to SOM or clay particles (which are both negatively charged) and thus is the principal form of nitrogen leached (Di and Cameron, 2000; Howden et al., 2013).

The routine application of P to grassland and arable fields have accumulated an average P surplus of *c.* 1000 kg ha^{-1} over the past 65 years (Withers et al. 2001) and 2013 had an estimated surplus of 7.2 kg ha^{-1} on managed agricultural land (DEFRA, 2014). This surplus has led to a build-up of soil total and easily-exchangeable P. Losses of P from soils have generally been attributed to the impact of cumulative surplus P inputs on soil total P and the degree of P saturation (Withers et al. 2001). The problem of P loss is further magnified by the fact that phosphate rock, from which P is derived, is a non-renewable resource, and global reserves may be depleted in 50-100 years (Oteino et al., 2015; Cordell et al., 2009).

Algae already present in freshwater, estuarine and coastal marine systems assimilate inorganic forms of nitrogen and phosphorus, and use these to synthesise toxins, fatty acids, polysaccharides and other biomolecules and as a result increase in biomass (Turner, 2002; Xu et al., 2010). Proliferation of algae results in lower dissolved oxygen concentrations, which promotes the death of other aquatic organisms (Camargo and Alonso, 2006) and causes other harmful effects up the food chain.

In the UK and EU, there exist strict laws on pollution prevention especially of water bodies. The EC Water Framework Directive (WFD) is one such example, which aims to tackle pollution at the source and set other environmental targets such as controlling diffuse nutrient pollution from agricultural land in the Nitrates Directive (Council Directive 91/676/EEC, 1991). The application of such laws has implications on the amount of fertiliser added to agricultural land, creating a need to minimise losses of nutrients as well as their recovery from water bodies. The need to recover these nutrients as an alternative source of chemical nitrogen or phosphorus fertiliser will not only be of great economic advantage, but also a strategic necessity (Akhter et al., 2002).

Today, the increased competition for land and the prioritisation of other activities such as safeguarding biodiversity and protecting the public goods provided by natural ecosystems (for instance the carbon storage capacity soils and of rainforests) are higher on the environmental agenda, making it increasingly difficult to convert natural ecosystems into land for agricultural purposes (Behnassi et al., 2011). Therefore, the alternative option is to better utilise the existing agricultural land space, restoring degraded soils and sustainably increasing productivity including more efficient use of nutrients. One potential contribution to this is to recycle nutrients lost from soil by applying algal biomass harvested from nutrient-rich water back onto agricultural land.

1.2 Thesis aim

The project aims were to explore the effects of algae as a soil conditioner, looking at algal effects on soil chemical, physical and biological properties.

1.3 Outline

This thesis is formed of 6 chapters

- Chapter 2 is a literature review looking at soil quality parameters as well as the role algae plays in improving soil quality.

Chapters 3 is a paper published in Geoderma. Co-authors include Prof. Jonathan Leake and Dr. Jagroop Pandhal.

- Chapter 3 presents experimental results in two parts: a greenhouse study looking at the effect of 5 chemically contrasting algal species on soil physicochemical properties. The field experiment builds on the greenhouse study, where the effects of algae on soil and crop nutrients are studied in the field under a wheat cropping system.
- Chapter 4 examines the impact of algae on various nitrogen pools through the use of a ^{15}N tracer experiment to study the fate, transformations and uptake of algal nitrogen.
- Chapter 5 delves into the identification of the bacterial community present following the addition of algal nitrogen, with the aim of understanding changes in community composition and structure over time as affected by algal additions.
- Chapter 6, the final chapter, provides a thesis summary as well as suggestions for further studies.

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Chapter II

Soil quality and algae

This chapter looks the importance of soil properties in relation to fertility as well as the properties of algae, their role in various biogeochemical cycles as well as examples of their uses and benefits to soil and plants.

2.1 Soil quality

Twenty per cent of the world's arable land (land suitable for crop production) has been lost as a result of soil degradation (Linner and Messing, 2012; Wu et al., 2018). As humans are dependent on crops for food directly, and via crops fed to livestock, we are required to utilise this declining, and essentially non-renewable resource, in a sustainable way if we are to support the demands of a rapidly increasing global population, which is estimated to pass 11 billion by the end of this Century (Bergaglio, 2017). Proper management of soil to maintain its quality is therefore crucial to our survival.

It is hard to define soil quality and up until now, there is no universal definition or measurement agreed upon by all scientists that defines the quality of the soil (Nortcliff, 2002). Attempts to define the quality of the soil have common intentions of preserving and safeguarding the soil ecosystem's ability to function properly, particularly for crop yields as well as recognising the crucial importance of the soil in providing other

ecosystem services. Ecosystem services are defined as the goods and ecosystem functions that provide benefits to human populations (Millennium Ecosystem Assessment, 2005). Soils offer provisioning services, where they support agricultural production through soil formation, nutrient cycling and primary production (Lavelle et al., 2006), as well as filtering and storing fresh water- including groundwater that is used for drinking and other human activities. They also provide regulation services by controlling greenhouse gas fluxes and C sequestration and flood control (Lavelle et al. 2006). They lastly offer cultural services although to a lesser degree and support the biodiversity of a very wide variety of organisms. In general, good agricultural soil quality can be relatively easily defined because of its primary function in food production.

Most definitions of soil quality are related to its function, for example, Doran and Zeiss (2000) defined soil quality as "the capacity of the soil to function to sustain plant and animal productivity, maintain or enhance water and air quality, and promote plant and animal health." This definition focuses on quantifying soil quality. So, within agriculture, soil quality would be defined as the 'fitness' of the soil to support crop growth without becoming degraded. This ties in with other definitions, which incorporate the quantity of crops produced in the definition of soil quality as suggested by authors such as (Kruse, 2007).

Authors such as Letey et al. (2003) believed that additionally it is important to also look at soil use and not just soil function, when trying to define its quality, as soils may be fit for one purpose but not another. For example, low levels of nitrogen in the soil in springtime are good for soil functioning to protect the environment e.g. reduce nutrient leaching but are bad for soil functioning in enhancing crop yield (Kruse, 2007; Letey et al., 2003) hence making it difficult to use a single index to measure soil quality.

Although the majority of the literature use similar indicators when measuring soil quality - there is no ‘standard’ set of indicators that are used and this is probably because, using a standard set of indicators will be adopting the ‘one size fits all’ approach which is inappropriate, as soil quality has various definitions according to its multiple uses and functions. In an attempt to come up with a complete set of indicators to determine good soil quality, soil attributes/indicators were looked at for their influence on soil function. This involved assessing the chemical, physical and biological properties of the soil – a method familiar to soil scientists and in the scientific literature, these soil attributes are commonly referred to as a minimum data set (MDS). Kruse (2007) put together a table of the soil quality indicators referenced by other authors (Table 2.1).

Table 2.1 Table showing soil quality indicators. Modified from Kruse, 2007

Soil Quality Indictors: Physical, Chemical and Biological		
Soil Quality Indicator-Physical	**Soil Quality Indicator-Chemical**	**Soil Quality Indicator-Biological**
Organic matter	Organic matter	Microbial biomass C
Texture	Nutrient supply	Microbial biomass N
Soil depth	pH	Total microbial biomass
Soil Structure	EC (Soil Electrical Conductivity)	Bacterial biomass
Aeration	Base saturation	Fungal biomass
Aggregate stability	CEC (Cation Exchange Capacity)	Potentially mineralizable N
Bulk density	Contaminant availability	Soil respiration
Clay mineralogy	Contaminant concentration	Enzymes - dehydrogenase
Colour	Contaminant mobility	Enzymes - phosphatase
Consistence	ESP (Exchangeable Sodium Percentage)	Enzymes - arlysulfatase
Depth to root limiting layer	SAR (Sodium Adsorption Ratio)	Biomass C:TOC
Hydraulic conductivity	Nutrient cycling rates	Respiration: biomass
Oxygen diffusion rate	Plant nutrient availability	Microbial community fingerprinting
Particle size distribution	Plant nutrient content	Substrate utilisation
Penetration resistance	Potentially mineralizable N	Fatty acid analysis
Pore connectivity	Heavy metal concentration	Nucleic acid analysis
Pore size distribution	Organic chemical concentration	Earthworm population
Soil strength	Soil test P	Invertebrate diversity
Soil tilth	Total and available P and K	Nematode maturity index
Structure type	Total Organic C	
Temperature	Total Organic N	
Total porosity		
Available water holding capacity		
Slope		
Infiltration		

Most assessment tools of soil quality incorporate a combination of biological, physical and chemical indicators. Most commonly used soil indicators for assessing the health of the soil include soil organic matter, macro aggregate stability (Andrews et al., 2002)

defined as the ability of soil to resist breakdown as a result of forces such as wind, water and cultivation and bulk density (Andrews et al., 2004). Chemical indicators mostly used are pH, salinity and forms of nitrogen (Andrews et al., 2004), while other authors use soil microfauna populations as an assessment of soil quality (Parisi et al., 2005) on the justification that they are sensitive to soil management changes and allow for the examination of species life cycle to gain greater insight into soil ecological conditions. While is it common to use different measurements for the assessment of soil quality, it is important to that the chosen parameters offer a balanced assessment of different important soil functions and services.

2.2 Some key components required to deliver soil quality with high level productivity:

Key measures of soil health required for increased crop productivity and other important ecosystem service functions of soil (Letey et al., 2003; Kruse, 2007)) include:

i) Soil organic matter/organic carbon content

ii) Nutrients (N, P, K)

iii) Structure (including water stable macroaggregates >1mm diameter)

iv) Microbial activity

2.2.1 Soil organic matter and carbon

Organic matter is a key factor constraining arable farms in the UK (Gregory et al., 2015). It originates from plant and animal residues; aboveground or living soil fauna

and micro flora belowground (Fernandez-Romero et al., 2016). Organic matter within the soil plays a vital role in enhancing soil productivity by carrying out several functions. These include acting as a "nutrient fund" as it is a storehouse of plant nutrients, especially organic nitrogen and phosphorus, which are released through decomposition. It provides cation exchange capacity, which helps to retain ionic forms of nutrients (Loveland and Webb, 2003). It also helps to improve soil structure, tilth and reduce erosion, and plays a major role in soil water storage (Wood et al., 2016). The physical breakdown and biochemical transformation of organic molecules (FAO, 2005) as well as the continual addition and decaying of plant residues all contribute to the carbon cycling process in the soil (Gregorich et al., 2006).

Carbon is the measurable component of soil organic matter (Loveland and Webb 2003) that is affected by farming management practices such as increased use of tillage practices (Haddaway et al., 2016). According to data compiled by The Countryside Survey, intensively managed arable soils in the UK experienced a loss of carbon between 1998 and 2007 (Emmett et al., 2010), where mean soil carbon concentrations in 2007 in the top 15 cm of UK arable land was 30.7 g kg^{-1}, a decrease from 33.5 g kg^{-1} in 1998 (Emmett et al., 2010).

Soil organic carbon is altered through organic inputs such as plants as well as the soil type, land use change, microbial activity and environmental variables e.g. moisture and temperature (Zhang et al., 2013; Gregory et al., 2016). Soil organic carbon inputs such as plant litter cause short term changes in soil organic matter turnover– these can either be positive changes, where decomposition is accelerated, or negative, where it is slowed down. These changes are primarily driven by the quality of the organic carbon, temperature as well as microbial activity (Zhang et al., 2013).

2.2.2 Soil macronutrients (NPK)

Soil nutrients play a vital role in crop nutrition and health. Macronutrients are significant determinants of crop yield, playing an important role in crop nutrition, with N, P, and K being the primary essential nutrients (Renuka et al., 2016). Nitrogen is needed in the greatest amounts by plants, as evidenced by the fact that plant growth, especially in cropped land is normally constrained by the availability of nitrogen (Kraiser et al., 2010). The largest store of nitrogen is atmospheric, as gaseous nitrogen, (~3.7 x 109 Tg) (Ward, 2012). Biological nitrogen is derived either from biological nitrogen fixation, atmospheric fixations or the Haber-Bosch process, which produces fertilizer (Kraiser et al., 2010). Biological nitrogen fixation in soils involves the conversion of atmospheric nitrogen to organic nitrogen by soil microbes. The breakdown and decay of these organisms by heterotrophic microbes releases ammonia in soils which is subsequently oxidised by nitrifying bacteria converting it into nitrate, which is the preferred form taken up by most plants, and is the main form used by arable crops (Chen et al., 2014). Inorganic fertilisers in the form of ammonium or nitrate created through the Haber-Bosch process are also added to soils to provide plant available nitrogen during the growing season, enabling rapid growth and high yields and supporting high protein content in grain. In agricultural soils in UK, there is not enough naturally occurring plant available nitrogen (DEFRA, 2017) and this is therefore supplemented in the form of organic residues such as slurry, or inorganic fertiliser, such as ammonium nitrate. Average soil nitrogen concentrations in UK in 2007 ranged from 0.25 – 1.78% N across all habitats, with arable soils (0-15 cm) holding the lowest soil nitrogen concentrations of 0.25% N (Emmett et al., 2010).

Phosphorus (P) is another key macronutrient required for crop growth. It exists in the soil mainly as insoluble P and total concentrations in top soils (0-15cm) world wide range from 50 – 3000 mg P kg^{-1} depending on a variety of factors such as soil management and parent material (Zhu et al., 2018). In UK arable soils, P concentrations in soil (0-15 cm) range between 2 and 380 mg P kg^{-1}, with an average across all UK habitats of 32 mg P kg^{-1} in 2007 – with arable land having a higher mean concentration of 44 mg P kg^{-1} (Emmett et al., 2010). Phosphorus exists in various forms (inorganic and organic) in soil. Fractions of both inorganic and organic phosphorus are made available to plants as a result of abiotic and biotic factors such as microbial mineralisation and pH. Only a small proportion of P concentrations in soils (0.01%) is plant available (Renuka et al. 2010). Phosphorus is taken up by plants, or their-root associated mycorrhizal fungal partners, as negatively charged orthophosphate ions from the soil solution (Zhu et al., 2018).

Potassium exists in four separate pools in the soil: water-soluble, exchangeable, non-exchangeable and structural (Blanchet et al., 2017). Plants take up potassium from the soil solution, from both the exchangeable potassium, which is readily available, and the non-exchangeable which is slowly plant available (Romheld and Kirkby, 2010).

2.2.3 Soil structure

Two of the most useful indices of soil structure are bulk density (inversely related to soil porosity) and aggregate stability, both of which are modified by organic matter, soil texture and land use (Gregory et al., 2015). For example, where there is little organic matter, bulk density is found to be high i.e. in sandy texture classes. Bulk density of UK soils across broad habitat classes ranged between 0.2 and 1.2 g cm^{-3},

with arable soils having the highest bulk densities (Emmett et al., 2010). Furthermore, aggregate stability decreases with declining soil organic matter, leading to increased bulk density as pore spaces fill with soil particles (Diaconno and Montecurro, 2010).

Components of soil organic matter such as polysaccharides, soil humic substances, root material and fungal hyphae play an important role in soil structural stabilisation by binding particles into larger aggregates (Six et al., 2004). Aggregation is essential for good soil structure, aeration, water infiltration and resistance to erosion and crusting (FAO, 2005).

The aggregation of soil particles is important in maintaining favourable conditions for soil microbial and faunal activity and plant growth (Six et al., 2004). Soil aggregation and aggregate stability also influence the vulnerability of soil to raindrop impact and consequently surface sealing and soil erosion (Zhang et al., 2016). They are also key to the storage of organic matter, which is mostly found in intra-aggregate spaces in microaggregates within macroaggregates (Six et al., 2004; Mizuta et al., 2015). These aggregate spaces (from macroaggregates) also provide pore space through which water drains – thereby reducing the extent to which soil becomes saturated and subject to surface runoff causing erosion when there is little or no vegetation e.g. after ploughing (Six et al., 2004). Gregory et al. (2015) reported a 10-40% decrease in aggregate stability of UK soils, linked to a 1% decrease in soil organic matter.

2.2.4 Soil organisms

Soil organisms are found where organic matter occurs. They represent 5% of the living portion of organic matter and include microorganisms (<0.2mm in length) such as bacteria, fungi, viruses, protozoa and algae, mesofauna representative of the phyla

Annelida, Nematoda, Arthropda and Mollusca and macrofauna such as moles (White, 2006).

Microbes play an important role in stabilising organic matter – by assimilating carbon compounds, from the mineral associated soil organic matter fraction in the soil, and subsequently converting it into microbial biomass which is stabilised on surfaces of mineral soil particles in the form of microbial necromass and microbial exudates (Wood et al. 2016).

Soil microbial activity requires a carbon source for metabolic activity, which it derives from soil organic matter, in turn impacting on nutrient fluxes and structure (Aislabie and Deslippe, 2013). Microbes decompose soil organic matter by excreting extracellular enzymes, which breakdown the compounds in the soil organic matter (Aislabie and Deslippe, 2013). This enzyme production process by the microbes requires nitrogen as well as energy and therefore is reliant on the elemental stoichiometry of available substrates (Wood et al., 2016).

A surplus of amino-N (which is not required by the microorganisms) can result in net mineralisation– this is dependent on the C:N ratio of the substrate as well as the characteristics of the decomposer organism. Usually a substrate C:N ratio of >20 will likely result in net N uptake causing immobilisation into microorganisms whereas a substrate C:N ratio of <20 will likely lead to net mineralisation (White, 2006). Table 2.2 shows the C:N ratios of freshly fallen litter, manure and soil. As seen from the table, the C:N ratio of fresh litter and waste is highly variable but as the organic matter passes through several cycles of decomposition, the C:N ratio reduces. In well drained soils of pH ≈ 7, the C:N is close to 10.

Table 2.2

C:N ratios of fresh leaf litter, manure, soil and algae. Modified from White (2006)

	RANGE	MEDIAN VALUE
Litter		
Herbaceous legumes	15-25	20
Cereal straw	40-120	80
Animal manure		
Farmyard manure	-	20
Soil		
Arable and Horticultural soils (UK) (Emmett et al., 2010)	-	11.3
Algae		
Freshwater microalgae (Baird and Middleton, 2004)	3-17	6.6
Marine macroalgae (Baird and Middleton, 2004)	---	18.3

2.3 Algae

Phytoplankton (suspended algae) are a major source of organic matter for other living organisms in aquatic systems and their growth is dependent on nutrient supplies (Kay et al., 2012). Increasing rates of nutrient supply from agricultural runoff has accelerated the rapid growth and accumulation of phytoplankton leading to the depletion of oxygen of affected waters in events termed blooms (Eom et al., 2017; Shaw et al., 2003). Algal blooms are a major problem of eutrophication and can lead to oxygen depletion from turnover of large amounts of biomass, but properly managed and harvested might offer opportunities for recapturing nutrients and producing soil and crop improver products.

There is still however a pressing need to better understand how harvested algae can best be deployed to reliably deliver crop and soil benefits. In order to do this, understanding

the effects of different algae, their phylogeny, biochemistry, role in certain biogeochemical cycles that impact soil processes, as well as their overall effects on agricultural soils and crops and their wider impact in non-agricultural ecosystems will be beneficial.

2.3.1 Algal diversity

Algae are the primary producers in aquatic ecosystems (Ramanan et al., 2016) and the primary colonisers in soil (Hastings et al., 2014) due to their photosynthetic abilities. Some of these algae, which are found on terrestrial as well as aquatic environments, range from 0.2 μm in diameter (microalgae) up to and exceeding 50 m in length (macroalgae or seaweeds). Algae are classified into 5 main groups: Bacillariophyceae, Chlorophyceae, Rhodophyceae, Cyanophyceae and Phaephyceae (Cheah et al., 2015). The groups of interest considered in this thesis include the Chlorophyceae, Rhodophyceae, Phaeopyceae and Cyanophyceae.

The class of Cyanophyceae (cyanobacteria) are common in eutrophic waters and have have been the most studied particularly in rice soil systems (see section 2.7.2). Common species include *Anabaena*, *Nostoc* and *Spirulina.* Chlorophyceae (green algae) are the largest and more diverse class of algae and are also most commonly found in eutrophic freshwaters and marine habitats as well as growing naturally in soils (Hastings et al., 2014). Algae in both Cyanophyceae and Chlorophyceae classes are also associated with the treatment of wastewater as they can thrive in waters high in N and P. As for macroalgae, there exists over 9000 species of macroalgae, which are roughly categorised into three main groups based on their pigmentation: brown (Class Phaeophyceae), green (Phylum Chlorophyta) and red (Phylum Rhodophyta) (Tierney

et al., 2010). The most abundant of these is the brown algae (Phaeophyceae), which encompass approximately 2000 species (Cheah et al., 2015) and range from kelps such as *Laminaria hyperborean* and *Macrocystis pyrifera*. Other species belonging to *Phaeophyceae* include, *L. digitata, Fucus vesiculosus, F. serratus, Ecklonia maxima* and *Durvillea potatorum*. Examples of red macroalgae include *Mastocarpus stellatus* and *Chondrus crispus*. They are generally smaller in size in comparison to brown macroalgae ranging from a few centimetres to roughly 1 metre in length. Commonly known species of green macroalgae include *Ulva* and *Codium* and they are similar in size to that of red macroalgae (Tierney et al. 2010)

2.4 Bioactive properties of algae

Algae contain various biologically active compounds such as polysaccharides and proteins, with potential agricultural significance (Chojnacka et al., 2012). Various biologically active compounds in algal extracts are presented in Table 2.3 along with their roles.

2.4.1 Polysaccharides

The many different polysaccharides present in macroalgae are related to the classification as well as cell structure of the algae (Wijesinghe et al., 2012). The most important algal polysaccharides include galactans, alginates, fucoidan and laminaran from brown algae (*Phaeophyta*) (Ferreira et al. 2012). Other seaweed polysaccharides include ulvans from green algae (Chlorophyta) and carrageenans and porphyran from red algae (*Rhodophyta*) (Michalak and Chojnacka, 2015). These polysaccharides have many functional properties. Sulfated polysaccharides are known to impede bacteria and

virus activity (Chojnacka et al., 2012). Fucoidan is an example of a sulphated polyscharride, whose chemical composition depends on the algal source and harvesting time. It comprises roughly 10% of dry mass in algae. Laminarin content in macroalgae makes up 10% of dry weight, but seasonally it can reach up to 32% (Holdt and Kraan, 2011). It is responsible for stimulating genes for producing protein that contribute in antimicrobial response (Rioux et al., 2007).

Table 2.3 showing bioactive compounds of algae and their functional properties. Adapted from Chojnacka et al. (2012).

Biologically active compounds in algal extracts	Activity
Polysaccharides (e.g. galactans, fucoidan, laminarin, alginate) Carbohydrates (e.g. agar, carrageenans)	• Antimicrobial activity • Growth-promoting activity • Antioxidants • Soil aggregate stability increase
Proteins (e.g. amino acids, peptides, lectins, phycobiliproteins)	• Source of essential amino acids • Elements of intercellular communication • Antimicrobial activity • Antioxidants
Poly Unsaturated Fatty Acids (PUFAs) (e.g. Phospholipids and glycolipids)	• Antifungal activity
Pigments (3 major groups: chlorophylls, carotenoids and phycobiliproteins)	• Antioxidants
Polyphenols (e.g. phenolic acid, flavonoids, isoflavones, cinnamic acid, benzoic acid, quereetin and lignins). Phlorotannins (e.g. eckol or dieckol)	• Host-defence activity • Strong antioxidants • Antimicrobial activity
Minerals (e.g. K, Mg, Ca, Na)	• Plant growth and health-improving activity
Plant growth hormones (e.g. Cytokinins, auxins, gibberellins, abscisic acid (ABA), betaines)	• Growth stimulants • Protective activities • Cell division controllers • Root formation stimulants • Host-defense activity • Source of Nitrogen for plants

Alginates constitute up to 47% of dry biomass in seaweeds (Holdt and Kraan, 2011), and possess general colloidal properties (Chojnacka et al., 2012). According to Michalak and Chojnacka, (2015) polysaccharides derived from marine Chlorella species are rich in functional groups which are able to bind to heavy metals (e.g. Zinc) important in plant nutrition. Galactans, carrageenans and agars are natural chelators found in the cell wall of red algae (*Rhodophyta*). They help promote root elongation

and improve the moistness of the soil (as hydrocolloids with antioxidant and gelling activity (Khan et al., 2009).

2.4.2 Proteins

The biological properties of proteins in algae have been less documented compared to polysaccharides, but they are potential sources of nitrogen once mineralized by soil microorganisms. According to Chojnacka et al. (2012), protein content in macroalgae is less than 5%. Examples of bioactive proteins extracted from macroalgae include lectins which are involved in many biological processes such as intercellular communication (Chojnacka et al., 2012). Phycobiliproteins, derived from macroalgae have been known to possess antioxidant properties. Other water-soluble proteins include phycocyanobilins (present in cyanobacteria) and phycoerythrobilins (present in certain algae, Rhodophyta) (Michalak and Chojnacka, 2015).

2.4.3 Pigments

There are three main groups of seaweed pigments: chlorophylls, carotenoids and phycobiliproteins, which are all lipid-soluble (Chojnacka et al., 2012). Carotenoids are organic pigments produced by marine algae, with different species of algae possessing different kinds of carotenoids. There are several types of carotenoids of which the most important are fucoxanthin, tocopherol and B-carotene. Phycobiliroteins are water soluble and mainly produced by cyanobacteria and red algae. Both carotenoids and phycobiliproteins are strong antioxidants (Praveena and Murthy, 2014).

2.4.4 Polyphenols

Polyphenols are produced by macroalgae and mainly consist of phenolic acids, flavonoids, isoflavones, cinnamic acid, benzoic acid, quercetin and lignans (Gupta and Abu-Ghannam, 2011). The measurement of polyphenols in algae is mainly dependent on the extraction method, however it is known that Ascophyllum spp. contain a larger amount of polyphenols in comparison to other macroalgae such as Ulva spp. which contain the lowest (Craigie, 2011). Phlrotannins, belonging to the group of polyphenols, have been found only in brown seaweeds (Gupta and Abu-Ghannam, 2011) and carry out biological activities in organisms, such as host defence mechanisms (Chojnacka et al. 2012) and they are also known antioxidants (Gupta and Abu-Ghannam, 2011) and have a high tendency to chelate metals (Haribabu and Sudha, 2011).

2.4.5 Plant growth hormones

Algae extracts contain plant growth hormones such as cytokinins, auxins, gibberelins, abscisic acid (ABA) and betaines, which help to stimulate plant growth and increase the intensity of photosynthesis (Chojnacka et al., 2012). Cytokinins, found in *Chlorella*, and *Scenedesmus* spp. protect plants from the consequences of temperature changes and are also responsible for controlling bud and cell division in plants (Tarakhovskaya et al., 2007). Auxins initiate the formation of roots and prevent its elongation (Tarakhovskaya et al. 2007). Gibberellins, found in extracts from *Fucus vesiculosus, Fucus spiralis* and *Ascophyllum nodosum* (Chojnacka et al., 2012) help start the process of seed germination. Abscisic acid (ABA) is made from carotenoids and produced by over 60 algal species (e.g. *Chlorella* spp., *Haematococcus pluvialis*). The main role of ABA is to synthesise proteins needed in response to drought (Craigie,

2011). Betaines act as a nitrogen source for plants and also help increase chlorophyll content in leaves by decreasing its degradation (Khan et al., 2009).

It is important to bear in mind that these biologically active substances are not fertilisers, but the mechanism of their action can sometimes stimulate plant growth, for example through use as foliar and seed stimulants (Karthikeyan et al., 2007) and they may be able to increase the efficiency of photosynthesis leading to higher crop yields, protection from abiotic (e.g. low temperatures) and biotic (pathogens) stress and aiding in the growth of symbiotic microflora in the rhizosphere (Chojnacka et al., 2012).

2.4.6 Minerals and algal nutrient stoichiometry

Minerals are classed into macronutrients and micronutrients based on the requirements of the algae. The growth of algae is often limited by the supply of nutrients, particularly nitrogen and phosphorus (Xu et al., 2010; Schade et al., 2011). The concept of limitation introduced by Von Liebig indicated that the growth of organisms is controlled by the nutrient available in the smallest quantity rather than the total nutrients available (Barsanti and Gualtieri, 2014). To determine whether a nutrient is limiting or not, one has to look at its supply rate or turnover time. For example, if the concentration of a nutrient is limiting, but its supply rate is a little less than its rate of uptake by the algae, then the algae will only be slightly nutrient limited (Barsanti and Gualtieri, 2014). Algae store the nutrients in their vacuoles as biomolecules, allowing the nutrient content of their cells to change as a result of the availability of nutrients in their surrounding environment (Schade et al., 2011). Alfred Redfield stated that phytoplankton have a constant C:N: P ratio of 106:16:1 (Redfield, 1958) and according to Baird and Middleton (2004), macroalgae have a larger median C:N:P ratio of

550:30:1. Other authors (Loladze and Elser, 2011; Geider and La Roche, 2002) have suggested that this ratio is in fact not fixed as previously thought. The C:N ratio is more constrained and does not vary much amongst species or nutrient replete/limited environments (Geider and La Roche, 2002;). On the other hand, the N: P ratio of 16:1 is often used to distinguish N limitation from P limitation where in waters with high nutrients N:P ranges from 5-19 mol N: mol P and C:N ranges from 3-17 mol C: mol N, due to interspecific variability amongst the species (Geider and La Roche, 2002). Under low nutrient conditions, the C:N and N:P ratio range tends to be larger, with C:N increasing and N:P decreasing, with P typically being a limiting nutrient in nutrient poor waters (Solovchenko et al., 2016).

Algae are also able to accumulate other minerals and heavy metals (As, Cu, Pb, Zn) in their biomass (Randrianarison and Ashraf, 2017). The extent to which they accumulate these metals is dependent on the alga, whether they are dead or alive, as well as the conditions of the solution (Randrianarison and Ashraf, 2017). High concentrations of potassium (2.71 g L^{-1}), magnesium (0.19 g L^{-1}), calcium (0.16 g L^{-1}) and sodium ions (1.21 g L^{-1}) have been found in extracts of seaweeds (*Sargassum ringgoldianum* subsp. *coreanum,* and *Codium fragile*) on Japanese beaches (Kuda and Ikemori, 2009). Ruperez (2002) observed that mineral content in brown (e.g. Laminaria digitata) and red seaweeds varied according to several factors including residence time in the ocean, harvesting and processing method as well as other physiological differences.

2.5 Role of algae in biogeochemical cycles

2.5.1 Algae and the phosphorus cycle

Phosphorus is the 11th most abundant mineral in the earth's crust (Barsanti and Gualtieri, 2014) and exists in inorganic (rock minerals from parent material) and organic forms (cells of living and dead organisms). There are three main forms of inorganic phosphorus: orthophosphate, metaphosphate and organically bound phosphate. Organically bound phosphate present as organic matter in autotrophs is only transformed to orthophosphate through the decomposition process (Barsanti and Gualtieri, 2014). Orthophosphate is preferably taken up by plants (Zhu et al., 2018) and is found in low concentrations in unpolluted waters as well as in soil (0.05-0.30 µg P mL^{-1}) (Zhu et al., 2018), making it a limiting nutrient in freshwater aquatic systems and often for crops in agricultural systems.

Algae (along with other autotrophs) play a small role in the phosphorus cycle in that they take up inorganic phosphate present in the environment, originating from chemically or biologically weathered rock, incorporate it into their biomass in the form of organic phosphorus, forming part of the lipid portion of cell membranes, many coenzymes, DNA, RNA and ATP (Barsanti and Gualtieri, 2014; Solovchenko et al., 2016). Some cyanobacteria (*Trichodesmium*) have been reported to directly take up P as naturally occurring phosphonate – this typically occurs in nutrient limiting conditions in marine environments (Solovchenko et al., 2016). Their death results in the release of phosphate back into the soil or water by decomposers (Barsanti and Gualtieri, 2014), where it is again assimilated by other autotrophs and used again. Algae also play a significant role in weathering rocks and therefore release of key

nutrients (Lababpour, 2016). It is not clear which strains play an active role in this process, but it is commonly accepted that lichens (symbiotic association between fungi, cyanobacteria and algae) are responsible for this process (Labapour, 2016). Apart from their role in rock weathering releasing nutrients including phosphorus and transferring phosphates to other living organisms, the significance of algae in the phosphorus cycle is related largely to the impact of the element on their growth (Barsanti and Gualtieri, 2014).

2.5.2 Algae and the nitrogen cycle

Nitrogen is an essential part of cellular constituents such as proteins, chlorophylls, and genetic materials (RNA and DNA) (Glass et al., 2009). It exists in various forms including nitrate (NO_3^-), nitrite (NO_2^-), nitric acid (HNO_3), ammonium (NH_4^+), ammonia (NH_3), atmospheric dinitrogen (N_2), nitrous oxide (N_2O), nitric oxide (NO) and nitrogen dioxide (NO_2).

