MICROBIOLOGICAL AND GEOCHEMICAL RESPONSE OF BIOSOLID AMENDED SOILS TO FLOODING

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DECLARATION

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

DEDICATION

To the memories of my grandfather Ged and grandmother Bernice, without whose inspiration I would not have found my love of the land and the natural world.

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ABSTRACT

More frequent extreme weather events induced by climate change could pose new challenges to agricultural production. Soil organic inputs, such as biosolids, could be an effective way to improve soil and crop resilience to short-term flooding. Biosolids are a valuable and sustainable organic input, containing high N, P and organic C contents which can act as a nutrient source for crops and soil microbes. However, these benefits could be offset if the contained N and P is lost to floodwater, leading to an increased eutrophication risk. To address this question a series of controlled laboratory growth box experiments were conducted to determine the impact of varying biosolid application rates and a 10-day flood on soil geochemistry, microbiology and crop performance.

Biosolid applications caused increased soil N, P and organic matter which mitigated poor crop establishment. However, there was no apparent impact on crop flood resilience, though at high application rates plants had greater dry weight biomass. The increased soil N content led to increased NO_3^- loss to floodwater, but no increase was observed in floodwater P with increased biosolid application despite higher soil P. This was attributed to the presence on NO_3^- in biosolid-applied soils acting as a terminal electron acceptor, preventing highly reducing conditions in soil porewater which would favour soluble P release. This effect meant biosolid-applied soils had no greater loss of soluble P than unapplied control soils. Soil bacterial populations characterised using 16s rRNA gene sequencing showed biosolid-applied soil populations still closely resembled those of control soils, though increased nutrient availability induced some changes in phylum relative abundances. There was evidence to show that some bacterial genera were transferred from biosolids to soils, and that flooding allowed some of these genera to increase in abundance. However, bacterial populations returned to pre-flood profiles after a 20-day recovery period.

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ABBREVIATIONS

AOA	Ammonia-Oxidising Archaea
AOB	Ammonia-Oxidising Bacteria
DNRA	Dissimilatory Nitrate Reduction to Ammonium
DO_2	Dissolved Oxygen
DWB	Dry Weight Biomass
FYM	Farmyard Manure
GHG	Greenhouse Gas
NGS	Next Generation Sequencing
NMDS	Non-Metric Multidimensional Scaling
NOB	Nitrite Oxidising Bacteria
NVZ	Nitrate Vulnerable Zone
OM	Organic Matter
ORP	Oxidation-Reduction Potential
OTU	Operational Taxonomic Unit
PAR	Photosynthetically Active Radiation
PLFA	Phospholipid Fatty Acid
PTE	Potentially Toxic Element
Redox	Reduction-Oxidation
SOC	Soil Organic Carbon
SOM	Soil Organic Matter
SRP	Soluble Reactive Phosphorus
TEA	Terminal Electron Acceptor
TKN	Total Kjeldahl Nitrogen
TIC	Total Inorganic Carbon
TIN	Total Inorganic Nitrogen
TOC	Total Organic Carbon
XRF	X-Ray Fluorescence

Chapter 1: INTRODUCTION

1.1 BACKGROUND

The application of digested sewage sludge (biosolids) to agricultural land has become increasingly popular due to improved wastewater treatment processes and its value as a soil conditioner. Historically there has been concern around the threat of introducing pathogenic microbes or the accumulation of harmful chemicals in soil, leading to the Code of Practice for Agricultural Use of Sewage Sludge (DoE, 1989). Improved waste water practices that reduce chemical contaminants going to sewer, advanced digestion processes that kill pathogens and thorough legislative controls around agricultural use means that biosolid application is now considered a safe and sustainable alternative to inorganic fertilisers (Smith, 2009b; Clarke and Smith, 2011; Al-Gheethi et al., 2018). Application of biosolids to land is a more sustainable and economically attractive alternative to either incineration or landfilling, with around 3.6 million tonnes of biosolids recycled to agricultural land each year, providing an estimated service value of £25 million to the UK farming sector (Assured Biosolids Limited, 2019). The value of biosolids come mainly from its high nitrogen (N) and phosphorus (P) content, which are major plant nutrients and essential inputs in arable crop production. The high organic N contained in biosolids can reduce the need for expensive mineral N fertiliser which has a high energy cost for its production (Basosi et al., 2014). The high P content in biosolids may also offer a long term and sustainable solution to globally depleting soil P stocks (Torri et al., 2017). Furthermore, biosolids are a source of organic carbon and increase soil organic matter (SOM) which is a key indicator of soil fertility and can lead to higher attainable crop yields (Singh and Agrawal, 2008; Johnston et al., 2009; Hijbeek et al., 2017).

Climate change has potential implications for the future management of soils in agriculture. Increases in overall annual rainfall, summer rainfall and flash flood events have been documented in the UK and EU in the last 5 years, and such changes are predicted to continue in the future (Lenderink and van Meijgaard, 2008; Falloon and Betts, 2010; Kendon et al., 2014; Centre for Ecology & Hydrology, 2016). Direct flash-flooding of fields, areas of sustained waterlogging or floodplain storage of water as an urban flooding alleviation measure (Wheater and Evans, 2009) are all possible scenarios resulting from extreme rainfall events. Flooding of farmland during the spring growing season when crops and soil microbes are more active due to rising temperatures and longer daylight hours could lead to increased environmental risk and negative impacts on crop production. Improved soil structure and resilience may be required to maintain agricultural production and

protect watercourses from nutrient leaching and soil particle runoff. One method of improving soil structure is to increase SOM from organic carbon inputs (Haynes and Naidu, 1998; Masri and Ryan, 2006), including biosolids. If land which has been amended with biosolids becomes flooded or waterlogged then any potential environmental impacts must be understood. The fate of N and P in biosolids, the health and resilience of crops, and the impact on soil microbial communities are all factors which must be considered.

N and P are the main causes of eutrophication in watercourses and runoff from agricultural land is a main contributor of both nutrients (Withers and Haygarth, 2007; Selman and Greenhalgh, 2010a). With N and P present in high amounts in biosolids there may be an increased eutrophication risk to watercourses from biosolid-amended land. Increased microbial activity from the large input of organic matter (OM) in biosolids could lead to increased mineralisation and release of soluble N and P under certain conditions (Heuck et al., 2015; Rigby et al., 2016). If there is a large increase in aerobes during any boost to microbial populations then this could quickly make floodwater in biosolid-amended soils anaerobic. The resultant die-off of aerobes in a flooded anaerobic system could then lead to the nutrients they contained also being released to the floodwater. Alternatively, the high OM content of biosolids and most of the nutrients being contained in organic forms could mean it improves soil structure and provides a nutrient source which is stable and resistant to loss in soluble forms during flooding. The increased nutrient content of a biosolid-amended soil may also help crops and microbial populations to survive water inundation by improving their numbers and resilience. The changes to soil and water from biosolid applications need to be understood for effective soil management and for environmental protection.

Usually the main loss of P to watercourses is from soil erosion as P is held strongly by soil particles and OM and has low aqueous solubility. However, if soil chemistry is significantly changed by the application of biosolids then it could lead to P becoming soluble and lost through leaching. Research using anoxic incubations has shown the release of P to soil solution with the reduction of ferric Iron (Fe^{3+}); although oxidising water can act as a barrier to P release if ferrous iron (Fe^{2+}) which has diffused from anoxic soil then sorbs or precipitates with P as it oxidises (Mortimer, 1941; Boström and Pettersson, 1982; Moore and Reddy, 1994; Hupfer and Lewandowski, 2008; Van Nguyen and Maeda, 2016). If surface water becomes anoxic, or if the concentration of P exceeds the mechanism for sorption and precipitation of Fe, then large quantities of P could remain soluble. These concepts are well understood in long term seasonal floods, paddy field irrigation and in river and lake sediments, however short-term flash floods and waterlogging have not received the same attention. Even if surface water remains oxidised the draining of large quantities of flood water from a field will result in the loss of surface water and potentially anoxic porewater containing soluble P.

The cycle of N in soil is well understood and the risk that nitrate (NO^{3-}) poses to watercourses is controlled by regulation in agriculture by limiting organic N applications to 250kg/ha per year and with Nitrate Vulnerable Zones (NVZs). NVZs are areas where total N fertiliser applications are limited to reduce environmental pollution risk, with the aim of keeping below a threshold of 50mg/l NO^{3-} in surface waters (DEFRA, 2018a). Mineral N applications are usually of most concern as they contain readily available and soluble N forms such as NO^{3-}, ammonium (NH_4^+) and urea (CH_4N_2O). Organic N in manures and biosolids however has the potential to become available through mineralisation to NH_4^+ and nitrification to NO_3^- through soil microbial processes. Mineralisation occurs when microbes decompose and release the N compounds within them. Mineralised N can be converted back to organic forms if there is enough carbon in the SOM to maintain an optimal C:N ratio of around 24:1 (USDA, 2011b). If there is a large increase in aerobe populations from the addition of biosolids this could lead to increased respiration and loss of C as carbon dioxide. This loss of C could then lead to an increase in N mineralisation due to an imbalanced C:N ratio and thus increase the risk of N loss to floodwater in soluble forms.

A key element which will affect soil nutrients and microbial populations is the presence of a growing crop which will also compete for resources in the system. Much previous research into soil flooding fails to account for cropped arable systems, instead opting for grassland or bare soil. This is because areas of repeated flooding are typically not used in arable production. However, as previously discussed, increased extreme rainfall events could alter this. Many of the mechanisms of nutrient mobilisation involved in flooded soils are understood well in isolation. Closely controlled laboratory jar experiments, anoxic incubations and closed column microcosm experiments have been previously studied (Rydin, 2000; Young and Ross, 2001; Suhadolc et al., 2010). While these experiments offer valuable insights into several individual aspects of soil systems many fail to account for key variables of real-world situations, such as vegetation growth and microbial community changes. Field scale trials and long-term or seasonal flood experiments have also received attention to try to better understand soil processes in live systems (Unger et al., 2009a; Unger et al., 2009b; Sánchez-Rodríguez et al., 2018). Field approaches can often lack the controlled conditions and ability to precisely measure and accurately evaluate small changes in soil and water biogeochemical properties. The constant changing of the natural environment means that outdoor experiments may lack repeatability and so results must be reported *in situ* with limited ability to assess and apply broader implications. To understand the complex geochemical and microbial interactions in natural ecosystems those systems must be closely simulated while allowing for control of critical variables and close monitoring. The soil-water-biosolid-microbe-crop system is intricate and intertwined and requires further study to explore its complexities.

1.2 RESEARCH AIMS AND OBJECTIVES

1.2.1 Project aim

This project is part of the wider 'Biochemical-physical-biological function of sludge in agricultural soils' (BIOSAS) project within the White Rose research network, consisting of a collaboration between the University of Leeds, University of Sheffield and University of York. Two other projects are run alongside this one. They are using similar soils and experimental parameters to understand the soil ecosystem's interaction with biosolids. One project is entitled 'Effects of sludge-rainfall interactions on soil quality and crop production' and the other 'Earthworms and water drainage – impacts of floods and sewage sludge amendments'. The common themes within all 3 projects, including this study, 'Microbiological and geochemical response of biosolid amended soils to flooding', is biosolid-soil interactions during extreme weather events leading to flooding in an agricultural setting.

The principal aim of this project is to assess whether biosolid applications are a suitable soil amendment for improving the resilience and recovery of soils and crops in an agricultural system, with consideration of any nutrient loss risk to the environment. To explore these effects a wide range of geochemical, microbiological and crop growth factors will be assessed in a biosolid-amended agricultural soil before, during and after a short-term flood event. Laboratory experiments will be designed to simulate field conditions as closely as practicable in a robust, controlled and repeatable manner. The experiments will test the hypotheses below relating to crop, geochemical and microbiological factors in the crop-soil-biosolid-water system.

1.2.2 Hypotheses

The following hypotheses will be investigated to achieve the project aim:

1. The addition of digested sewage sludge to an agricultural soil will positively impact crop growth and its resilience to a short-term stagnant flood event.

    Objective 1. Establish a cereal crop on soils treated with different rates of biosolids under controlled light, water and temperature conditions and determine differences in growth and establishment relating to biosolid application.

    Objective 2. Measure crop survival and recovery from a short-term flood event and determine to what extent biosolid application plays a role.

2. The addition of digested sewage sludge to an agricultural soil will increase total soil N and P content and create conditions which favour their solubility, therefore increasing the amount of soluble N and P lost to water during a flood event.

    Objective 3. Measure soil N and P content in soils treated with various rates of biosolid application before and after a short-term flood to determine the proportional soil nutrient changes.

    Objective 4. Monitor soil-water reduction-oxidation conditions during flooding to determine mechanisms for release of soluble N and P and how they are affected by biosolid applications.

    Objective 5. Measure the loss of soluble N and P to floodwater from soils treated with various rates of biosolids and determine the safe threshold for biosolid application in relation to environmental threat and regulations.

3. The addition of digested sewage sludge to soil will provide an energy source for existing microbial populations but will not change overall microbial composition.

    Objective 6. Measure changes in soil organic matter and nutrient content caused by different rates of biosolid addition to an untreated control soil.

    Objective 7. Determine the microbial populations in untreated soils and soils treated with different rates of biosolid application and analyse any differences which occur.

4. Flooding a biosolid-amended soil causes changes in soil microbial composition proportional to the biosolid application rate.

    Objective 8. Determine the microbial populations in untreated agricultural soils and soils treated with various rates of biosolid applications, then determine any changes in populations caused by a short-term flood event.

    Objective 9. Determine if microbial populations in biosolid-amended soil recover after being subjected to a short-term flood and to what extent.

1.3 THESIS STRUCTURE

This thesis is structured into seven chapters. The initial introduction chapter is followed by the literature review which explores the existing knowledge concerning the concepts explored in this project. This literature review was important to identify research gaps, develop the hypotheses and for determining the most appropriate methods to use for the design of the experiments. Chapter 3 presents the methodology employed in designing and constructing the experimental setup and the procedures used in running and maintaining the experiments. In chapters 4-6 the main results of the thesis are discussed, with specific methodologies for measurements relating to each area detailed in their respective chapters. Chapter 4 focuses on the geochemical response of flooded biosolid-amended soils, particularly the mechanisms governing N and P in soil and water. The chemical conditions created by flooding in the soil and water are explored, covering hypothesis 2, objectives 3-5. Chapter 5 focuses on the crop response to biosolid application relating to hypothesis 1, objectives 1 and 2. Chapter 6 focuses on soil microbiology, the methods used for analysing microbial populations and the results therein, which address hypotheses 3 and 4, objectives 6-9. Chapter 7 summarises the work presented in the thesis, discusses the overall conclusions and presents suggestions for future work.