Diazotrophic cyanobacteria (cyanophytes) play an important role in nitrogen fixation and assimilation (Giordano and Raven, 2014). They are able to fix atmospheric nitrogen to compounds such as ammonia (NH_3) and ammonium (NH_4^+), some of which is converted to amino acids subsequently proteins, which is their main N reservoir (Barsanti and Gualtieri, 2014). Microbiotic crusts (comprised of algae, fungi, bacteria, lichens and bryophytes) are reportedly able to fix roughly 40% of the estimated global biological nitrogen fixation (107 Tg a^{-1}) (Elbert et al., 2009). Eukaryotic algae such as Chlorophytes cannot fix atmospheric nitrogen however, they play a big part in assimilation (Giordano and Raven, 2014; Glass et al., 2009) where they convert ammonium or nitrate from soil and natural waters into organic nitrogen compounds.

N_2, organic N, NH_4^+ and NH_3 are preferentially taken up, more so than NO_2^- and NO_3^- in terrestrial and aquatic environments (Girodano and Raven, 2014). The ammonium is released back into the environment through the death and decay of these organisms, where is it is either recycled back into biomass, or oxidised to nitrite (NO_2^-) and then nitrate (NO_3^-) in a twostep process by nitrifying bacteria and archaea (Ward, 2012). The nitrogen is then released back into the atmosphere through a denitrification process, where NO^-_3 is converted to gaseous nitrogen (NO, N_2O N_2) by facultative anaerobic bacteria (Ward, 2012).

2.5.3 Algae and the carbon cycle

Phytoplankton growth reduces atmospheric CO_2 (through photosynthesis) (Moreira and Pires, 2016). Primary producers in marine environments reportedly contribute up to 50% of the total carbon fixed globally (Moreira and Pires, 2016). Marine macroalgae such as *Saccharina* and *Gracilaria* can reportedly fix 1.0×10^6 and 2.3×10^5 t_{dw} $year^{-1}$ (Moreira and Pires, 2016). Cheah et al. (2015) observed that the cultivation of 1 kg of microalgae was reportedly able to fix 1.83 kg CO_2. The free dissolved CO_2 joins with water molecules and ionizes to form bicarbonate and carbonate ions which form the highest amount of dissolved carbon dioxide in the water. Free CO_2 is removed by photosynthesis and the bound ionic forms release more CO_2, so CO_2 is never a limiting factor for photosynthesis and plant growth in rivers, lakes or oceans. Additionally, the respiration of algae releases carbon dioxide and hence more bicarbonate and carbonate ions (Barsanti and Gualtieri, 2014).

2.6 Algae in agriculture and other environments

The application of algae in agriculture is not new. The use of macroalgae as a soil conditioner (applied as compost) is widespread across coastal regions where the economic benefits of macroalgae are recognised (Monagail et al., 2017). This has allowed the seaweed industry to grow to an annual estimated production value of US $5.5-6 billion (Tierney, 2010). This estimate has now increased to US$10.1-16.1 billion (Monagail et al., 2017) as a result of expanding demand for seaweed products. According to Van der Voort and Vulsteke (2015), the microalgal biomass (including cycanobacteria) fertilizer market was worth an estimated US$ 440 million in 2012. The following section looks at the uses of algae in agriculture and their contributions to improving soil properties.

2.6.1 Impact of algae in rice paddy fields

Cyanobacteria typically reside in the rhizosphere where they influence plant growth and development (Singh, 2014). In an investigation carried out by Prasanna et al. (2009a) to characterise the quantity and diversity of the different genera of cyanobacteria in rhizospheres of rice in different regions in India, the isolates showed a high abundance (80%) of cyanobacterial strains belonging to the genera *Nostoc* and *Anabeana,* - also indicating the competitiveness of these filamentous heterocystous genera (Singh, 2014). These species carry out many functions in the soil ecosystem: they fix nitrogen and incorporate the products into amino acids, thus making a significant contribution to the N status of wet soils, with an estimated amount of nitrogen fixed in the range of 20-30 kg ha^{-1} in the rice fields (Prasanna et al. 2009b;

Singh, 2014). According to Akhter et al. (2002) blue green algae (BGA) can fix up to 25.30 kg N ha^{-1} per rice cropping season in flooded systems. In a rice experiment conducted by Pampolino et al. (2008) for a 15-year long period for zero-N treatments showed N fixation rates ranging from 19-44 kg N ha^{-1} $crop^{-1}$. Some of the mechanisms that could possibly explain this process include i) the release of nutrients from the living algae ii) microbial decomposition following the death of photosynthetic aquatic biomass (PAB) (algae and other flood water flora) - whereby nitrogen is released into the soil and taken up by the crop and lastly iii) when PAB die at the end of the crop flooding period where surface decomposition and subsequent tillage allow for the carbon and nitrogen to be integrated into the soil (Gaydon et al. 2012). In terms of crop productivity however, because PAB growth slowly increases soil fertility, this has a residual effect on, rather than an immediate advantage to the standing crop. So, for example, in the Philippines, there was no great increase in the yield of the standing crop however there was an increase of N in the soil surrounding the roots of the crop (Gaydon et al., 2012). Another symbiotic association in the case of nitrogen fixation is that between the aquatic fern *Azolla* and the cyanobacteria *Nostoc* (Prasanna et al. 2014). These cyanobacteria are also reported to colonise the roots of rice crops, where they are able to fix atmospheric nitrogen, which is subsequently taken up by the rice crop (Prasanna et al., 2014).

Cyanobacteria are also able to contribute to soil carbon. Studies on rice paddy fields have shown that under favourable conditions, cyanobacteria are able to produce 6-8 t ha^{-1} fresh biomass (Prasanna et al., 2014). Native cyanobacterial floras are able to increase soil organic carbon content by 0.03% (equivalent of 670 kg ha^{-1}) within six months under laboratory conditions. Halotolerant cyanobacteria are able to add 5.3-7.6 t C ha^{-1} in a cropping season when inoculated into sodic soils (Prasanna et al. 2014).

The synergistic action between rhizobacteria and cyanobacteria is beneficial to soils and crops in terms of increasing organic carbon and microbial biomass carbon (Prasanna et al., 2009b).

The role of cyanobacteria in increasing rice yields has also been widely documented. Tripathi et al. (2008) reported an increase plant height, grain and straw weight of rice plants after the addition of 12.5 kg ha^{-1} of blue green algae biofertilizer. Other authors have reported similar improvements to rice crop growth (Dhar et al., 2007; Chittapun et al., 2018). Growth promoting substances in cyanobacterial extracts have also been shown to have an effect on plant growth, with early evidence coming from Gupta and Lata, (1964) showing the accelerated germination of rice seeds soaked in extracts, compared to the later germination of untreated seeds.

2.6.2 Impact of algae in desert ecosystems

Microbiotic crusts comprise of algae, fungi, bacteria, lichen and bryophytes (Elbert et al., 2009). They are typically in the form of Biological Soil Crusts (BSCs) and are found throughout the world (Belnapp 2003) but are particularly common in desert environments, forming 'cohesive, thin, horizontal layer of the soil surface' (Gao et al. 2014).

Although not typically found in most agricultural ecosystems, Peng and Bruns (2018) for example have shown that cyanobacteria are able to grow on soil in agricultural fields as 'soil surface consortia' in humid regions in eastern North America, describing their growth as fast, ephemeral, yet recurrent. Thus, looking at, the formation of BSCs in desert regions gives insight into role and functions of algae and cyanobacteria when in a symbiotic relationship with other organisms.

BSCs sequester and accumulate CO_2 from the atmosphere through photosynthesis the CO_2 is captured by the free chlorophyll in algae and the chloroplasts in mosses through photosynthesis and stored in the form of carbohydrates in their tissues (Burgheimer et al., 2006; Gao et al., 2014). Photosynthetic rates (from algae and mosses) ranging from 0.1 ~11.5 umol CO_2 m^{-2} s^{-1} have been reported under natural and lab conditions – this variation is attributed to the differences in physical environments and the ability of different crust species to sequester carbon (Gao et al., 2014). According to Elbert et al. (2009), who collated previous studies on BSC photosynthetic rates, the median flux is 16 $g.m^{-2}$ a^{-1} for the net uptake of carbon by BSCs giving an estimated total of 1.0 Pg a^{-1} for carbon sequestration by BSCs in arid and semiarid environments globally (Gao et al., 2014).

BSCs also play an active role in fixing nitrogen as they do in sequestering carbon. Average fluxes of nitrogen fixation are reported in the range of 0.1-10 g m^{-2} a^{-1} contributing to a global total nitrogen fixation rate of roughly 30 Tg a^{-1} (Elbert et al. 2009; Gao et al., 2014). BSCs release low molecular weight organic N such as amino acids and ammonium into the soil, aiding the nutrition and growth of vascular plants. Decomposition (through enzymatic activities by the combined action of soil microorganisms and enzymes) of BSC input carbohydrates and organic nitrogen into the soil (Gao et al., 2014). Additionally, some algal crusts add carbon to the soil by releasing polysaccharides into the soil.

BSCs have also been investigated as a countermeasure to soil desertification as they have the ability to regulate water infiltration in desert soil with respect to the amount, position and time. They also possess certain attributes, which can control the water

distribution of soil profiles (Belnap et al. 2005). Lichner et al. (2013) also conducted an experiment comparing the effect of algae (coccal green alga (*Choricystis minor*), filamentous green alga (*Klebsormidium subtile*) and filamentous alga (*Tribonema minus*) isolated from glade soil and grown on pure sand (crusted sand). Results obtained were compared to pure sand and showed a decrease in water sorptivity of the dried crusts up to 10% that of pure sand, an increase in water drop penetration time of the dried crusts up to 14 times that of pure sand and hydraulic conductivity was about 7% that of pure sand (Lichner et al., 2013).

BSCs also help prevent soil erosion by protecting sandland surfaces against wind erosion. In an experiment using fractal scaling to determine topsoil properties influenced by BSCs, particularly cyanobacteria that were found to reduce topsoil loss through capturing the dust in airstreams using their filaments (Gao et al., 2014). To our knowledge there are no studied effects of BSCs on agricultural/arable soils.

2.6.3 Impact of algae on arable land

Unlike the influence of cyanobacteria in rice paddy fields and desert environments, there has been an extensive use of macroalgae on arable/agricultural soil to improve soil quality. Macroalgae are applied as compost and have been shown to increase soil macronutrients. Lopez-Mosquera and Pazos (1997) observed a 17% increase in total N under 80 t ha^{-1} of seaweed (mixture of red and brown algae) fertilised plots (0.34%) compared to unfertilised plots, as well as a significant increase (39%) in soil potassium levels (0.68 cmol kg^{-1}) compared to unfertilized plots following the addition to agricultural soil in northwest Spain. Other authors (Rathore et al., 2009; Arioli et al., 2015; Possinger and Amador, 2016) also reported an increase in soil macronutrients following the application of seaweed extract. Macroalgae as well as green algae have

also been reported to increase soil aggregate stability. Certain strains of green algae, mostly *Chlamydomonas* species are known to produce polysaccharides and grow quickly in environments ranging from pH 6.0 to pH 8.0 (Barclay and Lewin, 1985) and macroalgae such as *Laminaria digitata* have been observed to increase the aggregate stability of 4-7mm aggregates (Haslam and Hopkins, 1996). Polysaccharides produced are present as a mucilaginous external layer surrounding the cell. It is either organised as a well-defined sheath, a capsule closely associated with the cell surface, slime loosely associated with the cell surface, or a soluble polysaccharide released into the environment during cell growth (Singh, 2014). These exopolysaccharides not only help protect the cell, but also contribute to soil aggregation as a result of its gluing properties (Pereira et al., 2009) and they also help bind heavy metals and sodium ions in soil (Kaushik and Subhashini, 1985; Nisha et al., 2018).

Macroalgae extracts have also been reported to increase crop yield. Lopez-Mosquera and Pazos (1997) showed a 53% increase in potato yield after ~ 4months following the addition of 80 t ha^{-1} of a mixture (consisting of red and brown macroalgae) of seaweed applied to agricultural soil in northwest Spain. Eyras et al. (2008) and Possinger and Amador (2016) reported an increase in tomato and sweet corn yields in horticultural and conventionally cropped arable soils respectively. To our knowledge there are no recorded effects of macroalgae in wheat crop. Wheat is a major staple cereal for human and animal feed and is the main cereal crop in the UK. Today it is grown on about 2,000,000 ha nationally and has a value of roughly £1.2 billion (UK Agriculture, 2015). It is sown in both spring and autumn and harvested in August. Foliar applications of macroalgae have been reported to also increase wheat yields (Stamatiadis et al., 2015). There have also been some recorded impacts of cyanobacteria. In an experiment carried

out by Karthikeyan et al. (2007), in which the effects of three cyanobacterial isolates: *Calothric ghosei, Hapalosiphon intricatus* and *Nostoc* sp were applied to wheat crop in pot cultures under glass house conditions using unsterile soil, results showed an increase in plant height, dry weight and grain yields. In a similar experiment conducted two years later by Karthikeyan et al. (2009), where cyanobacterial strains isolated from the rhizosphere of wheat (*Calothrix ghosei, Westiellopsis, Hapalosiphon intricatus,* and *Nostoc* sp.) were shown to enhance wheat seed germination percentages from 90% germination rate under sterile water treatment to between 97 and 99% germination rate under treatment with cyanobacteria strains). The results however did not show any isolated effects of individual cyanobacteria.

With the increasing problem of nutrient loss from agricultural land as previously mentioned, the use of algae to capture those nutrients and recycle them back onto to soil is still a relatively new concept. As a result, research has not been as extensive as the use of cyanobacteria in rice paddy fields or the use of macroalgae on agricultural/arable soil. The Few studies that have assessed the ability of algae to capture nutrients in eutrophic waters such as agricultural drainage waters, confined animal facilities as well as municipal wastewater (Mulbry et al., 2008), have looked at other potentially useful algae – such as *Spirulina.* Mulbry et al. (2005) showed that dried algal biomass (type not specified) recycled from the treatment of dairy manure, increased plant (cucumber and corn) available nitrogen as well as soil phosphorus levels in sandy loam and silt loam soils. More recently, Wuang et al. (2016) demonstrated that *Spirulina* grown on aquaculture wastewater was able to increase the plant height by 55.3% and chlorophyll content (by 30.2%) of Arugula plants in a soil potted experiment. In this study the effects on soil characteristics were not measured.

This area is of growing interest for the application of recycled algal biomass (Mulbry et al., 2008), especially in the UK, where rice isn't grown and the impact of BSC (although existing on UK soils) cannot be applied on a large scale to replenish nutrients lost from agricultural land. The use of algae to recycle nutrients onto agricultural land has significant scope in the UK and in doing so could help mitigate environmental and economic issues related to eutrophication and soil degradation.

2.7 Conclusions

The research on algal biomass is extensive; there is plenty of research on their applications, especially of cyanobacteria in rice cropping systems as well as the influence of BSCs in desert environments. There is however a paucity of evidence on the impact of algal biomass added to soil in conventionally managed intensive arable land, which has suffered substantial declines in structure and organic matter content in recent years and is currently highly reliant on mineral fertilizer inputs to maintain crop yields. Of interest is the potential to recycle algal biomass to crop lands as a means of recycling nutrients lost from agricultural soils or from animal slurries into waterbodies in which algae grow and rapidly assimilate nitrogen and phosphorus, often originating from agricultural run-off. Whilst there has been some previous work on seaweeds as soil conditioners for crops such as tomatoes on degraded horticultural soil in Patagonia (Eyras et al., 2008) and seaweeds have been widely used in coastal communities to support crop production on sandy soils "Much of the information about agriculturally beneficial effects of seaweed added to soil is qualitative and/or anecdotal" (Haslam & Hopkins, 1996). Additionally, a lot of the research on seaweed looks at the impact of their extracts and therefore tend to focus more on their biostimulatory properties and

only few studies look at their effects when applied as compost (Lopez-Mosquera and Pazos 1997; Haslam & Hopkins, 1996). Even fewer studies document the impact of recycled algal biomass onto soil (Mulbry et al., 2005; Wuang et al., 2016). There is also currently little understanding of the comparative benefits of different algal species with respect to their effects on soil properties and crop growth and to our knowledge; no studies have compared effects of a wide variety of algal species. The mechanisms of how microscopic algae act to improve soil – especially soil nutrients are still uncertain, and their impact on soil microbial communities remains largely unexplored. This project will attempt to answer these questions in an attempt to objectively evaluate the use of algae as sustainable, soil conditioners on UK arable soils.

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Chapter III

[1]Circular economy fertilization: a greenhouse and field study

This chapter is based on a greenhouse and field experiment looking at the effects of different algal species on soil physicochemical properties. The field study, a follow up to the greenhouse study, looked at the impact of the algal biomass on soil nutrients as well as effects on yield and nutritional value of spring wheat.

Abstract

Nutrient losses from agricultural land to freshwater and marine environments contribute to eutrophication and the development of algal blooms, but the potential benefits of recycling algal biomass to agricultural land for soil quality and crop nutrition in a "circular-economy" has received little attention. We tested effects of algal additions to arable soil in greenhouse-grown garden peas, and field plots of spring wheat, on plant growth and nutrition and physical and chemical properties of the soil. Representatives of 4 chemically (elemental composition) contrasting algal groups were applied at 0.2, 2 and 4 g kg^{-1} in the greenhouse and at 24 g m^2 in the field, these included the cyanobacteria *Arthrospira platensis* (*Spirulina*), the unicellular green algae *Chlorella* sp., the red seaweed *Palmaria palmata*, and the brown seaweeds *Laminaria digitata* and *Ascophyllum nodosum*. In the greenhouse at the highest application rates (4 g kg^{-1}), *Chlorella* sp., and *Spirulina* increased

[1] This chapter is published in Geoderma.
Reference: Alobwede, E., Leake, R.J. & Pandhal, J. (2019) Circular economy fertilization: Testing micro and macro algal species as soil improvers and nutrient sources for crop production in greenhouse and field conditions. *Geoderma*. 334, p. 113-123.

soil total nitrogen and available phosphorus, and *Spirulina* also increased soil nitrate concentrations. *P. palmata* and *L. digitata* significantly increased soil inorganic (NH_4^+ and NO_3^-) concentrations under low (0.2 g kg^{-1}), medium (2 g kg^{-1}) and high (4 g kg^{-1}) application rates. *Chlorella* sp. significantly increased soil total P, N and C, available P, NH_4^+-N, and pea yield. Soil water-stable aggregates were unchanged by the algal additions in the greenhouse and field study. In the field, 4 species (*Chlorella* sp. *Spirulina*, *P. palmata* and *L. digitata*) increased soil inorganic nitrogen concentrations, confirming their potential to recycle mineralizable nitrogen to agricultural soils, but no significant effects were found on wheat yields under the application rates.

Keywords: Circular economy, Agricultural soil, Algae, Soil nutrient status, plant nutrition, yield

3.1 Introduction

Soil quality plays a critical role in crop productivity and both soil and crop resilience to drought and heavy rainfall, but there is increasing concern that intensive arable farming has degraded soil water and nutrient holding-capacity as a result of organic matter loss (DEFRA, 2009; Graves et al., 2015). Soil quality constraints are implicated in the yield plateaux seen in wheat and oilseed rape, the most important field-grown crops in the UK (Knight et al., 2012). Soil degradation is estimated to cost the UK between £0.9 billion and £1.2 billion annually, in onsite and offsite non-market 'external' costs (Graves et al., 2015). This value is mainly attributed to the loss of soil organic carbon (47%), compaction (39%) and erosion (12%) (Graves et al., 2015). These changes are reflected in soil physical and chemical attributes such as soil aggregate stability and nutrient status. Water-stable

aggregates are key indicators of soil quality since they deliver good soil structure and function by: (i) physically protecting soil organic matter against rapid decomposition, (ii) increasing soil water-holding capacity, (iii) providing pore space for root growth and water infiltration, (iv) enhance resistance to erosion, and ultimately reducing surface crusting and runoff, which leads to aquatic pollution (Paul et al., 2013).

Intensification of arable production with continuous annual cropping using high mineral nutrient inputs has depleted soil organic matter (Mulvaney et al., 2009), which is responsible for storing nutrients and maintaining soil structure, ultimately leading to nutrient losses to water bodies. This has been compounded by nutrient-rich topsoil being eroded from continuously cropped arable land at an average rate of 9.5 tonnes per hectare across the EU 28 countries (Eurostat, 2017). This has caused preferential loss of the finer particles, such as the nutrient-retaining organic matter and clays, exacerbating the risk of nutrient export from land to water bodies and eutrophication (DEFRA, 2014). Despite the implementation of the Water Framework Directive (WFD) and the active management of nitrate vulnerable zones, there has been a decrease in the overall number of water bodies in the UK being awarded high or good surface water status between 2011 and 2016 (JNCC, 2017). In England alone, 28% of failures to meet the WFD standards are directly attributed to diffuse water pollution from agriculture and rural land use (DEFRA, 2014). The urgency of this situation has grown with increasing awareness of the fossil-fuel energy costs in the production and use of chemical fertilizers. Each year, 100 million tonnes of fertiliser is used globally, contributing to greenhouse gas emissions. A recent study conducted by the Grantham Centre for Sustainable Futures at The University of Sheffield showed that more than half the environmental impact of producing a loaf of bread is attributed to the use of

ammonium nitrate fertiliser during the wheat cultivation process, which accounts for 43% of the sample loaf's greenhouse gas emissions (Goucher et al., 2017).

In order to reduce dependency on inorganic fertiliser use, organic fertilisers such as animal manure, biosolids from human wastes, anaerobic digestate, biochar and crop residues are used as alternatives (Farrell et al., 2014; Walsh et al., 2012; Rady, 2011). Of these, the manures, biosolids and digestates are potentially the most important nutrient sources, but these complex materials have caused pollution/ecological risks associated with veterinary antibiotics, use of growth promoting heavy metals such as (Cu and Zn) in pigfeed (Ciesinki et al., 2018) and other contaminants such as arsenic (Wuang et al., 2016; Heimann et al., 2015; Zhang et al., 2015). Alternative sources of organic fertilisers that can provide plants with an optimal mix of macro and micronutrients as well as benefit the structural characteristics of soil would be hugely beneficial for the agricultural industry. The European Commission disclosed a legislative proposal in March 2016 on organic and waste-based fertilisers as part of their Circular Economy Action Plan (European Commission, 2016). The aim is to promote resource efficiency with regards to the fertiliser sector in order to create new business opportunities for farmers, as well as help them become more competitive in recycling organic nutrients compared to purchasing inorganic fertilisers (European Economic and Social Committee, 2016). It seeks to reduce waste, energy consumption and environmental damage (Messenger, 2016).

Algae are the main primary producers in most water bodies, and their growth is naturally stimulated by organic effluents and mineral nutrients (Sen et al., 2013). As incidences of diffuse pollution increase due to anthropogenic activity, the size and frequency of algal blooms is on the increase. Furthermore, climate change has been predicted to exacerbate

the problem. One potential solution to limit the detrimental impacts of nutrient runoff from agriculture is to divert nutrients to water bodies where it is possible to exploit the natural ability of microalgae to grow much quicker than land plants (Wuang et al., 2016), and actively cultivate and harvest the biomass. The biomass can be used as a sustainable source of organic fertiliser, returning both nutrients and carbon to soil, potentially improving soil quality, crop growth and nutrition. Moreover, research in large-scale algal biomass production has increased in recent years, for diverse applications including biofuels, animal feed (Yaakob et al., 2014) and as nutrient scavengers in wastewater treatment processes (Zhu et al., 2013). This has also created opportunities for the development of by-products such as algal-based fertiliser that could contribute to a more sustainable circular-economy for nutrients in arable farming systems.

Chlorella sp. and *Spirulina (Arthrospira platensis* and *Arthrospira maxima)*, which are commonly used microalgal species in the treatment of wastewater (Aslan and Kapdan, 2006), are reported to have high nutrient (N and P) removal capabilities from effluents, making them suitable candidates as soil conditioners. *Spirulina platensis* biomass has been shown to improve soil macronutrients (nitrogen, phosphorus and potassium) (Aly and Esawy, 2008), act as a biofortification agent, enhance plant protein content (Kalpana et al., 2014) and increase crop growth, i.e. 5 g *Spirulina* in 500 g^{-1} soil increased the height of Bayam red (red spinach) by 58.3% as well as fresh and dry weights by 110.1% and 155.8% respectively, when compared to the control group (Wuang et al., 2016). Dried algal biomass grown on anaerobic digestate from dairy manure increased plant available N and P in soils within 21 days and thereby improved cucumber and corn seedling growth (Mulbry et al., 2005). Additions of 2-3 g dried *Chlorella vulgaris* kg^{-1} soil significantly increased ($p<0.0001$) fresh and dry weight of lettuce seedlings (Faheed and Abd-El Fattah,

2008). Extracts or composted marine algal seaweed species have been researched as amendments in crop production systems due to their biostimulatory potential on crop growth and their benefits as sources of organic matter and soil nutrients (Khan et al., 2009). Brown seaweeds (Phaeophyceae) have also been tested, with *Ascophyllum nodosum*, the most studied of the Phaeophyceae, shown to improve growth and drought stress tolerance when used as a soil drench or foliar spray in container-grown citrus trees (Spann and Little, 2011). Other positive responses include early seed germination and establishment, improved crop performance and yield, as well as elevated resistance to biotic and abiotic stress (Khan et al., 2009). Brown seaweeds contain high amounts of polyuronides such as alginates and fucoidans, which are known for their gelling and chelating abilities and their ability to combine with metallic ions in the soil. They form high-molecular-weight complexes that absorb moisture and result in better soil aeration and moisture retention, and in turn boost soil microbial activity (Khan et al., 2009). The application of another brown seaweed, *Laminaria digitata*, has been shown to also improve soil physical properties including total pore volume and aggregate stability of a sandy soil (Haslam and Hopkins, 1996).

Algae also represent a source of trace elements, which they acquire via biosorption and bioaccumulation (Michalak et al., 2017) and can therefore contribute to crop micronutrient uptake. Wheat, the second most important cereal crop globally, makes up about 28% of human dietary energy (Velu et al., 2016). It is the most important cereal crop in the UK where it is grown on 1.7 million hectares, yielding 15.2 million tonnes last year (DEFRA, 2017). The ability of algal-fertilizers to increase the often-suboptimal concentration in wheat grains of zinc, iron and selenium (Broadley et al., 2006; Stroud et al., 2010) which are essential for human nutrition, needs to be investigated, as this could provide a cost

effective, sustainable solution to micronutrient deficiencies (Velu et al., 2016).

There is increasing evidence that the deployment of algae biomass could act as a source of organic fertiliser. There are approximately 280,000 recognised algae species (Chojnacka and Kim, 2015), but the relative merits of different species and functional groups on soil quality and crop improvements, and their key attributes that control their effectiveness remain unclear. Algae vary greatly in their mineral and organic composition and consequently their impact on soil nutrients and aggregate stability are hypothesized to be strongly dependant on the initial concentration of nutrients in their biomass (Flavel and Murphy, 2006).

This study aims to investigate the use of chemically contrasting types (difference in elemental composition) of algal species biomass on soil aggregate stability, nutrients and ultimately growth and yields of crops. In addition, we explore the effects of different types of algae as soil amendments for improving micronutrient (e.g. zinc, iron and selenium) concentrations in wheat. To address these aims, bioassay greenhouse and field experiments were conducted with garden peas and wheat respectively. The five algal species chosen also represented different phylogenetic groups: the cyanobacterium *Spirulina*, the freshwater green alga *Chlorella* sp., a Chlorophyte, and three marine species namely *P. palmata* from the class Rhodophyta and *L. digitata* and *A. nodosum* both representing the class Phaeophyta.

3.2 Materials and Methods

3.2.1 Experimental set-up

The experimental site and location of soil collected for the greenhouse experiment was Wise Warren at Spen Farm, Tadcaster, England (longitude 1°20'32.9" W, latitude 53°51'40.7" N). The field had been subjected to continuous cropping since 1985, mainly growing winter wheat, spring and winter barley, oilseed rape, sugar beet, winter beans, and potatoes. The soil is in the Aberford series (Calcaric Endoleptic Cambisol; Cranfield University, 2017). Results for the characterisation of initial topsoil conditions are shown in Table 3.1. At the beginning of the greenhouse experiment, the soil had a total phosphorus concentration of 0.238 g kg^{-1}, total nitrogen of 1.732 g kg^{-1}, carbon content of 21.94 g kg^{-1} and a pH of 6.95.

The dry biomass of five algal species: *Arthrospira platensis,* (*Spirulina*), *Chlorella* sp. *Palmaria palmata, Laminaria digitata* and *Ascophyllum nodosum* were individually added to separate soil samples. The algal biomass used was purchased commercially with the exception of *Ascophyllum nodosum* which was obtained from the strandline of a beach on the west coast of Ireland, rinsed (to remove sand), oven dried at 60 °C, ground and ball milled to pass a 600 μm sieve and mixed to ensure homogeneity. The contents of carbon, nitrogen and phosphorus in the algae biomass are shown in Table 3.2 and the micronutrients and heavy metal content are shown in Table 3.3. No supplemental nutrients/fertilisers were added, in order to compare the benefits of the different algal biomass types and varying application rates.

Table 3.1

Physical and chemical characteristics of untreated soil used for greenhouse experiment

pH	**T.N**[A]	**T.P**	**T.C**	**P_{AV}**	**K_{AV}**	**NH_4^+**	**NO_3^-**	**C:N**	**Stability**[B] **(1-2mm)**	**WHC**[C]
	g kg^{-1}	g kg^{-1}	g kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}		%	%
6.95± 0.02	1.73± 0.02	0.24±0.08	21.9±0.2	18.1±1.1	16.3±0.2	14.7± 0.2	14.9±0.3	12.8 ±0.1	1.71± 0.08	49.8 ±0.3

[A] TN = Total nitrogen, TP = Total phosphorus, TC = Total carbon, P_{AV}= Available phosphorus, K_{AV}= Available potassium, NH_4^+ = Ammonium NO_3^- = nitrate, n=3
[B] Water stable aggregates of the 1-2mm size fraction
[C] WHC = water holding capacity of soil

Table 3.2

Carbon content and macronutrients found in the different algal species

Algae						**C:N**	**N:P**
	C	N	P	Ca	Mg		
	----------mg g^{-1} [A]----------						
Spirulina	543±9[a]	124±2[a]	2.08±0.03[a]	2.18±0.06[a]	2.12±0.03[a]	4.4	59.4
Chlorella sp.	511±54[a]	102±13[a]	2.67±0.05[b]	2.53±0.02[a]	2.07±0.02[a]	5.0	38.0
P. palmata	447±7[ab]	35.4±0.2[b]	0.87±0.04[c]	0.90±0.06[b]	1.7±0.1[b]	12.6	41.0
L. digitata	355±6[b]	18.8±0.3[c]	0.47±0.02[d]	7.0±0.2[c]	5.8±0.1[c]	18.9	39.6
A. nodosum	370±1[b]	16.3±0.2[c]	0.25±0.02[e]	19.8±0.7[d]	5.72±0.03[c]	22.8	65.4

[A] Mean ± standard error (n=3). Data were log transformed where assumption of normality was not met.
Values with different superscript e.g. a,b etc., in the same column are significantly different ($p<0.05$, One way ANOVA).

Table 3.3

Micronutrients and heavy metals found in the different algal species

Algae	Micronutrients and heavy metals										
	Zn	Fe	Se	B	Mn	Cu	Cd	Pb	As	Ni	Cr
	----------mg kg^{-1} [A]----------										
Spirulina	32±3[a]	1150±17[a]	0.15±0.03[a]	9.6±0.2[a]	31.2±0.4[a]	1.9±0.1[a]	0.05±0.003[a]	1.56±0.05[c]	2.6±0.1[a]	1.5±0.7[ab]	3.83±0.07[c]
Chlorella sp.	18±3[b]	886±12[b]	0.06±0.004[b]	1.9±0.5[b]	46.9±0.9[b]	1.94±0.06[a]	0.07±0.001[a]	0.04±0.02[a]	2.29±0.05[a]	1.1±0.2[ad]	1.33±0.05[a]
P. palmata	19.9±0.7[b]	146±14[ab]	0.83±0.05[c]	127±7[c]	8.7±0.5[c]	8.3±0.5[b]	0.95±0.06[b]	<0.01[a]	9.1±0.7[b]	4.4±0.3[c]	1.2±0.1[ab]
L. digitata	9±1[c]	43±4[c]	1.10±0.06[c]	87±4[c]	2.42±0.06[d]	1.10±0.01[c]	0.15±0.0009[a]	<0.01[a]	57.9±0.2[c]	0.11±0.09[bd]	0.97±0.08[b]
A. nodosum	69±7[d]	183±7[d]	0.91±0.05[c]	112.7±0.6[c]	75±1[e]	1.3±0.2[c]	0.70±0.02[c]	0.52±0.002[b]	35.7±0.5[d]	2.41±0.09[a]	1.32±0.05[a]

[A] Mean ± standard error (n=3). Data was log transformed where assumption of normality was not met.
Values with different superscript e.g. a,b etc., in the same column are significantly different (*p<0.05*, One way ANOVA).