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Chapter 2: LITERATURE REVIEW

This chapter explores peer-reviewed literature in several research areas which apply to this project. Areas of study are explored in detail which cover biosolids, flooding and climate change, soil organic matter, nitrogen behaviour in soils, soil phosphorus, plants and cropping, experimental design, and analysis methods.

2.1 BIOSOLIDS

2.1.1 Use and regulation in agriculture

'Biosolid' is a common term used in agriculture to include any organic fertiliser input derived after the treatment of sewage sludge. Sewage sludge is derived from the settled and treated organic and mineral solids from wastewater which can include domestic drain washings, human metabolic waste, food waste, rainwater runoff and industrial wastewater (DEFRA, 2012). Biosolids include the solid organic material and mineral content of wastewater which has undergone some form of digestion to break down organic matter (OM) and control any pathogenic microbes. Biosolids contain a high phosphate and nitrogen content and have an abundance of trace elements which are essential to crop growth making them valuable as fertilisers and soil conditioners (AHDB, 2017). As an organic input biosolids can also increase soil organic matter (SOM) which is a known way to improve soil structure, drainage and crop yields (Hijbeek et al., 2017).

Disposing of sewage sludge to agricultural land offers an economically and environmentally sustainable disposal method for waste from water treatment works. Historically there has always been concern about the use of sewage sludge on agricultural land due to potential problems with odour, contamination by heavy metals, threat to public health and environmental pollution (Sterritt and Lester, 1980). Modern wastewater management and treatment methods of sewage sludge such as digestion, thermal treatment, lime stabilisation and composting have greatly reduced the risks associated with sludge application in agriculture (Kidd et al., 2007; Singh and Agrawal, 2008; Smith, 2009b; Al-Gheethi et al., 2018). Improved monitoring and control of industrial wastewater discharge to sewers has also led to lower contamination of wastewater streams (DEFRA, 2012). The amount of sewage sludge disposed of to land in the UK has risen as it has become safer and more favourable than other disposal methods such as landfill, incineration and dumping at sea. In the UK in 2010 79% of sewage sludge produced was spread to agricultural land as biosolids (Ofwat, 2015) compared to just 40% in 1970 (Sterritt and Lester, 1980).

The disposal of biosolids to agricultural land is controlled by thorough legislative framework to minimise any risk from pathogens, excess nutrients and potentially toxic elements (PTEs). All biosolids applied to agricultural land must meet a range of guidance and legislation including RB209: Nutrient Management Guide (RB209) (AHDB, 2017), 'The Sludge (Use in Agriculture) Regulations 1989' (Department of the Environment, 1989), 'The Sludge (Use in Agriculture) (Amendment) Regulations 1990' (Department of the Environment, 1990), 'Sewage sludge on farmland: code of practice for England, Wales and Northern Ireland' (DEFRA, 2018b), 'The ADAS Safe Sludge Matrix' (ADAS, 2001), 'Biosolids Nutrient Management Matrix' (ADAS, 2014a) and the 'Biosolids Assurance Scheme' (Assured Biosolids Limited, 2018). These documents cover nutrient management, application methods and timings, soil and sludge testing procedures, limits for PTEs in sludge and soils, sludge treatment processes and requirements, record keeping and general good industry practice.

There are a variety of biosolids approved and available for use in agriculture, each coming from a different treatment process and having different physical and chemical properties. The treatment process and dry matter content of a biosolid product will determine how it is spread and handled and the nutrient content of the material. In UK agriculture fertiliser recommendations are typically based around guidance from RB209: Nutrient Management Guide (AHDB, 2017) and the industry standard nutrient values for biosolids from this are shown in Table 2.1.

Table 2.1 - Biosolid nutrient contents (AHDB, 2017)

	Dry matter %	**Total Nitrogen kg N/t**	**Total Phosphate kg P_2O_5/t**	**Total Potash kg K_2O/t**	**Total Sulphur Kg SO_3/t**	**Total Magnesium Kg MgO/t**
Digested Cake	25	11	11	0.6	8.2	1.6
Thermally Dried	95	40	55	2.0	23	6.0
Lime stabilised	25	8.5	7	0.8	7.4	2.4
Composted	40	11	10	3.0	6.1	2.0

Biosolids differ from most other organic fertilisers in that they have a lower N:P ratio, being around 1:1 in digested cake and 8:11 when thermally dried. Biosolids therefore tend to contain more P per tonne applied than other organic fertilisers, for instance cattle farmyard manure (FYM) which has a 2:1 N:P ratio (AHDB, 2017). In line with the EU Nitrates Directive (EU Commission, 1991), organic

fertiliser applications are limited by their total N content in UK agriculture, with a maximum 250kg total N applied per hectare in any 12-month period in Nitrate Vulnerable Zones (NVZ). This application limit would put the maximum application of digested cake at 22.7t/ha, thermally dried biosolids at 6.25t/ha, lime stabilised at 29.4t/ha and composted at 22.7t/ha. On most soil types approximately 15% of the nitrogen applied in the biosolids listed above is readily available to the crop as ammonium-N, the remainder is contained as organic-N which can become available slowly over time (AHDB, 2017). Only the available-N is considered in nutrient planning and any extra N needed to supplement crop growth is typically applied as inorganic fertiliser.

The large amount of P applied in biosolids will often need to be considered over multiple cropping cycles in nutrient planning. P application in organic fertilisers is only is prohibited when soil P is excessively high (>71 mg l^{-1} Olsen P, ADAS soil index 5) and is restricted to crop rotation requirements when soils have sufficient existing P levels (>25 mg l^{-1} Olsen P, ADAS soil index 2) (ADAS, 2014a; AHDB, 2017). In these cases, the time between biosolid applications must be increased based on soil type to prevent excess soil P build up over a rotation. However, this could still mean a large influx of P in one biosolid application to a soil already containing enough P for crop requirements. The amount of P contained in biosolid applications has caused concern in the past which has led to several academic studies focusing on the area (Krogstad et al., 2005; Withers and Flynn, 2006; Withers et al., 2009; Bøen et al., 2013; Withers et al., 2016; Torri et al., 2017). However, many of these studies only focus on the long-term build-up of soil P rather than acute loss from an extreme weather event. Less focus has been put on the possible acute loss of soluble reactive phosphorus (SRP) from an extreme weather event such as flash flooding, or the potential changes in soil conditions caused by such an event. The possible geochemical and microbiological changes in soils which could lead to the acute loss of P from a flooding event will be explored later in the chapter.

2.1.2 Potentially toxic elements

All sewage sludge being applied to land in the UK must conform with the limits of Zn, Cu, Ni, Cd, Pb, Hg, Cr, Mo, Se, Ar and F set out in the Code of Practice for Agriculture Use of Sewage Sludge (Department of the Environment, 1989). These limits include maximum permitted levels of PTEs in soil and the maximum addition allowed over 10 years, shown in Table 2.2. Modern treatment processes ensure that biosolids produced for use in agriculture are well within these limits and methods continue to improve. Levels of PTEs in soils now rarely reach toxic levels except in exceptional circumstances or where there has been a history of poorly treated sludge application. Table 2.3 details how the composition of treated sewage sludge has changed since the 1970s and how loading of PTEs has now become less of a problem.

Table 2.2 - Maximum permissible concentrations of PTEs in soil after application of sewage sludge and maximum annual rates of addition (Department of the Environment 1989)

PTE	Maximum permissible concentration of PTE in soil (mg/kg dry solids)				Maximum permissible average annual rate of PTE addition over a 10 year period (kg/ha)[2]
	pH 5.0<5.5	pH 5.5<6.0	pH 6.0-7.0	PH[3] >7.0	
Zinc [1]	200	200	200	300	15
Copper [1]	80	100	135	200	7.5
Nickel	50	60	75	110	3
	For pH 5.0 and above				
Cadmium[1]	3				0.15
Lead [1]	300				15
Mercury	1				0.1
*Chromium	400				15
*Molybdenum[4]	4				0.2
*Selenium	3				0.15
*Arsenic	50				0.7
*Fluoride	500				20

Table 2.3 - Comparison of metal content in archived and current sewage sludge (ADAS et al., 2007)

Metal	Archived sewage sludges**	Currently produced sewage sludges***
Zinc	6000	608
Cadmium	44	1.4
Copper	5050	300
Lead	1240	138
Nickel	n/a	39
Chromium	n/a	82
Mercury	n/a	1.36

***Late 1970s-early 1980s information*
***Data for England & Wales 2005 (Source: Ofwat MD109)*
n/a = not available

Several studies have investigated the application of biosolids and sewage sludge to land with regards to PTE accumulation (Mantovi et al., 2005; Evanylo et al., 2006; Kidd et al., 2007; Singh and Agrawal, 2008; Carbonell et al., 2009; Delibacak et al., 2009; Smith, 2009a). These studies have generally found that there is some accumulation in soils of PTEs from biosolid applications, but their bioavailability to plants and microorganisms was low except in cases where very high application rates were used (Singh and Agrawal, 2008). In several cases the application of biosolids caused soil pH to lower but this only became a problem when left unchecked and redcuing below pH 5.5 in which case some phytotoxicity did occur (Evanylo et al., 2006). However, in well managed soils this would not become a problem, and in all experiments biosolid applications led to increased SOM, total N, available P and other important plant nutrients. In most cases these soil nutrient increases

also led to improved crop growth and yields. These findings would indicate that high nutrient contents rather than high PTEs are the main influencing factor on soil chemistry and crop growth.

Though the effect of biosolid PTE accumulation on soil chemical properties and crop performance seems well understood the impact on microbial populations may be less so. A long term experiment in Sweden conducted by Börjesson et al. (2014) investigated the uptake of PTEs to microbial biomass from biosolid-applied soils over different time frames. In one of the sites which had received biosolid applications in the early 1970s the concentrations of Cd, Ni and Pb were high due to their high content in the biosolid used at the time. However, since the reduction in PTE content of sewage sludge the soils had no further significant accumulation of those PTEs from biosolid application, with any hightened levels due to the historic application only. In the three other sites the accumulation of PTEs was only slightly significant due to the lower levels in modern treated biosolids. An exception to this was in Cu and Zn which were high and continued to increase across all sites due to the continued high content of these in biosolids. No toxic effects were found on microbial communities when anaylsed using phospholipid fatty acid (PLFA) analysis. Higher Cu and Zn accumulation in biosolid-applied soils is a commonly observed theme across biosolid application studies (Mantovi et al., 2005; Evanylo et al., 2006; Singh and Agrawal, 2008; Suhadolc et al., 2010; Börjesson et al., 2014) and zinc accumulation has been identified as the main concern relating to soil microbial activity. However, a critical review of heavy metal bioavailability from biosolid application by Smith (2009a) showed that in all but one study there was no tangible effect on soil microbial processes from PTEs, and only positive effects relating to soil microbial status and soil fertility were reported. Despite accumulation of PTEs in the soils of these studies the concentrations never exceeded safe levels and only led to any toxicity effects in plants when other factors such as soil pH were poorly manged.

Though Börjesson et al. (2014) did not find influences on soil microbial populations from biosolid application a study by Suhadolc et al. (2010) also investigated these effects. Suhadolc et al. (2010) did observe changes to soil microbial communities due to biosolid applications using DNA extraction and sequencing of the 16s rRNA gene in bacteria, and using the ITS1/ITS4 region as a marker in fungal communities. Results indicated that bacterial communities were more influenced by biosolid application than fungal communities, but that both communities showed signs of recovery after 3 months. The results from the Suhadolc et al. (2010) study compared to the Börjesson et al. (2014) study would indicate that PLFA analysis may not be sensitive enough to observe changes in microbial composition and other methods such as sequencing of the 16s rRNA gene are more suitable. The sequencing carried out by Suhadolc et al. (2010) only investigated the microbial communities using a Restriction Fragment Length Polymorphism fingerprint analysis to demonstrate differences between

populations, rather than what those differences might signify. Due to this restriction it was concluded that biosolids do have an effect on soil microbial communities, but whether this was due to PTE accumulation or the nutrient application was not determined. However, recent advancements in Next Generation Sequencing (NGS) technology have further improved the identification of microbes and the quality and quantity of data that can be obtained. Recent reviews by Wang et al. (2019) on anaerobic digestate microbial communities, and Zhao and Liu (2019) on pathogen control in biosolids, suggest that high-throughput 16s rRNA sequencing could be the most effective method of interpreting biosolid microbial communities. The utilisation of these methods could therfore be a useful tool for understanding changes to soil microbial communities from biosolid applications in this project.

2.1.3 Digestion processes

Before discussing the use of NGS to characterise biosolid microbial populations the digestion processes used in biosolid treatment first need to be understood as these can heavily influence the microbes present. To produce biosolids some form of OM digestion is carried out on treated sewage sludge, which can include aerobic digestion, composting, lime stabilisation or anaerobic digestion (AD). The aim of all these processes is to reduce the OM of the material using bacteria which favour the conditions created to break down organic carbon compounds. Aerobic digestion is the breakdown of OM in the presence of oxygen, this is generally quicker than AD and requires less capital cost. However, the energy consumption of keeping the digestate aerated means it is not as energy efficient as AD. The rapid oxidation of organic C during aerobic digestion also generates carbon dioxide (CO_2), which has negative environmental impacts as a greenhouse gas (Demirbas et al., 2017). Composting also involves the aerobic digestion of sewage sludge but it is also mixed with green waste including wood chips, crop residues or other waste plant material. As the sludge is digested microbes also digest the OM from the plants. This process is useful for controlling high nutrient or PTE concentrations which may be present in treated sewage sludge. The higher OM content of the compost due to the plant material means that the total concentration of potential contaminants is reduced. These changes mean that the risk of N leaching, excessive soil P, or high levels of PTE loading from biosolid compost applications are reduced. High core temperatures during the composting process also eliminates pathogenic bacteria (Mello Leite Moretti et al., 2015). However, composting produces the greenhouse gases CO_2, methane (CH_4) and nitrous oxide (N_2O) (Sánchez et al., 2015) and can have high transport costs for land application due to its bulk and lower nutrient concentrations. Lime stabilisation involves the addition of calcium oxide (CaO) and calcium hydroxide ($Ca(OH)_2$) to raise the pH of sewage sludge to 12 or greater and heat to between 55^{o}C and 70^{o}C. These high pH and temperature conditions are unfavourable to pathogens and result in their

deactivation or death (Al-Gheethi et al., 2018). AD is the breakdown of OM in the absence of oxygen. In the UK 73% of biosolids are produced using AD (Assured Biosolids Limited, 2019), making it the predominant production method. AD though taking longer than aerobic digestion is more energy efficient as the digestion of organic C by this method generates CH_4 which can be collected and reused for energy production as biogas. A focus on biosolid derived from AD within this project may therefore be the most relevant approach for representing UK biosolid use.