A pot experiment, using soil taken from the field at Wise Warren, was conducted in a GroDome greenhouse at the Arthur Willis Environment Centre (AWEC), The University of Sheffield, for 90 days (starting on 2nd April 2015 and ending on 1st July 2015) with pea maincrop (*Pisum sativa)*. Prior to starting the experiment, the soil was air dried and homogenized by mixing in one large basin before being put into separate pots in equal amounts of 1 kg (± 0.05). The pots and plants were maintained under 12 h photoperiod, 200 μE m^{-2} s^{-1} light intensity, 21°C:15°C day:night temperatures. Dried algal biomass was added at low (0.2 g kg^{-1}) medium (2 g kg^{-1}) and high (4 g kg^{-1}) application rates, accompanied by controls with no algal additions. Application rates were chosen according to previous studies (Akhter at al., 2002; Nisha et al., 2007; Obana et al., 2007). Each pot was sown with four pea seeds, with four replicate pots of each treatment.

The field experiment was conducted the following year at Wise Warren Farm, from 27th April 2016 to 26th September 2016. The experiment was divided into 21 plots with three replications of each treatment. The square plots were 1 m^2 and each plot was divided equally into two. The algae were applied only once as topdressing, with application rates of 8 g and 16 g per half a metre square. This was equivalent to 29.81 kg N ha^{-1} for *Spirulina*, which had the highest N concentrations, down to 3.91 kg N ha^{-1} for *A. nodosum*, which had the lowest N concentrations. For phosphorus, algae application rates ranged from 0.65 kg P ha^{-1} under *Chlorella* sp., which had the highest P concentrations, down to 0.06 kg P ha^{-1} under *A. nodosum*. The plots were sown with spring wheat (*Triticum aestivum* L.) (Tybalt high yielding variety purchased from Limagrain) in April and harvested at maturation after 5 months in September. The measured response variables included soil

total phosphorus, carbon and nitrogen, available phosphorus, potassium and nitrogen, water stable aggregates, crop yield and grain micronutrient content.

3.2.1.1 Analysis of soil physico-chemical properties

Quantitative analyses of specific soil physico-chemical properties were carried out for the greenhouse experiment before the start of the experiment and at the end after 90 days (~13 weeks). In the field experiment, in order to gain a better understanding of the nutrient dynamics of the algae biomass following addition onto soil, analysis was carried out on soil 2, 8- and 20-weeks following algae addition. Soil samples were air-dried and sieved (2 mm) prior to analysis of soil nutrients and pH.

i) pH and soil nutrients

Soil pH was determined with 20 g of air-dried soil, mixed with 20 ml of distilled water to form a 1:1 ratio and measured using a pH electrode (Kalra, 1995). Total carbon (C) and nitrogen (N) were determined using a CN elemental analyser (Vario EL Cube, Langenselbold, Germany). Total soil phosphorus (P), including both organic and inorganic P (Carter and Gregorich, 2008), was determined for homogenised subsamples (20 - 50 mg, ± 0.001 mg). A catalyst of $LiSO_4$ and $CuSO_4$ (1:1) was added and the mixture digested in 1 ml of concentrated sulphuric acid at 365 °C for 6 hours. Once cool, 9 ml of 18.2 MΩhm.cm (UHP) water was added to samples and the samples analysed colorimetrically using the ammonium molybdate-antimony potassium tartrate-ascorbic acid method of Murphy Riley (1962). Soil-available P was determined using the sodium bicarbonate ($NaHCO_3$) extraction method by extracting 2.5 g soil with 50 ml 0.5 M $NaHCO_3$ (Olsen et al., 1954), orthophosphate was then determined using the Murphy Riley method. Available

nitrogen was analysed following extraction of 10 g soil with 40 ml 2 M KCl on a shaker for an hour. Samples were filtered using Whatman No.1 paper and then ammonium (NH_4^+-N) determined spectrophotometrically by means of a modified Berthelot reaction (Krom, 1980) and nitrate (NO_3^-N) using a rapid colorimetric determination by nitration of salicylic acid measured by absorbance at 405 nm (Cataldo et al., 1975; Matsumura and Witjaksono, 1999).

ii) Physical properties

Aggregate stability was measured using the sequential wet sieving method adapted from Cambardella and Elliott (1993), to derive five size classes: >2000 μm, (large macroaggregates) 1000-2000 μm (medium macroaggregates), 250-1000 μm (small macroaggregates), 53-250 μm (large microaggregates) and less than 53 μm (small microaggregates and silt or smaller-sized particles. For each sample, 50 g ± 0.005 of soil was placed in a 2000 μm sieve and submerged in a bowl of distilled water filled up to 15 mm above the sieve mesh for 5 minutes, to allow for slaking. Subsequently, the sieve was moved up and down in 50 strokes over the period of approximately 2 minutes. Stones, roots and other organic material were removed, and the aggregates placed into a pre-weighed tin cup. The remaining soil and water that passed through the 2000 μm sieve was then poured through a 1000 μm sieve and moved vertically for 40 strokes and transferred to a pre-weighed aluminium cup. The same steps were repeated for the 250 μm sieve (30 vertical strokes) and 53 μm (10 vertical strokes). The remaining water was allowed to settle overnight and poured into aluminium cups representing the <53 μm fraction. Aluminium cups with soil samples were left in the oven at 105 °C for 24 hours and the soil dry weight obtained.

3.2.1.2 Algae elemental ratio, crop biomass and grain micronutrient analysis

i) Algae elemental ratio

A Flash 2000 Elemental Analyser was used to obtain total C and N values and the total P of the algae and shoot (straw) biomass was measured as previously described for soil total P concentration.

ii) Pea and wheat biomass

Harvested pea plants were weighed immediately to obtain fresh weight and then oven dried at 70 °C to obtain dry weights. Pea yield was analysed by counting the total number of pods per plant in each pot. Dry weight of wheat ears, straw and grain was obtained after drying in an oven at 70 °C for 3 days. Wheat ears were threshed by hand and the grain subsequently passed through a riffle box to obtain a representative 10 g sample for nutrient analysis. Straw was powdered using a Retch s100 mill and subsamples analysed for total nutrients: C and N by CN elemental analyser, total P using the method as previously described for soil analysis.

iii) Micronutrient analysis in wheat grain

Grain total micronutrients were analysed in wheat grains imbibed in UHP water and chopped into small pieces. 0.25 g was digested in aqua regia solution (3:1, HCl:HNO_3). The digested solution was filtered using a 0.2 µm syringe filter and diluted using UHP water to a fixed volume of 25 ml and the solution analysed using ICP-MS (Perkin-Elmer, Elan DRCII). All sample vessels were acid washed prior to analysis.

3.2.1.3 Statistical analysis of data

The means of the replicates for the 5 treatments ± standard error are presented. Statistical analyses were conducted using the RStudio software version 3.1.0 and Graphpad Prism. To compare the effect of different algal species and application rates on measured soil characteristics, the Anderson-Darling test was used to check for distributional adequacy of the data and data were log transformed prior to analysis if they did not follow a normal distribution. The impact of the algae treatments on soil nutrients was tested using one-way ANOVA and Tukey post-hoc analysis to see how the treatments compared against each other and the control and a two-way ANOVA to see whether the treatments and their application rates had an impact on soil nutrients. Differences were considered significant at a probability level of ($p<0.05$).

3.3 Results

3.3.1 Algae biomass elemental characterisation

The elemental composition of the algal species varied considerably and for some elements these differences were greatest between freshwater and marine algae (Table 2). For example, C concentrations were significantly higher in *Chlorella* sp. and *Spirulina* compared to *L. digitata* ($p<0.01$) and *A. nodosum* ($p<0.01$). Total N concentrations also varied significantly ($p<0.001$) among the algae species but were similar between *Chlorella* sp. and *Spirulina* and between *L. digitata* and *A. nodosum* ($p>0.05$). All algae species differed in their total P concentrations ($p<0.001$), while *A. nodosum* had the lowest C, N and P concentrations. Mg concentrations varied among all 5 species, with *L. digitata* and

A. nodosum having higher concentrations in their biomass (5.8 and 5.7 mg g^{-1} respectively) compared to *P.palmata,* which had the lowest concentrations (1.7 mg g^{-1}).

For the micronutrients analysed (Table 3), all the algae species differed significantly in their Zn, Ca and Fe concentrations ($p<0.0001$), except for *P. palmata*, *Spirulina*, *Chlorella* sp. and *A. nodosum*, which all had similar Zn, Ca and Fe concentrations, respectively. Se concentrations were significantly higher in macroalgae species compared to microalgae ($p<0.0001$), with *L.digitata* having the highest concentration at 1.1 mgkg^{-1}.

Heavy metals analysed were cadmium (Cd), lead (Pb), arsenic (As), nickel (Ni) and chromium (Cr). Apart from in *P. palmata* and *A. nodosum*, the algal species contained negligible concentrations of Cd. The Pb concentrations in *P. palmata* and *L.digitata* were both below limits of detection, but low concentrations (0.04 mg kg^{-1}) were detected in *Chlorella* sp. *Spirulina* was found to have significantly higher concentrations of Cr (3.83 mg kg^{-1}) than the other algal species, and *L. digitata* had high concentrations of As (57.9 mg kg^{-1}), just above the lower guideline value of 50 mg kg^{-1} for agricultural land (Toth et al., 2015).

PART I GREENHOUSE EXPERIMENT

3.3.2 Effect of algal biomass on soil total nitrogen, carbon and phosphorus concentrations

Total P under both *Chlorella* sp. and *P. palmata* treatments was significantly lower ($p<0.01$) in comparison to the control (Figure 3.1). There was a significant interaction between treatment and application rate ($p<0.01$), which was only evident under low

application rates (0.2 g kg^{-1}) of *P. palmata* treatment, which had significantly lower concentrations of total P ($p<0.05$) in comparison to low and high (4 g kg^{-1}) application rates of *A. nodosum*. Highest amounts of total N in the greenhouse experiment were observed under *Chlorella* sp. and *Spirulina* (both 1.9 g kg^{-1}) treatments, which both increased by approximately 12% from initial soil N concentration of 1.7 g kg^{-1}. Both these treatments were found to have significantly higher ($p<0.001$) concentrations of total N than the control treatments. High application rates (4 g kg^{-1}) of *Spirulina* and *Chlorella* sp. significantly ($p<0.05$) increased soil total N concentrations in comparison to the control treatments. Total soil C concentrations significantly increased under both *Chlorella* sp. and *P. palmata* treatments in comparison to the control ($p<0.05$), with concentrations under highest application rates of *Chlorella* sp. treatment increasing by 17 % from the initial soil C concentrations. Additionally, under high application rates of *Chlorella* sp. soil C concentrations were significantly higher ($p<0.05$) than they were under low application rates of *A. nodosum* and *L. digitata*.

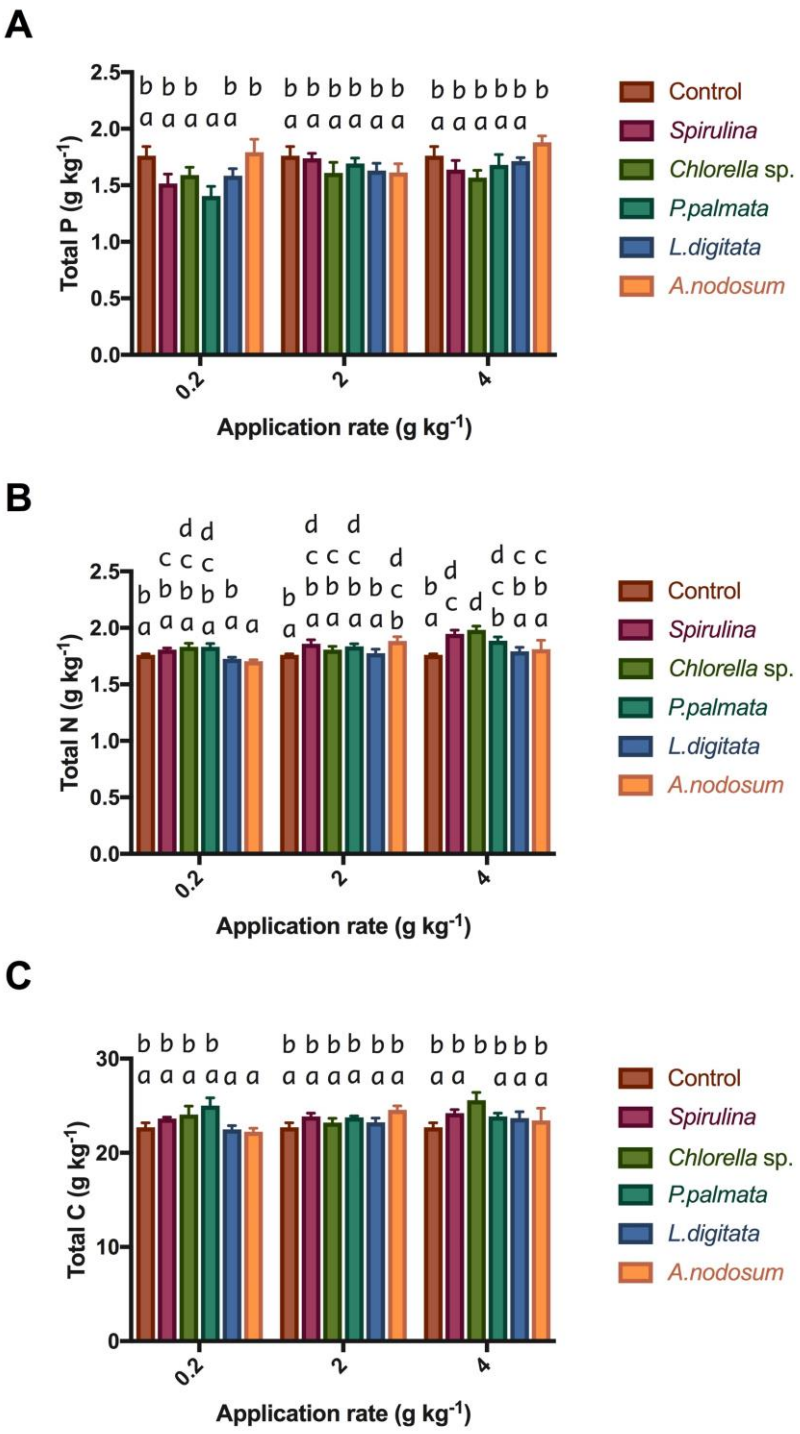

Fig. 3.1 (a) Soil total phosphorus ([F (17,54) = 2.607, p=0.004] two-way ANOVA) **(b)** total N ([F (17,54)=4.956, p<0.0001] two-way ANOVA) **(c)** total C concentrations ([F (17,54)=2.255, p=0.01] two-way ANOVA) at harvest (13 weeks) as affected by application of different algae species at 3 application rates (0.2, 2 and 4 g kg^{-1}). Boxplots represent mean concentrations, with the bars on the columns representing standard error of the mean, n = 4. Means which do not share the same letter e.g. a,b etc., are significantly different (p<0.05, two way ANOVA).

3.3.3 Effect of algal biomass on available nitrogen and phosphorus concentrations

After 13 weeks (Figure 3.2), *Chlorella* sp., *Spirulina*, and *P. palmata* treatments significantly increased ($p<0.05$) soil available P in comparison to the control. Both *L. digitata* and *A. nodosum* had the lowest increases in soil available P concentrations. A significant interaction ($p<0.0001$) was also observed between application rate and algae treatment where under high (4 g kg^{-1}) application rates, *Chlorella* sp. was observed to have increased soil available P by ~ 50% from 18.1 mg kg^{-1} at the start of the experiment to 27 mg kg^{-1} at harvest (13 weeks). Medium application rates of *Spirulina* were also found to significantly improve soil available P concentrations in comparison to the control ($p<0.0001$).

At harvest, the concentration of NH_4^+ - N in the soil significantly increased ($p<0.0001$) under *Chlorella* sp., *P. palmata* and *L. digitata* treatments, which were found to have higher concentrations than the control. An increase from initial soil concentrations of 180%, 200% and 200% was observed respectively under *Chlorella* sp. *P. palmata* and *L. digitata*. There was no difference in NH_4^+ - N concentrations between *Spirulina* treatment and *A. nodosum* in comparison to the control. None of the algae treatments had any significant impact on soil NO_3^- -N except for *Spirulina*, where high application rates increased NO_3^- -N concentrations by 42%. This increase was significantly higher ($p<0.05$) than the NO_3^- -N concentrations measured under the lowest application rates of *A. nodosum*, *P. palmata* and the control.

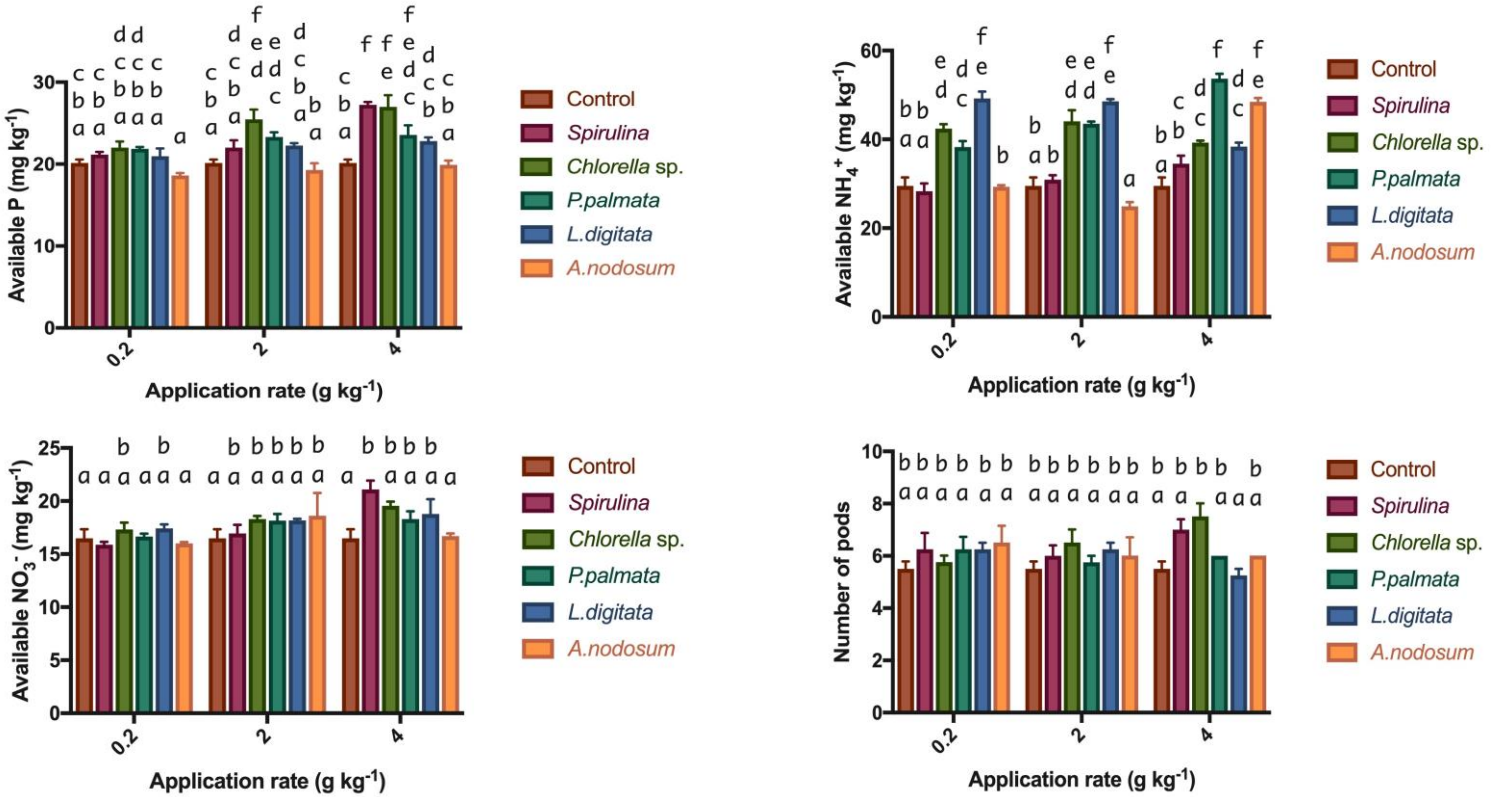

Fig. 3.2 (a) Soil available phosphorus ([F (17,54) = 11.66, p<0.0001] two-way ANOVA) **(b)** available NH_4^+-N ([F (17,54)=40.05, p<0.0001] two-way ANOVA) **(c)** available NO_3^-N concentrations ([F (17,54)=2.763, p=0.002] two-way ANOVA) as affected by application of different algae species at 3 application rates (0.2, 2 and 4 g kg^{-1}) and**(d)** yield at harvest (13 weeks) ([F(5,66)=2.478, p=0.04] One-way ANOVA) as affected by different algae species. Boxplots represent mean concentrations, with the bars on the columns representing standard error of the mean, n = 4. Means which do not share the same letter e.g. a,b etc., are significantly different (p<0.05, two way ANOVA).

3.3.4 Effect of algae biomass on soil aggregate stability

There was no significant difference between any of the treatment means and the control. Data showed a lot of variability under all the different algal treatments and application rates.

3.3.5 Effect of algae biomass on pea yield

Chlorella sp. treatment had the highest average pea yield with 6.6 pods, which was significantly higher ($p<0.05$) than yields under the control treatments. The control had the lowest overall average yield of 5.5 pods. Pea yield under a high application rate of *Chlorella* sp. were significantly higher ($p<0.05$) than yields under high application rates of *L. digitata* treatment.

PART II FIELD EXPERIMENT

In the field experiment with spring wheat (*Triticum aestivum* L.) the focus was to see whether the effects on soil available nutrients were replicable and more specifically how this would affect crop yield and nutritional value. Furthermore, to gain a better understanding of the degradation of the algae biomass in soil, temporal measurements were taken after: 2, 8 and 20 weeks (at harvest), when the wheat crop had reached maturation.

3.3.6 Temporal effects of algal biomass on soil available phosphorus concentrations

Soil available P concentrations 2 weeks after the addition of the algae biomass were highest under the control and *Chlorella* sp. treatments in comparison to *A. nodosum* ($p<0.05$). However, application rate did not have any significant impact on available P concentrations between any of the algal treatments (Table 3.4).

Table 3.4

Soil available phosphorus dynamics in arable soil during 20 weeks of wheat crop growth, as affected by application of different algal species.

Treatment	Time [A]		
	2	8	20
	----------mg kg^{-1} [B]----------		
Control (1)	54±8[a]	67±7[a]	44±3[a]
Control (2)	53±7[a]	67±6[a]	40±4[a]
Spirulina (8g)	50±3[a]	65±6[a]	35±4[a]
Spirulina (16g)	48±2[a]	60±4[a]	33±2[a]
Chlorella sp. (8g)	53±5[a]	66±4[a]	33±2[a]
Chlorella sp. (16g)	55±3[a]	65±8[a]	38±3[a]
P. palmata (8g)	50±2[a]	62±3[a]	33±3[a]
P. palmata (16g)	48.3±0.7[a]	58±2[a]	33±3[a]
L. digitata (8g)	48±4[a]	58±4[a]	32±4[a]
L. digitata (16g)	46±4[a]	56±6[a]	31±5[a]
A. nodosum (8g)	45±2[a]	54.90±0.002[a]	32±1[a]
A. nodosum(16g)	47±3[a]	56±1[a]	31±3[a]

[A] Weeks after addition of algal biomass. Initial available P concentrations= 63±2 mg kg^{-1}.

[B] Mean ± standard error (n=3). Values with different superscript e.g. a,b etc., in the same column are significantly different (*$p<0.05$*, two way ANOVA).

After 8 weeks, soil available P concentrations increased from their concentrations at 2 weeks. However, there was no significant difference between any of the treatments means

and no effect of application rates on soil available P concentrations ($p>0.05$). After 20 weeks, P concentrations were found to have decreased and were highest under control treatments and significantly higher ($p<0.05$) in comparison to *A. nodosum* and *L. digitata* treatments under which the lowest concentrations of available P were recorded. There was also no significant interaction between application rate and treatment.

3.3.7 Temporal effects of algal biomass on soil available NH_4^+-N concentrations

Soil NH_4^+-N concentrations under *Chlorella* sp. increased by 8% two weeks after the addition of algae treatments (Table 3.5) and were significantly higher ($p<0.01$) than the NH_4^+-N concentrations under the control, *L. digitata* and *A. nodosum* treatments. Both low (8 g) and high (16 g) application rates of *Chlorella* sp. and high application rates of *Spirulina* significantly increased ($p<0.0001$) NH_4^+-N concentrations in comparison to low and high application rates of *A. nodosum*. 8 weeks after the addition of algae, *P. palmata* increased NH_4^+-N by 5%, significantly higher ($p<0.05$) than concentrations under the control, *Spirulina* and *A. nodosum* treatments. Low application rates of *P. palmata* had higher ($p<0.001$) NH_4^+-N concentrations in comparison to low application rates of *L. digitata* and both low and high application rates of *A. nodosum*. By harvest at 20 weeks, soil NH_4^+-N concentrations decreased under all treatments, to values lower than at the beginning of the experiment. There was no significant difference between any of the treatment means and no significant interaction between treatments and application rates on NH_4^+-N concentrations.

Table 3.5

Soil available nitrogen (NH_4^+) dynamics in arable soil during 20 weeks of wheat crop growth, as affected by application of different algal species.

Treatment	Time [A]		
	2	8	20
	----------mg kg^{-1} [B]----------		
Control 1	16.6±0.7^{ab}	11.9±0.6^{ab}	12.0±0.3^{a}
Control 2	16.8±0.6^{ab}	13 ±2^{ab}	11.8 ±0.3^{a}
Spirulina (8g)	17.4+0.8^{ab}	12.3±0.5^{ab}	12.10±0.007^{a}
Spirulina (16g)	19.1±0.1^{a}	12.7±0.6^{ab}	12.1±0.3^{a}
Chlorella sp. (8g)	19.1±0.6^{a}	18±2^{ab}	12.4±0.4^{a}
Chlorella sp. (16g)	19.0±0.8^{a}	18±3^{ab}	12.2±0.3^{a}
P. palmata (8g)	17.1±0.4^{ab}	19±2^{a}	11.6±0.3^{a}
P. palmata (16g)	17.6±0.7^{ab}	18±2^{ab}	12.2±0.5^{a}
L. digitata (8g)	16.8±0.4^{ab}	20±2^{a}	12.5±0.3^{a}
L. digitata (16g)	16.6±0.8^{ab}	10.1±0.6^{b}	12.5±0.4^{a}
A. nodosum (8g)	15.5±0.4^{b}	10±1^{b}	11.8±0.3^{a}
A. nodosum(16g)	15.5±0.8^{b}	10.7±0.8^{b}	12.20±0.08^{a}

[A] Weeks after addition of algal biomass. Initial NH_4^+-N concentrations = 17.6±0.2 mg kg^{-1}.
[B] Mean ± standard error (n=3). Values with different superscript e.g. a,b etc., in the same column are significantly different ($p<0.05$, two way ANOVA).

3.3.8 Temporal effects of algal biomass on soil available NO_3^--N concentrations

After 2 weeks, soil NO_3^--N concentrations increased significantly under *Spirulina* and *Chlorella* sp. treatments ($p<0.05$) in comparison to the control (Table 3.6). Under high application rates of *Spirulina*, NO_3^--N was higher ($p<0.001$) in comparison to NO_3^--N concentrations under both high and low application rates of control, *L. digitata*, *P. palmata* and *A. nodosum*. After 8 weeks**,** NO_3^--N concentrations were highest under *P. palmata* treatments and were significantly higher ($p<0.05$) in comparison to the control. There was

no interaction between the treatments and their application rates on soil NO_3^--N concentrations ($p>0.05$). Twenty weeks after the addition of algae treatments, soil NO_3^--N concentrations increased again under all treatments. The lowest increase was observed under *Spirulina*, where NO_3^--N concentrations were significantly lower ($p<0.01$) than control, *A. nodosum*, *L. digitata* and *P. palmata*.

Table 3.6

Soil available nitrogen (NO_3^-) dynamics in arable soil during 20 weeks of wheat crop growth, as affected by application of different algal species.

Treatment	**Time** [A]		
	2	8	20
	----------mg kg^{-1} [B]----------		
Control 1	18.4±0.2[b]	7.62±0.01[a]	20.3±0.5[a]
Control 2	18.3±0.3[b]	8.0±0.2[a]	19.4±0.3[ab]
***Spirulina* (8g)**	19.9±0.2[b]	8.1±0.3[a]	18.0±0.6[b]
***Spirulina* (16g)**	23±1[a]	7.9±0.3[a]	18.2±0.2[ab]
***Chlorella sp.* (8g)**	20.2±0.7[ab]	8.0±0.3 [a]	19.3±0.8[ab]
***Chlorella sp.* (16g)**	20.8±0.1[ab]	7.9±0.1[a]	18.6±0.5[ab]
***P. palmata* (8g)**	18.2±0.4[b]	8.3±0.2[a]	20.3±0.2[a]
***P. palmata* (16g)**	18.4±0.3[b]	8.5±0.1[a]	19.9±0.6[ab]
***L. digitata* (8g)**	18.3±0.1[b]	8.22±0.05[a]	20.0±0.3[ab]
***L. digitata* (16g)**	18.1±0.4[b]	8.4±0.2[a]	19.4±0.2[ab]
***A. nodosum* (8g)**	18.6±0.4[b]	8.0±0.2[a]	20.12±0.07[ab]
***A. nodosum*(16g)**	19.0±0.2[b]	7.90±0.05[a]	19.8±0.4[ab]

[A] Weeks after addition of algal biomass. Initial NO_3^--N concentrations = 18.1±0.5 mg kg^{-1}.

[B] Mean ± standard error (n=3). Values with different superscript e.g. a,b etc., in the same column are significantly different (*$p<0.05$*, two way ANOVA).

3.3.9 Temporal effects of algal biomass on soil aggregate stability

One-way ANOVA analysis looking at the difference between treatment means showed that two weeks after the addition of algae, the highest increase in water stable macro-aggregates (250 - 2000 μm) was observed under *L. digitata* treatment and was found to be significantly higher ($p<0.05$) than under *A. nodosum* treatment. However, the % dry weight of water stable macro-aggregates under *L. digitata* were found to be no different from the control and all the other algae treatments. After 8 weeks, water stable macro-aggregates appeared to increase following the addition of *A. nodosum*, and *P. palmata*, however, there were no significant differences between any of the treatment means. After 20 weeks, under *L. digitata* treatment, water stable macro-aggregates increased significantly ($p<0.05$) in comparison to *A. nodosum* treatment. However, there was no difference between *L. digitata* and other algae treatments or the control. There was no apparent relationship between treatment and application rate on water stable macro-aggregates after 2, 8 or 20 weeks (Table 3.7).

Table 3.7

Water stable aggregates (250μm->2000μm) dynamics in arable soil for 20 weeks of wheat crop growth, as affected by application of different algal species.

Treatment	Time [A]		
	2	8	20
	----------% dry weight [B]----------		
Control 1	52±1[a]	58±2[a]	47±1[a]
Control 2	55±2[a]	59±2[a]	46±2[a]
Spirulina (8g)	57±1[a]	59±1[a]	48±1[a]
Spirulina (16g)	55±4[a]	59±4[a]	46±2[a]
Chlorella sp. (8g)	56±1[a]	61±2[a]	50±4[a]
Chlorella sp. (16g)	53±3[a]	58±1[a]	49±3[a]
P. palmata (8g)	55±2[a]	62±2[a]	55.1±0.6[a]
P. palmata (16g)	52±2[a]	61±1[a]	48±3[a]
L. digitata (8g)	62±1[a]	59±0.9[a]	54±1[a]
L. digitata (16g)	57.3±0.8[a]	63±1[a]	51±3[a]
A. nodosum (8g)	52±3[a]	54±2[a]	46±3[a]
A. nodosum(16g)	41±10[a]	58±2[a]	45±2[a]

[A] Weeks after addition of algal biomass. Initial % dry weight of macro aggregates = 57±1.

[B] % dry weight of soil macro aggregate (250-2000 μm) fraction. Mean ± standard error (n=3). Values with different superscript e.g. a,b etc., in the same column are significantly different (*p<0.05*, two way ANOVA).

3.3.10 Effect of algal biomass on wheat parameters and micronutrients

The effects of the algae on wheat parameters are presented in Table 3.8. Total shoot biomass was calculated from the combined dry weight of the harvested straw and wheat ears. Total shoot biomass was highest under *Chlorella* sp. treatment (444 g m^{-2}). There were no significant differences in total shoot biomass between *Chlorella* sp. and the control, *Spirulina*, *P. palmata* and *L. digitata* treatments, but there was between *Chlorella* sp. and *A. nodosum*. Wheat ear count was also highest under *Chlorella* sp. (172, n=3),

though there was no significant difference between any of the other treatments. The highest grain yield was obtained under *Chlorella* sp. with an average yield of 5 t ha^{-1} and the lowest was observed under *A. nodosum* (4.2 t ha^{-1}). The control treatment had an average grain yield of 4.6 t ha^{-1}. There was no significant difference in yield between any of the treatments. Total N and P measured in the aboveground biomass, which included the wheat shoot and grain (Table 3.9), showed no significant differences for either macronutrient amounts between any of the algal treatments and control. Despite this, *Chlorella* sp. had the highest amounts of N and P in its aboveground biomass in comparison to all the other treatments and the control.