Though the aforementioned digestion processes have long been considered effective ways of removing pathogens and other bacteria transferred from sewage sludge to biosolids and soils some recent studies have questioned its effectiveness. Al-Gheethi et al. (2018) reviewed sewage treatment methods in the Middle East including AD, aerobic digestion, lime stabilisation and heat treatment of sewage sludge for pathogen removal. When reviewing the effectiveness of AD there appeared to be potential for the survival of some pathogenic bacteria within solid materials. These surviving bacteria then had the ability to regrow during storage due to the high nutrient resources available and the more favourable environmental conditions, such as the reintroduction of oxygen (Chen et al., 2011). Another review by Zhao and Liu (2019) also raised concern about the potential survival of pathogenic microorganisms in AD biosolids. The study reported that AD was useful for controlling some pathogens but not all. *Escherichia coli, Salmonella, Yersinia enterocolitica, Enterococcus* were found to be vulnerable to AD, but *Campylobacter jejuni*, and *Streptomyces, Collinsella aerofaciens, Streptococcus salivarius* and *Gordonia bronchialis* were hardly removed by AD and were much more resistant to the process. The resistance to acute stress of *Clostridium* and *Bacillus* species was also highlighted as a possible problem with these species able to survive AD conditions. *Clostridium* and *Bacillus* both contain important human pathogenic species (Turnbull, 1996; Wells and Wilkins, 1996). The study states that in order to assess the potential environmental risks of infectious pathogens a more holistic approach is required, suggesting that investigation of the whole soil-biosolid-environment system would be beneficial. An aerated soil environment could offer favourable conditions for surviving aerobic pathogenic organisms to regrow after AD. Also, flooding of soils leading to anaerobic conditions could mean that any resilient and adaptable microbes which survive the AD process may persist in soils and outperform native populations for resources upon relief of the flood event. Further investigation would be required to assess this risk.

2.1.4 Biosolid microbial populations

As discussed in section 2.1.2 the most appropriate method for assessing the microbial populations in biosolids, and in soils influenced by their application, appears to be the used of NGS, particularly amplification of the hypervariable 16s rRNA gene. 16s rRNA gene amplification of bacterial DNA can be used to determine the identity of the bacteria present in soils and biosolids and determine how

they persist between samples as conditions change (Sanschagrin and Yergeau, 2014). The fast development and enhancement of high-throughput NGS technologies in recent years means an increasingly broad range of soil microbes can be identified and are continually being explored (Levy and Myers, 2016). Several studies have used NGS to characterise the bacterial compositions of biosolids and anaerobic digestates to various levels of detail and with different focuses.

The review by Wang et al. (2019) previously mentioned in section 2.1.2 characterised a range of different AD methods and organic materials using NGS. Hydrolytic bacteria in the phyla *Bacteroidetes* and *Firmicutes* were important in the digestion of polysaccharides, proteins and lipids during hydrolysis but only accounted for around 6% of microbes in AD. Fermentative bacteria made up around 70% of AD microbes and were present in the phyla *Proteobacteria, Bacteroidetes, Firmicutes* and *Chloroflexi,* with *Proteobacteria* associated with the breakdown of amino acids and *Bacteroidetes* and *Firmicutes* preferring monosaccharides and fatty acids. The relative abundances of each bacterial phyla and the prevalence of different species within them depended heavily on the feedstuff used during the AD process. A study by Liu et al. (2016) which focused specifically on sewage sludge AD found that bacterial communities were dominated by *Firmicutes, Bacteroidetes, Candidate Division WS6, Proteobacteria* and *Chloroflexi.* This study varied the total solids content of the materials used. *Firmicutes* dominated in 10-15% total solid materials, *Bacteroidetes* became more prominent alongside *Firmicutes* as solids rose to 17%, and then *Candidate Division WS6* rose in abundance alongside *Bacteroidetes* at 19% solids. *Proteobacteria* and *Chloroflexi* changed little in relative abundance between treatments and were always secondary to the other major phyla. When assessing bacterial phyla across a range of studies investigating AD from different farmyard wastes and sewage sludge a common trend appears to be the dominance of the *Firmicutes* phylum (Luo et al., 2013; Sun et al., 2015a; Liu et al., 2016; Jiang et al., 2019; Wang et al., 2019). Secondary dominant phyla found in AD processes included *Bacteroidetes, Proteobacteria* and *Chloroflexi.* Other phyla which were common in many of the studies, but which tended not to dominate, include *Synergistetes, Actinobacteria, Planctomycetes* and *Saccharibacteria.* A point of note is that the genera *Clostridium* and *Bacillus*, which were highlighted in section 2.1.3 as potentially being resistant to the AD process and potential pathogens (Zhao and Liu, 2019), belong to the *Firmicutes* phylum. Furthermore, in many of the studies *Clostridium* was identified as dominating *Firmicutes* populations (Sun et al., 2015a; Liu et al., 2016; Wang et al., 2019) and this genus is known to contain a number of important human pathogens (Wells and Wilkins, 1996). How the bacterial phyla identified in biosolids overlap with soil bacterial populations, and the potential impact of their application to soils, will be a point of exploration of this project. Reviewing the typical characteristics of soil bacterial phyla for comparison to biosolid bacterial populations will be explored later in this chapter.

2.2 SOIL ORGANIC MATTER

2.2.1 Organic inputs

SOM is vital to the soil ecosystem, containing all organic components of the soil including plant detritus, animal remains, animal excreta, active and dead microbes, humic substances and various organic compounds for use in microbial metabolisms. Increased SOM is a known way to improve soil drainage, increase water holding capacity, retain and release nutrients, supply substrate to beneficial microorganisms, and ultimately increase crop yields (Hijbeek et al., 2017), with SOM often considered a reliable indicator of soil fertility (Reeves, 1997; Johnston et al., 2009). Increasing SOM is therefore desirable for farmers and several options are available for achieving this including the use of cover crops or grass leys in crop rotations, reduced tillage practices, incorporating crop residues or applying organic inputs (Reeves, 1997; Ding et al., 2006; Koch and Stockfisch, 2006; Nascente et al., 2013). In UK arable soils the SOM% is usually in the range of 3-5%, increasing to 4-7% with incorporated crop residues or OM inputs, and to 5-10% when using grass leys in rotation (Soffe, 2003). Direct additions of OM through organic inputs are a practical option for improving SOM both in the short-term and for inclusion as part of long-term management plans (Magdoff and Weil, 2004). Different options for adding OM to soil are available including incorporation of crop resides such as straw or additions of FYMs, composts or biosolids, each of which will impact soil physical, chemical and biological properties in different ways (Haynes and Naidu, 1998; Fuentes et al., 2012; Kaleeem Abbasi et al., 2015).

Biosolid additions are the focus of this project but understanding how different forms of OM are broken down is beneficial to understanding the broader effects of SOM on the soil system. Nutrients are released to crops from SOM through decomposition of organic nutrients to inorganic, readily plant available forms. Decomposition of SOM is carried out by a variety of soil organisms at different rates which can be affected by crop management and environmental conditions. In aerated soil conditions decomposition of soil organic carbon (SOC) by microbes results in the loss of some of the SOC as carbon dioxide (CO_2) through aerobic respiration. An example of this process is displayed in equation 2.1, which shows respiration involving glucose.

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6H_2O + 6CO_2$$

Equation 2.1

Other more complex molecules containing C and other nutrients will be present in soils at different quantities depending on soil inputs and native SOM composition. Starches and sugars which are highly soluble and shorter chain molecules can be broken down very quickly in a matter of days, whereas fats, waxes and lignin which are more complex and less soluble can take months or years to

decay (Soffe, 2003). Warm, moist and well aerated soils increase microbial activity and decomposition rates, as these conditions favour the oxidation of SOC to CO_2 through microbial respiration. Waterlogged, cool soils will reduce decomposition rates due to a restricted oxygen supply for SOC oxidation, and cooler temperatures lowering microbial activity (Magdoff and Weil, 2004). Much of the variation between organic inputs and their decomposition rates comes from the differences in the C:N ratio of those inputs.

2.2.2 C:N ratios

Soil microbes have a C:N ratio of around 8:1, however they require a diet of around 24:1, with the excess C used as a source of energy in respiration and much of it oxidised as CO_2 rather than being retained in microbial cells. C:N ratios of organic inputs vary considerably from animal manure slurries (6-9:1) with high N and lower C content, to straw with high C and low N (80:1). If the C:N of the organic input is below the 24:1 ratio then the excess N not used in the decomposition process will be released to the soil, known as N mineralisation. If the organic input ratio is above 24:1 then there will be net consumption of N from inorganic sources, known as immobilisation, as this will be required by microbes to offset the low organic N and complete the decomposition process (USDA, 2011a). With biosolids having a relatively low C:N ratio of around 10:1 in a dewatered digestate then net N mineralisation is expected to occur which will become available to the crop at a rate based on the activity of the microbial populations (Gärdenäs et al., 2011; Kaleeem Abbasi et al., 2015). Any N mineralised from biosolids in excess of what the crop can use may be released to the soil solution and lost to surface waters by leaching or released to floodwater in the case of an extreme weather event. In comparison, FYMs have a C:N ratio in the region of 17-20:1 so will have a lower N mineralisation potential than biosolids. Though the structural impacts on soil and the management considerations of FYM and biosolids may be similar, the difference in potential for the mineralisation of N and other nutrients needs to be considered, especially when reviewing and comparing any literature which focuses on FYM applications.

Though the C:N ratio of organic inputs to soils in relation to the soil native C:N ratio is important, the actual C:N ratio of the soil may not be a good indicator of N mineralisation rates. A study of forest soils by Bengtsson et al. (2002) suggested that the mineralisation rate of N was not due to the C:N ratio of the soil but was instead related to the respiration rate in the soil. The mineralisation or immobilisation of N in the soils was therefore deemed to be in response to microbial density and activity, and the potential for SOC turnover. A possible explanation for this is differences in C lability in different fractions of the SOC. SOC can exist in several forms in the soil, one of which is as plant material such as cellulose, starch and lignin, which can be broken down by microbes through respiration with carbon being lost to the atmosphere as CO_2. Other carbon may end up in the soil

humic substances pool containing humus, humic acid and fulvic acid which are less available to microbes. The soil humic fraction is much older and in more stable, polymerised molecular form than the small chain cellulose and starch molecules from fresh plant materials which are more easily broken down and favoured by soil microbes (Kramer and Gleixner, 2006; DiDonato et al., 2016; Masoom et al., 2016). A soil with more fresh, labile SOC may therefore have higher N mineralisation rates that soils with more stable, native humic SOC, despite both having similar total SOC contents. This means the lability of C in organic inputs, particularly biosolids for this study, will also be important in N mineralisation.

A study by Smith et al. (1998) showed that C:N ratio and availability of C and N in different biosolids resulted in different N mineralisation rates from the biosolids. In liquid biosolids where the C:N ratio was low, and the available N content was high, around 90% of the total N was made available as nitrate (NO_3^-) in soils after 20 days of incubation. In dewatered, digested biosolids, where C:N ratios were closer to the soil optimal and available N was lower, mineralisation was moderate with around 20-40% conversion to NO_3^- after 73 days of incubation. In dewatered, undigested biosolids C:N ratios were higher and exceeded 20:1, available N was lower, and available C was higher. These biosolids caused a net immobilisation of N in soils. These results would partially support that C:N ratios play an important part in N mineralisation, but also indicate that the availability of both N and C will have a large influence on its rate and quantity. This is likely due to the availability of those nutrients to soil microbes for both mineralisation and immobilisation processes. Assessment of digested air dried biosolids within the same study would support this. These biosolids had the lowest N mineralisation level at 7% of total N after 73 days of incubation. The available N of these biosolids was low, and the available C is also assumed to have been low due to the digestion process, resulting in a material which was relatively resistant to mineralisation by soil microbes.

2.2.3 The priming effect

A well explored research area in relation to the difference in SOC lability and turnover is the so called 'priming effect' (PE). The PE is the phenomenon by which the addition of fresh, highly labile organic carbon causes an overall decrease in SOC stocks due to the decomposition of native SOC through increased microbial activity. A very large number of studies have explored this area with mixed results and explanations of the mechanisms governing the PE, fortunately meta-analyses have also been conducted on this literature to identify the main trends and conclusions. A meta-analysis by Zhang et al. (2013) found that fresh organic additions increased the microbial decomposition of native SOC by an average of 26.5%, though this figure varied greatly from 95.1% inhibition to 1,207% stimulation. The PE was found to be enhanced in soils with higher SOC, lower N, higher C:N ratios and lighter textures. However, although this study assessed the substrate inputs based on their C

quality and content, no N figures or C:N ratios of substrates were assessed. Given that the PE was found to be responsive to soil N contents, as soil microbes appeared to scavenge N from the native SOM when extra SOC was introduced, then N content of the organic input would also be important to the PE. The importance of N was also supported by control soils, with higher N content having higher decomposition rates. A review by Luo et al. (2015) also showed that fresh carbon inputs increased the rate of decomposition in a range of soils, but placed this at a 14.2% average increase. This review investigated in more detail the properties of organic inputs and of the lability of SOC and their importance in the PE. For instance, forest soils were found to have lower PE despite having higher SOC because of the turnover of forest litter in the topsoil acting as a labile 'high energy' C source. This access to 'high energy' C was identified as the main driver of the PE and, in soils other than forest soils, meant that fresh C inputs had a positive correlation to microbial activity. This link to energy rich substrates increasing the PE due to increased microbial activity is supported in several other individual studies as the driving factor of C and N turnover in soils (Fontaine et al., 2004; Chowdhury et al., 2014; Wang et al., 2015). Evidence therefore indicates that one of the main influencing factors on whether a PE occurs is the quality of the OM input as a microbial feedstock.

Understanding the composition of the OM in biosolids is important for understanding how the material will impact soil microbial populations and in turn how this will impact soil nutrient contents. A study by Wijesekara et al. (2017) found that although biosolid applications increased SOC, only 27% of the carbon added was labile. This would suggest that most of the carbon added through biosolids is not readily available to microbes. Despite this, microbial activity still increased at both sites in the study with biosolid additions to a point where SOC loss occurred. A study by Pan et al. (2017) investigating C sequestration from biosolid applications found that C storage potential was high in biosolids, with 91% being retained in soils. This ability for biosolid C to be stored was linked to the C:N ratios of the soil and biosolid. The lower C:N ratio of the biosolids led to an increased loss of N through mineralisation and a retention of the remaining C in the microbes. This retained C was found to be from both labile and non-labile pools, with labile C becoming native SOC over time. Evidence from these studies would therefore suggest that biosolid applications could lead to increased microbial activity in soil, but increases in SOC, rather than losses by the PE, due to high levels of non-labile C. Increases in N losses due to high N mineralisation should also be expected due to the low C:N ratio of the material and quick turnover of its labile C.