Table 3.8

Effect of algae biomass on various parameters of spring wheat.

Treatment	**Total shoot biomass**		**Ear number per m^2**		**Yield**	
	Mean[A]	SE [B]	Mean	SE	Mean	SE
	(g^{-1})				(t ha^{-1})	
Control	359[ab]	14	156[a]	9	4.6[a]	0.2
Spirulina	377 [ab]	20	145[a]	14	4.2[a]	0.3
Chlorella sp.	444[a]	13	172[a]	9	5.0[a]	0.3
P. palmata	403[ab]	25	158[a]	17	4.6[a]	0.5
L. digitata	385[ab]	33	150[a]	17	4.4[a]	0.6
A. nodosum	341[b]	25	143[a]	10	4.2[a]	0.4

[A] Mean values (n=3), with different superscript e.g. a,b etc., in the same column are significantly different ($p<0.05$, one way ANOVA).
[B] Standard error

Table 3.9

Input and output amounts of total N and P in field experiment

Treatments	Total nitrogen		
	Input [B]	**Output** [C]	**Δ mg m^{-2}**
	mg N m^{-2} [A]	mg N m^{-2}	mg N m^{-2}
Control		24380±1411^{a}	24380±1411^{a}
Spirulina	2980±4.3^{c}	27312±1786^{a}	24332±1389^{a}
Chlorella	2446±302^{c}	31241±1094^{a}	28795±825^{a}
P. palmata	850±6^{b}	26472±1912^{a}	25621±2876^{a}
S. latissima	450±7^{a}	26033±2480^{a}	25582±2265^{a}
A.nodosum	391±5^{a}	23895 ±1505^{a}	23504±1781^{a}
	Total phosphorus		
	Input	**Output**	**Δ mg m^{-2}**
	mg N m^{-2} [A]	mg N m^{-2}	mg N m^{-2}
Control		536±28^{a}	536±28^{a}
Spirulina	50.1±0.8^{a}	520±26^{a}	470±29^{a}
Chlorella	64±1^{b}	561±58^{a}	497±64^{a}
P. palmata	21±1^{c}	533±49^{a}	512±58^{a}
S. latissima	11.4±0.5^{a}	504±63^{a}	493±53^{a}
A.nodosum	6.0±0.5^{a}	487±35^{a}	481±48^{a}

[A] Mean ± standard error (n=3).
[B] Total N and P in the amount of algae added
[C] Total N and P in aboveground (shoot and grain) dry biomass after harvest

Specific micronutrients were measured in the wheat grain at harvest, 20 weeks after algal biomass additions (Table 3.10). There were no significant differences in wheat grain micronutrient concentrations, namely, Zn, Fe, Mn, and Mg between any of the algae treatments. Ca concentrations in wheat grain were similar under all treatments except for control, which was significantly higher ($p<0.05$) than *Chlorella* sp. B concentrations were highest under *Chlorella* sp. and *P. palmata* in comparison to the control and *A. nodosum* treatments ($p<0.001$). Se concentrations in wheat grain were found to be significantly higher ($p<0.0001$) under *Spirulina* treatments in comparison to all the other treatments. Se concentrations were 0 mg kg^{-1} under the algae treatments and control, but

was 0.13 mg kg^{-1} under *Spirulina*.

Table 3.10

Micronutrient concentrations in wheat grain

Treatment	Micronutrients and heavy metals												
	Ca	Mg	Zn	Fe	Se	B	Mn	Cu	Cd	Pb	As	Ni	Cr
	mg kg^{-1} [A]												
Control	336±12^{a}	<0.01^{a}	27±2^{a}	35±1^{a}	<0.05^{b}	6.4±0.5^{b}	29.3 ±0.9^{a}	3.7±0.3^{a}	0.02±0.001^{a}	0.08±0.02^{a}	8.6±0.2^{ab}	2.1±0.6^{ab}	1.87±0.03^{a}
Spirulina	289±28^{ab}	<0.01^{a}	29±2^{a}	32±2^{a}	0.13±0.05^{a}	12.8±0.6^{ab}	31.0 ±0.9^{a}	3.7 ±0.4^{a}	0.03 ±0.003^{a}	0.27±0.06^{a}	8.0 ±0.4^{a}	2.6±0.7^{ab}	2.19±0.07^{b}
Chlorella sp.	251±8^{b}	<0.01^{a}	26±1^{a}	33±1^{a}	<0.05^{b}	16±2^{a}	31.3 ±0.9^{a}	3.0±0.1^{a}	0.02 ±0.003^{a}	0.17±0.05^{a}	8.4±0.1^{ab}	3±1^{b}	2.22 ±0.05^{b}
P. palmata	285±9^{ab}	<0.01^{a}	25±1^{a}	35±2^{a}	<0.05^{b}	15±2^{a}	30±1^{a}	3.1±0.2^{a}	0.02 ±0.003^{a}	0.11±0.06^{a}	9.17±0.09^{b}	0.5 ±0.1^{a}	2.22±0.06^{b}
L. digitata	322±15^{ab}	<0.01^{a}	25±1^{a}	35.0±0.5^{a}	<0.05^{b}	9±2^{ab}	31.1±0.6^{a}	3.27 ±0.08^{a}	0.04 ±0.02^{a}	0.6±0.6^{a}	9.2 ±0.1^{b}	1.7±0.7^{ab}	2.17±0.04^{b}
A. nodosum	310±21^{ab}	<0.01^{a}	27±1^{a}	36±1^{a}	<0.05^{b}	7±2^{b}	29.4 ±0.6^{a}	3.3±0.1^{a}	0.02 ±0.002^{a}	0.2 ±0.1^{a}	8.6±0.2^{ab}	0.6±0.2^{a}	2.1±0.1^{ab}

[A] Mean ± standard error (n=3). Values with the same letters in a column are not significantly different ($p<0.05$, One-way ANOVA).

3.4 Discussion

The present study compared the effect of five chemically different algae species on restoring soil physicochemical properties and improving crop yield. Prior to the start of the experiment, the concentrations of total C, N, P, and micronutrients in the algae biomass were quantified, based on the hypothesis that their initial nutrient composition would have an impact on nutrient concentrations in the soil.

Net mineralisation and immobilisation of nutrients are dependent on whether the C:N ratio of the substrate (biomass) is above or below the critical value of *c*. 20, where ratios >20 indicate net immobilisation and ratios <20 favour net mineralisation (White, 2006). The C:N ratios of the algal biomass (Table 2) show that *Spirulina*, *Chlorella* sp., *P. palmata* and *L. digitata* all have C:N ratios <20. *A. nodosum* was the only algae to have C:N ratio above 20. C:N ratios of both freshwater and marine algae are reflective of their individual growth conditions and indicate whether they have been grown in nutrient replete or deficient conditions (Geider and La Roche, 2002). The C:N:P ratio of marine algae is tightly linked to the inorganic pool of C, N and P in the ocean interior (i.e. the Redfield ratio) and this ratio may differ within and among taxa in response to variation in the abiotic environment (Yvon-Durocher et al., 2015). The composition of microalgae typically found in freshwater lakes is also highly variable: the ratio of C:N:P varies with the ratio supplied in the water as well as the pH of the water (Krebs, 2008).

In the greenhouse study, *Chlorella* sp. and *Spirulina* were shown to increase total soil N. Evidence of algae increasing soil N is not uncommon: certain cyanobacteria e.g. *Nostoc* and *Anabaena* have been recognised as significant contributors to soil N through their atmospheric N-fixing abilities (Akhter et al., 2002). This property has been predominantly observed in cyanobacteria species and in experiments carried out using *Nostoc muscorum*, total N has been reported to increase by 111-120% (Rogers and Burns, 1994), under inoculum rates ranging from equivalents of 2 kg ha^{-1} to 5 kg ha^{-1}. Akhter et al. (2002) also reported an increase in total N in rice soil inoculated with 2 g of a mixture of five cyanobacterial species, and an increase of 50% total N after inoculation with live *Nostoc* cells was reported by Maqubela et al. (2009). Our results show that the green alga *Chlorella* sp. was just as effective, highlighting its possible use in increasing soil total N concentrations, particularly in UK agricultural soils.

Chlorella sp. and *P. palmata* also significantly increased soil total C. Algae are known to help in the accumulation of C in the soil, for example, *Nostoc* strains added to soil at a rate of 0.02 g cm^{-2}, were shown to increase soil organic C after 90 days in an experiment conducted by Obana et al. (2007). In another previous experiment conducted by Rogers and Burns (1994), smaller doses of live *Nostoc muscorum* (4.04×10^5 equivalent to 5 kg ha^{-1} cell dry weight) recorded an increase of 50-63% of total C in a poorly structured silt loam soil. This is a much larger increase in comparison to the increase observed in the present experiment under high application rates of *Chlorella* sp. treatment, which increased by 17% under highest application rates (4 g kg^{-1}). Nevertheless, the benefits of adding *Chlorella* sp. to improve soil C concentrations are evident. This was also expected as *Chlorella* sp. had the second highest concentration of C stored in its biomass.

Total P in soil decreased significantly under both *Chlorella* sp. and *P. palmata* treatments in the greenhouse. There was no significant difference between any of the algae treatments and their impact on soil total P concentrations in comparison to the control, suggesting that the soil was already rich in phosphorus (0.24 g kg^{-1} ± 0.08) and the algae treatments had little impact on altering the natural concentrations in the soil. Due to factors such as adsorption, precipitation or conversion to the organic form (Moonrungsee et al., 2015), only a very small portion of total P is available to plants in the form of orthophosphate or easily mineralized organic P. The addition of algal biomass was expected to increase mineralisation of organic P by soil microbes thereby releasing orthophosphate anions (HPO_4^2 and $H_2PO_4^-$) into the soil solution (Richardson et al., 2009). Medium and high application rates of *Chlorella* sp. and *Spirulina* significantly increased soil available P concentrations in the greenhouse compared to the control. Under field conditions however, soil available P concentrations under the algae treatments did not change significantly at 2, 8 or 20 weeks after algal addition. The significance of their impact in the field could have been lost as a result of the larger variability in soil conditions. Results from a flask study conducted by Mulbry et al. (2005) showed increasing available P in soils with increasing algal additions, with responses being affected by existing soil P concentrations. In the present study, available P concentrations had declined by the end of the field experiment, most likely due to depletion by the crop growth. The control soils had higher P concentrations compared to the algal treatments, which was possibly due to microbial immobilization as a result of the carbon supplied by the algal necromass.

Soil NH_4^+-N concentrations in the greenhouse study increased under *Chlorella* sp., *P. palmata* and *L. digitata*. Similar results were observed in the field, where high NH_4^+-N concentrations were also recorded under both *Chlorella* sp. and *P. palmata,* suggesting

mineralization of algal necromass N. *Spirulina*, along with *Chlorella* sp. and *P. palmata* also increased soil NO_3^--N concentrations in the greenhouse and field experiment. Soil NO_3^--N decreased from 2 to 8 weeks most likely due to plant uptake. Concentrations increased again by week 20 possibly as a result of both nitrification and due to the crop N demand decreasing as it reached maturity and stopped growing, leaving higher concentrations in the soil as residual nitrogen. With the addition of nitrogen-rich organic matter, soil NO_3^--N concentrations would be expected to increase. Most studies have focused on the impact of algal amendments on soil total nitrogen concentrations, while only few have looked at available N, particularly NO_3^--N, which is the preferred form of N taken up by crops like wheat. One of these studies was conducted by Possinger and Amador (2016), where the addition of a seaweed mixture including *A. nodosum* and *L. digitata*, amongst others, elicited a decrease in soil NO_3^- concentrations over time. The study concluded that the addition of seaweed did not improve NO_3^- concentrations. This contrasts with our findings where algae, particularly *Spirulina*, caused an increase in soil NO_3^--N concentrations both in the field and greenhouse study.

Algal biomass had little effect on soil aggregate stability in the greenhouse experiment after 13 weeks. In the field soil, % dry weight of soil macro aggregates (250->2000 μm) increased under *L. digitata* after 2 weeks, however this was not significantly different to the control. Maqubela et al. (2009) reported an increase in soil macroaggregates when live *Nostoc* was added to non-cropped soils compared to cropped soils, implying that the addition of dried biomass, as undertaken in this study, has minimal impact on soil aggregate stability. Other studies support this, where improvements in soil aggregate stability were observed when adding live cultures, producing a subsequent enmeshing

effect of the growing inoculated cyanobacterium filaments and the gluing effect of excreted polysaccharides (Maqubela et al., 2009).

In terms of micronutrients, Se was only found in wheat grain grown under *Spirulina* treatment. Se is a micronutrient normally deficient in wheat crops. UK grown wheat consumption has increased, but Se concentrations remain low, or exist in forms not chemically available to the crop in the UK (Hart et al., 2011). Increased Se concentrations in wheat will be beneficial for human nutrition and health since it is often deficient in UK diets (Hart et al., 2011). Other studies have shown the capacity of *Spirulina* to take up micronutrients including Se (Wuang et al., 2016), thus highlighting its potential as a source of Se for wheat crop.

It is clear that the addition of algal biomass, particularly at higher application rates of 4 g kg^{-1} have significant effects on soil total C and N as well as available P, NH_4^+-N and NO_3^--N. The algal amendments did show consistently significant improvements on soil NH_4^+-N in the greenhouse and field. However, in terms of available P, where improvements were seen only in the greenhouse and not in the field, a higher application rate may have altered this. Conversely, the purpose of soil conditioning is not only to improve the characteristics of the soil, but should also be translatable to crop growth and yield. *Chlorella* sp. was the only algal species to significantly increase pea yield in comparison to the control in the greenhouse, although in the field, the effects on wheat were not as significant. *A. nodosum* had no significant impact on any of the soil characteristics. This was most likely because it was applied as a dried amendment and had low N and P concentrations in its biomass. Materials with high C:N (low N) ratios tend to decompose more slowly as the N is less readily available to plants due to

it being immobilized in microbial biomass. Previous studies on the impacts of *A. nodosum* on plants and soil typically use an extract rather than the dried biomass, possibly allowing for the nutrients to be released and taken up more rapidly by the plants.

3.5 Conclusions

There is a growing interest in the use of algal-based biofertilisers to increase crop productivity. Capturing nutrient run off using algae could also counter eutrophication of natural water bodies (Michalak et al., 2016), a more sustainable method for "closed-loop" nutrient cycling. Algae have previously been shown to improve soil characteristics such as C content and aggregate stability, and cyanobacteria (e.g. *Nostoc muscorum*) have been shown to improve soil N in desert environments as well as rice paddy fields through their N-fixing abilities. In the present study, it was shown that the algae had a significant impact on agricultural soils, through the addition of soil nutrients. However, they did not show any significant improvement on soil aggregate stability under the conditions tested and it is suggested that the addition of live algal biomass needs to be investigated for effects on soil aggregation. *Chlorella* sp. and *Spirulina* had immediate impact on inorganic N, with *Chlorella* sp. increasing NH_4^+-N concentrations and *Spirulina* increasing NO_3^--N; *P. palmata* was also shown to influence soil available N concentrations at a later stage during crop growth. The outcome of both experiments highlights the importance of chemical composition of algae in supplying plant available nutrients, providing insights into selecting appropriate species for arable soil nutrient management strategies. Overall, the results show the benefits and potential of using algae as a sustainable organic fertiliser with

the aim of increasing soil total N content and in particular improving N mineralization rates in the soil.

3.6 References

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Chapter IV

Dynamics and transformations of algal nitrogen

This chapter looks at the effect of Chlorella vulgaris as an organic fertiliser, by studying its distribution and uptake in the soil-plant system.

4.1 Introduction

Large amounts of inorganic fertilizer application have helped drive agricultural intensification, with global N application rates increasing from 10-81.7 million tonnes between 1960 and 2000 (Heijboer et al., 2016; Fixen and West, 2002). Although in the UK, rates of inorganic fertilizer applications to tillage crops are decreasing (DEFRA, 2017), global N inputs are predicted to exceed 200 million tonnes by 2050 (Tilman et al., 2001). Intensive agriculture, including high inorganic N fertilizer inputs, has contributed to increased losses of nitrogen to the environment (Muller and Clough, 2014). Recent estimates suggest that only 47% of the nitrogen added globally onto cropland is recovered in harvested crops, and this efficiency has remained fairly constant over the past 20 years, during which time mineral N fertilizer use has increased by over 40% (Lassaletta et al., 2014). Organic amendments and the use of legumes are commonly suggested as alternatives to increased use of inorganic fertilizer and can result in reduced rates of N loss (Fixen and West, 2002; Lassaletta et al., 2014).

The effects of algae on soil quality and crop growth have been tested extensively and the positive impacts on soil nitrogen have been acknowledged and exemplified in the

previous chapter. However, the fate of algal nitrogen added to conventionally managed arable soils, its distribution into the main soil nitrogen pools, and the extent of its utilization by crop plants has not previously been explored.

Tracer studies using nitrogen isotopes have been used to study the pathways of N transformations in soil and plants and the fate of added nitrogen in both terrestrial ecosystems and agriculture (Haynes et al., 1997; Kramer et al., 2002; Chalk et al., 2015; Heijboer et al., 2016). Nitrogen has two stable isotopes of two different masses: ^{14}N, which is the most abundant form of N_2 in our atmosphere (99.6337%), and ^{15}N which is the remaining 0.3663% (Bedard-Haughm et al., 2003). The low natural abundance of ^{15}N means that it is a sensitive tracer for studying nitrogen cycling processes, and nitrogen dynamics in organisms and ecosystems. The two most common ways of using ^{15}N are by providing ^{15}N-enriched substrates, the fate and transformation of which can be quantified, or by measuring the natural abundance of ^{15}N. The natural abundance of ^{15}N varies as a result of slight discrimination between the two isotopes in biological processes- with preferential processing of the lighter isotope by most biological enzymes. Consequently, ^{15}N generally accumulates in organisms, and becomes progressively slightly more enriched through food-webs (Perkins *et al.,* 2014), up to the highest trophic groups of organisms as exemplified by polar bears (Hobson & Welch 1992). Natural abundance of ^{15}N is measured relative to international standards, and normally expressed as delta values, which effectively uses the difference between the $^{15}N/^{14}N$ ratio of N source being investigated in comparison to the $^{15}N/^{14}N$ already present in the system, to trace N through different pools. This method has its benefits in that it can be used in any ecosystem and does not require purchasing artificially enriched ^{15}N tracers, which can be costly (Barraclough, 1991). It does suffer, however,

from analytical and interpretative limitations (Bedard-Haughm et al., 2003). The same processes of isotopic discrimination seen in marine food webs take place in soil food webs, but the wide diversity of microorganisms performing different functions in the nitrogen cycle and the diverse and complex mix of organic materials present in soils makes the study of pathways of N transformations based on natural abundance ^{15}N especially challenging in soils. By contrast, applying ^{15}N enriched nitrogen sources to soil, for example in the form of inorganic fertilizer, amino acids or more complex materials like ^{15}N enriched plant litter, enables the added ^{15}N to be traced and quantified through the various N pools (Bedard-Haughm et al., 2003). The ^{15}N enrichment approach although more expensive, is less likely to produce analytical errors due to the ^{15}N isotopic signature being distinct from the background N already in the system – which is absolutely necessary for it to work. Additionally, the extent of isotopic discrimination in biological processing of N is sufficiently small that it is normally ignored in the ^{15}N enrichment-tracer studies (Harmsen, 2003).

Studies conducted on the fate of algal-derived N on the soil, microorganisms or plant pools have focused largely on the use of cyanobacteria, under rice field growing conditions (flooded soils). Mian and Stewart, (1985) looked at the uptake as N from blue-green algae (*Anabaena variabilis* and *Nostoc muscorum*) by rice plants in pots submerged in 4cm flood water for 60 days. They observed at the end of the 60 days, an increase by 176 and 215% in N uptake by rice plant derived from *Anabaena* and *Nostoc* respectively, whilst 51 and 47% of the undecomposed biofertilizer was retained in the soils. The increases in N uptake was largely attributed to the nitrogen fixation by the blue green algae. Another study conducted by Tirol et al. (1982), showed that rice crop incorporated 27% of *Nostoc*-N, and Thind and Rowell (1999) reported that 42% of

added algae (type not specified) was mineralized 8 weeks after addition to sandy loam soil. These studies show the potential of algae to supply plant available nitrogen, despite the conditions under which these were investigated being flooded soils. To our knowledge, no studies exist on the use and uptake of algal N by plants on agricultural soils. Other studies using different fertilisers such as grass/clover residues and wheat straw also recorded recovery rates ranging from 12% to 80% (Haynes, 1997; Powlson et al., 1986; 1992).

Decomposition of organic substrates (in non-flooded soils) is governed by microbial depolymerisation of macromolecules such as proteins, using the carbon as an energy source (Gallardo and Schlesinger, 1994; Schimel and Bennett, 2004). The addition of organic substrates as an N source could also increase the belowground C availability through root exudates thereby providing an additional source of C for the microbes in addition to the C already present in the soil organic matter (Riggs and Hobbie, 2016) The resulting products e.g. peptides and amino acids are assimilated by the microbes for the formation of new cells (Piatek, 2011, Ward, 2012). Eventually the labile C is used up and the N in the microbial biomass declines as the macromolecules (i.e. proteins) are degraded, returning to inorganic form in the process of mineralisation (Ward, 2012). The NH_4^+ is oxidised by nitrifying bacteria to nitrite and subsequently nitrate. It would be useful to see in the case of *C. vulgaris*, how this mineralised N is used up, particularly by the plants. Therefore, the pools of interest are the soil, plant, inorganic N, as well as microbial biomass N pools.

In the work reported earlier in this thesis, *Chlorella* sp. demonstrated potential in both greenhouse and field experiments to act as an organic fertilizer increasing the total N

and NH_4^+-N in soil concentrations. In this chapter the focus of the research was to follow up these observations by using ^{15}N-labelled *Chlorella* to trace and quantify, and thereby improve understanding of the dynamics, transformations and fate of algal nitrogen in relation to soil N pools and plant available N uptake by wheat grown on arable soil. The goals were to measure the effects of the addition of ^{15}N-labelled *C. vulgaris* on the main soil nitrogen pools, differentiated by chemical analysis and measurement of ^{15}N, and to assess the main sinks/pathways of *Chlorella* N. The experiments tested the hypothesis that addition of *C. vulgaris* will contribute significantly to plant N uptake, because of rapid microbial degradation and mineralization (due to low C:N ratio) following its addition. To evaluate the effectiveness of the algal-derived N against inorganic fertilizer that is used on UK agricultural soils growing wheat, urea, a widely used fertilizer was supplied in a set of controls that were compared to the wheat plants and soil supplied with algal-N only.

4.2 Materials and methods

4.2.1 Maintenance, cultivation and harvesting of C. vulgaris

4.2.1.1 Maintenance of stock cultures

The algae biomass used was *Chlorella vulgaris*, CCAP 211/12 obtained from CCAP (Culture Collection of Algae and Protozoa, UK). The liquid stock culture was centrifuged (at 4000 g, 4 ºC for 20 minutes) and the media discarded. The pellet was then suspended in liquid Bold's basal medium (Bold, 1949; Anderson, 2005) (Table 4.1) and 1 ml of this stock culture was inoculated in 100 ml of autoclave-sterilized

Bolds basal media and maintained in an incubator at 20 ± 1 ºC without shaking, and continuously illuminated by light tubes from above at 259 µmol m^2s^{-1}.

4.2.1.2 Cultivation of ^{15}N enriched algal biomass

C. vulgaris was cultivated in Bold's basal medium substituted with ^{15}N sodium nitrate (98% $Na^{15}NO_3$) purchased from Sercon. $NaNO_3$ was substituted with $Na^{15}NO_3$, by dissolving 5g $Na^{15}NO_3$ in 200 mL distilled water. Six flasks each containing ~5 litres each of media solution were autoclaved before being inoculated with *C. vulgaris* biomass from stock cultures and maintained at 20 ± 1ºC, 24 hr light at 259 µmol $m^2 s^{-1}$, with air bubbled through the flask to increase CO_2 flow from the air to the culture (Rocha et al., 2003).

Table 4.1

Culture medium composition (Culture Collection for Algae and Protozoa, Bold's Basal Medium (BBM))

Stock solution	**Chemical**	**Concentration (g 400 ml^{-1})**
1	$NaNO_3$	10.0
2	$MgSO_4.7H_2O$	3.0
3	NaCl	1.0
4	K_2HPO_4	3.0
5	KH_2PO_4	7.0
6	$CaCl_2.2H_2O$	1.0
		Concentration (g l^{-1})
7	Trace elements solution (autoclave)	
	$ZnSO_4.7H_2O$	8.82
	$MnCl_2.4H_2O$	1.44
	MoO_3	0.71
	$CuSO_4.5H_2O$	1.57
	$Co(NO_3)_3.6H_2O$	0.49
8	H_3BO_3	11.42
9	EDTA	50.0
	KOH	31.0
10	$FeSO_4.7H_2O$	4.98
	H_2SO_4 (conc)	1.0 ml
Medium	**per litre**	
Stock solutions 1-6	**10.0 ml each**	
Stock solutions 7-10	**1.0 ml each**	
Make up to 1 litre with deionised water.		

4.2.1.3 Harvesting of ^{15}N-labeled *Chlorella* for use as N source.

The algal biomass was harvested by centrifugation (at 4000 g, 4 ºC for 20 minutes). Centrifuged biomass was placed in 50 ml Falcon tubes and was frozen at -20ºC before being freeze-dried and stored in a desiccator until further use.

4.2.1.4 Assessment of the ^{15}N isotopic enrichment of algae and soil.

The ^{15}N content of the algae as well as the soil to be used, was measured using an Isotope Ratio Mass Spectrometer (IRMS) (ANCA GSL 20-20 Mass Spectrometer, Sercon PDZ Europa, Cheshire).

Due to the small quantities of biomass harvested, the labelled *Chlorella vulgaris* was mixed with unlabelled, commercially purchased *Chlorella*, in a ratio of 1:1.5 (80 mg: 120 mg) respectively. The isotopic signature of the labelled biomass before and after mixing as well as the isotopic signature once applied to the potting system was measured and the results recorded in Table 4.2. This ratio was attained based on gaining a detectable isotopic signature once the *C. vulgaris* was applied to the soil plant potting system to make sure there was a significant difference between the isotopic signature of the source compared to the background levels.

Table 4.2

Isotopic signature of algae and soil before experiment (n=3)

Algae	**Atom % ^{15}N**
Soil	0.363
Unlabelled *Chlorella*	0.363
Labelled *C. vulgaris* (^{15}N)	82.79
Labelled and unlabelled *C. vulgaris* (1:1.5)	55.75
Labelled and unlabelled *C. vulgaris* with soil	2.56

4.2.2 Experimental design

The pot experiment was carried out at the Arthur Willis Environment Centre, at the University of Sheffield, from the 18th April 2017 to the 18th May 2017, in a GroDome greenhouse under a 12 h photoperiod, 200 μE m^{-2} s^{-1} light intensity, and 21°C:15°C day: night temperatures. The soil used in the experiment was taken from Wise Warren, at Spen Farm, Tadcaster, England (longitude 1°20'32.9" W, latitude 53°51'40.7" N), Quarry Field 1, which is long-term arable soil that has been cultivated and cropped every year for at least 20 years. The initial soil characteristics are shown in Table 4.3. The soil was transported back to Sheffield and subsequently riddled and air dried in the greenhouse and then mixed to homogenise, before being placed into pots. Each pot consisted of 500 ± 1g soil.

Table 4.3

Table showing initial soil characteristics. Mean ± standard error (n=3).

Total carbon	**Total nitrogen**	**C:N**	**NH_4^+-N**	**NO_3^--N**	**pH**
--mg g^{-1}--	--mg g^{-1}--		--mg kg^{-1}--	--mg kg^{-1}--	
21.3 ± 0.36	16.6 ± 0.01	12.8 ± 0.23	1.48 ± 0.04	7.27 ± 0.21	7.4 ± 0.06

4.2.2.1 Measuring soil field capacity

In the experiment the soil moisture content was maintained close to 40% field capacity. This value was chosen not only because most arable soils are normally significantly below field capacity during the growing season (Brown et al., 2017), but also maintaining the same level of moisture in each pot would have allowed for comparisons

of microbial processes between soils following harvest, so as not to introduce any differences that might be caused by differences in moisture (Fierer et al., 2007). According to Brookes et al. (1985) prior to measuring soil microbial biomass, soils should be adjusted to 40%. Soil moisture plays a significant role in microbial activity and the availability of nutrients in the soil (Rutting et al., 2011; Braun et al., 2018). The field capacity of the pots was determined using the method described in Manmathan and Lapitan (2013). Air-dried soil (500 g ± 1 g) was added to each of 10 pots, and the pots watered until completely saturated and left to drain by gravity for 3 hours. After 3 hours, the pots were weighed and then left to drain by gravity for a further 3 days. After 3 days, pots were weighed and the field capacity of the soil in each pot was calculated by subtracting the mass of soil drained after 3 days from the mass after 3 hours (after gravitational drainage). The values for the 10 pots were averaged to give the mass of water needed to achieve field capacity (100%), and this was used to guide the quantity of water needed to be added to achieve 40% field capacity. The pots were watered every two days to 40% field capacity before the start of the experiment (before addition of N treatments). The day before harvest, they were not watered, to allow them to dry so that at harvest, they could be adjusted to 40% FC.

4.2.2.2 Experimental treatments

Algal biomass (*C. vulgaris*) was supplied to soil in pots with and without wheat (n=18) in each case; inorganic (urea) fertilizer (representing a conventional cropping system) was supplied to soil with and without wheat (n=18) in each case, and the control treatment had no fertilizer and was fallow (n=12) or with wheat (n=18). The total carbon and nitrogen contents for treatments and control soil are shown in Table 4.4.

Comparisons between soil with and without wheat were made as the plants are likely to affect soil nitrogen transformations by the addition of carbon residues into the rhizosphere and change soil microbial communities (Barraclough, 1991; Turner et al., 2013).

Prior to the incubation, all treatments (algae and urea) were added at a rate of 0.2 g pot^{-1}. The *C. vulgaris* labelled with ^{15}N stable isotope and mixed with unlabelled *Chlorella* was dissolved in 5 mL of distilled water and added at 55.75 atom % ^{15}N enrichment.

Spring wheat seeds (*Triticum aestivum* L.) (Tybalt high yielding variety purchased from Limagrain) were pre-germinated on moist filter paper for 3-4 days at room temperature in the dark, prior to the start of the experiment and transplanted to the pots once seedlings had emerged and allowed to grow for 3 weeks, with distilled water added as necessary. This ensured that each pot contained exactly 3 plants. Pots without plants were also set up as controls and treated the same as the pots with plants.

Pots were randomized and incubated in the greenhouse and were destructively harvested at 6 time intervals, on days 1, 3, 5, 10, 20 and 30 (to measure the changes to the pool sizes over time), with 3 replicates of each treatment removed from the greenhouse at each time point for analysis.

Table 4.4

Table showing total carbon and nitrogen concentrations in treatments (n=3)

Treatment	Total carbon	Total nitrogen
	---------mg g^{-1}---------	---------mg g^{-1}---------
Initial soil (control)	21.3 ± 0.36	1.66 ± 0.01
Unlabeled algae	482.9 ±0.47	95.5 ± 0.45
Labelled algae	477 ± 0.59	66.9 ± 0.05
Mixed algae[a]	478.5 + 1.99	79.0 ± 2.28
Mixed algae in soil[b]	22.3 ± 0.96	1.69 ± 0.04
Urea	0.10 ± 0.06	353.9 ± 0.14

[a] Labelled ^{15}N algae mixed with unlabeled algae in a 1: 1.5 (80 mg: 120mg) ratio
[b] Labelled and unlabeled algae were mixed in a 1:1.5 ratio and then added to 20g soil reflecting the application rate of 0.2 g algae per 500 g soil

4.2.3 N pools and ^{15}N isotope analyses

4.2.3.1 Wheat shoots

At harvest, aboveground biomass was cut just above the soil surface, washed to remove soil contamination and the fresh weights taken. The wheat shoots were oven dried at 70ºC for 3 days and their dry weights obtained. The shoots were then ground (using a heavy duty analytical mill, IKA –WERKE, Germany) into a fine powder to homogenise them and enable subsampling for determination of N content (16 mg ± 1) using a CN elemental analyser (Vario EL Cube, Langenselbold, Germany) and ^{15}N analysis (2 mg ± 0.5) using the Sercon PDZ Europa IRMS.

4.2.1.1 Bulk soil nitrogen

At the end of the experiment, after carefully picking out the roots (which were air dried and ground), bulk soil was mixed by hand to homogenise and then ~50 g ± 0.05 was subsampled. The subsamples were oven dried at 105 ºC overnight and homgenised again using an agate ball mill (Fritsch Pulverisette, Germany) before being analysed for total C and N concentrations and atom % ^{15}N as described in 4.2.3.1 above.

4.2.3.2 Microbial biomass C and N

Bulk soil samples were mixed to homogenize before samples were taken for measurement of microbial biomass carbon and nitrogen. Three subsamples (10 g each) of fresh soil were taken from the bulk soil – one subsample was used to determine moisture content by oven drying at 105 ºC to a constant weight. Microbial biomass C (C_{mic}) and N (N_{mic}) was extracted using the chloroform fumigation-extraction method (Brookes et al. 1985) from another subsample. One set of soil samples was exposed to ethanol free chloroform and fumigated for 24 hours in a desiccator. The chloroform was then removed by evacuation and the soils extracted with 0.5 M K_2SO_4 (1:5 soil: extractant ratio). The other set of soil samples served as a control and were just extracted with 0.5 M K_2SO_4 in the same ratio. The solution was placed on a shaker at 120 rpm for ~1 hour, after which the soil suspension was filtered using Whatman No. 42 paper and the extracts frozen at -20 ºC prior to analysis. Samples were analysed for C_{mic} and N_{mic} using a CN elemental analyser (Elementar VarioMICRO cube, detection limit of 0.03%) by subtracting the concentrations of total C and N in the fumigated samples from the controls and using a correction factor: $K_C = 0.45$ (Vance et al., 1987)

and K_N = 0.68 (Brookes et al., 1985). Concentrations were determined on a dry weight basis.

4.2.3.3 Inorganic nitrogen

A 2.0 M KCl solution was used to analyse inorganic N (NH_4^+ and NO_3^-) using 10g of soil in 40 mL KCl solution. The solutions were placed on a shaker for ~1hr and filtered using a prewettted Whatman No. 1 filter paper. The extracts were frozen at -20ºC prior to analysis. Upon thawing, samples were analysed using a Skalar San++ Continuous Flow Analyser (CFA), where the nitrate was measured colourimetrically using the cadmium reduction-diazotisation method (BS EN ISO 13395:1996) and the ammonium was measured colourimetrically using the salicyclate method (BS EN ISO 11732:2005). Atom% ^{15}N measurements of NH_4^+ and NO_3^- were determined using a modified version of the diffusion technique as described by Brooks et al. (1989) and Stark and Hart (1996). The KCl extract (15 mL) was transferred into scintillation vials. Fixed stainless steel hooks were added to the lids of the scintillation vials and filter paper discs were impaled onto the hooks. For samples to be diffused for $^{15}NH_4$, the filter discs were acidified with 10 µl of 2.5 M $KHSO_4$ and 200 mg of MgO was added into each scintillation vial and capped immediately. The contents were mixed carefully and allowed to sit for 6 days. After 6 days, the filter discs were removed and placed in a desiccator overnight to dry. For diffusion of $^{15}NO_3$, 0.1 mL of 30% Brij-35 was then added to the vials with 10 ml KCl and another 10 µl of 2.5M $KHSO_4$ added to fresh filter discs before weighing 400 mg of Devarda's alloy into vials. The vials were then capped, mixed and allowed to sit for another 6 days before being removed and dried in the desiccator.

Diffused standards were treated in the same way as samples, where 15 mL of 2M KCl were added to scintillation vials and then spiked with $^{15}NH_4NO_3$ stock solution to give concentrations ranging from 10 µg to 60 µg at 1 atom% ^{15}N. 10 µl of 2.5M $KHSO_4$ were pipetted onto filter discs and left for 60 days after adding 200 mg MgO and a further 6 days after adding 400 mg of Devarda's alloy. All dried filter discs were then wrapped in tin cups and analysed for ^{15}N using the IRMS.

4.2.4 Calculations

An IRMS was used to determine the ratio of ^{15}N:^{14}N and calculate the atom% ^{15}N abundance of samples using the following expression:

(1)

$$\text{Atom\% } 15\text{N} = \frac{15\text{N}}{14\text{N} + 15\text{N}} \times 100$$

^{15}N enrichment values are expressed in Atom Percent Excess (APE), which was calculated by subtracting ^{15}N measured in the specific N pool, from the ^{15}N in natural abundance of the control (Bagherzadeh et al., 2009; Braun et al., 2018).

4.2.4.1 ^{15}N calculations of budgets and pools

All N calculations were made in relation to soil in the plant pots (500 g). For determination of total N of a particular pool per pot, N concentrations in mg g^{-1} were multiplied by total soil mass per pot. For shoots, N concentrations were multiplied by total shoot dry weight to give total N amount per wheat shoot.

To calculate the N balance, changes in soil total N and total N outputs were subtracted from the total N inputs at the start of the experiment (Sainju, 2017).

4.2.4.2 ^{15}N recovery in plant and soil pools

The concentration of N in soil, plant, NH_4^+-N and NO_3^--N pools derived from the ^{15}N source once applied and mixed with the N in the system was calculated using the following equation, taking into account total N in pools:

(3)

$$\text{Total 15N content} = \frac{\text{atom\%15N}_{\text{excess}}}{100} \times \text{ total N in pool}$$

The following equation was used for estimation of % recovery of ^{15}N tracer found in the pools (Braun et al., 2018)

$$\text{Total } ^{15}\text{N content x } \frac{\text{excess } ^{15}\text{N initial abundance (at.\%)}}{100}$$

4.2.5 Statistical analyses

Statistical analyses were conducted using Graphpad Prism version 7.0c and R Studio version 3.5.1. The means of the replicates for the treatments ± standard error of the mean are presented as results. Differences in N total amounts of N in soil and plant pools between the control, algae and urea treatments in relation to duration of the experiment, were analysed using a 2-way analysis of variance (ANOVA) followed by a Tukey's post hoc test. The effect of time on total ^{15}N content and % recovery rates in the different N pools were also tested using one-way ANOVA followed by a Tukey's post hoc test. Differences were considered significant at a probability level of ($p<0.05$).

4.3 Results

4.3.1 Total N in pools (algae and control)

Total nitrogen in different pools was calculated per pot and is presented in Figure 4.1, showing the effect of the different treatments over the period of 30 days. The N pools in the treatments with *C. vulgaris* tended to follow those of the controls without added N. The effects of growing wheat on the soil, compared to the fallow pots had modest, but nonetheless important effects on the N pools and their dynamic changes over the 30-day experiment when supplied with algae or no added N. The plants in these treatments generally depleted the most available soil N pools (NH_4^+ and NO_3^-) over this time (Figure 4.1 a-g).

Effects of *C. vulgaris* on total N in wheat shoot increased significantly with time (Figure 4.1 a), from day 1, at 6.7 mg N and was highest on day 20 at 13.5 mg N. Similarly, shoot N also increased in the control treatments, with no significant differences observed between the *C. vulgaris* and control treatments and their effects on shoot total N.

Added *C. vulgaris* did not significantly change the amount of total N in the soil with wheat (Figure 4.1 b). Although the values seemed to fluctuate between days 1 and 30, there was no difference in total soil N between the days, and additionally, the effects on total N were similar to the control treatments, where no significant changes were observed. In pots without wheat (Figure 4.1 c), *C. vulgaris* had a more significant effect on soil total N, with the effect being more significant with time. Total soil N under *C. vulgaris* treatments decreased significantly ($p=0.01$) between day 1 and 20 from 903.7

mg N to 836.9 mg N (± 19.2 and ±20.5, SE). In control treatments, the opposite effect was observed, where total N in soil followed an increasing trend after day 3. There was however no difference between the two treatments and their effects on soil total N.

C. vulgaris significantly increased soil NH_4^+-N in wheat pots over time in comparison to the control (Figure 4.1 d). Following the addition of the alga, NH_4^+-N fluctuated significantly ($p<0.001$) between all the time points, increasing between day 1 and 3, and then dropping between 3 and 5, before increasing again by day 10 and dropping again by day 20. NH_4^+-N was also significantly higher than control treatments on day 1 ($p=0.04$), 3 ($p=0.004$), and 5 ($p=0.0001$). In pots without wheat (Figure 4.1 e), total NH_4^+-N was lower under *C. vulgaris* treatments in comparison to the controls, although with the large variations in the control treatments, there were no significant differences between the two treatments at the different time points.

Total NO_3^--N in pots with wheat increased after the addition of *C. vulgaris,* from 3.63 (±0.1) mg N in the initial soil (per pot) to 7.84 (±0.97) mg N on day 3 (Figure 4.1 f). After day 3, NO_3^--N decreased significantly ($p<0.001$) to 0.97 (±0.04) mg N on day 20. There was no significant difference in NO_3^--N between the *C. vulgaris* treatment and the control at any time point except for day 10. In pots without wheat (Figure 4.1 g), between day 1 and 20, total NO_3^--N was higher under *C. vulgaris* treatments compared to the control, with the differences being most significant ($p=0.04$) on day 10. Similar to the pots with wheat, soil NO_3^--N under *C. vulgaris* increased up until day 10, however unlike the pots with wheat where NO_3^--N decreased after this time point, in the fallow pots, amounts in the soil remained more or less the same till the end of the experiment.

The addition of *C. vulgaris* had very modest effects on the total N in the plant, soil and mineral N pools (NH_4^+ and NO_3^-) relative to the much larger total N added in the form of urea. In wheat pots, urea had a significantly larger effect on the total N in wheat shoots, soil, and inorganic N pools and both in the wheat and fallow pots. These effects were also consistent over time (Fig.4.2 a-g). In soil, plant and NH_4^+-N pools, both wheat and fallow pots amended with urea followed a similar trend, with significant increases in plant, soil and inorganic N pools in comparison to both the *C. vulgaris* and control treatments.

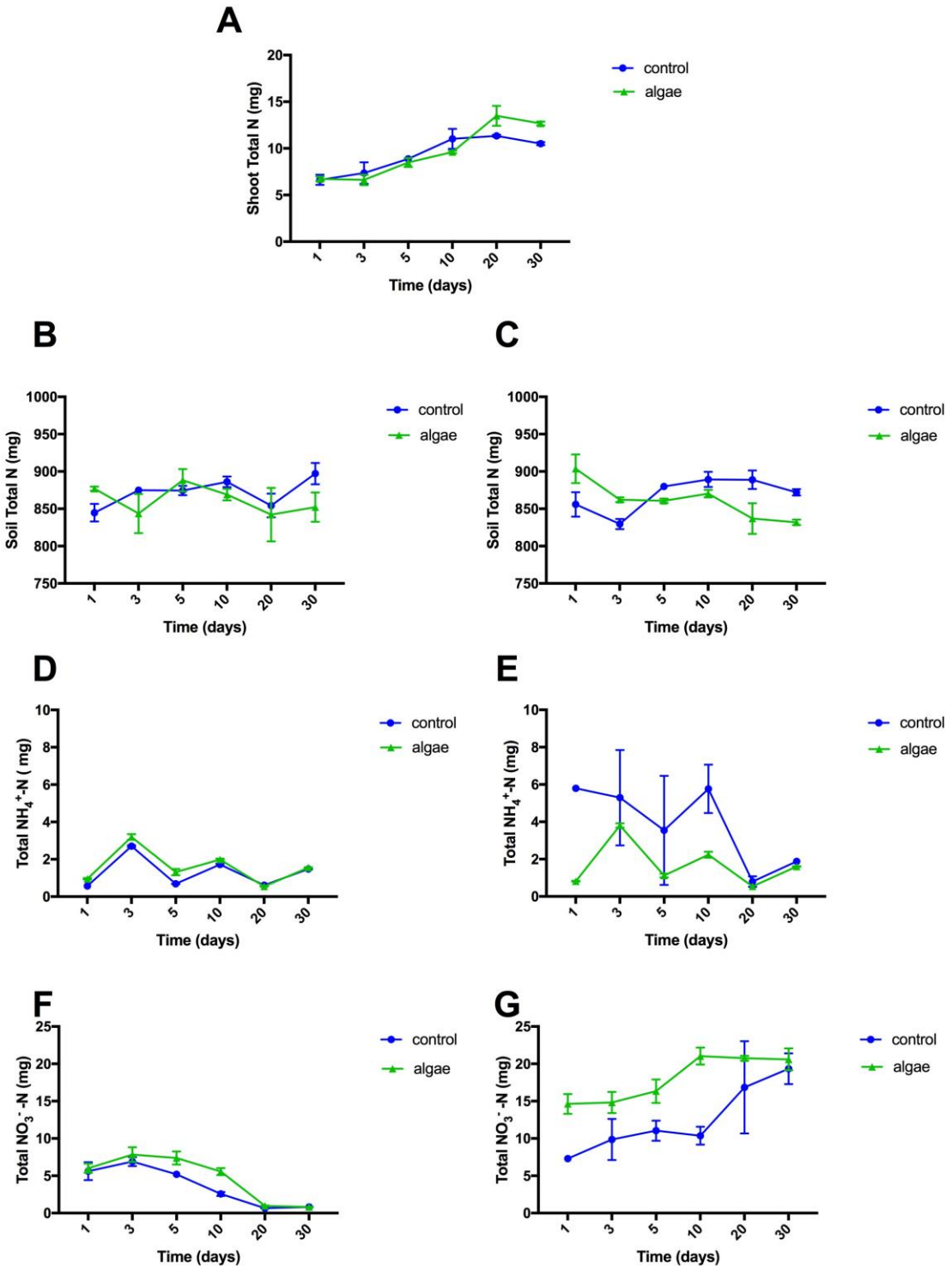

Fig. 4.1 Total N for control and *C. vulgaris* treatments in (a) wheat shoots (b) soil with wheat (c) soil without wheat (d) NH_4^+-N with wheat (e) NH_4^+-N without wheat (f) NO_3^--N with wheat (g) NO_3^--N without wheat. Data points represent the mean of 3 replicates, with bars representing S.E.

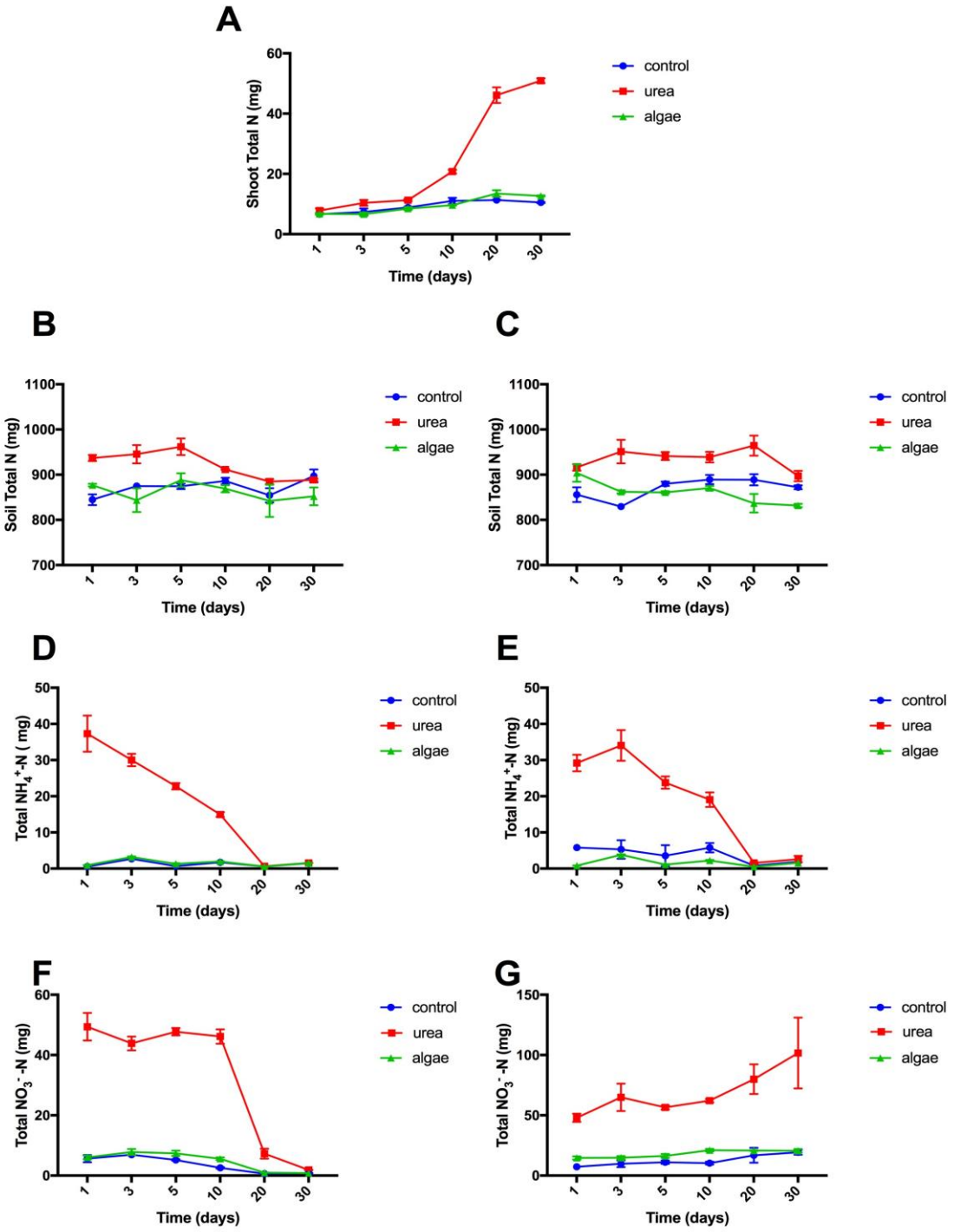

Fig. 4.2 Effect of urea and *C. vulgaris* (algae), and control (unamended) treatments on (a) wheat shoot N, (b & c) total soil N, (d & e) NH_4^+-N (f & g) NO_3^--N. Plates a, b, d and f refer to planted pots and c, e and g to fallow (unplanted) pots. Data points represent the mean of 3 replicates, with bars representing S.E.

4.3.2 Nitrogen balance and plant uptake

At the beginning of the experiment, 15.8 mg of algal nitrogen was added to soil, already containing 829.82 mg N/pot, giving a 1.9% increase in total N on day 0 compared to the control (Table 4-5). For urea, the total N amount added was 70.78 mg N, giving it a soil total of 900.60mg N/pot on day 0, an increase of 8.5% in total N compared to the control.

After 30 days, total N in soil with wheat increased under algae treatments by 16.38 mg N/pot (~2% increase) and in soil without wheat, a slightly smaller increase of 15.35 mg N/pot (1.8% increase) was observed (Table 4.5). In the control pots with wheat, soil total N increased by 42.12 mg N/pot (5% increase) after 30 days and in the control soils without wheat, a lesser increase of 39.46 mg/N (4.8%) was observed. The increase in soil total N under the control, both with and without wheat, were significantly higher ($p=0.02$) than in the algae treatments. For the urea treatment, after 30 days, soil total N increased by 20.85 mg N/pot (2.3% increase) in soil with wheat and by 34.17 mg N/pot (3.8% increase) in soil without wheat. There was no significant difference in total N increases between the urea and algal additions in soil with wheat. In soil without wheat however, both urea and controls soils had significantly higher total N after 30 days compared to the soils treated with algae.

Table 4.5

Changes in soil total N within the control with (n=18) and without wheat (n=12), algae with (n=18) and without wheat (n=18) and urea with (n=18) and without wheat (n=18), over the period of 30 days.

Treatment	**N input -soil**	**N output -soil**	**Δ soil total N**
	mg N/500 g soil	mg N/500 g soil	mg N/500 g soil
Control (wheat)	829.83±6.37	871.95±5.69	42.12±5.68
Control	829.83±6.37	869.29±7.04	39.46±7.04
Algae (wheat)	845.62±0.46	861.99±8.32	16.38±8.32
Algae	845.62±0.46	860.96±7.02	15.34±7.02
Urea (wheat)	900.60±0.03	921.45±8.07	20.85±8.07
Urea	900.60±0.03	934.77±7.74	34.17±7.74

The nitrogen uptake by plants is shown in Table 4.6. A larger number of replicates (n=18) were used due to the high variation in total N in the different pots (as a result of inherent soil heterogeneity) after each sampling time point. The control soils with no added nitrogen had a total 9.29 mg N/shoot at the end of the experiment. The urea treatments had 156% more nitrogen in the wheat shoot biomass than in the algae amended pots. In the algae amended pots, 9.60 mg/N was measured in the wheat shoot

biomass - a total of, 3% more nitrogen than control soils, assumed to be due to uptake of N from algal necromass. Calculation of the efficiency of utility of the algal nitrogen showed that only ~2% of the added amount of algal N was taken up by the plant, compared to 21.6% N taken up by the plant from the urea treatment.

Table 4.6

N input, total shoot uptake, and total balance of N within the control with (n=18) and without wheat (n=12), algae with (n=18) and without wheat (n=18) and urea with (n=18) and without wheat (n=18), over the period of 30 days.

Treatment	N-input	N output (plant uptake)	Proportion of added N in plant
	mg N/500g soil	mg N/plant	(%)
Control	0	9.29±0.50	---
Algae	15.81±2.28	9.60±0.68	1.98±2.8
Urea	70.78±0.03	24.55±4.26	21.6±5.6

4.3.3 Total ^{15}N content in different pools

This section focused on the algae and its ^{15}N contribution to the measured N pools. Comparing the values of ^{15}N retained in pots with wheat, the ^{15}N content measured in the wheat shoots were generally low over the duration of the experiment (Figure 4.3a). Low ^{15}N (0.02 mg ^{15}N) values were observed at the beginning of the experiment (day 1-3) before increasing from day 5 onwards, with the highest increase observed on day 20, where ^{15}N amounts were at an average of 0.5 mg ^{15}N, significantly higher ($p=0.0002$) than all the previous time points. The ^{15}N contents decreased slightly between day 20 and 30 to 0.4 mg ^{15}N but there was no significant difference between

these two time points. ^{15}N tracer amounts in soil with wheat plants (Figure 4.3b) were high at the beginning of the experiment, where on day 1 and 3 concentrations were 16.4 and 17.4 mg/pot ^{15}N tracer retained respectively. Concentrations decreased significantly ($p<0.0001$) thereafter down to 6.5 mg ^{15}N on day 5 and remained more or less the same, only decreasing slightly to 5.9 mg ^{15}N on day 20. $^{15}N\text{-}NH_4^+$ concentrations were very low on day 1 (Figure 4.3c), at 0.0003 mg ^{15}N, and increased to their highest concentrations of 0.03 mg ^{15}N before decreasing again on day 20 to 0.002 mg ^{15}N. There were no significant differences in $^{15}N\text{-}NH_4^+$ between any of the time points. $^{15}N\text{-}NO_3^-$ concentrations (Figure 4.3d) were low (0.1 mg $^{15}N\text{-}NO_3^-$) at the start of the experiment. Concentrations significantly increased ($p=0.0024$) on day 5 to 0.5 mg $^{15}N\text{-}NO_3^-$ and decreased significantly ($p<0.0001$) by day 20, to 0.02 mg $^{15}N\text{-}NO_3^-$.

Concentrations in pots without wheat (Figure 4.4a) were high on day 1 at 18.8 mg ^{15}N and similar to the soil in pots with wheat, concentrations decreased significantly ($p<0.0001$) after day 3 to 7 mg ^{15}N and remained more or less the same for the rest of the experiment. $^{15}N\text{-}NH_4^+$ varied over the 30-day experimental period. $^{15}N\text{-}NH_4^+$ concentrations (Figure 4.4b) on day 1 were at 0.001 mg $^{15}N\text{-}NH_4^+$, but then increased significantly ($p=0.0002$) by day 3 to 0.08 mg $^{15}N\text{-}NH_4^+$. On day 20, concentrations had decreased significantly ($p=0.0036$) from day 10 right down again to 0.002 mg $^{15}N\text{-}NH_4^+$. $^{15}N\text{-}NO_3^-$ concentrations in pots without wheat (Figure 4.4c) were the same as the concentrations in pots with wheat on day 1 (0.1 mg $^{15}N\text{-}NO_3^-$). Thereafter, $^{15}N\text{-}NO_3^-$ concentrations continued to increase significantly at each time point and were highest on day 30 at 1.2 mg $^{15}N\text{-}NO_3^-$.

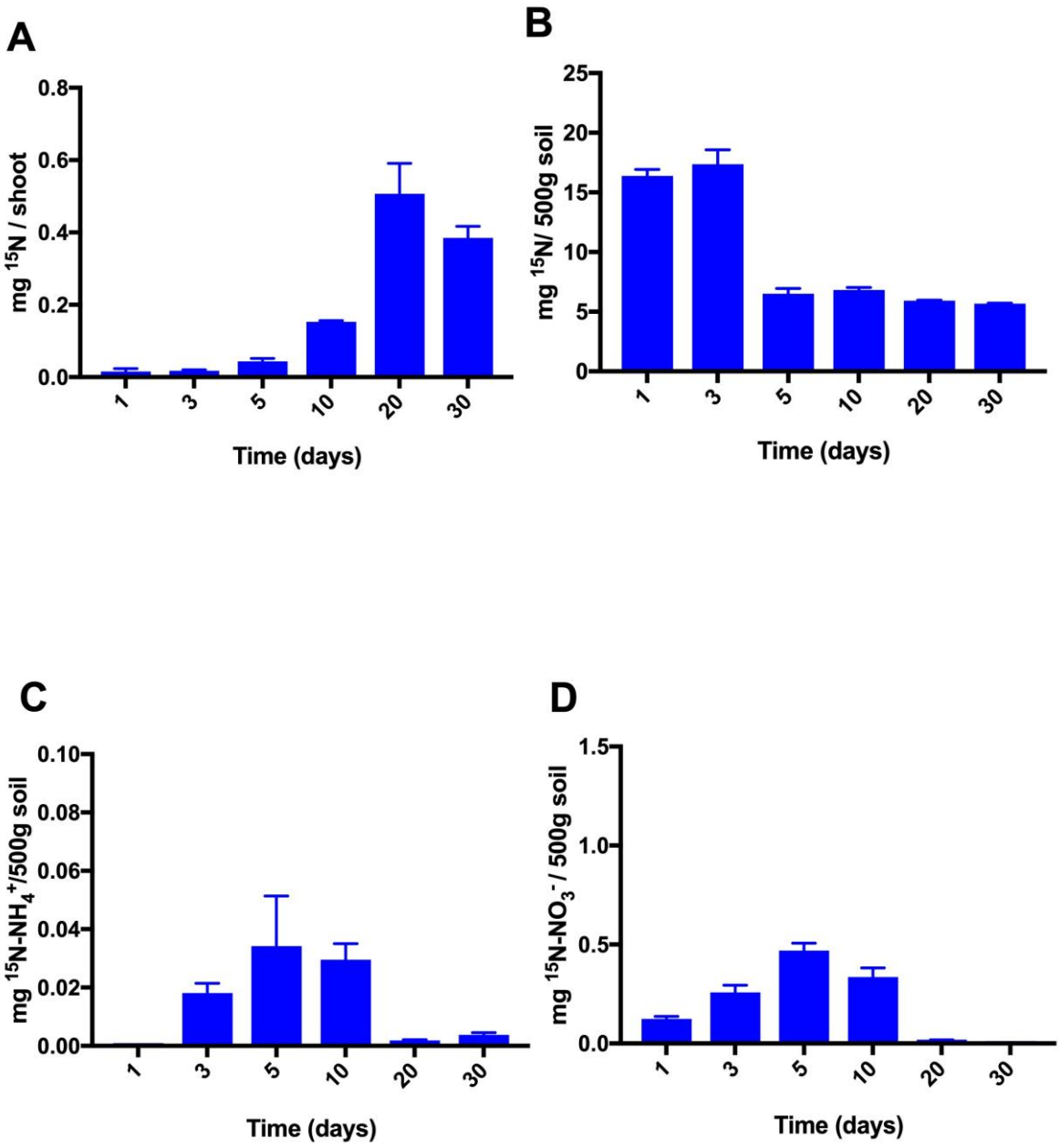

Figure 4.3 Total algal ^{15}N content in (a) shoots (b) soil with wheat (c) NH_4^+-N in soil with wheat (d) NO_3^--N in soil with wheat. Boxplots represent mean concentrations, with the bars on the columns representing S.E.

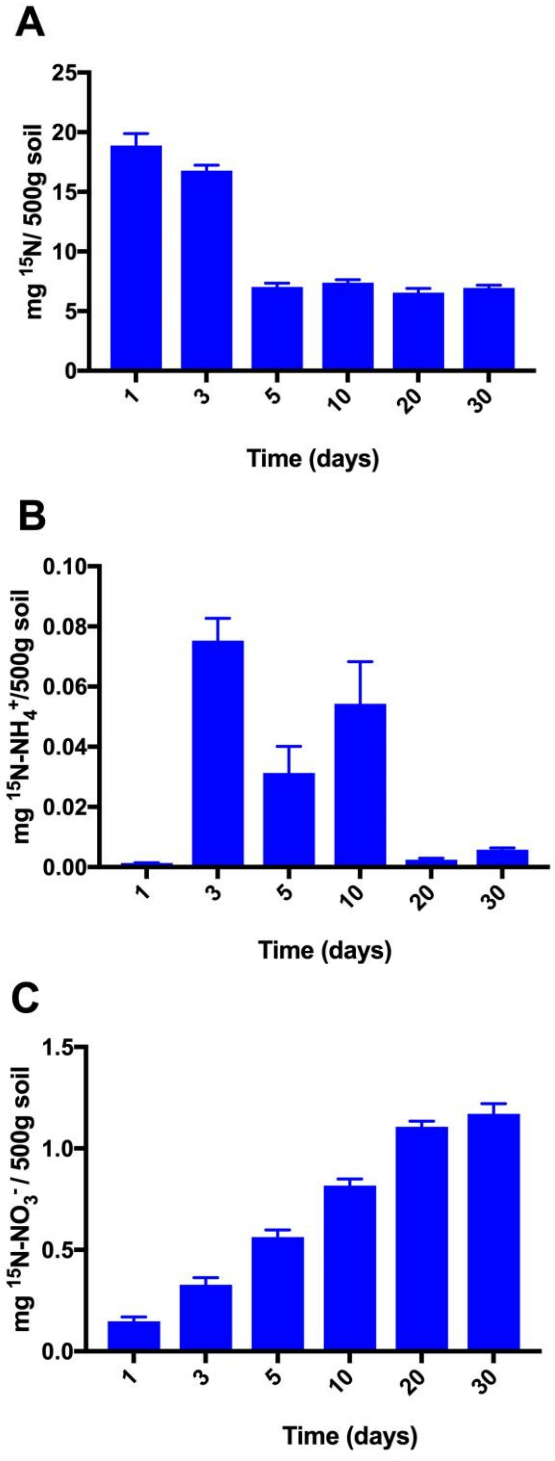

Figure 4.4 Total algal ^{15}N content in (a) soil without wheat (b) NH_4^+-N in soil without wheat (c) NO_3^--N in soil without wheat. Boxplots represent mean concentrations, with the bars on the columns representing S.E.

4.3.4 % recovery of ^{15}N from algal necromass in measured N pools

Table 4.7 compares the values of ^{15}N tracer recovered in pots with wheat. Highest ^{15}N recoveries were observed in the bulk soil, at the beginning of the experiment on day 3 at 31.4% and gradually decreasing to 10.3% by day 30. Recoveries on days 1 and 3 were significantly higher ($p<0.0001$) in comparison to recoveries at the other time periods.

^{15}N recoveries in shoots were very low, where at the beginning of the experiment, recovery rates of 0.03% were observed. This gradually increased to 0.3% on day 10, but was not significantly higher than on days 1 or 3. ^{15}N tracer recoveries then significantly increased ($p=0.0002$) between day 10 and 20, to the highest rates of 0.9% and then decreased slightly, but not significantly by day 30, to 0.7%. The amount of the added ^{15}N that was recovered in the soil extractable NH_4^+ was also very low, with no significant differences between any of the time points. Recoveries of 0.0006% were observed at the start of the experiment. This increased to 0.05% on day 5, before decreasing back down again to 0.007% by day 30. For ^{15}N recovered in soil extractable NO_3^-, the values increased gradually increased from 0.2% on day 1 to 0.5% on day 3. The values significantly increased ($p=0.0024$) to their highest recoveries on day 5 at 0.8% and then decreased significantly thereafter by day 30, to 0.004%.