2.2.4 SOM influence on crop yield

Although the benefits of organic additions and increased SOM are well documented, the reasons for any improvement in crop performance cannot be attributed to merely increased SOM, but rather the factors that SOM has an impact on. Increased SOM includes the physical benefits of increased

water holding capacity, porosity, infiltration capacity, hydraulic conductivity and water stable aggregation, as well as decreased bulk density and surface crusting (Haynes and Naidu, 1998). These structural improvements also come alongside increased nutrient supply and holding capacity and increased microbial biomass and its associated benefits. Isolating which is the main influencing factor on any yield benefits from improved SOM is therefore difficult, as increases in SOM rarely exist in isolation. A meta-analysis by Hijbeek et al. (2017) investigated whether there was a direct yield response from SOM additions and found no significant improvements, but found that attainable yields increased. The study instead suggested that the limiting factor in crop production was nutrient supply rather than SOM. A study by Oelofse et al. (2015) also found results that suggested nutrient supply rather than SOC content was a more important contributor to increased crop yields. This study suggested that an SOC content of just 1% was enough to maintain yields when nutrient supply is sufficient. This conclusion is supported in a review by Loveland and Webb (2003) which found that there was no critical level of organic matter in UK soils, and that crop yields could be maintained as long as nutrient supply was adequate. However, in the Oelofse et al. (2015) study several confounding variables exist including soil type, local climate and altitude which provide limitations to the study's broad generalisations on SOM impact over a wide range of studies. It is stated that a range of factors need to be considered when assessing the impact of SOM on crops as it is difficult to separate individual influencing factors. Site-specific details can therefore be lost with such broad approaches.

Despite the studies mentioned above suggesting that SOM content was not important in influencing crop yield, in soils where the crop is stressed the case could be otherwise. Increased SOC has been found to improve crop production in drought-risk areas due to soil structural improvements and improved water holding capacity (Iizumi and Wagai, 2019). A study by Alvarez (2002) found that increased wheat yields in a semi-arid region were more associated with SOC than crop N supply due to increased soil water holding capacity causing improved crop resilience to drought stress. Structural changes from increased SOM improving crop production in drought conditions could also be relevant for improved crop resilience in flooded conditions. Increased aggregate stability and aeration in the soil may mean that roots remain aerated for longer under flooding due to trapped air pockets. After flooding, improved drainage may also allow soils with higher SOM to return to normal conditions more quickly and reduce the duration of crop stress and improve yields. However, studies showing direct arable crop yield benefits from SOM after flooding are difficult to identify and this area could call for further investigation in this project.

In situations of high crop drought or flood stress then SOM itself may be the main influencing factor on crop performance. Where crops are not stressed however, then nutrient supply could be the

determining factor. Biosolid applications are known to increase a number of factors in the soil including SOM, N, Olsen P, K and a variety of other important plant nutrients (Kidd et al., 2007; Suhadolc et al., 2010; Börjesson et al., 2014). This could mean that it is difficult, if not impossible, to isolate which are the main influencing factors in any crop performance differences. Whether crops are more resilient due to increased vigour from higher nutrient supply, or due to better soil structure, may be irrelevant though, as the factors will never exist in isolation when considering biosolid applications.

2.3 NITROGEN

2.3.1 The nitrogen cycle in soil

As has previously been established in section 2.1.1 the maximum application rate for organic fertiliser inputs, including biosolids, is based on 250kg per hectare total N content as a field limit. This limit is in place to protect surface waters from leaching of NO_3^- from soils, which can occur from both the addition of readily available N contained in the organic input and by mineralisation from organic N in the input. The behaviour of N in soils and SOM is not straightforward, with the rates of change between different N compounds governed by soil properties, microbial processes and environmental conditions. The 'nitrogen cycle' is the term often used for describing N processes in soils and the environment. The nitrogen cycle includes N at several different stages of transformation, including fixation, assimilation, mineralisation, nitrification and denitrification, all of which involve oxidation-reduction reactions in the soil mitigated by microbes or plants. Soil N can appear as many different compounds during the nitrogen cycle including as organic N, nitrate (NO_3^-), nitrite (NO_2^-), ammonia (NH_3), ammonium (NH_4^+), urea (CH_4N_2O), atmospheric nitrogen (N_2), nitric oxide (NO) and nitrous oxide (N_2O). Understanding the nitrogen cycle in detail and how biosolid applications and soil flooding affect it a vital part of this project.

In soils nitrogen compounds can exist as organic N within SOM or as inorganic forms, mainly NH_4^+, NO_2^- and NO_3^-. When biosolids are applied to the soil they contain nitrogen mostly in organic forms which is added to the SOM pool, but they also contain some readily available N in the form of NH_4^+ and NO_3^- (AHDB, 2017). In the first stage of the nitrogen cycle organic N in the SOM is mineralised, also known as ammonification, into NH_4^+ by soil microorganisms. NH_4^+ can either be used as a plant nutrient, used by microbes and immobilised back into organic N, or can be used as a substrate in the nitrification process. Nitrification is the two-stage biological oxidation of NH_4^+ to NO_2^- and then to

NO_3^- and is displayed in equations 2.2 and 2.3. The rate of nitrification of NO_2^- to NO_3^- is typically faster than NH_4^+ to NO_2^-, and so NO_2^- is usually only present in small quantities in soil. NO_3^- can be easily taken up as a plant nutrient and is the favoured form in most UK agricultural crops (Soffe, 2003). NO_3^- is very mobile in soils, is highly soluble and can be easily lost by leaching run-off into surface waters or lost to gaseous forms by denitrification. An outline if the nitrogen cycle is detailed in Figure 2.1 and is discussed in greater detail in this section.

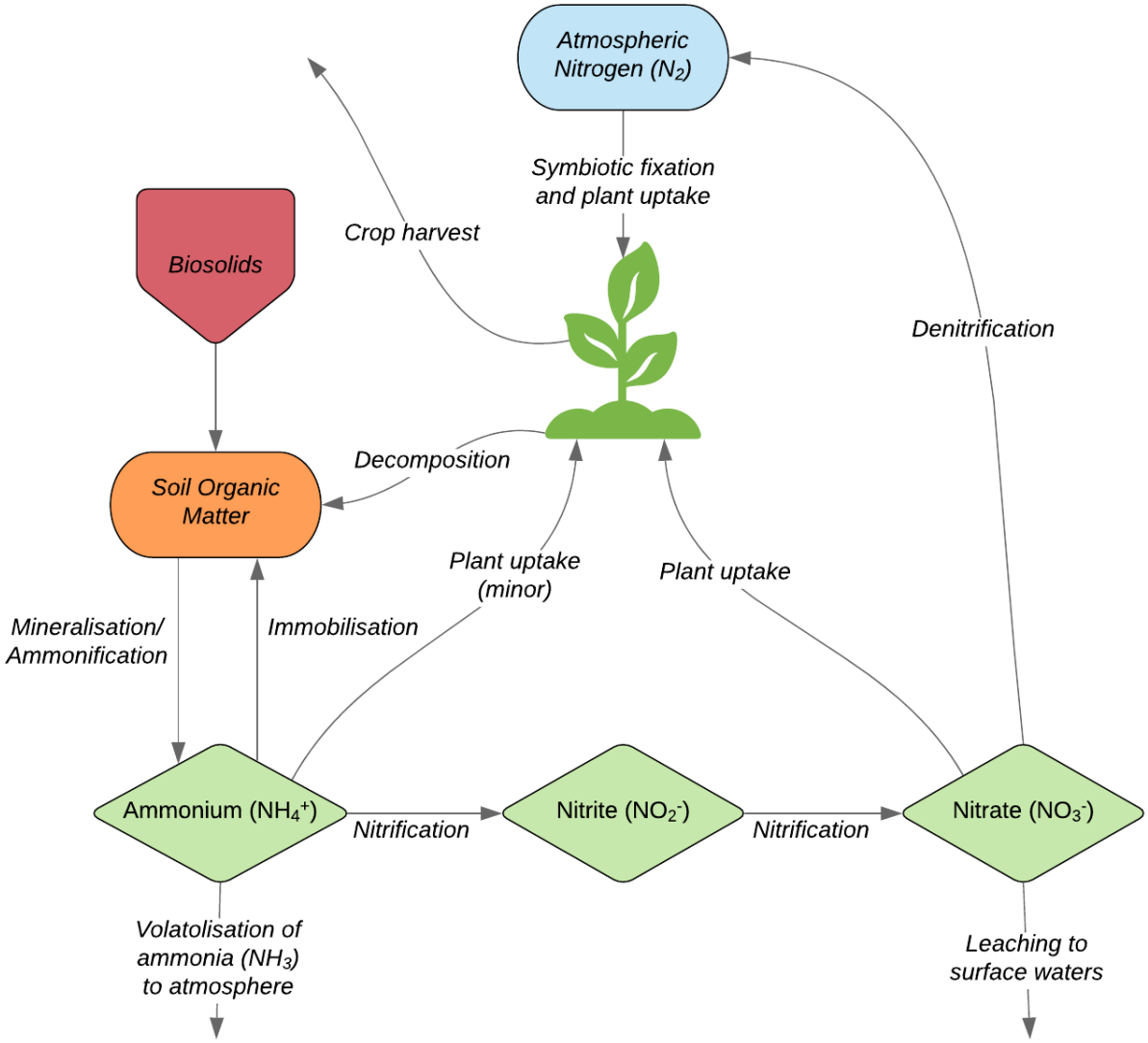

Figure 2.1 - The nitrogen cycle in soil, after Soffe (2003).

2.3.1.1 Nitrogen additions

Nitrogen can be introduced to the soil through several different methods. Additions of mineral fertiliser adds N directly to the mineral pool in the forms of NH_4^+, NO_3^- or CH_4N_2O where it is available to be taken up directly by plants and soil microbes. Nitrogen fixation is another way for N to enter the soil and is the process of atmospheric nitrogen being converted to nitrogen compounds

in the soil by microbes. Many nitrogen fixing bacteria have symbiotic relationships with plants, particularly legumes, which are often used in crop rotations to enhance soil N contents (Ledgard and Steele, 1992). The main form of nitrogen addition to soil which is focused on in this project is through organic N. Organic N is contained in plant litter and other organic compounds which are encompassed in organic inputs including biosolids. This organic N is broken down through the process of mineralisation/ammonification by decomposer microbes in the soil microbiome and released to the soil as NH_3 or NH_4^+. As discussed in section 2.2.1 the total amount of N available for mineralisation and the rate of its release is governed by the C:N ratios of any organic input and that of the soil microbiome. After N is released as NH_4^+ through mineralisation it is competed for in the soil microbiome by immobilisers, which assimilate the NH_4^+ back to organic N, and nitrifiers. In aerated agricultural soils nitrification is considered the main fate of NH_4^+ over immobilisation (Burger and Jackson, 2003) and occurs in two stages. The first stage of nitrification is the oxidation of NH_3 or NH_4^+ to NO_2^- by ammonia-oxidising bacteria (AOB) and ammonia-oxidising archaea (AOA). The second stage is the oxidation of the NO_2^- from the first stage into NO_3^- by nitrite-oxidising bacteria (NOB) (Prosser, 1990). Ammonia-oxidation is the rate-limiting step of the nitrification process as the nitrite-oxidation stage depends on the amount of NO_2^- oxidised from NH_4^+. The nitrification process can be affected by many different factors including substrate supply, environmental conditions, presence and abundance of nitrifying organisms, and plant and microbial interactions with those organisms (Norton and Ouyang, 2019). Understanding this process is therefore important for determining the fate of the N contained in biosolids and the risk of its loss as NO_3^- after nitrification. The nitrification process is demonstrated in equations 2.2 and 2.3.

$$2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 2H_2O + 4H^+$$

Equation 2.2

$$2NO_2^- + O_2 \rightarrow 2NO_3^-$$

Equation 2.3

he activity of AOB and AOA varies depending on soil properties and environmental conditions over which group are dominant in the ammonia-oxidation process. A review of studies by Shen et al. (2012) exploring AOA and AOB in a variety of Chinese soils showed that there was a tendency for higher AOA abundance and activity in low-nutrient, acidic soils. AOB on the other hand dominated the nitrification process in high-nutrient soils with a neutral or alkaline pH. Banning et al. (2015) conducted a study into semi-arid agricultural soils in Western Australia and focused on the distribution of AOA and AOB in the soil profile. AOB were found to have higher abundance than AOA in all soils but were concentrated in the top 10cm of the soil profile, while AOA abundance increased with soil depth. SOM tends to be concentrated in the top 5-10cm of undisturbed or min-till soils (Hernanz et al., 2002; Wright et al., 2007; López-Fando and Pardo, 2011; Nascente et al., 2013) and

so this increase in AOB abundance in the more nutrient rich surface layer would support the conclusions of Shen et al. (2012). Banning et al. (2015) also showed correlations of increasing AOB with increased SOC and higher pH, though the pH in the soils only ranged from acidic to neutral. The study concluded that AOB were most likely responsible for most of the nitrification activity in those soils, and the correlations also support that AOB dominate nitrification in soils with higher nutrient content and higher pH. Other studies have shown that with nitrogen and organic fertiliser additions to agricultural soils the activity of AOB is increased more than that of AOA (Carey et al., 2016; Ouyang et al., 2016). AOB in agricultural soils typically include the genera of bacteria 'Nitrosomonas' and 'Nitrosospira' in the *Proteobacteria* phylum (Norton and Ouyang, 2019).