In pots without wheat (Table 4.8) bulk soil still had the highest ^{15}N recovery rates, ranging from 11.8% to 34.1%. Recovery rates were highest on day 1 and then significantly decreased ($p<0.0001$) between day 3 and 5 from 30.3% down to 12.7%. Subsequently recovery rates did not vary much for the remaining period of the experiment, remaining at approximately 12%. ^{15}N-NH_4^+ recovery rates were lower than

bulk soil, ranging from 0.002% on day 1 to 0.01% on day 30, with the highest recovery rates observed on day 3 and 10 at 0.1%. Recovery rates decreased significantly ($p=0.0036$) between days 10 and 20 from 0.1% to 0.004%. ^{15}N tracer recovered in the NO_3^--N pool was also very low. Recovery rates on day 1 were 0.3% and continued to increase significantly up till day 30, where highest recovery rates were achieved at 2.1%

Table 4.7

Table showing recovery of ^{15}N tracer added as algal necromass to pots of soil with wheat (n=3)

N pool	Time days	^{15}N recovery %
Wheat shoot	1	0.03±0.01
	3	0.03+0.006
	5	0.08±0.01
	10	0.3±0.006
	20	0.9±0.2
	30	0.7±0.06
Bulk soil	1	29.6±1
	3	31.4±2.2
	5	11.7±0.8
	10	12.3±0.3
	20	10.7±0.07
	30	10.3±0.08
Soil NH_4^+-N	1	0.0006±0.0002
	3	0.03±0.006
	5	0.06±0.03
	10	0.05±0.01
	20	0.003±0.0004
	30	0.007±0.001
Soil NO_3^--N	1	0.2±0.02
	3	0.5±0.07
	5	0.8±0.07
	10	0.6±0.08
	20	0.03±0.004
	30	0.004±0.002

Table 4.8

Table showing recovery of ^{15}N tracer added as algal necromass to pots of soil without wheat (n=3)

N pool	Time	^{15}N tracer recovery
	days	%
Bulk soil	1	34.1±1.8
	3	30.3±0.9
	5	12.7±0.5
	10	13.3±0.5
	20	11.8±0.6
	30	12.5±0.4
Soil NH_4^+-N	1	0.002±0.0005
	3	0.1±0.01
	5	0.06±0.02
	10	0.1±0.03
	20	0.004±0.0009
	30	0.01±0.001
Soil NO_3^--N	1	0.3±0.04
	3	0.6±0.06
	5	1.0±0.06
	10	1.5±0.06
	20	2.0±0.05
	30	2.1±0.09

4.4 Discussion

The use of ^{15}N isotopic enrichment technique allowed for accurate tracing of algal derived N in the soil inorganic N pools, bulk soil and plant biomass over the time period of 30 days. In the previous chapter, soil NH_4^+-N and NO_3^- -N were shown to increase significantly 2 weeks after the addition of algal biomass under field conditions, therefore 30 days was chosen as an adequate time frame for assessing the degradation and subsequent uptake of algal-N by plants. The effects of algal additions were also compared against a conventional fertilizer (urea), as well as against a control.

C. vulgaris was shown to have modest effects on the soil and plant N pools. It was hypothesised that the algal N would contribute significantly to plant nitrogen uptake as a result of increased mineralisation due to its low C:N ratio (C:N=6), however only a small percentage (~2%) was taken up by the wheat plant. This was smaller in comparison to the control (5%) and urea (2.3%) treatments. These results were unexpected, especially with respect to the control where no nitrogen was added and can be explained by either nitrogen fixation (in the control pots) by free-living bacteria in the soils, and inherent errors involved in sampling and measuring soil N pools, including soil heterogeneity, extraction and analytical errors.

^{15}N tracer recovery in shoots increased with time till their peak at 20 days, with low recoveries at the beginning and significantly higher recoveries on day 10 and 20. Despite this, the amount of ^{15}N recovered in the shoots was lower in comparison to other studies. Mian and Stewart (1985) reported recovery rates of 35 and 40% in rice crop following the addition of *Anabaena* and *Nostoc* respectively (both species were

added in amounts to supply 40 mg N per 0.5 kg soil). Meanwhile, Tirol et al. (1982) reported a 28% recovery rate of ^{15}N *Nostoc* sp. in rice plants. However, the high recovery rates in these studies could be attributed to the nature of the experiment: i.e. the use of blue green algae once added to the soils in submerged water, experience growth of biofilms and therefore are able to contribute to soil nitrogen process through N-fixation (Thind and Rowell, 1999). In other studies using other organic substrate types, Holbeck et al. (2013) recorded 60% of added mushroom compost N (61.4 kg N/ha, C:N = 12.04) recovered in mustard cover crop after 42 days. Meanwhile Haynes et al. (1997) reported a 14% and 12% uptake of ^{15}N labelled grass/clover residue (150 kg N/ha, C:N = 17.9) by winter and spring sown wheat respectively and Heijboer et al. (2016) added organic amendments at the equivalent rate of 200 kg N/ha with varying C:N ratios ranging from 12 (lucerne silage) to 132 (wheat straw). It was shown that after 69 days, plant uptake was highest (71-78% ^{15}N recovery) following the addition of low C:N ratio amendments (lucerne silage) compared to recovery rates of 8-45% under high C:N ratio amendments (wheat straw). This high percentage recovery was attributed to the low C:N ratio (12) of the substrate. The C:N ratio of the algae biomass in our study was also low, yet low recovery rates in the plant were obtained. The lower recovery rates observed could be due to several processes, including leaching (potential loss through drainage), NH_4^+ volatilization, denitrification, and gaseous emissions (NO_X). Moisture is a key factor affecting nitrogen transformation rates (Rutting et al., 2011), with higher moisture content (80% field capacity) reported to increase soil N mineralisation (Guntinas et al., 2012). In this experiment, there was an attempt to control moisture, by maintaining the pots at 40% field capacity before harvesting. However, control soils towards the end of the experiment had more moisture in them, particularly the soils without wheat – this might have been affected by the weather as

there was less sun during the last days of the experiment, causing the soil to lose moisture less quickly. Also, the absence of plants meant there was no transpiration, leaving higher soil moisture content in the pots. Higher soil moisture content would have led to increase N mineralisation and therefore increased potential losses through denitrification and leaching of nutrients. Lower recovery rates could also be attributed to the uptake of inorganic N by roots and mycorrhizal fungi within roots, although the ^{15}N in mycorrhizal hyphae in soil and other soil microorganisms should have been detected in the soil total N pools (Rutting et al. 2011; Holbeck et al., 2013). Several authors have reported loss of >50% of ^{15}N tracer immediately (minutes after application (Morier et al., 2008)). No losses were measured from any of the pots however in fallow pots for example; a build-up of NO_3^- occurred, therefore increasing the possibility of denitrification. Braun et al. (2018) and Jones et al. (2013) have also recorded microbial uptake of both organic and inorganic compounds several minutes after the addition of ^{15}N tracer. Alternatively, the apparent losses of N may arise from sampling or measurement errors - the measurement of N in soil being notoriously difficult as a result of soil heterogeneity. It is also likely that wheat plant uptake and ^{15}N recovery rates were underestimated. Although roots were collected, the sampling process had compromising effects on the data: the roots were not washed or weighed after collection and therefore were mixed with soil when ground. Additionally, in an attempt to grind the roots, different methods were tried (using an agate ball mill and heavy duty analytical mill) as a result; some samples were lost in the process. Furthermore, because the root weights were not obtained, it was impossible to calculate the total amount of N. This process highlighted one of the advantages of conducting pilot studies to allow for such mistakes. Nevertheless, according to Heijboer et al. (2016) and Bagherzadeh et al. (2009), roots take up a large proportion of the total plant ^{15}N uptake, where when

studying the nitrogen partitioning of ^{15}N tracer, of the 19% recovered in the whole plants, the stems had the largest proportion of ^{15}N uptake (6.40%), followed by the coarse roots (5.75) and fine roots (4.33%).

The highest recovery of applied *C. vulgaris*-N was assimilated into the plant biomass after 20 days, suggesting that decomposition started within a few days following their incorporation into the soil. On day 20, wheat shoots had the highest ^{15}N tracer recovery, meanwhile in fallow pots on day 20, the lowest ^{15}N recovery was observed in the soil and NO_3^--N pools. This indicated that ^{15}N was actively being taken up by the plant around this time and most likely in the form of NO_3^- -N. Evidence of mineralisation could be seen in the increase in soil NH_4^+ at the start of the experiment as well as evidence of nitrification through the increase in NO_3^- between days 3 and 10 and a subsequent increase in plant uptake by day 20. It is also likely that soil microbes assimilated some of the NH_4^+ and NO_3^- produced (Mian and Stewart. 1985).

There was a higher utilisation of urea N compared to algal N, as was expected due to the fact that urea is a readily available N source for microbes and plants, whereas the *C. vulgaris* is composed of various macromolecules (Safi et al., 2014) including proteins and takes longer to decompose (through the action of microbes) which assimilate the by-products (e.g. amino acids) into their biomass (Ward, 2012). This process, and the greater amount of N provided in the urea treatment, therefore meant that plant N uptake was significantly higher in this treatment after only 5 days in comparison to adding *C. vulgaris* necromass. *C. vulgaris* necromass increased NO_3^- concentrations during the early stages (day 1-5) of decomposition before the incorporation of the algal N into the wheat shoot peaked. This was expected as

conventionally, once added to the soil, the algal N is decomposed and undergoes microbial transformation to produce inorganic fractions (Piatek, 2011). It is also possible that the high NO_3^- concentrations at the start of the experiment could have resulted from the dissolved organic matter leaching of water-soluble algal components (Scheibe and Gleixner, 2014). In fallow pots, total NO_3^- stabilised after 10 days, due to the fact that the absence of a plant meant that no nitrate was taken up. The same trend was not observed under the urea treatment however, where in fallow pots, NO_3^- continued to increase – signifying its build up in the system, which is usually associated with increased risk of leaching.

Total N in the soil did not change significantly at any time point throughout the experiment and was similar between the *C. vulgaris* and control treatments. The soil represents a significant N pool (Schimel and Bennett, 2004; Piatek, 2011) and the amount of added algal N was perhaps so small that this did not affect the already large pool of nitrogen already present in the soil. This is further supported by the fact that urea significantly increased the soil N pool as it was added at a higher N application rate. Thus highlighting the importance of algal quantity on impacting soil N pools. The soil retained the highest amount of ^{15}N tracer. Studies such as Nadelhoffer et al. (1995, 1999) have shown than soils are stronger sinks for ^{15}N addition than plants – and there was strong evidence for this in our experiment. Tirol et al. (1982) also observed that the soil retained more ^{15}N when ^{15}N from blue green algal nitrogen (57.3%) was added than when ^{15}N from ammonium sulphate (30.9%) was added. The high recovery rates in the soil could possibly be due to the rapid rates of consumption (immobilisation), as a result of biotic processes, following the application of algae. It was expected that the addition of algae will induce an increase in mineralization, due to its low C:N, however

the high recovery rates in the soil at the beginning of the experiment, gives strong evidence that the added algae induced immobilization of organic N and nutrient retention by the organisms. After this stage the added algae was then mineralized from day 5 onwards, transforming it into forms available for plant uptake. Perhaps a longer experimental timeframe would have allowed for the observation of differences within these pools i.e. an increase in mineralisation and subsequent increase in plant uptake.

Soil organic N represents another pool that could have provided further insights into the transformations of algal N as it acts as a pathway for the assimilation of organic matter into soil and as a short-term sink for nutrients (Templer et al. 2003; Zogg et al., 2000). Values for microbial biomass C and N are not presented. Microbial biomass C and N measured using an Elementar VarioMICRO cube for the few samples analysed, were below the machine detection limit of 0.3%, therefore further analysis was stopped. Obtaining microbial biomass values would have also provided a clearer picture in terms of whether and how much of the nitrogen was indeed being immobilised and retained by soil microbes. Haynes (1997) observed that 95% of ^{15}N recovered was stored in organic form and mineral forms of N had little ^{15}N.

4.5 Conclusions

The experiment attempted to understand the fate and distribution of algal N–input into different components of a soil-plant system. The results of the present study identified the soil as the major sink for algal N, demonstrating the high immobilisation capability of the soil microorganisms (Bagherzadeh et al., 2009). After 20 days, there was a significant increase in algal N uptake by the plant, establishing that the use of *C. vulgaris* as a nitrogen source. In order to achieve a more detailed understanding of the

fate of the ^{15}N tracer, it would have been useful to have ^{15}N isotope enrichment root as well at microbial biomass data to get a fuller account of the partitioning of *Chlorella*-N. In any case, the strong evidence of immobilization of algal N highlights the significant role of soil microbes in N cycling from organic N inputs. On this basis, the following chapter will describe a microbial community analysis, where changes in abundance and community structures of key bacterial taxa could provide useful insights into the degradation and cycling of *C. vulgaris* added to agricultural soils.

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Chapter V

Algal necromass effects on soil microbial communities

This chapter focuses on the effect of algal addition on soil bacterial communities, including their abundance and diversity.

5.1 Introduction

Biological processes govern many ecosystem functions, including the breakdown of organic matter and formation of soil aggregates (Sarker et al., 2018; Stark et al., 2008), plant growth as well as nutrient cycling (Leloup et al., 2018; Wang et al., 2018). Soils are very microbially diverse, with bacteria present in the highest numbers (2000-18000 per gram of soil), archaea being 10-fold less and fungi contributing the most to the total soil microbial biomass (Aislabie and Deslippe, 2013). Soil microbial diversity has reportedly reduced in the UK as a result of soil degradation (Robinson and Sutherland, 2002; Gregory et al., 2015).

Strong links have been observed between the addition of various types of organic matter (Mader et al., 2002) and increases in soil microbial richness and diversity compared to intensive agriculture with high mineral fertilizer and pesticide and fungicide use (Van der Heijden and Wagg, 2013). Furthermore, studies comparing organic versus inorganic fertilizer regimes have shown strong evidence of the positive impacts of organic fertilizers on soil biological processes, namely an increase in functional

diversity (particularly regarding carbon utilisation) of heterotrophs as well as an increase in species richness (Liu et al., 2007; Kolton et al., 2011; Val-Moraes et al., 2011; Nielsen et al., 2014; Hartmann et al., 2015). Examples of organic fertilisers used in these studies include biochar, compost, wheat straw and manure. Hartmann et al. (2015) reported an increase in species richness, decreased evenness in organic farming (farmyard manure and slurry application) compared to conventionally managed soils under exclusively mineral fertilization in a study conducted for more than two decades. The study also observed that organically fertilized systems were characterized by "specific microbial guilds" involved in the decomposition of complex organic materials such as manure and compost, whereas a scattered and functionally versatile microbial community of mainly oligotrophic organisms was observed in systems not fertilized by manure (Hartmann et al., 2015). Plant protection measures (herbicide, fungicide and insecticides) were however applied to the conventionally managed systems, and it is possible that the differences between the organic and conventional systems were greater than just the form of nutrients used. The addition of sewage sludge to soil plots from an experimental field in Brazil showed an increase in soil functional diversity where low application rates (25 kg N ha^{-1}) increased the abundance of certain phyla associated with organic matter breakdown, namely Proteobacteria, whereas the addition of high doses (200 kg N ha^{-1}) of sewage sludge increased the abundance of Epsilonproteobacteria (associated with sulfuric environments (Val-Moraes et al., 2011). Kolton et al. (2011) reported a significant difference in microbial community composition following the application of biochar to soil in a greenhouse experiment compared to control soils. The study reported a significant increase in relative abundance of the Bacteroidetes phyla of the Flavobacterium genus under biochar (30%) versus control (12%). This genus is known to mineralise diverse (carbohydrates,

proteins and polysaccharides) types of organic matter. Bastian et al. (2009), observed that the addition of 1.0 g dry wheat straw packed in soil cores (7 cm dia., 4.8 cm height, ~268.4 g dry soil) resulted in a succession of bacterial communities, during residue decomposition, comprised predominantly of copiotrohic taxa (*Pseudomonas* sp.) at the beginning (14 and 28 days) and an increase in relative abundance of oligotrophic organisms (Actinobacteria and Deltaproteobacteria) from 56 to 168 days.

A few studies have reported the effects of algal additions on soil microbes (Haslam and Hopkins, 1996; Haroun & Hussein, 2003; Alam et al., 2013, 2014; Grzesik et al., 2017). Alam et al. (2014), studied the effect of *Ascophyllum nodosum* extract on soil microbial activity in the root zone of carrots and in a separate study looked at the microbial community of strawberry as affected by the addition of *Ascophyllum nodosum* extract (Alam et al., 2013). They reported an increase in soil microbial activity/respiration (increase in CO_2 production), bacterial colony counts and rhizosphere microbial diversity (measured using the BIOLOG plates technique and subsequently calculating for substrate diversity) compared to the control treatments. These studies, despite providing useful information on overall microbial processes, failed to provide insight into changes at the community or species level (Chou et al., 2017). Additionally, these studies used old techniques of serial dilution and bacterial cultures on agar plates, which have several disadvantages, namely that only a small percentage of bacteria can be cultivated in laboratory conditions in monoculture. These methods have since been superseded by molecular sequencing techniques. A more recent study conducted by Wang et al. (2018) using 16S rRNA gene amplicon sequencing, investigated the responses of microbial communities following the addition of seaweed (*Sargassum horneri*) fertilizer. An increase in bacterial alpha diversity was observed, with an

observed OTU of 2830-3050 and Shannon indices of 9.45-9.90 significantly increased 60 days after the application of seaweed.

DNA-based microbial community analysis in relation to algal communities has been widely applied to investigate the dynamics of bacterial and algal community interactions in aquatic environments (Gonzalez et al., 2000; Park et al., 2015; Su et al., 2017). Similarly, there have been studies of algal and microbial communities within wastewater treatment systems (Limayem et al., 2018) and those responsible for algal degradation in anaerobic conditions i.e. highly eutrophic environments. According to Morrison et al. (2017), algae type is a significant factor in shaping microbial communities, where they compared the microbial communities responsible for the degradation of *C. vulgaris* and *A. nodosum* in a highly enriched, (with lake water and sludge from waste water treatment plant) anaerobic environment. Results showed a distinction in bacterial communities responsible for the degradation of both species, with the phyla Bacteroidetes, Firmicutes and Delta-Proteobacteria being the most abundant in the *C. vulgaris* microcosms (120 ml serum bottles), meanwhile the community enriched on *A. nodosum* comprised mainly of Firmicutes (>70%). The study attributed this to the structural and chemical complexity of the substrates to be degraded therefore selecting for a wide range of organisms, each responsible for the degradation of specific substrate within the algal biomass (Morrison et al., 2017).

To date, studies on how algal biomass turnover in soils impacts on the microbial community in agricultural soils are relatively scarce. This is despite the fact that algae have been shown to be useful soil amendments by increasing soil nutrients, including nitrogen and phosphorus (Alobwede et al., 2018; Grzesik et al., 2017; Mulbry et al.,

2005), as well as the established role of microbes in nutrient cycling from organic matter (Leloup et al., 2018). The impact of *C. vulgaris*, as an organic fertilizer, on soil microbial communities has never been explored. The previous chapter showed high soil retention of ^{15}N from algal biomass leading to the conclusion that once added to the soil, the algae is immediately immobilized by soil microorganisms within the first 3 days, before subsequently being mineralized into plant available inorganic forms, highlighting the important role of soil microorganisms.

The aim of this chapter was to examine the impact of algal N on soil microbial communities. The overall objectives of this study were to assess the difference in microbial communities following algal addition with comparisons made with urea, a typical inorganic fertiliser, to a representative arable soil, to characterize the shifts in microbial communities and to identify bacterial taxa responsible for the degradation of algal N. We hypothesized that algae would increase soil microbial diversity as the additional carbon source will stimulate an increase in bacterial taxa responsible for degradation and mineralisation of the algal biomass, namely Bacteroidetes, Firmicutes and Deltaproteobacteria (Morrison et al., 2017).

5.2 Materials and methods

5.2.1 Site description and experimental design

Plant growth and sampling

Soil was collected in April 2017, from Wise Warren, at Spen Farm, Tadcaster, England (longitude 1°20’32.9” W, latitude 53°51’40.7” N), an agricultural field that had been

cultivated and cropped for the past 20 years. Soil pH was 7.4, total C 21.3 mg g^{-1}, total N, 16.6 mg g^{-1}, and ammonium (NH_4^+-N) and nitrate (NO_3^- -N) concentrations were 1.48 and 7.27 mg kg^{-1} respectively.

The plant used was spring wheat (*Triticum aestivum* L.). The seeds were pre-germinated and transplanted to pots with 500 ± 1 g soil and allowed to grow for 3 weeks before treatments (0.2 g of *C. vulgaris* and urea were added). Pots without wheat were also used as controls and given the same treatment as in the wheat-sown pots. See section 4.2.1 for site description and detailed experimental design.

5.2.2 Soil sampling and DNA extraction

As described in the previous chapter, section 4.2.2.2, the soil was sampled at six different time points. However for the analysis of bacterial communities, soils from 3 different time points were used: day 3, 10 and 30. After removing the wheat shoot and picking out all the roots, the bulk soil was mixed by hand to homogenise and then sub sampled (~4 g) from the bulk soil in the pots, flash frozen using liquid nitrogen (LN_2) and subsequently stored at -80°C for DNA extraction.

Before DNA extraction, ~4 g of soil was ground using a mortar and pestle to homogenise. The mortars and pestles were sterilized by autoclaving, heating in at oven at 200 °C and allowed to cool before cleaning with 70% ethanol. In a laminar flow cabinet, LN_2 was then poured in the mortars, followed by the soil sample, and subsequently ground for 5 min. This was repeated 3 times, before the sample was transferred to a 1.5 mL Eppendorf tube. The same procedure was followed for all

samples and the samples were then frozen at -80 °C in preparation for DNA extraction. Soil DNA was extracted from 0.25 g soil using the MoBio® PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), following the manufacturer's instructions. The final 100 µl of eluted DNA was then checked for quality using 1% agarose gel electrophoresis collected and stored at -20 °C.

5.2.3 PCR amplification of DNA

5.2.3.1 Amplicon PCR

MiSeq Amplicon Sequencing of the extracted soil DNA was carried in a laminar flow cabinet using the KAPA HiFi PCR kit (Kapa Biosystems, Inc., Boston, MA, USA) and the Mastermix shown in Table 5.1, using 799F (5'-AACMGGATTAGATACCCKG-3') (Chelius and Triplett, 2001) and 1193R (5'-ACGTCATCCCCACCTTCC-3') primers (Bodenhausen et al., 2013). A negative control without DNA template was also included with every batch of PCR reaction. The PCR amplification was carried out using a Life-ECO thermal cycler (Bioer Technology Co., China) under the conditions of 94 °C for 2 mins initial denaturation; 27 cycles of denaturing at 94 °C for 30 seconds, annealing at 60 °C for 30 secs and elongating at 72 °C for 30 secs, with a final elongation of 72 °C for 1min and then held at 10 °C. The presence of bacterial DNA was detected by running a 1% (w/v) agarose ethidium bromide gel using 4 µl of product. Following this stage, a further 3 amplicon PCRs were carried out in order to reduce amplification biases, and the products 9 µl from each PCR pooled together. After each of the PCRs, a 1% (w/v) agarose ethidium bromide gel using 4 µl of product was carried out.

Table 5.1
Master mix for amplicon PCR

Reagent	**Volume (µl)**
PCR Grade (nuclease free) water	12.8
KAPA HiFi Fidelity Buffer (x5)	4.0
$MgCl_2$ (25mM)	0.4
KAPA dNTP Mix (10mM each)	0.6
Amplicon PCR Forward Primer (10 µM)	0.4
Amplicon PCR Reverse Primer (10 µM)	0.4
KAPA HiFi HotStart DNA polymerase (1U/µl)	0.4
DNA extract	1.0
TOTAL	20.0

5.2.3.2 PCR cleanup of DNA

The pooled triplicate PCR products were than cleaned using bead purification, where 24 µl of the pooled product was transferred to a well of a new 96 well PCR plate, this was done for all 51 samples. AMPure XP beads (Beckman Coulter Inc., USA) were vortexed for 30 seconds (to ensure even dispersion) and 20 µl added to each sample in the well plate, before pipetting the entire volume up and down 10 to ensure adequate and more reproducible mixing. The mixed products were then incubated at room temperature for 5 mins before being placed on an Agencourt SPRI Super Magnet Plate (Beckman Coulter Inc., USA) till the supernatant cleared (~2 mins). The supernatant (~50 – 60 µl) was then carefully removed and discarded using a multichannel pipette,

taking care not to touch the ring of separated magnetic beads. The beads were then washed by adding 200 µl of 80% (v/v) ethanol to each well and the plate left to stand for 30 seconds, before removing and discarding the ethanol. This was repeated once more, and the beads then left to air dry for 10 minutes. The plate was then removed from the magnetic stand and 52.5 µl of 10 mM Tris (pH 8.5) added to each well and mixed gently by pipetting up and down 10 times to resuspend the beads, before leaving at room temperature for 2 minutes. The PCR well plate was then placed back onto the magnetic stand and left to sit for ~ 2 mins, before transferring 50 µl of the clear supernatant to clean 1.5 mL Eppendorf tubes and subsequently frozen at -20 ºC.

5.2.3.3 Indexing PCR

A mastermix was made according to Table 5.2, in separate PCR tubes, with a negative control prepared for each set of PCR reactions. Each reaction was then pipetted up and down 10 times to mix, before centrifuging at 1000 g at 20 ºC for 1 minute. PCR amplification was then carried out using a program of 95 ºC for 3 mins initial denaturation, 8 cycles of denaturing at 95 ºC for 30 seconds, annealing at 55 ºC for 30 seconds and elongating at 72 ºC for 30 seconds, with a final elongation of 72 ºC for 5 minutes and then held at 10 ºC. A 1% (w/v) agarose ethidium bromide gel using 4 µl of product was carried out to check for the presence of DNA. The Index PCR product was then cleaned again using the bead purification method described in section 5.2.3.2, except this time, 45 µl of index PCR product and 50.4 µl of AMPURE XP beads, with 25 µl of supernatant collected at the end. The supernatant was transferred to 1.5 mL Eppendorf tubes and frozen at -20 ºC.

Table 5.2
Mastermix for Index PCR

Reagent	**Volume (µl)**
PCR Grade (nuclease free) water	21.5
KAPA HiFi Fidelity Buffer (x5)	10.0
MgCl2 (25mM)	1.0
KAPA dNTP Mix (10mM each)	1.5
KAPA HiFi HotStart DNA Polymerase (1U/ µl)	1.0
Nextera XT Index Primer 1	5.0
Nextera XT Index Primer 2	5.0
DNA	5.0
Total	50.0

5.2.3.4 Quantification, dilution and pooling

Prior to DNA quantification, the DNA concentrations of a few samples were measured using the NanoDrop to get an approximation of their concentration, making sure that they fell within a standard curve. For more accurate quantification, the QuantiFlour® dsDNA Dye was used. The TE buffer (provided in the kit) was diluted (1ml TE buffer: 19 ml ddH_2O) and filtered using a 0.2 µm syringe filter to eliminate any particles that could cause interference. The QuantiFlour® dsDNA Dye was then diluted 400-fold using the filtered TE buffer and wrapped in foil as it is light sensitive. In a black 96 well microplate, 2 µl of DNA samples were added, followed by 200 µl of the diluted QuantiFlour solution. The standards were prepared by dilution of the 100µg/ml DNA standard provided with the kit and a standard curve (0 to 200 ng/ul) prepared. The microplate was wrapped in foil and kept at room temperature for 5 minutes. The

fluorescence of each well was then measured using a FLUOStar OPTIMA spectrofluorimeter (BMG LabTech, USA) (excitation at 485nm, fluorescence emission measured at 545nm).

All 51 DNA samples, each with a final concentration of 20nM, were then pooled together (2 µl) each to produce a single library and sent off on dry ice to Earlham Institute, Norwich Research Park, Norwich for Illumina MiSeq Amplicon sequencing.

5.2.4 Data processing/bioinformatics

The demultiplexed sequencing data files were downloaded from the sequencing centre and the overall quality of the 16S Illumina Miseq sequencing forward and reverse reads were checked using Fast QC (Babraham Bioinformatics). Using a QIIME pipeline (Caporaso et al., 2010), the forward and reverse reads were then merged with quality filtering using USEARCH8.1 (Edgar, 2010) to remove low quality reads (minimum read length of 350 bp for prokaryotes). The amplicon primers were then stripped out from the reads and chimeras subsequently removed. The quality-filtered sequences were clustered into operational taxonomic units (OTUs), where sequences that were 97% identical were clustered together. Taxonomy assignment to the OTU was carried out using the Ribosomal Database Project (RDP) database (Wang et al., 2007) version 16. The OTU table along with the taxonomy tables were then used for downstream analysis using Rstudio.

5.2.5 Statistical analysis

Statistical analysis was performed in R studio version 3.5.0, using the phyloseq package (McMurdie and Holmes, 2013). OTU counts and associated taxonomy tables, in the form of biom files, along with mapping files with details of samples, were read into phyloseq. A rarefaction analysis was carried out using the Operational Taxonomic Units (OTUs) at 97% similarity, to observe sampling efficiency of each sample. The richness and diversity of total soil microbial communities for each treatment was measured using the Shannon and Simpson's indices. One-way ANOVA followed by Tukey post hoc tests were used to assess differences between treatments, as well as the effects of wheat at different time points, with statistically significant differences reported at probability levels of $p<0.05$. Percentage relative abundance of OTUs was plotted showing the OTUs present in the different treatments at different time points at the phyla and class level. A 2-way ANOVA was carried out to test for any significant differences in % relative abundance between the difference treatments. Differences were considered statistically significant at probability levels of $p<0.05$.

5.3 Results

5.3.1 Diversity of soil microbial communities

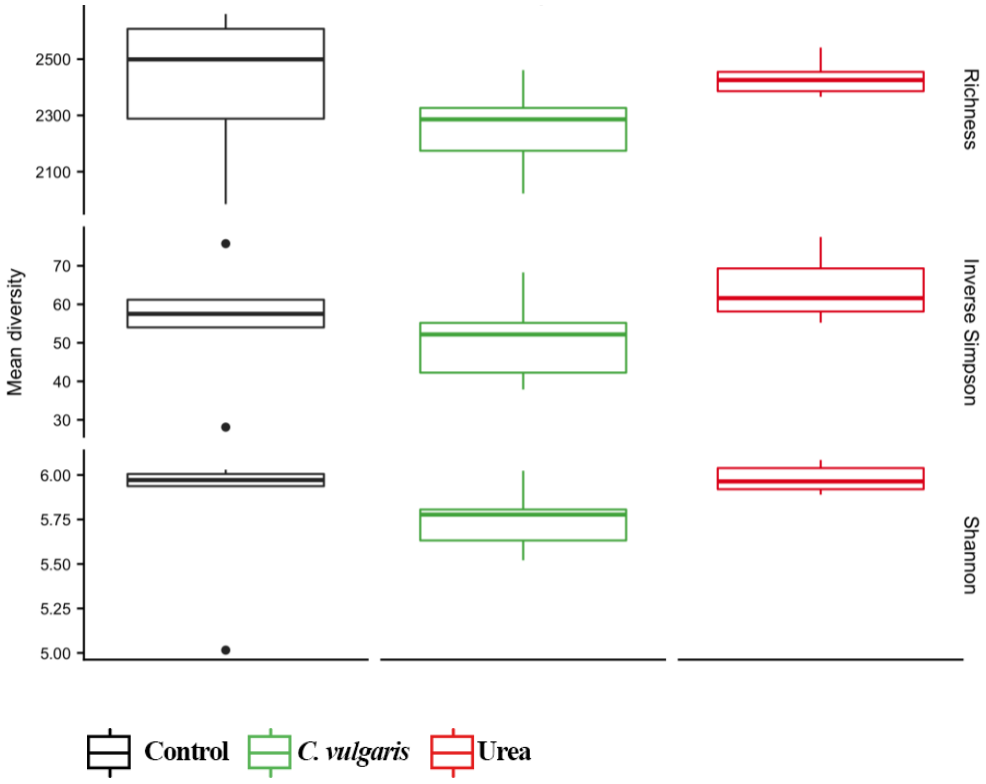
A total of 3,201,159 sequence reads were identified after filtering and clustering the data, with a minimum read of 21956 and a maximum of 97802 sequence reads. Alpha diversity indices including observed OTU richness, inverse Simpson and Shannon index (Simpson, 1949; Shannon, 1948) were used to assess the impact of the different treatments on the diversity of microbial communities at different time points throughout the experiment. Both the Shannon and Simpsons indices provide information regarding

the microbial diversity by assessing species richness (number of different species in a community), evenness (similarity in numbers of each species in an environment) as well as the relative abundances of the different species (Kim et al., 2017). An increase in species richness and evenness leads to an increase in diversity. The Shannon index places greater emphasis on species richness, where its value increases with an increase in the number of species. The Simpson index focuses more on species evenness, where the index reveals the chances of two individuals of the same species being randomly chosen and its value increases as the diversity decreases (Kim et al., 2017).