The activity of NOB in the nitrification process, as previously mentioned, is limited by the rate of ammonia-oxidation. The most important and predominant NOB in terrestrial ecosystems belong to the 'Nitrobacter' genus of the *Proteobacteria* phylum, or the 'Nitrospira' genus of *Nitrospirae* (Han et al., 2018; Norton and Ouyang, 2019). The rate of activity of NOB in terrestrial soil systems means that free NO_2^- does not persist for long in the soil and is rapidly oxidised to NO_3^- (Carey et al., 2016; Norton and Ouyang, 2019). A study by Han et al. (2018) investigating the effect of fertiliser applications on NOB populations in soil found that NOB increased in abundance with fertiliser use. 'Nitrospira' were more affected by fertiliser application than 'Nitrobacter' in terms of both population size and diversity. 'Nitrospira' may therefore be more sensitive to nutrient additions to soils from biosolid application. A study by Yao and Peng (2017) into nitrifying communities in wastewater treatment plants found that NOB dominated the nitrifying communities in terms of population size and activity. This suggests that NOB populations can be larger than AOB populations, though they are still limited by the ammonia-oxidation step. However, in the study the rate of nitrification from AOB did not account for all the required electron transfer for microbial growth in the NOB populations, and the act of nitrate-reducing bacteria in the denitrification process could also be supporting NOB populations in the experiment. This may not be replicated in agricultural soils under normal conditions but may affect flooded soils in the right circumstances. NOB populations were dominant in the system and had ample capacity for metabolising all free NO_2^- from AOB activity, supporting that NO_2^- does not persist for long in nitrifying systems. Another factor which alters how AOB and NOB interact in the soil is the recent discovery that some 'Nitrospira' members can act as catalysts to both the ammonia- and nitrite-oxidation steps of nitrification and are complete ammonia oxidisers or 'comammox' organisms (Daims and Wagner, 2018). This comammox behaviour of 'Nitrospira' means that it could act to enhance the activity of AOB or NOB as required for equilibrium of the nitrification process and make the system more robust to change if present in large numbers. The nitrification process is complex, as highlighted by

the literature, but is vital for providing N to crops in the readily available form of NO_3^-. Though useful for crop growth the potential for leaching loss of NO_3^- is also high, especially in flooded conditions, and is a key focus of this project.

2.3.1.2 Nitrogen losses

NO_3^- leaching is typically the greatest source of N loss in terrestrial ecosystems (Robertson et al., 2013; Sebilo et al., 2013). NO_3^- leaching occurs when rainfall or other excess water input to the soil exceeds the rate of evapotranspiration and crop uptake leading to drainage runoff, carrying with it highly soluble NO_3^-. The use of mineral fertilisers such as ammonium nitrate (NH_4NO_3) can increase the risk of NO_3^- leaching as they are more readily soluble than organic N inputs (Soffe, 2003). A long-term study by Sebilo et al. (2013) found that over 30 years only 61-65% of applied N fertiliser was taken up by plants, 8-12% was lost by leaching, 12-15% was contained in SOM, and any remaining was lost to the atmosphere by volatilisation and denitrification. The use of organic fertilisers and FYM can reduce acute leaching loss of NO_3^- as organic N is mineralised more slowly to the soil solution from SOM. However, as mineralisation is a continuous process NO_3^- leaching can still occur in significant amounts if mineralisation does not align with crop uptake (Kirchmann and Bergström, 2001). As discussed previously this loss of NO_3^- to surface waters can have significant negative environmental effects through driving eutrophication (Michael Beman et al., 2005; Sebilo et al., 2013; Huang et al., 2017). The potential for NO_3^- leaching from biosolid applied soils is a crucial point of understanding for this project. If excess NO_3^- is mineralised and nitrified from biosolids then a flood event could allow it a path of easy access to surface waters. Alternatively, the organic N in biosolids could provide a more stable N form than mineral applications and mitigate NO_3^- loss from flooded agricultural soils.

Loss of N from soils does not occur only by leaching. Other significant losses of N can occur to the atmosphere by denitrification and volatilisation. NH_3 volatilisation can occur when N is added to the soil in CH_4N_2O or NH_4^+ containing fertilisers. CH_4N_2O is broken down by the urease enzyme in soil and then released as NH_3 gas to the atmosphere and NH_4^+ can convert to NH_3 in high pH conditions (Freney et al., 1983). CH_4N_2O is found in animal urine and most FYM, being especially high in poultry litter (Schilke-Gartley et al., 1993; AHDB, 2017). Mineral CH_4N_2O applications also suffer from high volatilisation losses if not managed correctly. A meta-analysis by Pan et al. (2016) found that globally an average of 18% of applied mineral N was lost to volatilisation with a high of up to 64%. Volatilisation is an important route of N loss in soils, however with the focus on loss to watercourses rather than atmospheric loss it may not be of great relevance to this project. Denitrification on the other hand, while being a source of atmospheric N loss, is caused by the reduction of NO_3^- and can occur in wet and flooded soils so is highly relevant to the studied system of this project.

Denitrification in the microbially facilitated reduction of NO_3^- to NO_2^-, NO, N_2O and ultimately N_2, and can occur under even moderately reducing conditions (McBride et al., 1994). The half-reactions which constitute the denitrification process are shown in equations 2.4-2.7.

$$NO_3^- + 2H^+ + 2\,e^- \rightarrow NO_2^- + H_2O$$

Equation 2.4

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O$$

Equation 2.5

$$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O$$

Equation 2.6

$$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$$

Equation 2.7

During the process of denitrification N_2O can be lost to the atmosphere before being fully reduced to N_2. Though the NO_3^- lost by leaching is lessened by the denitrification process, the N_2O released is a potent greenhouse gas (GHG) which has significant negative environmental effects (Wang et al., 2014). With denitrification occurring in reducing conditions many studies have found significant NO_3^- loss in flooded soils where the oxygen supply is restricted (Unger et al., 2009b; Gardiner and James, 2012; Rubol et al., 2012). A study by Sánchez-Andrés et al. (2010) found that denitrification increased significantly after around 7 days of flooding in semi-arid flood plain soils. High organic soils have been shown to have high rates of denitrification, with SOC and microbial biomass C strongly correlated to denitrification rates; with C also being the limiting factor rather than NO_3^- (Drury et al., 1991). A study by Kramer et al. (2006) also showed that organic additions to orchard soils in the form of poultry manure increased denitrification rates over the same soils applied with equivalent mineral N. This supports that SOC and microbial activity are the limiting factors of denitrification rather than the amount or source of NO_3^- present. In a recent study by Sánchez-Rodríguez et al. (2018) soils amended with OM and flooded showed increased denitrification rates and overall GHG emissions. In that study a long-term flood saw NO_3^- in organic-treated soil floodwaters was lower than that of control soils due to increased NO_3^- reduction and denitrification. If soil floodwaters in the experiments of this project become reducing more quickly due to biosolid application then that could lead to a reduced loss of NO_3^- to floodwaters through increased denitrification. This would be despite the added readily available and organic N contained in the biosolids. This is one of the main points for investigation in this project.

2.3.2 Nitrate limits in surface waters

The loss of NO_3^- to surface waters is one of the main concerns around the application of any N fertilisers to agricultural land. Various restrictions are in place to minimise the risk of leaching of N from agricultural soils, including closed periods and maximum application limits based on soil types and crop selection. As previously discussed in section 2.1.1 the maximum application limit for organic fertilisers is 250kg N/ha per year based on NVZ restrictions as part of the EU Nitrates Directive (EU Commission, 1991). The Nitrates Directive, of which NVZs are a key part, was put in place to maintain the quality of surface water and minimise the risk of eutrophication, of which NO_3^- is a main driver alongside phosphate (Selman and Greenhalgh, 2010b). The limits for application of N fertiliser for crops are based on maintaining watercourses at concentrations below 50mg/l nitrates in NVZs. However, the definition of 'nitrates' in the Nitrates Directive refers to all NO_3^- and nitrogen compounds at equivalent NO_3-N concentrations (DEFRA, 2016). This definition means that it is not just the NO_3^- content of water which needs to be measured but also the NH_4^+ and NO_2^- content to give total inorganic nitrogen (TIN). This is of importance to this project when assessing the risk level of any N losses from biosolid applied soils under flooding as all nitrogen compounds need to be considered. The conversion from TIN to NO_3^- can be done by finding the weight of N in TIN compounds and comparing to the equivalent nitrogen in 50 mg/l NO_3^-. Using the atomic number of nitrogen (14) and the total weight of the NO_3^- molecule (62) gives the proportional weight of NO_3^- as 22.6% nitrogen. To calculate the weight of the NO_3^- molecules from TIN content then you must multiply the measured N in the TIN by 4.43 (22.6%). This calculation means that a measured N content of 11.3 mg/l TIN in water would equal the limit of 50 mg/l NO_3^- and should be considered in any measurements taken in this project.

2.3.3 Oxidation-reduction reactions

Many of the nitrogen reactions, and other important reactions, which happen in soil and water are oxidation-reduction (redox) reactions. Redox reactions involve the transfer of electrons between different chemical species in a system. The species losing/donating an electron is considered the reducing agent and undergoes oxidation, and the species gaining/accepting an electron as the oxidising agent undergoing reduction. Half-reactions representing either the oxidation or reduction part of redox reactions are often used to simplify the reactions and highlight changes in oxidation state of individual substances involved. Table 2.4 shows a summary of the important redox transformations that can happen in soil and the Eh levels at which they can occur. The Eh displayed is based on the standard-state reduction potential, which is defined relative to a hydrogen reference electrode, having a potential of 0 volts, measured at 25 °C, 1 atmosphere, and with a pH of 0 in aqueous solution. These reduction potentials refer to conditions where the activity of molecules in

the system and free protons are in unity, which is not realistic in soils where conditions of non-equilibrium are common. The actual reduction potentials of the transformations displayed in Table 2.4 can therefore vary considerably. The order of reduction in waterlogged soils will be coupled to O_2 in the system, which will likely maintain ORP of the soil in a strongly oxidising state even with only residual presence (McBride et al., 1994). The lack of equilibrium in the system, and the fact that redox reactions can be slow, means that different chemical species can exist in the system at once at various levels of reduction. Table 2.4 is therefore an indicator of the half-reactions that can happen in the system as it becomes reducing but is not an absolute measure. In addition, taking reliable and repeatable ORP measurements in environmental systems is often difficult due to variations in methodology, the potential for electrode poisoning, spatial variations in soil systems, chemical disequilibrium and microbial mediation of redox reactions (Husson et al., 2016).

Table 2.4 - Significant half-reactions in soil and water and their standard-state reduction potentials (McBride et al., 1994). Actual reduction potentials in soil systems may vary considerably from those suggested here as soils do not exist in standard-state conditions.

Transformation	**Reaction**	**E_h^0 (volts)**
Denitrification	$NO_3^- + 6H^+ + 5e^- = 0.5N_2 + 3H_2O$	1.245
Mn reduction, Mn(IV) to Mn(II)	$MnO_2 + 4H^+ + 2e^- = Mn^{2+} + 2H_2O$	1.230
O_2 depletion	$0.5O_2 + 2H^+ = H_2O$	1.229
Fe reduction, Fe(III) to Fe(II)	$Fe(OH)_3 + 3H^+ + e^- = Fe^{2+} + 3H_2O$	1.057
Sulphate reduction, S(IV) to S(-II)	$SO_4^{2-} + 10H^+ + 8e^- = H_2S + 4H_2O$	0.303
Methanogenesis, C(IV) to C(-IV)	$CO_2^- + 8H^+ + 8e^- = CH_4 + 2H_2O$	0.169
H_2 generation, H(I) to H(0)	$H^+ + e^- = 0.5H_2$	0

As standard-state conditions are not realistic in soils more useful than Table 2.4 for visualising redox transformations in soil and water may be the graphical representation displayed in Figure 2.2 which accounts for both Eh and pH.

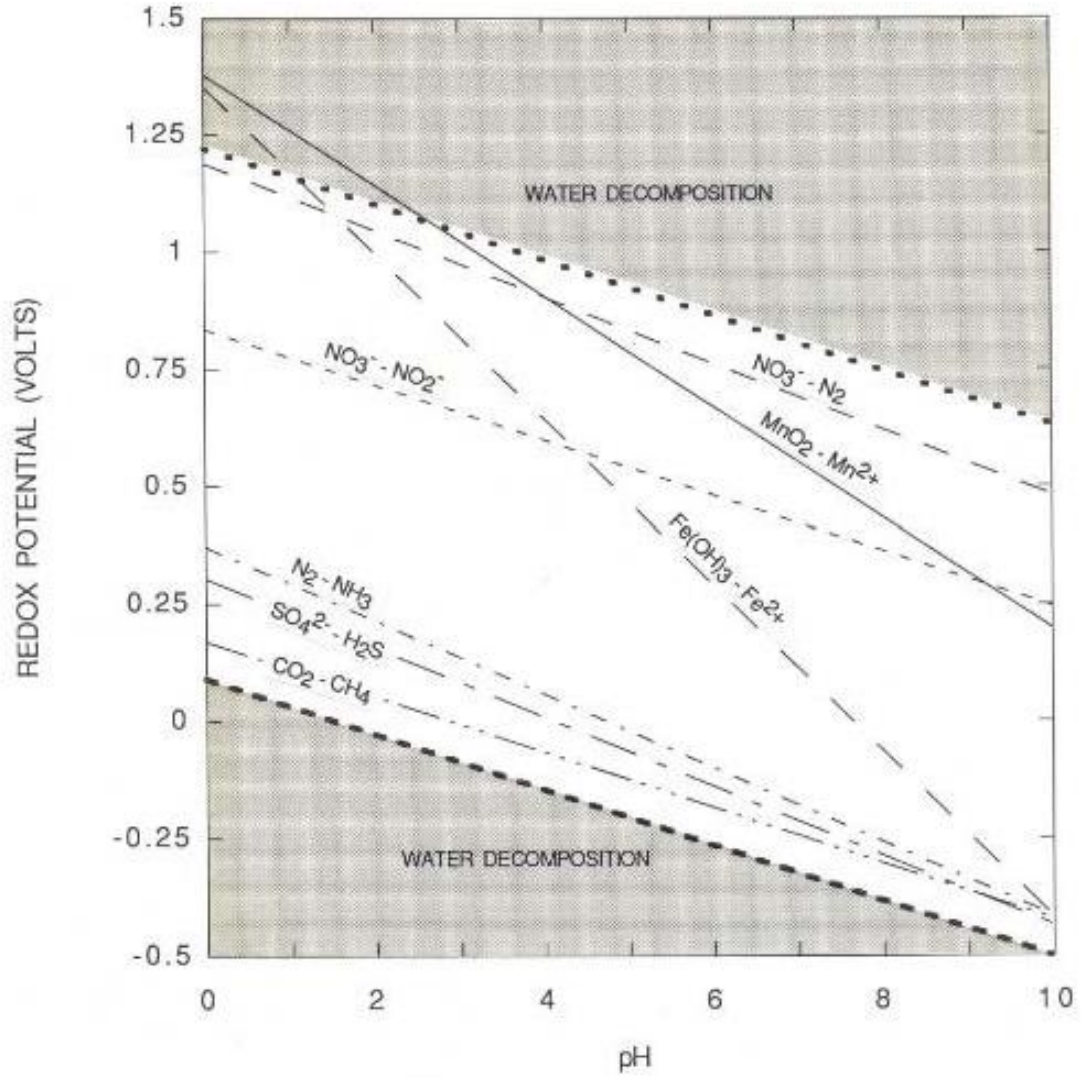

Figure 2.2 - Relationship of Eh to pH for important half-cell reactions in water. The upper bold broken line denotes the Eh at which water is oxidised to O_2, and the lower where it is reduced to H_2 (McBride et al., 1994)

2.3.4 Nitrogen redox chemistry

Ammonification of organic nitrogen compounds is a reduction reaction with organic NH_2 groups being reduced to NH_3 or NH_4^+ (Strock, 2008). Nitrification is then a two-stage oxidation process with NH_4^+ being biologically oxidised in the presence of oxygen to NO_2^- and then again to NO_3^-. In aerated soils or oxygenated waters where O_2 is present then it acts as the dominant terminal electron acceptor and maintains the oxidation-reduction potential (ORP) of the system. In soils which become flooded and have a limited O_2 supply then different compounds become the dominant TEA and are usually reduced after O_2 in the order NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2+} as ORP lowers (McBride et al., 1994; Groenenberg, 2018). This means that in the absence of O_2 then NO_3^- could become the dominant TEA in a flooded system and would maintain the ORP at a certain level. The ORP at which NO_3^- would be maintained is difficult to determine in environmental systems as there are many

variables, as discussed in section 2.3.4. However, a study by Bailey and Beauchamp (1971) found that in a system spiked with NO_3^- the ORP was maintained at +200mV Eh until all the NO_3^- was depleted. Reddy and Delaune (2008) found an Eh of +300mV was maintained in wetland soils when NO_3^- was present between 25-50 mg l^{-1}. Regardless of the exact ORP maintained, these Eh levels suggest that the presence of NO_3^- would prevent any flooded soil system which had become anoxic from becoming highly reducing. If any NO_3^- is added directly in biosolid applications to the soil, or is nitrified from mineralised NH_4^+, then it could alter other geochemical reactions occurring in the flooded soils until it was depleted. The length of the flood, the rate of O_2 loss and the amount of NO_3^- in the system, either natively or added directly or indirectly through biosolid application, and the rate of loss of this NO_3^- could therefore be very important in a number of other geochemical redox reactions in the flooded soil system.