The diversity of the soil microbial communities was examined at different time points. Three days after the start of the experiment, no significant differences were detected in microbial diversity between any of the treatments (Fig 5.1 a). The control soils had the highest overall species richness at ~2500 OTUs, while the *C. vulgaris* amended soils had a total of 2300 OTUs and urea treated soils had ~2400 OTUs. The soils under the different treatments were also similar in the relative abundance and number of species present. After 10 days, no significant differences were detected in microbial diversity between all three treatments (Fig 5.1 b). Species richness increased under both treatments and the control after ten days, with the control again having a higher number of OTUs, followed by the urea treatment and then the *C. vulgaris* treatment. All three treatments were also similar in species evenness. At the end of the experiment, after 30 days, the *C. vulgaris* amended pots had a significantly higher microbial diversity compared to the control and urea treated pots (Fig 5.1 c), with a significantly higher even distribution and relative abundance of species (inverse Simpson, $p=0.001$) and (Shannon, $p=0.01$) in comparison to both control and urea amended pots.

Figure 5.2a compares the effects of wheat on microbial diversity. Three days after algal necromass and urea were added to soil in pots with wheat and without wheat (fallow treatment), results showed no significant differences in microbial diversity between the two differing potting systems. The difference in species richness in wheat and fallow pots was also the same after 10 days (Fig 5.2 b). However, there was a significant difference ($p=0.03$) in evenness, where pots with wheat had significantly higher species evenness than pots without. After 30 days (Fig 5.2 c), there was no significant difference in the microbial diversity in all wheat and fallow pots.

A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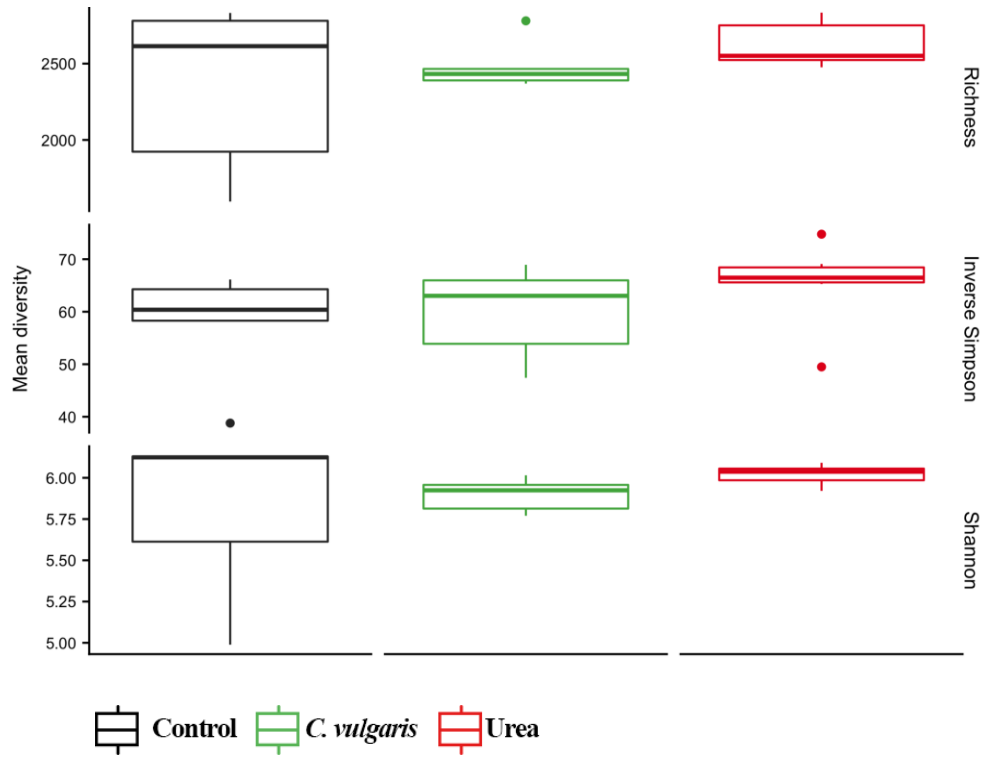
Richness
Inverse Simpson
Shannon
Control
C. vulgaris
Urea

B


Mean diversity
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C. vulgaris
Urea

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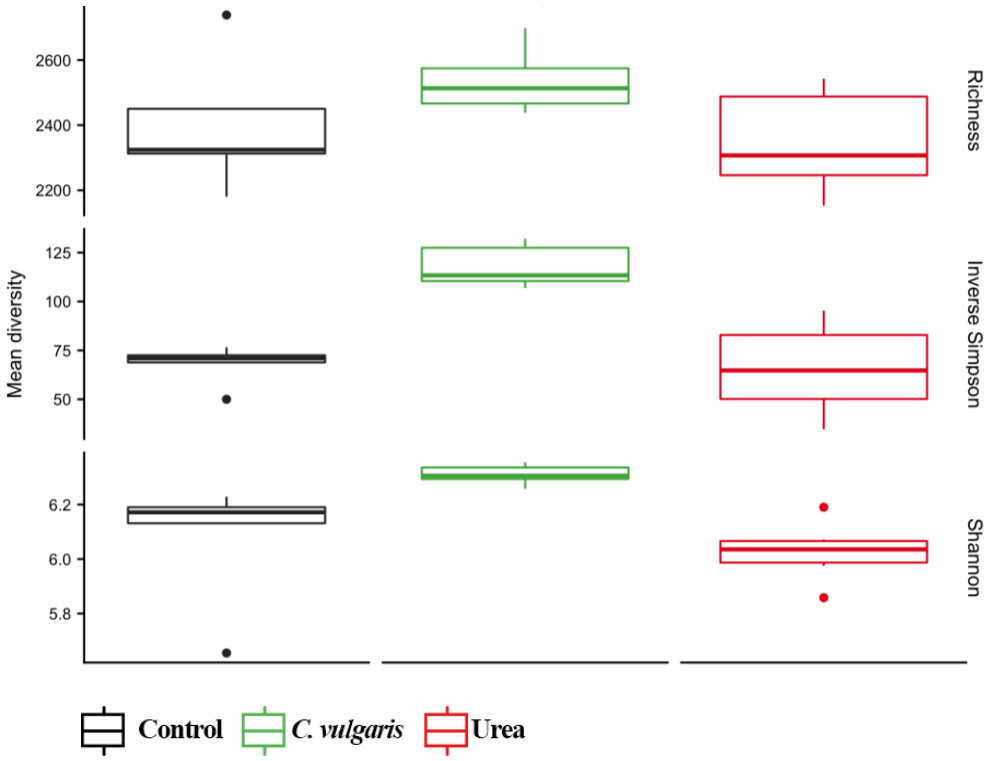
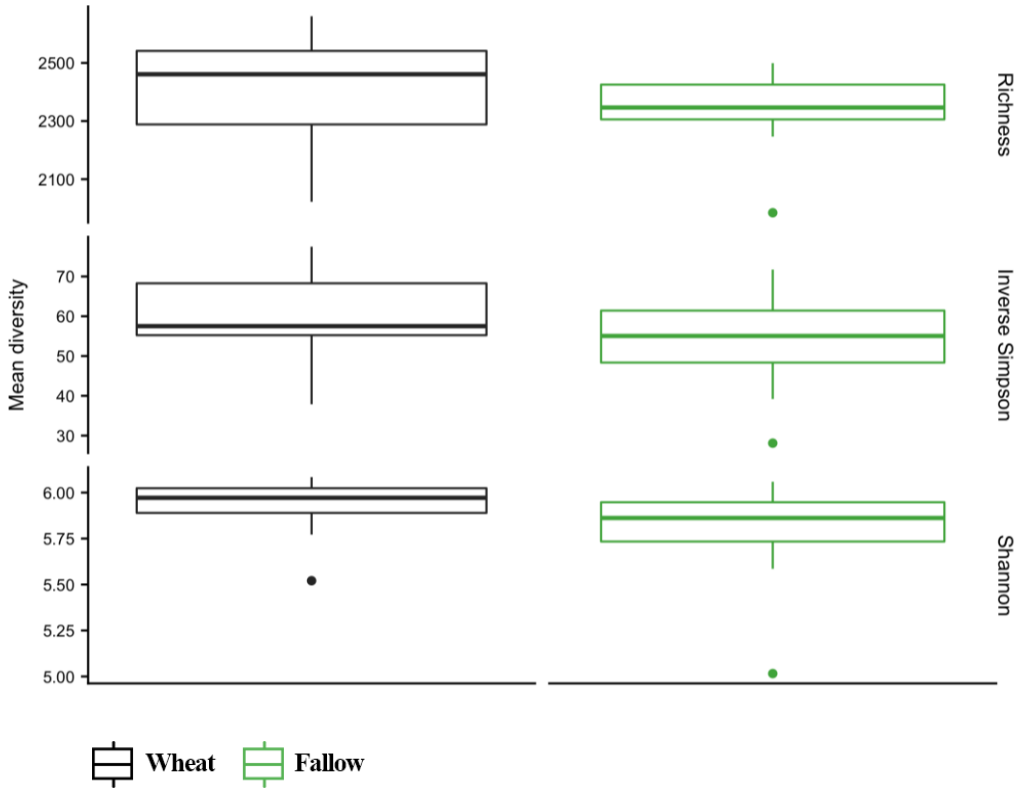


Figure 5.1 Box and whisker plot showing species richness, inverse Simpson and Shannon diversity indices for the different treatments after 3 days (a), 10 days (b) and 30 days (c). Boxes represent the interquartile range, with the middle line representing the mean and the dots representing the outliers.

A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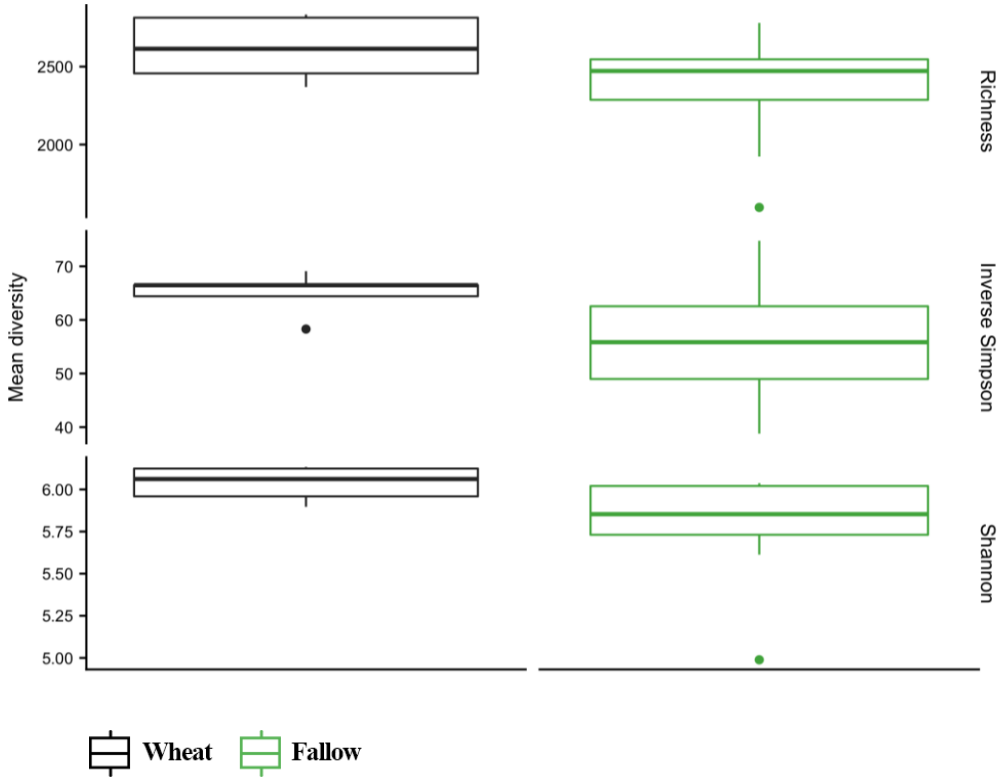
Richness
Inverse Simpson
Shannon
Wheat
Fallow

B


Mean diversity
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Richness
Inverse Simpson
Shannon
Wheat
Fallow

C

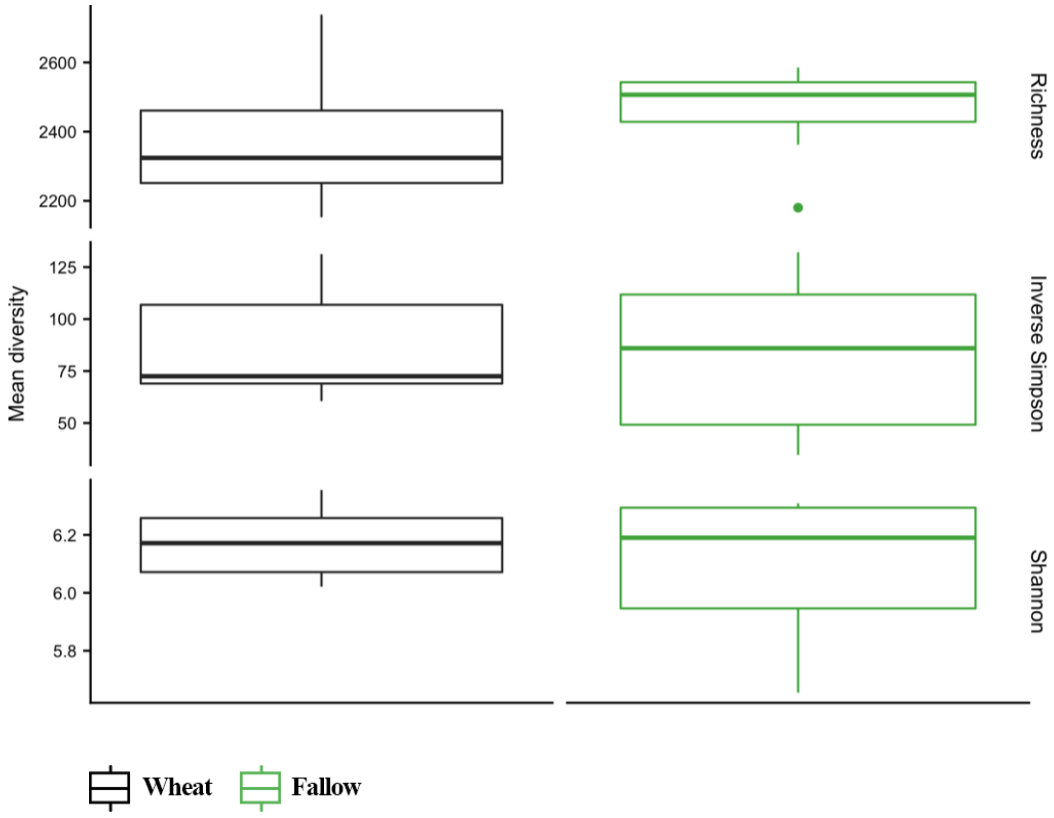


Figure 5.2 Box and whisker plot showing species richness, inverse Simpson and Shannon diversity indices in wheat and non-wheat pots after 3 days (a), 10 days (b) and 30 days (c). Boxes represent the interquartile range, with the middle line representing the mean and the dots representing the outliers.

5.3.2 Taxonomic composition

Table 5.3 shows a list of all 38 phyla that were identified, and Figure 5.3 shows the identified phyla with a relative abundance of more than 1%. The most abundant phylum was Proteobacteria (33.05%), followed by Actinobacteria (22.66%), Firmicutes (20.28%), Bacteroidetes (13.26%), Acidobacteria (4.10%), Verrucomicrobia (2.11%) and Chloroflexi (1.31%).

Table 5.3
List of all identified bacterial phyla

1) Fibrobacteres	11) OD1	21) Tenericutes	31) OP11
2) Thermotogae	12) MVP-21	22) Armatimonadetes	32) Acidobacteria
3) Chlorobi	13) [Thermi]	23) TM6	33) Cyanobacteria
4) Firmicutes	14) Verrucomicrobia	24) WS2	34) Chlamydiae
5) OP3	15) Bacteroidetes	25) BH180-139	35) GN02
6) Chloroflexi	16) BRC1	26) Gemmatimonadetes	36) GN04
7) Actinobacteria	17) WS4	27) Kazan-3B-28	37) WPS-2
8) FBP	18) WS3	28) Elusimicrobia	38) NKB19
9) gal15	19) TM7	29) Proteobacteria	
10) Nitrospirae	20) Planctomycetes	30) Spirochaetes	

The relative abundance of Proteobacteria was significantly higher under *C. vulgaris* treatments throughout the duration of the experiment (Figure 5.4). After 3 days,

Proteobacteria dominated the *C. vulgaris* amended soil in pots with wheat and had a significantly higher abundance (38.15%) compared to the control pots (30.28%, p=0.03) and urea amended pots (29.63%, p=0.02). The relative abundance of Proteobacteria under *C. vulgaris* treatments remained the same more or less the same for the duration of the experiment, only decreasing slightly on day 10 to 35.91% and increasing slightly again by day 30 to 37.64%. Nonetheless, a significantly greater abundance of this phylum was found under *C. vulgaris* treatments compared to the control and urea treatments, on both days 10 and 30. In fallow pots, a similar relative abundance of Proteobacteria was observed under *C. vulgaris* treatments on days 3 (36.21%), 10 (39.53%) and 30 (36.77%). These relative abundances were similar to the urea-amended pots on the 3 and 30 of the experiment, but significantly higher (p=0.0009) on day 10 and additionally were higher than the abundances in the control pots on day 3 (p=0.01).

Relative abundance of Actinobacteria and Firmicutes, the next most abundant phyla, decreased after 3 days from 24.07% and 23.03%, to 22.05% and 17.66% respectively after 30 days; this decrease was also reflected in the *C. vulgaris* treatments in both pots with and without wheat. On day 30, the relative abundance of *Firmicutes* decreased significantly (p<0.0001) under the *C. vulgaris* treatments added to pots with wheat in comparison to both control and urea treatments. The same trend of a significantly lower Firmicutes abundance on day 30 was also observed in fallow pots.

The relative abundance of Bacteroidetes increased from 12.81% after 3 days to 13.51% after 30 days under experimental treatments and control, including with *C. vulgaris* added to wheat and fallow pots. This increase was significantly higher under *C. vulgaris*

treatment than in the controls on both day 10 ($p=0.02$) and 30 ($p=0.007$) in wheat pots, and no differences in abundance observed between all 3 treatments in the fallow pots.

The relative abundance of Acidobacteria increased under both treatments and control from days 3 to day 30. There were however no significant differences in the both wheat and fallow pots between any of the treatments.

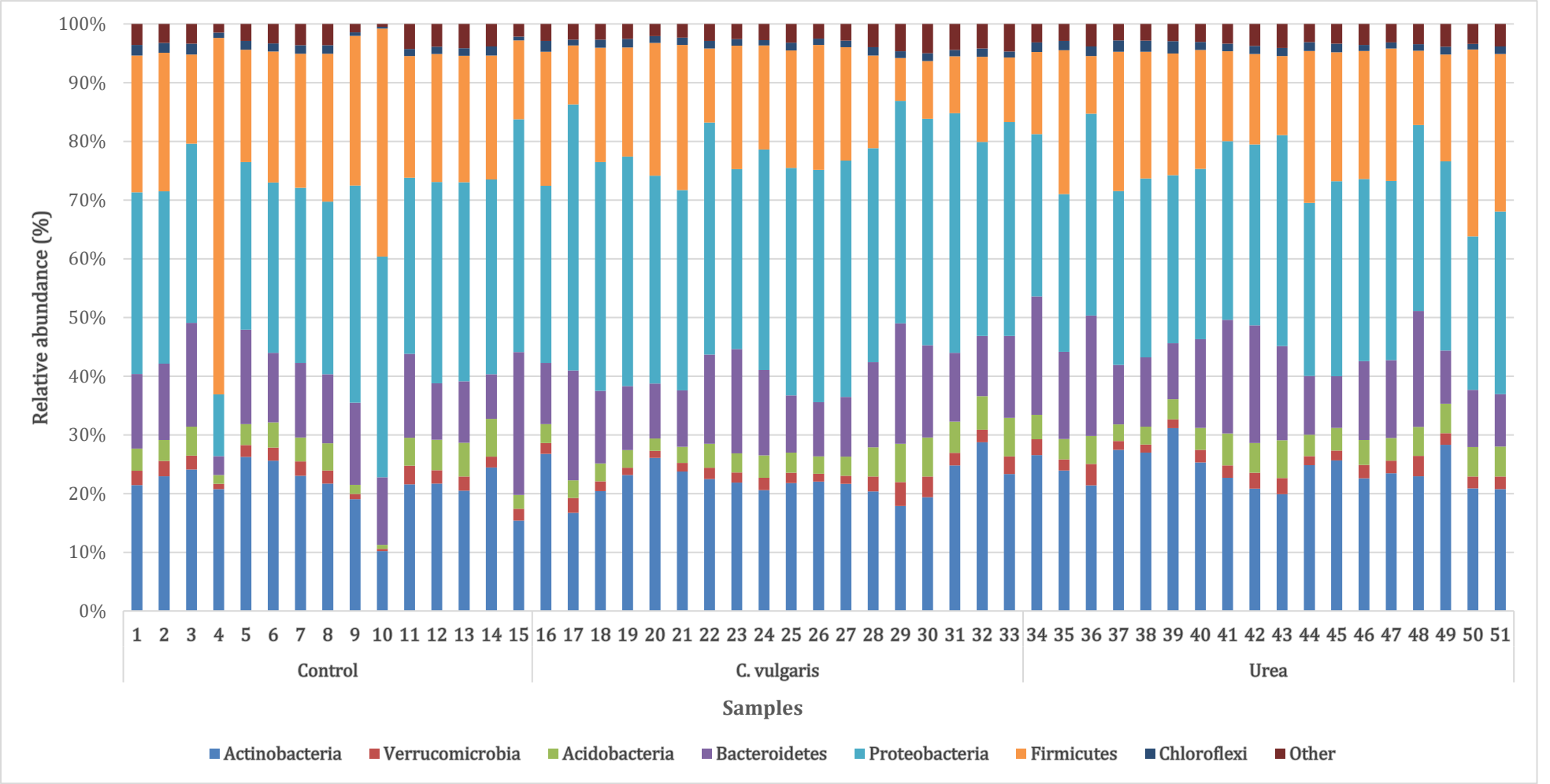
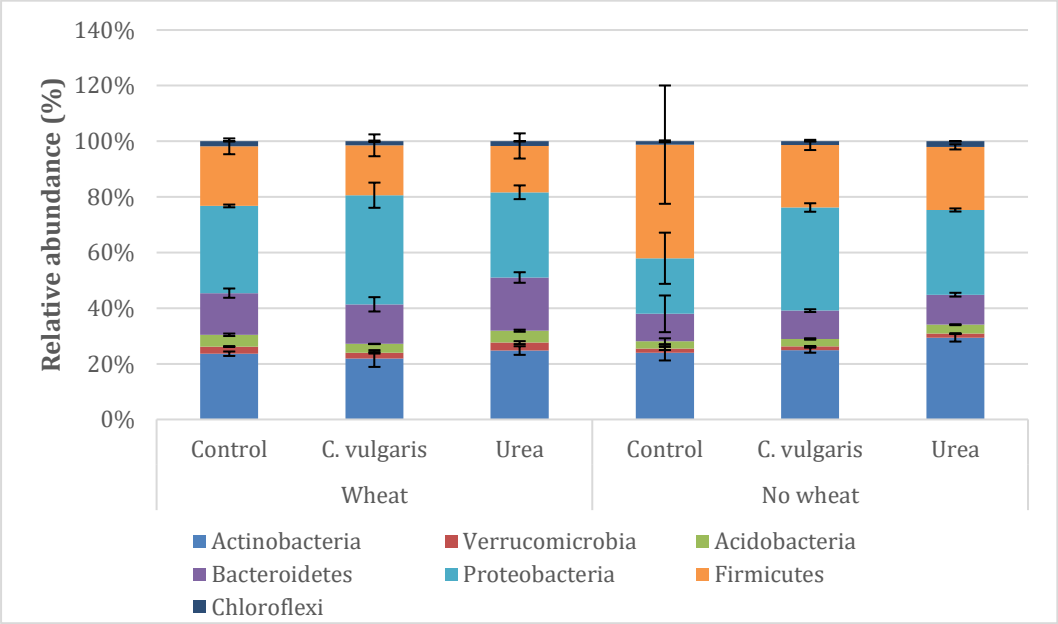
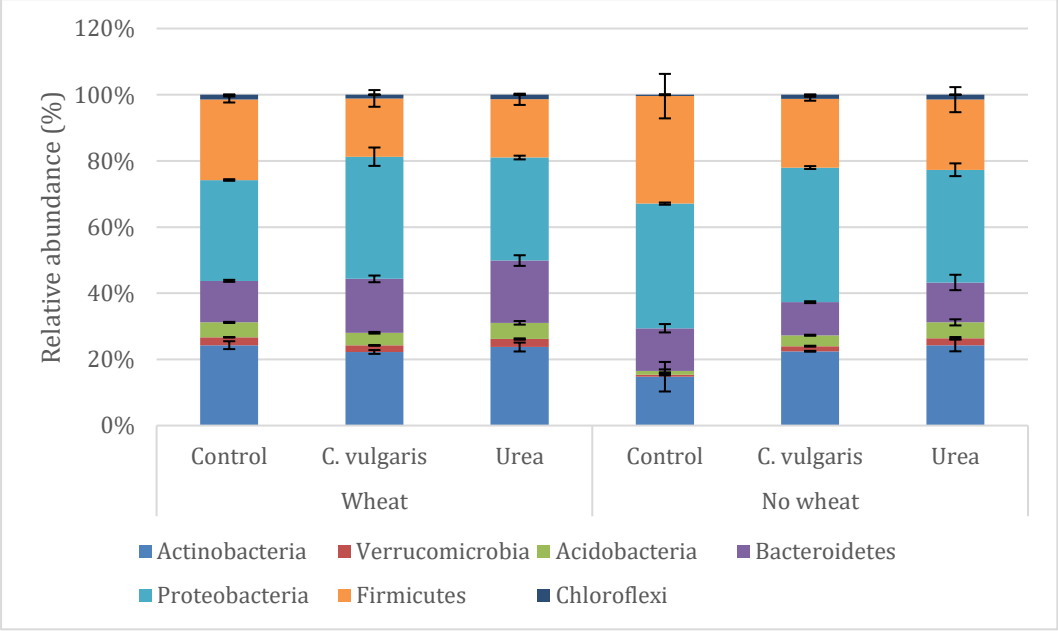


Figure 5.3 Relative abundance of prokaryotic reads found in all samples (numbers on x-axis correspond to sample number). Only microorganisms with a relative abundance of >1% are shown. All other phyla have been grouped to represent the 'Others' category.

A


140%
120%
100%
80%
60%
40%
20%
0%
Relative abundance (%)
Control
C. vulgaris
Urea
Control
C. vulgaris
Urea
Wheat
No wheat
Actinobacteria
Verrucomicrobia
Acidobacteria
Bacteroidetes
Proteobacteria
Firmicutes
Chloroflexi

B


120%
100%
80%
60%
40%
20%
0%
Relative abundance (%)
Control
C. vulgaris
Urea
Control
C. vulgaris
Urea
Wheat
No wheat
Actinobacteria
Verrucomicrobia
Acidobacteria
Bacteroidetes
Proteobacteria
Firmicutes
Chloroflexi

C

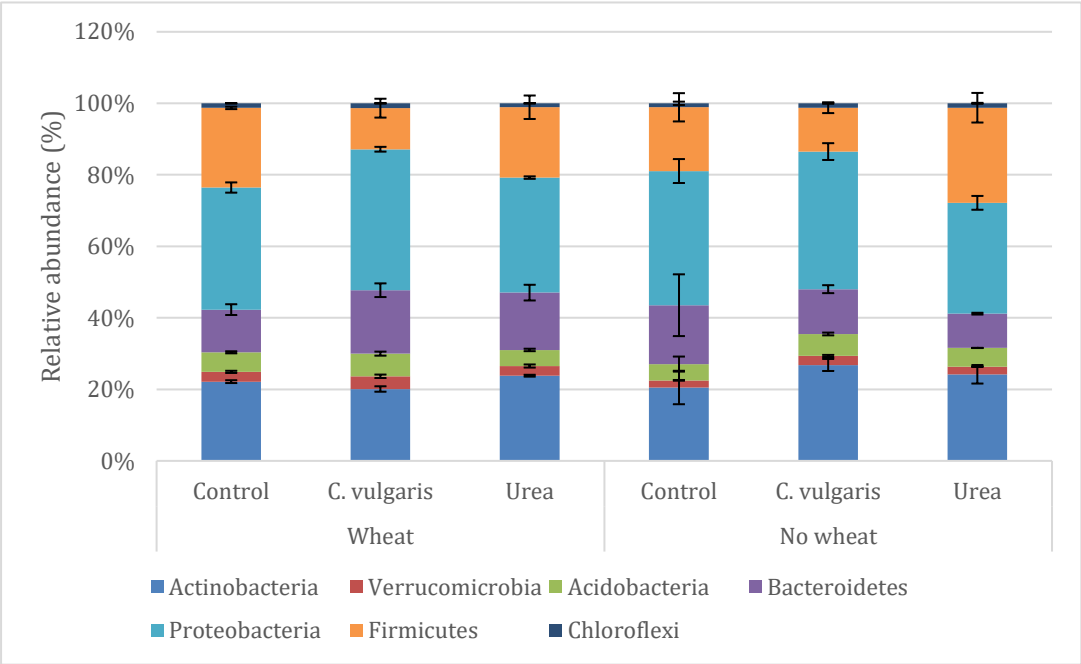


Figure 5.4 Relative abundance of OTUs at the phylum level found in the different treatments after 3 days (a) 10 days (b) and 30 days (c).

Within the 38 phyla, 114 classes of bacteria were identified, of which, 14 had a relative abundance above 1% (Figure 5-5). The most abundant class was *Bacilli* (18.51%), followed by Actinobacteria (15.43%), Betaproteobacteria (15.14%), Alphaproteobacteri*a* (10.69%), Cytophagia (9.17%), Thermoleophilia (4.73%), Deltaproteobacteria (4.04%), Flavobacteriia (3.22%), Gammaproteobacteria (3.20%), Clostridia (1.80%), Pedosphaerae (1.77%), Chloracidobacteria (1.54%), *MB-A2-108* (1.11%) and Acidimicrobiia (1.10%).

Bacilli, the most abundant class of bacteria, from the phyla Firmicutes, was found in high abundance under control conditions in wheat pots throughout the duration of the experiment. Whereas the *C. vulgaris* treatments had the lowest relative abundance of *Bacilli*, decreasing from 15.43% on day 10 to 9.87% on day 30. The relative abundance

on day 30 was significantly lower ($p<0.0001$) than the other two treatments. In fallow pots, the relative abundance of Bacilli also decreased under *C. vulgaris* treatment from 20.17% on day 3 to 10.68% on day 30, again significantly lower than the other two treatments.

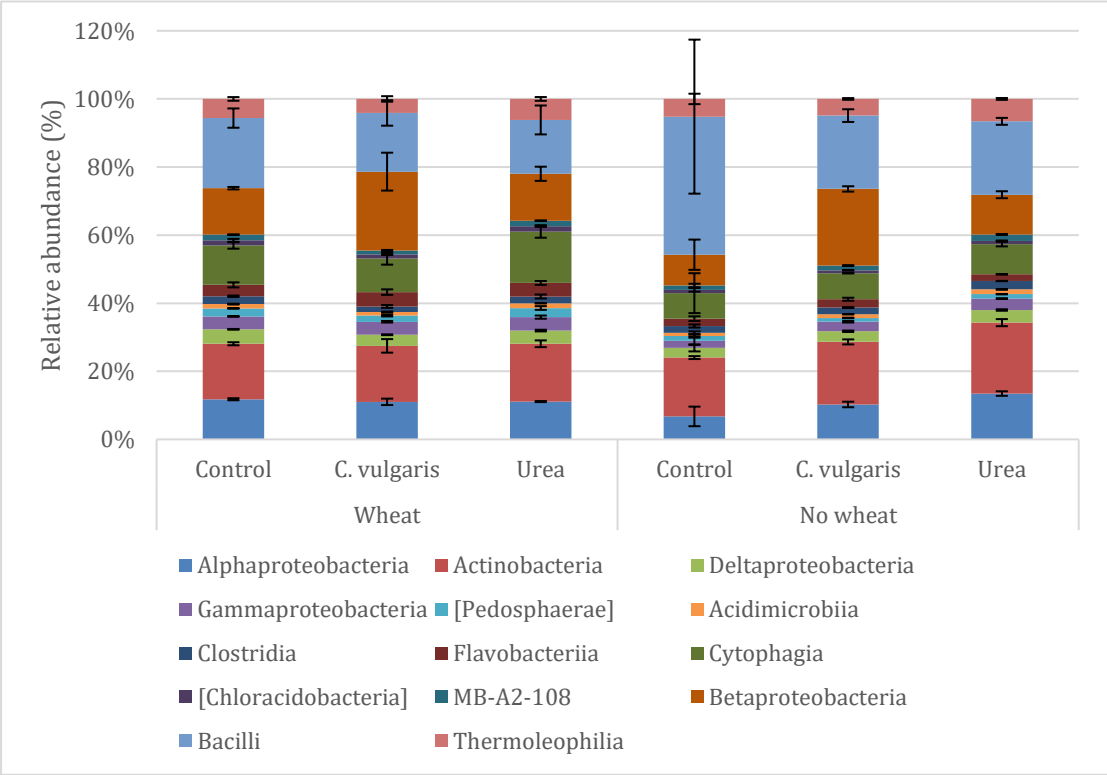
Actinobacteria, the next abundant class of bacteria, decreased in abundance under all treatments from 16.32% on day 3, to 15% by day 30, with no significant differences in abundance between any of the treatments in both wheat and fallow pots. Within the Proteobacteria, the Betaproteobacteria were the dominant class, followed by the Alphaproteobacteria, Deltaproteobacteria and the Gammaproteobacteria, which had the lowest abundance among the four. Relative abundance of Alphaproteobacteria, Deltaproteobacteria and Gammaproteobacteria increased throughout the experiment from day 3 to day 30, with no significant differences between any of the treatments.

The relative abundance of Betaproteobacteria, in pots with wheat, decreased under *C. vulgaris* from 21.42% (day3) to 17.35% (day 30) but was still significantly higher in comparison to the urea treatment and the control on day 3 ($p<0.0001$), 10 ($p<0.0001$) and 30 ($p=0.0004$). In the fallow pots, a similar trend was observed, where again relative abundance of Betaproteobacteria under *C. vulgaris* remained the same between day 3 and 10 at ~ 21%, where it was significantly higher than the urea treatments on both these days, but decreased by day 30 to 14.51% and had a similar relative abundance to urea treatment.

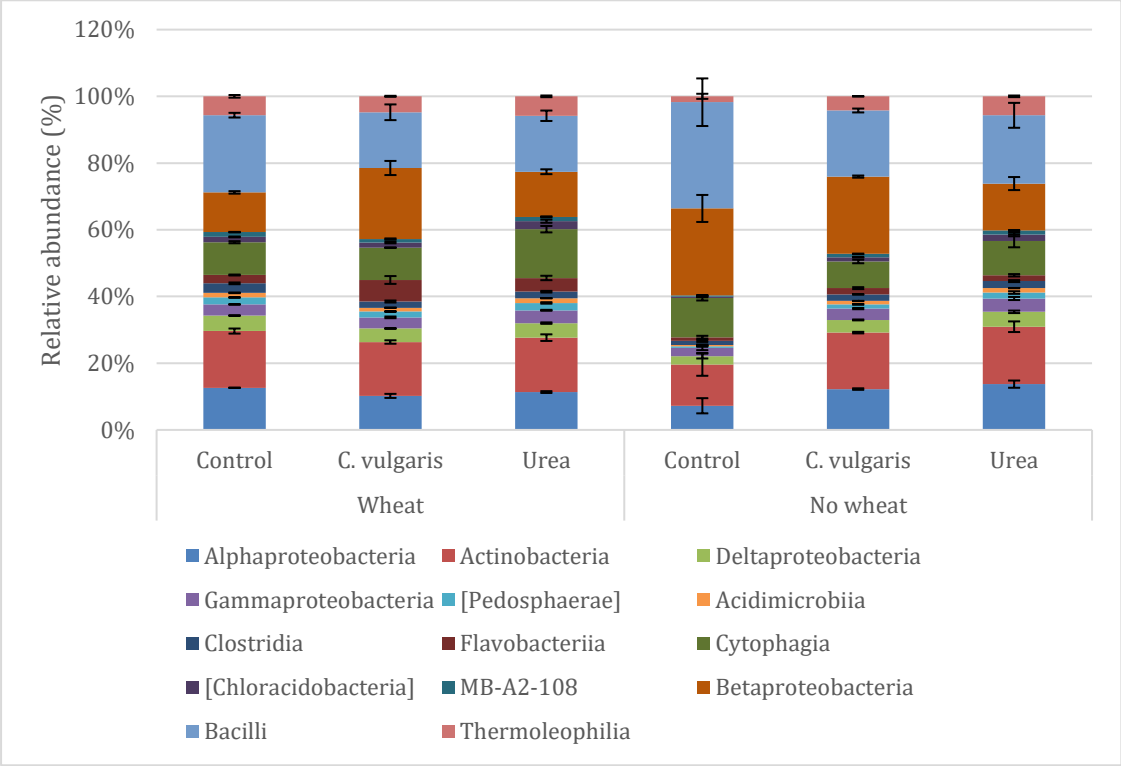
C. vulgaris was observed to have increased the relative abundance of Flavobacteria in pots with wheat from 3.90% on day 3 to 5.96% and 6.52% on day 10 and 30,

respectively. This increase in abundance was significantly higher than both control (p=0.0002) and the urea (p=0.04) treatment on day 10.

A



B


120%
100%
80%
60%
40%
20%
0%
Relative abundance (%)
Control
C. vulgaris
Urea
Control
C. vulgaris
Urea
Wheat
No wheat
Alphaproteobacteria
Actinobacteria
Deltaproteobacteria
Gammaproteobacteria
[Pedosphaerae]
Acidimicrobiia
Clostridia
Flavobacteriia
Cytophagia
[Chloracidobacteria]
MB-A2-108
Betaproteobacteria
Bacilli
Thermoleophilia

C

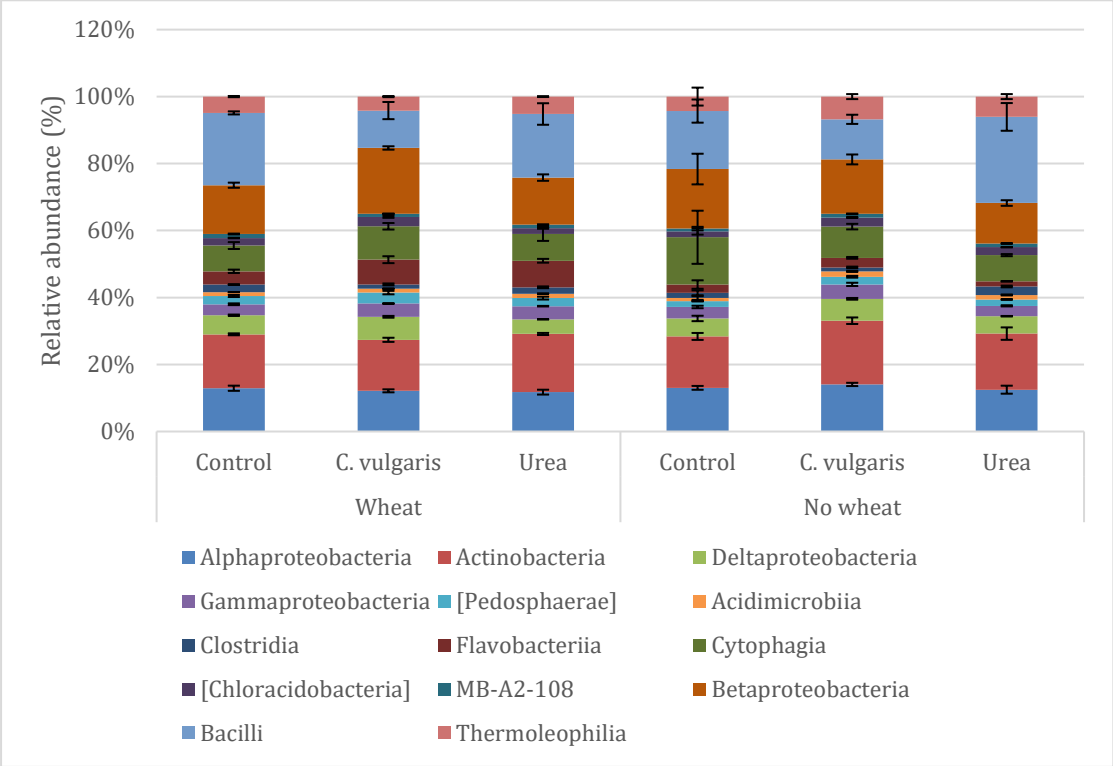


Figure 5.5 Relative abundance of OTUs at the class level found in the different treatments after 3 days (a) 10 days (b) and 30 days (c).

5.4 Discussion

C. vulgaris necromass has been shown to increase mineralizable nitrogen in a conventionally managed agricultural soil (Alobwede et al. 2018). To date however, the microbial processes involved in this response have not been characterized. Here a 16s rRNA study was carried out to assess the effect of *C. vulgaris* necromass on soil bacterial communities as well as identify the bacterial communities and taxa involved in the degradation and cycling of its biomass.

The pots with wheat had a significantly higher microbial diversity (evenness) compared to the fallow pots, with both treatments and the control after 10 days. It was assumed that roots influence the taxonomic diversity of soil microbial communities as they modify the soil rhizosphere chemistry through their secretion of carbon, fatty acids, carbohydrates as well as other organic compounds (Cheng and Coleman, 1990; Rohrbacher and St-Arnaud, 2016), which attract microorganisms through chemotaxis and allow them to utilize these substrates. We hypothesized that the addition of *C. vulgaris* to soil would also increase microorganism diversity. The temporal component showed significant differences between the treatments only after 30 days, where differences in microbial community diversity increased significantly where algal necromass was added, in comparison to the control and urea treatment. A high diversity of bacterial taxa able to colonise the organic residue might also be linked to the biochemical composition of the residue, as suggested by Bastian et al. (2009) and Drenovsky et al. (2004). *C. vulgaris* has a cell wall composed of a chitosan like layer, cellulose, hemicellulose, proteins lipids and minerals (Griffiths and Griffiths, 1969; Safi et al., 2014), which is resistant to breakdown and poses a major barrier for digestibility and extraction processes of all internal components (Safi et al., 2014). In soil, it is also likely that these complex substrates are recalcitrant to degradation (Bastian et al., 2009), thus requiring the need of a more diverse microbial community to carry out this process (Bastian et al., 2009).

The significant increase in bacterial diversity after 30 days was likely due to the changes in relative abundance of certain taxa. The phylum level profile under all treatments was composed of mostly Proteobacteria, followed by Actinobacteria, Firmicutes and Bacteroidetes. *C. vulgaris* treatment led to a significantly higher abundance of

Proteobacteria and Bacteroidetes, when compared to urea treatment and the control, which is likely to have driven the increase in diversity. The microbial community composition following the addition of *C. vulgaris* is similar to what was seen previously by Morrison et al. (2017), who reported significant increases in Bacteroidetes, Firmicutes and Proteobacteria associated with the addition of *C. vulgaris* in a highly nutrient enriched environment. The study did not specify the relative abundance nor the shifts in abundance of these taxa, so it is impossible to compare further. Other studies on soils have shown a similar phylum level profile, with Proteobacteria being the dominant species. The addition of wheat straw residue to loamy soil (Bastian et al., 2009) increased the relative abundance of Proteobacteria (69%), Bacteroidetes (25%) at 28 days of residue decomposition. Meanwhile biochar addition to soils (Nielsen et al., 2014) showed Proteobacteria to be the most dominant bacterial phyla followed by Acidobacteria and Verrucomicrobia. Suleiman et al. (2016) also showed that the degradation of swine slurry was driven by Proteobacteria (32%), Bacteroidetes (28.9%) and Firmicutes (25.9%). In Wang et al. (2018), the addition of seaweed (*Sargassum horneri*) fertilizer increased the relative abundance of Proteobacteria (28.24-32.08%), Actinobacteria (21.68-26.16%), Firmicutes (11.95-18.07%), Acidobacteria (8.25-9.99%) and Chloroflexi (4.47-6.62%) immediately after application – this phyla profile and abundance was reported to have only changed slightly after 60 days, with Actinobacteria and Firmicutes decreasing slightly and Acidobacteria and Chlorofexi increasing slightly. Despite the difference in algae (*C. vulgaris,* versus seaweed), this profile was very similar to our study and therefore also did not seem to agree with Morrison et al. (2017) who asserted that different types of alga would lead to slightly different community compositions due to their structural/chemical compositional differences. Although in their study, they observed a higher relative abundance of

Firmicutes (70%) under Kelp compared to *C. vulgaris*. This was attributed to its unique cell wall structure made of cellulose microfibrils of alginates and fucans. It assumed that this kelp (brown seaweed) was similar in cell composition to the brown algae used by Wang et al. (2018), therefore suggesting that perhaps other factors could have influenced the differences in community composition and abundance e.g. quantity and quality.

The ecological characteristics of the different taxa were compared in an attempt to explain their potential roles in the degradation of *C. vulgaris* biomass. According to Bastian et al. (2009), the microbial community is dominated by copiotrophs following the addition of fresh organic matter, and over time, as the organic matter quantity and quality declines, the community shifts resulting in an increase in relative abundance of oligotrophs. A similar trend was observed in this study. Proteobacteria are classed as copiotrophs and generally found in resource abundant conditions (Fierer et al., 2007). Not only were they the most abundant phyla present under all three treatments, their relative abundance was highest under *C. vulgaris* treatment in both wheat and fallow pots at all three time points of the experiment. Additionally, Betaproteobacteria of the phyla Proteobacteria, had a high relative abundance at the beginning of the experiment. Fierer et al. (2007) observed an enrichment of Betaproteobacteria following the addition of soluble carbon e.g. sucrose. The decrease in relative abundance of Betaproteobacteria by day 30 also suggests that most of the soluble carbon source had been degraded. Bacteriodetes abundance was also significantly higher under *C. vulgaris* treatment, with the abundance increasing throughout the experiment. Bacteroidetes are part of the copiotrophic bacterial population and have also been shown to favour conditions with high substrate availability (Fierer et al., 2007; Hou et

al., 2017). The significantly higher abundance of these phyla under *C. vulgaris* treatments suggests their ability to degrade carbon substrates. Acidobacteria, from oligotrophic populations are more abundant under conditions of limited substrate availability (Chen et al., 2014; Fierer et al., 2007; Bastian et al., 2009; Pascault et al., 2013). This was supported by our study, where their relative abundance increased under *C. vulgaris* throughout the duration of the experiment. Actinobacteria are also found in environments with limited nutrient availability and are responsible for the breakdown of recalcitrant organic matter (Suleiman et al., 2016; Ventura et al., 2007). Actinobacteria abundance was high under all treatments and decreased under *C. vulgaris* treatment by day 30, suggesting that any recalcitrant macromolecules had already been broken down before then. Firmicutes significantly decreased by day 30 and also had the lowest observed abundance under *C. vulgaris* treatment. Bacilli, belonging to the phyla Firmicutes, was shown to be significantly higher under control treatments on days 10 and 30, and lowest under the *C.* vulgaris treatments, which was surprising, as Bacilli are best known for their ability to breakdown macromolecules such as cellulose (Hou et al., 2017).

Microbial abundance is informative in assessing differences in microbial communities between treatments however it would be interesting and more informative to look at whether or not these changes affect a change in the functioning of these taxa. Although inferring the functional processes of these taxa in algal amended soil provides some insight, it would be more accurate to use alternative molecular techniques, such as real time quantitative PCR (Xu et al., 2014) or metaproteomics (Jia et al., 2017) in order to identify and quantify N-related functional gene transcripts and proteins, respectively, as well as decipher the functions of these microbial communities, and their role in the

breakdown of *C. vulgaris* biomass. It would also be interesting to quantify bacterial compositional changes and associated functions based on the amount of algae added. As some studies have shown a change in community composition following the addition of different amounts of organic amendments. In an experiment conducted by Val-Moraes et al. (2011), low application rates (25 kg N ha^{-1}) of sewage sludge increased the numbers of members of various phyla (Acidobacteria, Bacteroidetes, Firmicutes), whereas high application rates (200 kg N ha^{-1}) resulted in a reduction in the relative abundance of members of almost all the phyla, with the Proteobacteria being the most affected. Additionally, Nielsen et al. (2014) showed that high biochar application rates of 5.44 t/ha induced a lower relative abundance of Bacteroidetes, and Proteobacteria compared to lower (1.1 t/ha) biochar treatments. Despite the current study showing some differences in relative abundance of the 3 of the dominant phyla (Proteobacteria, Bacteroidetes and Firmicutes) between *C. vulgaris*, urea and control treatments, it is possible that the quantity of *C. vulgaris* could change the associated microbial community structure and possibly functions.

5.5 Conclusion

The use of modern techniques such as amplicon sequencing, rather than culture-dependent techniques, provides not only greater accuracy, but also a more comprehensive measure of soil microbial diversity and composition (Bastian et al., 2009). This can help foster greater understanding of the responses of soil microorganisms to the impacts of management processes, such as in this case the addition of *C. vulgaris* necromass. The results of this study showed an increase in bacterial alpha diversity after 30 days following the addition of *C. vulgaris* necromass,

driven by an increase in the presence of bacterial taxa conducive in breaking down the biomass.

Future work should look at factors affecting the microbial processes for algal decomposition such as moisture content, and especially those pertaining to algal quality and quantity, all of which could affect the bacterial colonization and decomposition patterns. This study provides the opportunity to exploit the potential of algal residue degradation, however a more comprehensive understanding, using targeted techniques, is needed on the functional diversity of microbial populations.

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Chapter VI

Conclusions and future research

This chapter summarises the work of the entire thesis and provides concluding remarks and suggestions for future research.

6.1 Conclusions and summary of findings

This thesis aimed to investigate the effect of algae as sustainable organic fertilizers to conventionally managed agricultural soils, particularly when applied as dried biomass, in order to improve soil chemical, physical and biological properties, and reveal features that could be exploited for future use. The main objectives were to 1) assess the effect of chemically contrasting algal species on soil physical and chemical properties and crop yield 2) trace and quantify the fate of N derived from *C. vulgaris* into soil and crop plant N pools 3) characterize the microbial communities responsible for the degradation and cycling of *C. vulgaris*.

Chapter 3 provided an experimental investigation of the effects on soil physical and chemical properties of five phylogenetically different algal species with varying C:N ratios and at 3 different application rates, under both field and greenhouse conditions. Previous works carried out on the effects of algae have made no comparisons on the effects of species that are phylogenetically different and therefore differing in their composition (in this case elemental composition). Results support the idea that algae

(micro and macro) are significant in providing mineralizable nitrogen to agricultural soils. Both greenhouse and field experiments showed evidence of N mineralization under algae treatments with lower C:N ratios (*Spirulina*, *Chlorella* sp. and *P. palmata*) – where *Spirulina* mostly affected NO_3^- -N and *Chlorella* sp. and *P. palmata* affected NH_4^+-N concentrations. Additionally, *Chlorella* sp. was the only alga to significantly improve crop (garden pea) yields under greenhouse conditions. There was no effect of treatment application rate on soil characteristics in an arable field (except for *Spirulina* effects on NO_3^- -N concentrations) whereas in the greenhouse, higher application rates tended to have more significant effects. The application of dried algae biomass was observed to have no significant impact on soil aggregate stability under field conditions after 20 weeks.

Chapter 4 focused on *Chlorella vulgaris* – the alga that had the most impact on soil chemical properties, namely total N and NH_4^+-N as well as pea yields in the greenhouse. ^{15}N *C. vulgaris* was added to the soil to monitor its fate and quantify the amount of algal N in plant, soil and mineral N (NH_4^+ and NO_3^-) pools. Following its application, the *Chlorella* ^{15}N was assumed to be immobilized–– as reflected in the high soil ^{15}N tracer recoveries. ^{15}N recoveries decreased in the soil after day 5 onwards, while recoveries simultaneously increased in the wheat shoot by day 20, indicating that the ^{15}N had been mineralized and taken up by the plant by this time.

Chapter 5 focused on the soil biotic parameters (e.g. diversity and abundance) to assess the impact on *C. vulgaris* on soil microbial communities, firstly to see whether there were any differences in microbial communities compared to an inorganic substrate (urea) and to identify the taxa responsible for the degradation and cycling of *C. vulgaris*

in a conventionally managed agricultural soil. This work presented the first survey of microbial communities mediating the turnover of *C. vulgaris* necromass in agricultural soils. The *C. vulgaris* amendment was shown to significantly increase microbial diversity in comparison to urea and control treatments. This increase in alpha diversity was likely driven by an increase in abundance of specific bacteria taxa conducive in breaking down its biomass namely Betaproteobacteria and Bacteroidetes. Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes were the most abundant taxa found under all three treatments, however both Bacteroidetes and Proteobacteria had a significantly higher abundance under *C. vulgaris* compared to both urea and control treatments. Both these taxa are identified as copiotrophs and are strongly implicated in the mineralization of *C. vulgaris* necromass. There was some evidence of shifts in community composition under *C. vulgaris* treatments with the communities gradually moving from one dominated by copiotrophs (Proteobacteria and Bacteroidetes) at 3 days, to one steadily becoming more dominated by oligotrophs (Acidobacteria) by 30 days.

6.2 Future work

The significance of the results obtained in this thesis, are likely to have important implications for the selection of algal species in nutrient management strategies. However, further studies would need to be carried out to compare the effects of the state of the biomass applied i.e. fresh wet biomass (and therefore live) as opposed to dried biomass (as was investigated in this thesis). This study did not focus on the use of live algal biomass due the fact that it was easier to attain dried biomass. However, in the field, this may not be the case, as additional processes will be needed to get the biomass into a dried form. The effects of live biomass were not studied and could also

be an important factor in improving soil characteristics, but also perhaps more convenient for the farmer.

Further studies are also required to look at N loss pathways (e.g. nitrous oxide emissions) as a result of *C. vulgaris* addition effects on immobilization and mineralization within the soil, in order to improve N management strategies. It would also be useful to look at the ^{15}N isotopic enrichment in the roots as well as the organic matter to gain a fuller understanding of soil nitrogen transformations, as both these pools, particularly the organic matter pool, are also key in determining plant nitrogen availability through their competition for both organic and inorganic sources of N within the soil (Rutting et al. 2011).

Future examination of the interactions between the abundant taxa identified as likely to be important for the degradation *C. vulgaris* would need to focus on how quality (i.e. algae species, different carbon compounds present in biomass, C:N ratio) and quantity could affect differences in community composition. Additionally, the use of more in depth and targeted techniques such as qPCR (to identify and quantify N-related functional genes) and metaproteomics (to identify functional attributes of microbial communities), is needed in order to gain a better understanding of the functional roles of the taxa involved in the degradation and cycling of *C. vulgaris*. This would also provide greater insights into the functional significance of *C. vulgaris* biomass on soil microbial communities and how these can affect carbon storage, nutrient cycling and essentially agricultural productivity.

6.2 Commercial potential of algae as a biofertilizer

Setting up an integrated system (Figure 6.1), whereby the algae is harvested from eutrophic water body types, using various technologies, then processed before being applied back onto soil in a 'circular economy fertilization' system has significant potential as a nutrient management strategy on agricultural farms. Water bodies with high nutrient contents could include wastewater treatment facilities, agricultural animal facilities (such as dairy facilities), as well as agricultural drainage and ditches (Randrianarison and Ashraf, 2017) as these all represent sources of water pollution from agriculture. This circular economy fertilization practice will provide a holistic solution to nutrient management by recovering the nutrients lost as a result of leaching, through the cultivation of algae, which have a high capacity of accumulating N and P (Singh and Dhar, 2007; He et al., 2015), and conserving them into biologically available forms in their biomass.

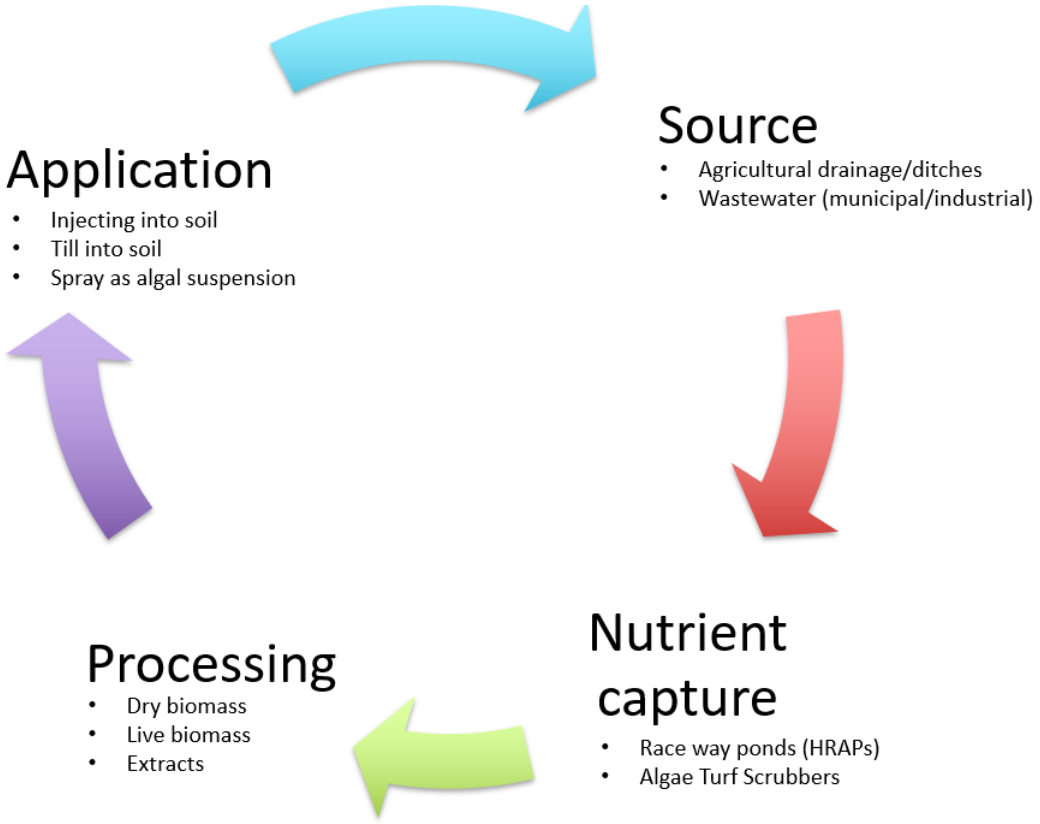

Figure 6.1 Circular economy fertilization

Devices used for nutrient removal could include either algae ponds/raceway ponds (Randrianarison and Ashraf, 2017; Shilton et al., 2012) usually used in treating wastewater, or algal turf scrubbers (Wilkie and Mulbry, 2002). Raceway ponds (Figure 6.2), are oval shaped ponds usually between 0.2 to 0.5 m deep, with paddle wheels used for circulation of nutrients and stabilization of algal growth (Brennan and Owende, 2010). Some ponds are designed to add CO_2 into the system, however algae are also able to get their CO2 requirement from the open air (Brennan and Owende, 2010). These ponds will allow the growth of planktonic algae and are cheap to run (Randrianarison and Ashraf, 2017) however they present several disadvantages in that they suffer from contamination which could result in low amount of biomass being

harvested, as well as poor mixing and poor light distribution (Radmann et al., 2007; Randrianarison and Ashraf, 2017).

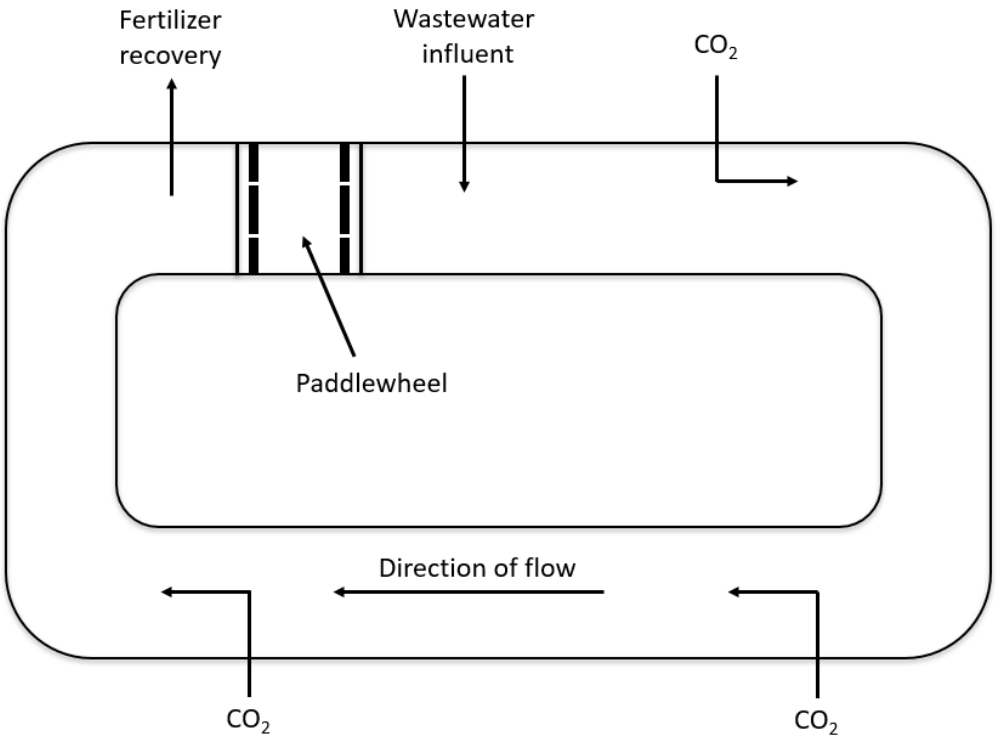

Figure 6.2 Side view of an opened raceway pond (Adapted from Brennan and Owende, 2009)

An alternative to open raceway ponds is the use of algal turf scrubbers which could be installed closer to farms as their space requirements are not as large as open raceway ponds. The use of algal turf scrubbers initially developed by Adey and Hackney (1989) to clean up marine waters is one of the more promising ways in which agricultural draining could be contained and the nutrients recovered (Mulbry et al., 2008; Mulbry et al., 2010; Kanga et al., 2014; D'Aiuto et al., 2015; Ray et al., 2015; Lui et al., 2016). An algal turf scrubber (Figure 6.3) consists of a solid support on which the algae can grow on. The mat is at an incline allowing water to flow from one end to the other, where it is either reintroduced back into the system, or discharged (D'Aiuto et al., 2015)

This system is more advantageous for the growth of benthic algae (attached) as opposed to planktonic (suspended) algae as the benthic algae are more easily recovered and separated (Wilkie and Mulbry, 2002). And unlike the open raceway ponds, it does not require an initial algal inoculum as the algae can also naturally grow on the surface along with other organisms including bacteria, fungus and other small microorganisms (D'Aiuto et al., 2015). This "periphytic" community is able to scavenge nutrients from the eutrophic water as well as heavy metals and can be subsequently harvested by either using a vacuum (Mulbry et al., 2010) or scraping (D'Aiuto et al., 2015) from the surface using shovels or rakes, to be used as fertilizer.

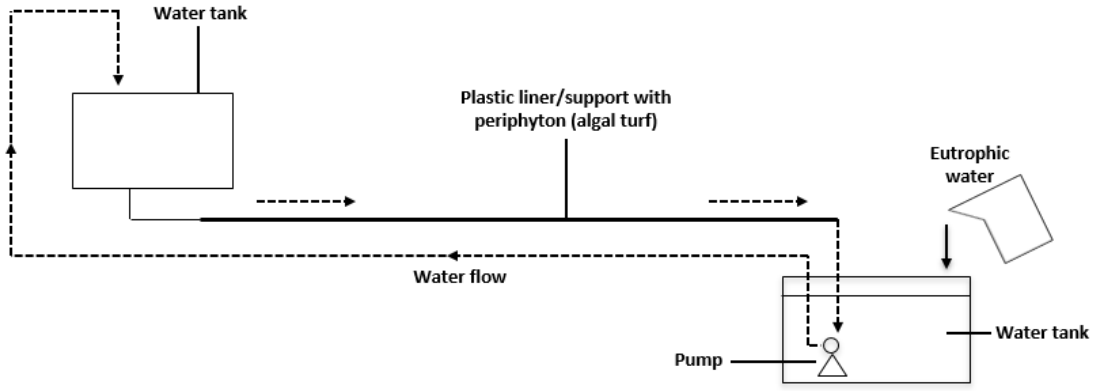

Figure 6.3 Side view schematic of an ATS (Adapted from Lui et al., 2016)

Once harvested, the algal biomass could be applied as fresh biomass, sprayed as a suspension of applied directly onto soil, depending on the farmer. The use of umbilical applicators used for slurry spreading (injecting) into soil could be used to apply the algae to the soil. This might involve the tanker parking at the edge of a field and the tractor pumping the liquid into the soil across the whole field using a hose reel system. This avoids soil compaction and nutrient volatilization is reduced by direct injection. The algal material, if filamentous, might need to be macerated to avoid blocking

injectors - in order to do this, it would be easier if it is dried first. Dried biomass application has shown promising effects on soil nutrients and additionally it is more easily handled, can be stored for longer periods (Malik, 1993; Wilkie and Mulbry, 2002) and has a reduced weight for transportation and distribution onto soil. Drying the harvested algae however could prove costly (Slade and Bauen, 2013) and therefore may present a drawback. In our study, *C. vulgaris* was freeze dried however on farming systems, other dewatering techniques could be assessed, in particular to reduce the cost of this operation in the system. Examples of possible drying techniques include the use of industrial fans and well as simply spreading the algae on a surface to air dry. For filamentous algal species, perhaps milling or shredding would be needed as well as drying to provide a powder for distribution.

6.3 References

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