Understanding the pathways for loss of NO_3^- in soil floodwater and the evolution of ORP will be important for understanding the flooded soil system. Depending on the extremity of any reducing conditions induced in the soils during flooding then different nitrogen transformations can occur. Both denitrification and Dissimilatory Nitrate Reduction to Ammonium (DNRA) can occur in reducing conditions, with anaerobic microbes utilising NO_3^- as an electron acceptor for respiration. Denitrification, as discussed in section 2.3.1, is the biological reduction of NO_3^- to NO_2^-, NO, N_2O and ultimately N_2, DNRA is the biological reduction of NO_3^- to NO_2^- and then NH_4^+. Denitrification and DNRA tend only to be significant reactions in reducing conditions, with nitrification dominating N chemistry at higher Eh levels (Giles et al., 2012). DNRA can aid in the retention of soil N, as the N remains in the system as NH_4^+, rather than during denitrification where it is lost as gaseous N_2. The Eh threshold required the reduction of NO_3^- by denitrification, and for it to become the dominant biological process in soil N chemistry over nitrification, has been previously identified as +200mV (Bell, 1969; Kralova et al., 1992; Chatterjee and Saha, 2018). For DNRA to occur the Eh required is lower than that needed for denitrification (Yin et al., 2002; Rütting et al., 2011), though both DNRA and denitrification can occur concurrently. A study by Yin et al. (2002) found that in two different rice paddy soils DNRA accounted for 5% and 14.9% of all N reduction. The large difference between the two soils was attributed to greater DNRA microorganism populations in the higher rate soil and tenfold fewer denitrifying microorganisms. The levels of labile C in the soils were determined to be the main difference between the microbial activity in each soil, with the higher labile C content seeing the higher DNRA rate. However, this could be more an indication of overall increased microbial activity in the soil rather than microbes favouring DNRA, as both denitrification and DNRA are widespread metabolic functions in soil microbial communities (Rütting et al., 2011; Robertson and Groffman, 2015; Wang et al., 2017). Denitrification has also been shown to increase

substantially with increased microbial biomass and increased SOC, even more so than with N inputs, indicating that increased microbial activity is also the determining factor of denitrification rates (Drury et al., 1991; Bárta et al., 2017). Evidence therefore suggests that ORP alone may not control the rates of denitrification and DNRA in soils, but that the soil microbiome is inherently linked to the rate of both and needs to be understood in depth to obtain a full picture of any system.

The +200mV Eh level which is maintained by NO_3^- in the system and the level at which denitrification occurs could be a key threshold for the evolution of any flooded soil system. The NO_3^- content and the rate of reduction could determine how quickly and to what extent the system becomes more highly reducing. However, denitrification and DNRA are not the only routes for loss of NO_3^-, and as previously discussed NO_3^- leaching is usually the major source of loss. Though the leaching of NO_3^- is not a redox reaction the presence of NO_3^- in a system can alter ORP as has been established. Depending on the conditions of the flood in any experiments which are utilised then this could be a point of interest. For instance, in a flowing flood NO_3^- could be washed away or diluted in surface waters. If this flowing flood then became stagnant it could become reducing more quickly due to the lack of NO_3^- maintaining a high ORP. If the flood were stagnant from initiation on the other hand then NO_3^- could freely accumulate in floodwaters and prevent highly reducing conditions, but factors such as subsurface drainage in field could provide a path for NO_3^- loss. Crop uptake needs to also be considered in a field situation and introducing plants into the system could significantly alter redox conditions, either by uptake of NO_3^- or by photosynthesis increasing floodwater O_2 levels and preventing or delaying the +200mV Eh threshold from being reached. Many variables within any studied system therefore need to be considered and controlled as necessary. This area will be discussed further in the experimental design section later in this chapter.

2.4 PHOSPHORUS

2.4.1 The phosphorus cycle in soil

The other major nutrient of focus for this project is P, which is important for energy transfer in plant cells and for root growth, and so is important in a plant's ability to take up other nutrients and for overall health (Hart et al., 2004; AHDB, 2017). P in soils can be present in both organic and inorganic forms, the dynamics of which are complex. Organic P is contained in the SOM as plant or microbial P and can be found in the soil solution as P compounds made up of combinations of C, H and P atoms. Inorganic P in solution is mostly made up of orthophosphate as PO_4^{3-}, but orthophosphate is also used as a general term to refer to HPO_4^{2-}, $H_2PO_4^-$, H_3PO_4 or $CaPO_4^-$ which are all forms of soluble reactive P (SRP). Organic and inorganic polyphosphates also exist in addition to these, but must be hydrolysed to SRP before they can be taken up by plants and microbes (Darch et al., 2014). P itself has low solubility, and so P available to plants is often limited as it is bound tightly to the soil and moves only slowly into solution, typically with <1% of total inorganic P in solution at any time (Bünemann, 2015). Plant uptake of SRP from the soil solution leads to its depletion, and P is then released from labile inorganic forms to replace it, but the soluble pool remains small under normal field conditions. Due to this small soluble pool and the tight binding of P to soil particles the main risk of P loss therefore tends to be from soil erosion rather than as leaching of SRP which is minimal (Sharpley et al., 2001; Withers et al., 2009). However, additions of fertiliser which contain large amounts of SRP can lead to small but significant losses via leaching in conditions which favour SRP release (McDowell et al., 2001; Hart et al., 2004). Synthetic fertiliser additions add P to the soil as SRP in the form of P_2O_5 (AHDB, 2017). Organic fertilisers, such as biosolids, add P in different quantities of both organic and inorganic forms which will depend on the chemical composition and characteristics of the material. With biosolids containing a range of different mineral and organic compounds due to its composite nature, then the availability of the contained P could be complex. Understanding how the different forms of P are released to soil solution for plant uptake is therefore key.

Organic P is released slowly through mineralisation from SOM by soil microbes, but any excess P released to solution is generally subject to rapid adsorption to soil particles (Bünemann, 2015). Mineralisation of organic P can occur at different rates based on the rate of decomposition of the material, the mechanisms of which were discussed in section 2.2. Inorganic P can be contributed to the SRP pool via several routes. Weathering from primary P minerals in the soil, the most common being apatite, happens very slowly over time and has a small contribution (Frossard et al., 1995). Secondary minerals such as calcium phosphates in calcareous soils, and iron and aluminium

phosphates in acid soils can release P through weathering, but SRP can also precipitate to these minerals and become unavailable. In addition to being contained in mineral forms, SRP can also be adsorbed to the surfaces of minerals, clay and colloidal materials. Orthophosphates can be bound to Mn^{4+}, Fe^{3+}, Al^{3+} or Ca^{2+} and form secondary P compounds (Moore and Reddy, 1994; Frossard et al., 1995), or bind to the small number or anion exchange sites in soils (Soffe, 2003). Desorption and adsorption of secondary P compounds happen more quickly than precipitation and dissolution of mineral P, and so account for most of the labile inorganic P which exchanges with the soil solution and is available to plants. Changes in ORP can also lead to the desorption of P from secondary P compounds if they become reduced. Exploring these interactions will be key to this project and are further discussed in this section. The phosphorus cycle as described above is illustrated in Figure 2.2.

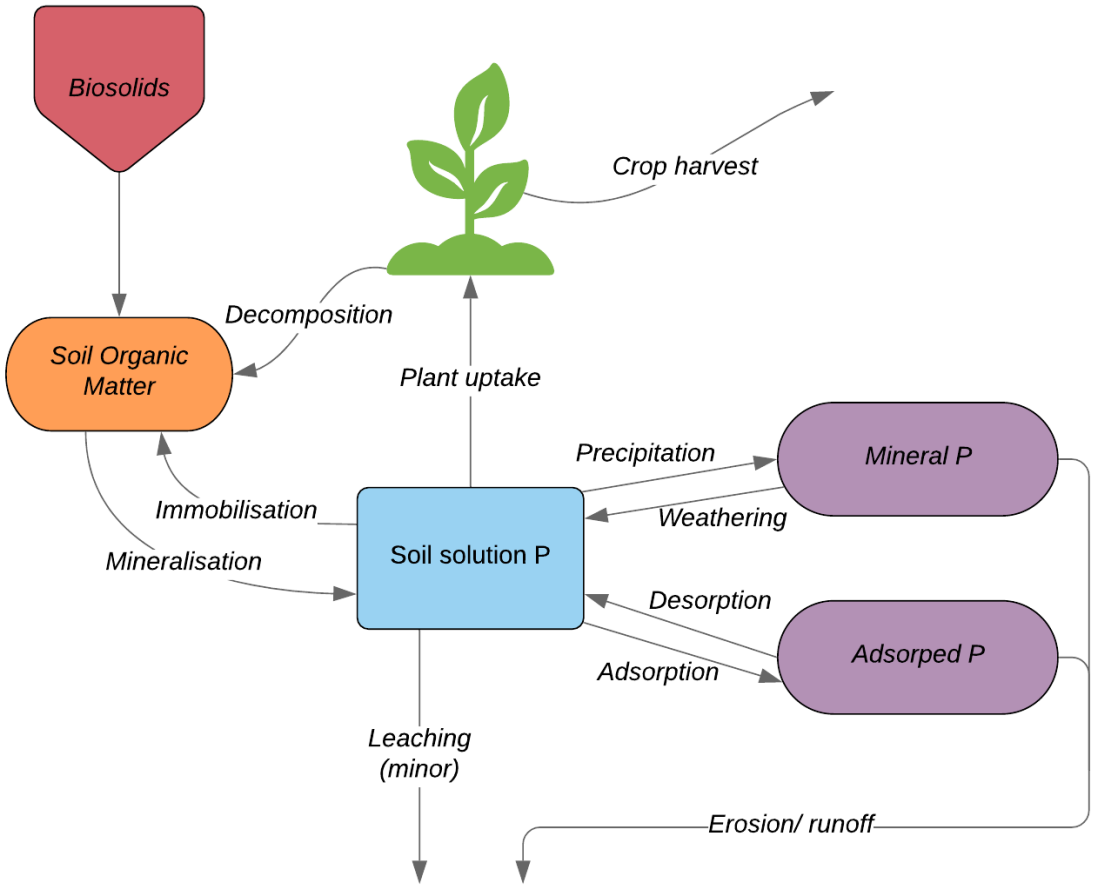

Figure 2.3 - The phosphorus cycle in soil, after Soffe (2003).

2.4.2 Phosphorus solubility

A lot of literature exists on the decreasing the loss of P from agricultural land to watercourses due to the environmental risk of eutrophication. Most of this focuses on avoiding the build-up of P in soils beyond what is required by crops and reducing soil erosion to avoid large losses of P contained on soil particles to surface waters where it can be released (McDowell et al., 2001; Sharpley et al., 2001; Hart et al., 2004; Withers et al., 2009; Hart and Cornish, 2012). The literature also highlights that under normal field conditions P is not readily soluble, and so SRP loss is not a major concern in well maintained soils. However, this project seeks to understand the geochemical response of flooded soils with a large P addition through biosolid application, which does not constitute normal field conditions. Should flooded soils become reducing then this could favour P solubility and lead to a high risk of SRP loss. As previously discussed in section 2.3.3, after the reduction of O_2 and NO_3^- then Mn^{4+} and Fe^{3+} become the TEA can be reduced to Mn^{2+} and Fe^{2+}. This reduction can lead to the P associated with inorganic minerals such as reddingite ($Mn_3[PO_4]_2)\cdot 3H_2O$), hureaulite ($Mn_5H_2[PO_4]_4\cdot 4H_2O$), trivalent Mn phosphate ($MnPO_4\cdot 1.5H_2O$), strengite ($FePO_4 2H_2O$) and vivianite ($Fe_3[PO_4]_2\cdot 8H_2O$) to be released as SRP (Boström and Pettersson, 1982; Moore and Reddy, 1994; Hupfer and Lewandowski, 2008; Van Nguyen and Maeda, 2016). Any inorganic additions of P, or mineralised P from the organic fraction, will also be subject to these redox reactions if they are adsorbed as secondary P compounds, as discussed in section 2.4.1. The level of reduction reached, the time taken, and the available P in any flooded soil will be important factors in determining the potential P loss. The occurrence of reducing conditions in different environmental systems and situations needs to be explored to gain a better understanding of these mechanisms. Valuable research has been conducted in areas including studies of lake sediments, rice paddies, wetland soils and seasonally flooded agricultural and forest soils (Moore and Reddy, 1994; Young and Ross, 2001; Zhang et al., 2003; Hupfer and Lewandowski, 2008; Unger et al., 2009b; Rakotoson et al., 2015; Amarawansha et al., 2016; Dharmakeerthi et al., 2019). Much of the knowledge gained can provide insights into the mechanisms governing SRP release to porewater, groundwaters and floodwaters. The information gained from such studies can then be applied to an extreme weather event leading to a short-term acute flood or waterlogging, which is the focus of this project.

2.4.2.1 SRP release from porewater to surface water

A key area of understanding relating to SRP release to watercourses from soils is not only the potential for SRP release to the soil solution but also its release to overlying floodwater which may have a different ORP. A landmark study on the importance of ORP on SRP release from lake sediments systems was by Moore and Reddy (1994), with many of the mechanisms observed applicable to other flooded soil systems. In this study a contrast was found between surface water,

which remained oxidised and porewater which became reducing a few centimetres deep. Eh and pH were found to play an important role in the regulation of SRP at the interface between reduced sediments and oxygenated overlying surface waters. More reducing and more acidic conditions were highly correlated with increased SRP in sediment porewaters. Under acidic conditions the dissolution of calcium phosphates, to which P was strongly precipitated, was attributed to the rise in SRP, with the sediments used in the experiments having a high Ca content. SRP was also found to be highly correlated with water soluble Fe. This was attributed to the dissolving of ferric phosphates under highly reducing conditions which led to reduced Fe^{2+} in solution. As this Fe^{2+} diffused into oxygenated surface water it oxidised back to Fe^{3+} and precipitated with SRP. The more oxidised surface waters therefore acted as a barrier to SRP release from the sediment. SRP at the sediment-water interface was governed by the geochemistry of Fe, despite the sediments used being calcareous and precipitation to Ca likely the dominant mineral P pool. Figure 2.3 clearly demonstrates the mechanisms controlling the release of SRP from lake sediments in the Moore and Reddy (1994) study.

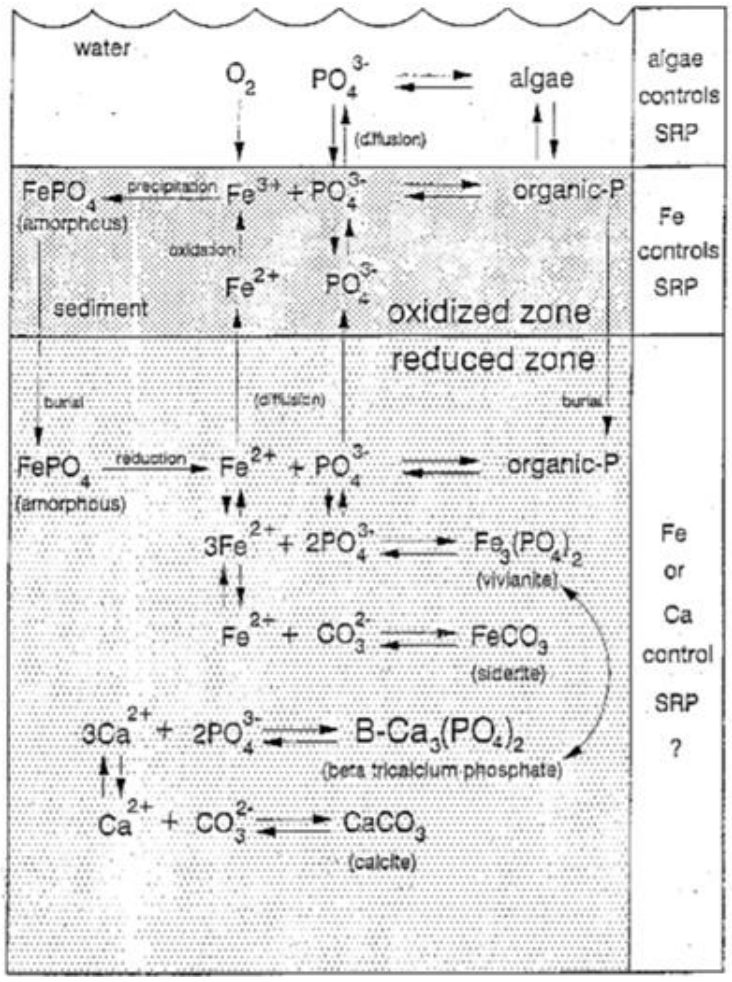

Figure 2.4 - Schematic diagram of the processes controlling phosphate release in lake sediments (Moore and Reddy, 1994)

A study by Young and Ross (2001) also supports the findings of Moore and Reddy (1994). It was observed that an increase in ferrous Fe^{2+} in microcosm seasonally flooded soil porewaters showed increased SRP, but that Fe^{2+} was never detectable in overlying floodwaters which remained oxygenated with an Eh of +350mV and saw much lower SRP content. These results indicated the presence of a redox threshold caused by oxygenated surface waters overlying reduced, anoxic porewaters. High and low P soils were studied, and it was found that higher soil P led to higher floodwater P due to the limited capacity for Fe to reprecipitate or sorb the P released. However, even low soil P systems had SRP in surface waters which exceeded that of commonly reported eutrophication thresholds (0.01-0.03 mg/l). Soil P content therefore does appear to influence the potential for release of SRP, but if conditions lead to ORP becoming low enough to lead to the reduction of P compounds then this can increase considerably over normal levels.

2.4.2.2 SRP mobilisation in flooded agricultural soils

The redox reactions responsible for SRP release in sediments also appears to be relevant in flooded agricultural soils, but variables of flood length and soil inputs can also become important factors. A study by Amarawansha et al. (2015) investigated P release from a selection of calcareous agricultural soils which were either treated with cattle manure or left untreated. Soil Eh decreased rapidly in the first 3 weeks of flooding in all treatments and SRP release followed similar trends in all soils. However, SRP release was much higher in manure-amended soils. Porewater Fe^{2+} and Mn^{2+} concentrations also increased over the course of the flood in all soils, with Mn^{2+} levels responding much faster than Fe^{2+} due to the higher Eh level required for Mn reduction. The increased SRP release from higher P, manure-amended soils is also supported by the previously discussed Young and Ross (2001) study which saw soils with higher native-P release more SRP to porewaters and floodwaters. Reducing soil conditions therefore appear to still be the determining factor of P release, even when available soil P has been increased with organic inputs, but these reducing conditions do then allow more P to be released from higher P soils.

Soils with higher available P were also found to release more SRP to floodwaters in a study by Dharmakeerthi et al. (2019). This study performed incubated lab experiments to assess the release of SRP from pig slurry amended soils treated and untreated with gypsum over a 56-day flood. Again, in this study the release of SRP was dependent on the levels of reduction reached in flooded soils. Gypsum amendments were found to prevent the drop of Eh in soils below +200mV, decreased porewater pH, and increased Ca, Mn and Mg concentrations, all of which were suggested as being possible reasons for the limitation of SRP release. The maintenance of the +200mV threshold for longer in amended soils over unamended is not explained in this study. However, based on the literature previously reviewed in this section, the presence of the +200mV Eh threshold preventing

SRP release could suggest that the NO_3^- was present and acting as a TEA, but this was not measured in the experiments. The reason that NO_3^- may have persisted in the floodwaters of soils which had been amended with gypsum is unknown, with gypsum being a complex composite material. The +200mV threshold may not represent NO_3^- presence in this case, but another TEA which was contained in the gypsum, and with the ORP measurement being skewed due being relative to the individual system.

2.4.2.3 Iron reduction threshold

The problems with ORP measurements being relative between systems is evident when trying to determine the reduction threshold of Fe^{3+} in soils, which could be responsible for a large release of SRP from soils if it is reached. The actual Eh at which Fe^{2+} solubility increases within soil systems has been suggested in a variety of studies to be between 0mV and +200mV depending on the pH of the system. Patrick (1964) found in a soil with pH 6-7 that Fe^{2+} and SRP increased below an Eh of +200mV. Other studies show microbially remediated reduction of Fe^{3+} to Fe^{2+} in soil solutions at ~+100mV Eh with a pH of >7 (Petruzzelli et al., 2005; Amarawansha et al., 2015). In the experiments of Moore and Reddy (1994) increased Fe^{2+} in solution coincided with an Eh of around 0mV in sediments with pH ranging from 6.5 to 8.5. Christophoridis and Fytianos (2006) found that in flooded lake sediments the release of SRP from Fe^{2+} in pH 7-9 sediments occurred at ~+100mV and was at its maximum at -200mV in pH 9 soils, but above +300mV SRP release was negligible. The study also mentioned that Mn can be liberated under the same reductive conditions and via similar mechanisms as Fe, but dissolves far more rapidly than Fe^{3+} at a higher Eh. The sensitivity of Mn to ORP changes at high Eh means that it could be a good indicator of O_2 depletion in flooded systems. This study did also highlight that microbial remediation may raise redox thresholds, but that in lake sediments this was likely negligible. It also found large variation in P release and solubility of Fe, Mn, Ca and Al, and therefore their tendency to release associated SRP, based on sediment pH. These results suggest that the Eh threshold for Fe^{3+} reduction may be higher than expected from a strictly chemical standpoint, even when accounting for reactions occurring outside standard-state conditions, due to the presence of soil microbes. The variation in results may also be due to Eh measurements being relative to each system and dependent on pH. Eh measurements are useful for tracking changes in experiments but not necessarily reliable for comparisons between studies (Husson et al., 2016). Regardless of the exact measurement, reducing conditions appear to be more influential on SRP release than P application. ORP should therefore be closely monitored in any experiments carried out.

2.4.3 Phosphorus release from biosolid applications

A report by Withers and Flynn (2006) written for DEFRA, UKWIR and the Environment Agency assessed the extent of P runoff from agricultural soils treated with different biosolids. This report was written with the intent of creating a set of operational guidelines for biosolid application to minimise the risk of P loss, including from heavy rainfall events. The main risk of P transfer to watercourses from agricultural soils was identified as being from P attached to soil particles and their loss through water erosion and runoff. For this reason, the focus of the report was mainly runoff from subsurface drains and surface waters. Most soil erosion and P loss occurred in the first major 'storm event' or large influx of flowing water. This suggests that any loose soil particles and SRP already in solution are lost in the first instance of water flow, but that new SRP is not mobilised during these events. P applied in biosolids was found to be generally less available than that which is available in inorganic fertilisers and other manures due to sewage treatment processes removing a lot of the water-soluble P. However, the low N:P ratios found in biosolids could give it a greater long-term potential for release of SRP as OM is consumed by microbial populations. The Withers and Flynn (2006) report found that less than 15% of the total P of many biosolids was available to plants when measured as Olsen-extractable P (Olsen et al., 1954), and water extractable P was less than 5% of the total P content. Olsen P was also lower in biosolids with higher Fe contents. These results could therefore indicate that biosolids as a material would be highly susceptible to high SRP release under reducing conditions, even when plant available Olsen P in soils appeared to not be dramatically increased. The level of reduction reached in any flooded biosolid-applied soils is therefore a very important factor for determining SRP release, with the type of flood and its duration needing to be carefully considered.

2.5 FLOODING

2.5.1 Flood risk and occurrence

Increases in overall annual rainfall, summer rainfall and flash flood events have been documented in the UK and EU in the last 5 years, and such changes are predicted to continue into the future (Lenderink and van Meijgaard, 2008; Falloon and Betts, 2010; Kendon et al., 2014; Centre for Ecology & Hydrology, 2016). Increased extreme rainfall events could pose a risk through direct inundation of agricultural soils, or through floodplain water storage on farmed land as an urban flood alleviation measure (Wheater and Evans, 2009). Several examples of extreme rainfall leading to flooding have been observed in the UK in recent years which highlight the impact and extent of these flood risks. Most recently, in February 2020, the UK was hit by Storms Ciara, Dennis and Jorge which caused an estimated £297 million in property damage (Floodlist, 2020). In December 2015 Storms Desmond

and Eva caused an estimated £1.3bn in damages (Association of British Insurers, 2016). The report by Withers and Flynn (2006) discussed in section 2.4.3 which assessed the potential P release from biosolid-applied soils focused mainly on P loss from a large influx of water causing soil particle loss by erosion. However, extreme rainfall events may not immediately drain from soils as demonstrated by the previously mentioned floods. Soils which are not usually prone to seasonal flooding could become inundated with river overflow water which could lead to NO_3^- loss, anoxic soil conditions and SRP mobilisation. Alternatively, the occurrence of unseasonal rainfall in spring could lead to waterlogging during crop growth periods. A variety of different flood conditions could therefore persist from an extreme rainfall event, each leading to different flood durations and types of flood.

2.5.2 Flood type and length

Studies of flooded soils often focus on seasonally flooded land or land prone to flood pulses, flooded rice paddy growth systems and marshland. These flood types are typically characterised by extended inundation where reducing conditions are very likely to occur, arable crops cannot survive, and on soils which may have adaptations to these conditions. Other studies which focus on lake sediments, as discussed in section 2.4.2.1 (Moore and Reddy, 1994), can offer valuable insights into flooded soils but are dissimilar to soils used for crop production experiencing an acute flood event. The data gathered from many of these studies can offer insights into the mechanisms for SRP release, N transformations and behaviour, and crop and microbial response. These factors will depend on the specific conditions achieved in each experiment but should be explored to identify possible gaps in the literature.

The length of the flood in the previously discussed study by Amarawansha et al. (2015) was eight weeks long and was specific to regularly flooded soils rather than cropland, despite the soils selected being in agricultural production. The experiment did not mention any vegetation growth which would have died over the flood duration but may have altered oxygen conditions, nutrient uptake and microbial populations early in the flood. The weekly frequency of porewater sampling and ORP measurements may also not have been enough to gain a detailed picture of the system and its geochemical evolution, especially early in the flood. Indeed, the study itself highlights that most of the drop in Eh happened within the first 3 weeks and most SRP release occurred in the first week of flooding. Dharmakeerthi et al. (2019) performed incubated lab experiments to simulate flooding of and agricultural soils over a 56-day flood and did not include crop growth. Measurements were taken on the day of flooding and weekly thereafter, including ORP and porewater samples for chemical analysis. Large changes in ORP occurred in the first week and continued to rise until day 42 when they stabilised or decreased in some soils, possibly showing readsorption of SRP. The experiments of Young and Ross (2001) ran over a period of 60 to 85 days of floodwater inundation to

simulate seasonal flooding. Soil microcosms were used without vegetation growth, with porewater and surface water sampled every 3-4 days and Eh measured weekly. In this study the time taken for Fe^{2+} to be present in porewater and so release SRP was 15 days, with most of the highest floodwater SRP readings occurring in the first 20 days. All these studies focused on a flood period of longer than 50 days which would allow reducing conditions to develop but would not allow a growing arable crop to survive. This project seeks to assess a short-term, acute flood caused by an extreme weather event and the effect biosolid application has on crop and microbial recovery from this flood. To achieve this aim then the crop must survive in some capacity after a period of stress caused by the flood. The studies focused on longer-term floods are useful for assessing the impact of how floodwater chemistry evolves as flooded conditions persist but is not so useful for assessing the recovery of soils and crops from acute flooding events. These studies also only measured Eh weekly but found that the biggest changes in ORP happened in the first few weeks of flooding. This then highlights the need for frequent monitoring of flooded soils in the early stages of flooding, as changes in the system could take place quickly and be missed otherwise.

Unger et al. (2009b) conducted a field study of the chemical response of soils and water to different flood types, including stagnant, flowing and intermittent flooding in greenhouse and field experiments. Field experiments were carried out in channels with the different flood treatments for 3-5 weeks. ORP and pH measurements were taken continuously during the flood at 30s intervals, dissolved oxygen measurements were taken twice per week and soil samples were taken pre- and post-flood. SOC and total N were not significantly affected by N, but NO_3^- was lower, attributed to denitrification and chemical reduction, and NH_4^+ was higher, attributed to the reducing conditions of the flood. During the flood ORP dropped over time in all treatments, decreasing to its lowest in the 5-week flooded soil. Flowing or stagnant conditions in the overlying water did not affect ORP or the anaerobic status of the soils, despite the hypothesis that flowing floodwater would allow more mixing and diffusion of O_2 to porewaters to maintain a higher Eh. This is important when considering experimental design as the duration of the flood would appear to be more important in determining ORP change than the conditions of the flood.

2.5.3 Microbial response to flooding

The geochemical response of soils to flooding has been discussed in sections 2.3 and 2.4 when exploring the redox chemistry of N and P release in flooded soils and sediments. However, the response of soil microbes to flooding has not yet been properly discussed. A key study exploring the response of soil microbes under flooded field conditions was conducted by Unger et al. (2009a). PLFA analysis was used to characterise soil microbial communities and assess their responses to flowing, intermittent and stagnant floods, and to different residue treatments, in field and

greenhouse experiments. All flood durations were 56 days, with intermittent treatments including two rounds of a two-week flooding period and a two-week drying period. Vegetation growth was not included in the experiments. Both composition and function of microbial communities were affected greatly by flooding, but no significant changes were found from different residue types and nutrient loading. Additionally, the study found that soil microbial biomass and activity was related to soil depth, with most of the microbial biomass concentrated in the top 10cm of soil due to nutrient stratification and therefore most of the microbial changes were concentrated there. Microbial biomass and markers for aerobic bacteria, gram-positive bacteria, gram-negative bacteria and mycorrhizal fungi were all reduced by flooding, with greater effects observed in the greenhouse trials than the field. Intermittent flood treatment was found to be closer to aerated control soils than to the longer flowing and stagnant flood treatments. This demonstrated that the length of oxygen deprivation to the soil was more important than the type of flood for influencing microbial populations, and that they recovered quickly after a short-term flood event. However, a study by Sánchez-Andrés et al. (2010) which investigated soil microbial respiration rates after flood pulses found that a return to equilibrium may not be as quick as suggested by a return to pre-flood microbial activity levels. In this study the respiration rates of soils returned to control conditions 1-2 weeks after the end of flooding, but significant fluctuations in CO_2 release based on flood pulse duration were still observed 5-8 weeks later. Time take to return to equilibrium was also linked to the duration of the preceding flood. The conclusion that flood duration is more important than flood type is also in line with the geochemical conclusions of the Unger et al. (2009b) study which found flood duration was a bigger influence on geochemical conditions than flood type. The effect of flooding and oxygen deprivation also appears to be greater than that of organic input type and substrate supply to soil microbes (Unger et al., 2009a).

2.6 SOIL MICROBIAL CHARACTERISATION

2.6.1 Unflooded soils

A key component of this project is to understand the influence of the combined effects of flooding and biosolid applications on soil microbial communities. The potential influences of biosolids on soil microbes was discussed in section 2.1, but the influence of flooding also needs exploring in the literature. Using NGS to characterise bacterial populations in soils offers an effective and appropriate method for assessing which microbes are present in soil and biosolid communities (Sanschagrin and Yergeau, 2014; Levy and Myers, 2016). Several studies exist which have characterised the microbiomes of soils and sediments using 16s rRNA gene sequencing. Gaining an overview of what to expect in a field-state soil from these studies is important first step in understanding of 'normal' soil conditions before biosolid application or flooding.

A study by Miyashita (2015) characterised different tropical forest soils in Southeast Asia and found the dominant phyla were *Acidobacteria, Proteobacteria, Verrucomicrobia, Planctomycetes, Bacteroidetes* and *Actinobacteria. Proteobacteria* and *Acidobacteria* were the most dominant of all phyla in all the soils tested, though diversity within the phyla varied between soils. *Proteobacteria* was also found to have a greater variety of species than that of *Acidobacteria.* A study by Zeng et al. (2016) investigated bacterial communities in soils in China in response to different vegetation growth. The dominant bacterial phyla in this study were *Actinobacteria, Proteobacteria, Chloroflexi, Acidobacteria* and *Planctomycetes*, with *Actinobacteria* and *Proteobacteria* the most dominant in this case. As soils changed from forest to sandy *Actinobacteria* were found to increase in relative abundance, while *Proteobacteria* decreased. Mean annual rainfall significantly correlated with phyla relative abundance changes in this study, suggesting soil water availability has a large influence on populations. Feng et al. (2018) carried out a study to assess the diversity of microbial populations in Cd contaminated soils. *Proteobacteria* were the most abundant phylum in the study, followed by *Gemmatimonadetes, Acidobacteria* and *Thaumarchaeota*. Cd had some effects on taxonomic diversity of soil populations but, as discussed in section 2.1.2, PTE toxicity is not a focus of this project and is not expected to occur with the use of well-treated biosolid products. The taxonomic makeup of the bacteria within these soils was however useful to know.

2.6.2 Digestate-applied soils

Studies using NGS to study biosolid applications to soils were difficult to identify, but a study by Sapp et al. (2015) explored digestate and mineral fertiliser applications to soils. *Proteobacteria* (24.05%), *Actinobacteria* (19.17%) and *Acidobacteria* (15%) dominated the soils of the experiment, with *Bacteroidetes, Chloroflexi* and *Gemmatimonadetes* also present in all samples. This study found only

small changes in diversity from digestate application and found that the most significant changes came from nutrient addition and plant growth and there was no introduction of new bacterial species due to digestate application. A study by Podmirseg et al. (2019) also supported these finding in a study assessing the effects of raw and digested cattle slurry applications on soil microbial populations. The study found that applications of both treatments resulted in no significant changes to microbial population composition, but that native microbes were able to capitalise on nutrient additions to increase metabolism rates. The bacterial populations consisted of the eight main phyla of *Proteobacteria, Acidobacteria, Actinobacteria, Chloroflexi, Bacteroidetes, Planctomycetes, Nitrospirae* and *Verrucomicrobia.*

Though the above studies did not find any changes to bacterial composition from digestate applications Hou et al. (2017) studied the application of fermented food waste to Japanese impoverished grassland soils and had interesting results. *Proteobacteria, Acidobacteria* and *Actinobacteria* were the dominant phyla in the soils. However, a large influence was observed from the application of the fermented food waste inputs, in particular the increase in relative abundance of *Firmicutes* in the applied soils which then became one of the dominant phyla. This is an important observation, as *Firmicutes* were found to be a dominant phylum in biosolid samples as discussed in section 2.1.4. The increase in relative abundance of the *Firmicutes* phylum in soil after the application of fermented food waste where it was not dominant before is important. This could indicate that biosolid applications also containing a large volume of *Firmicutes* could significantly affect soil bacterial populations. However, the soils of this study were SOM impoverished and so may have had a relatively low abundance of native species due to lack of substrate for food. This factor may mean the shift in bacterial phyla relative abundances is exaggerated over what would occur in a well-maintained, temperate arable soil. From these results the bacterial populations of digestate applied soils would therefore appear to be very similar to the soils characterised in the other studies in section 2.6.1 (Miyashita, 2015; Zeng et al., 2016; Feng et al., 2018).

2.6.3 Flooded soils and sediments

As well as characterisation of soils under 'normal' unflooded field conditions as discussed in section 2.6.1.1 the bacterial populations of soils and sediments which are adapted to flooded conditions have also been characterised in several studies. This information will be useful to understand any overlaps or shifts in population which may occur under flooded conditions. A study by King and Henry (2019) characterised soil microbial communities to assess methane fluxes under flooding in meadow soils. The soils were mainly dominated by the phyla *Acidobacteria* and *Proteobacteria,* followed by *Verrucomicrobia* and *Bacteroidetes*. Though some differences in methane flux activity was observed from flooding no significant changes in relative abundances of soil bacterial phyla

were observed. This indicated that the methane fluxes were from changes in microbial activity rather than composition. However, the flood carried out was only 72 hours long, which was likely too short for bacterial populations to shift, a longer flood may see more substantial changes in populations. This study was therefore useful for soil bacterial population characterisation, but not for observing flood effects and potential shifts towards a more anaerobic and reducing environment.

Rice paddy soils were investigated by Breidenbach and Conrad (2015) and the largest phylum by far in those soil was *Proteobacteria* at 36-40% relative abundance, followed by *Acidobacteria* with 14-18% relative abundance. Draining of these soils saw little change to the population relative abundances. However, these soils were already adapted to flood conditions, and so may take an extended amount of time to see shifts from draining, as opposed to a normally aerated soil becoming flooded. A study by Pittol et al. (2018) also supported the dominant phyla of rice paddy soils being *Proteobacteria,* but also saw high relative abundances of *Bacteroidetes, Actinobacteria* and *Firmicutes.* An investigation of bacterial diversity in river mudflats and sediments by Vidal Dura et al. (2018) gave high-quality data for the characterisation of the bacterial population composition present. An average of 51% of reads across all samples belonged to the *Proteobacteria* phylum making it the dominant phylum in all samples. *Acidobacteria* represented 11% of reads, *Bacteroidetes* 10% and *Chloroflexi* 9%. A study by Huang et al. (2019)compared bacterial communities in lake sediments, lake floodplains and estuary sediments and also found *Proteobacteria* was the dominant phylum across all samples with >30% relative abundance on average. *Nitrospirae, Bacteroidetes, Firmicutes* and *Acidobacteria* were also present as dominant phyla in many of the samples, though this varied considerably between samples. Randle-Boggis et al. (2018) investigated flood pulse effects on soil bacterial communities, with all soils flooded for two weeks prior to any flood pulse treatment. This means the soil bacteria may have already adapted to flooded conditions. The dominant phylum in all samples was *Proteobacteria,* with other major phyla including *Actinobacteria, Acidobacteria* and *Verrucomicrobia.* Functional and compositional changes were observed in the bacterial populations relating to different flood pulses and they were attributed to differences in redox conditions induced by the flooding. However, the dominant bacterial phyla remained relatively unchanged overall.

The evidence from all the studies in this section would indicate the main dominant bacterial phyla in those soils tends to belong to the *Proteobacteria* and *Acidobacteria* phyla. Other important, but less dominant phyla, include *Chloroflexi, Verrucomicrobia, Planctomycetes, Bacteroidetes* and *Actinobacteria*. The most dominant phyla in all flooded soil studies was *Proteobacteria*, which was also present in the unflooded soils as one of the dominant phyla in most cases. This would point to *Proteobacteria* being an adaptable and diverse phylum, and this is supported in literature exploring

the its phylogeny (Gupta, 2000; Spain et al., 2009). *Acidobacteria, Actinobacteria, Verrucomicrobia* and *Bacteroidetes* all also appeared as dominant phyla across both flooded and unflooded soil bacterial population characterisations. Some flooded soils also showed increases in the *Firmicutes* phylum which was not prominent in unflooded soil, except those soils where fermented food waste had been applied (Hou et al., 2017). *Firmicutes* are also highly abundant in biosolid samples, as discussed in section 2.1.4. If *Firmicutes* persist in soils after biosolid application, then flooding may see an increase in their abundance, based on the literature reviewed.

2.6.4 Microbial community statistical analysis

Considerations need to be made for the handling of high-throughput NGS data as this generates a very large number of 16s rRNA reads which need to be analysed. The data will need to be statistically interpreted before it can be used to give an accurate representation of sample microbial communities and to draw accurate conclusions based on the results. Both Alpha diversity metrics, which is the diversity of bacterial populations within each sample, and Beta diversity metrics, which is the diversity between different samples, need to be understood. Further to the diversity metrics characterisation of bacteria needs to be carried out to understand what species are present and in what abundances.

2.6.4.1 Alpha diversity

When considering Alpha diversity within samples it is easy to assume that the number of species within a sample would be an appropriate measure of biodiversity. However, if the diversity were determined simply by the number of bacterial species in a sample then increasing the sample size would inevitably lead to an increased bacterial diversity. This would continue indefinitely as sample size increased, with a disproportionate weighting of rare species adding to the diversity measure, when in fact the overall proportion of species within the sample would remain the same. This is particularly a problem in NGS as determining bacterial diversity is challenging due to technological issues and the intrinsic properties of bacteria, such as hyperdiversity and variability in 16s rRNA gene copying leading to overestimation of rare taxa (Kang et al., 2016). Metrics therefore need to be used which can measure species diversity proportionally across samples from different environments and which may vary in size. It is also important when evaluating samples to identify not only the overall diversity of a species in a sample, but also the number of *effective* species within a sample. Having a measure of effective species can allow for determination of the main species which are significantly influencing their environment, rather than merely present. One method for measuring diversity as proposed by Hill (1973) was to use three common measures of diversity to give a broader overview and understanding of species within a sample, which are the species richness, common species and

dominant species. Hill numbers (D_q) provide a unified family of diversity indices which weigh taxa based on abundance and therefore compensate for the disproportionate impact of rare taxa in the dataset. The measures have subsequently been reintroduced and updated for use as biodiversity measures for NGS applications (Jost, 2007; Chao et al., 2010; Kang et al., 2016). Hill numbers can be expressed as shown in equation 2.8, where 'S' is the total number of species in the sample, and 'P_i' is the proportion of species belonging to the i^{th} species of the dataset.

$$D_q = \left(\sum_{i=1}^{S} p_i^q \right)^{\frac{1}{1-q}}$$

Equation 2.8

In equation 2.8 'q' is the order of diversity, which is the sensitivity of diversity to rare versus abundant species. At q=0 the species weight would equal the species proportional abundances, and so D_0 represents the actual number of species in the sample, or the richness of the sample. When q=1 each species is weighted by its proportional abundance which gives D_1, the common species in the sample. When q=2 the weight given to abundant species is exaggerated when compared to rare species, this gives D_2 as the dominant species. Hill numbers can also be converted to more traditional diversity indices, with D_0 representing species richness, D_1 is the equivalent of the exponential of Shannon entropy and D_2 corresponds to the inverse Simpson concentration (Kang et al., 2016).

2.6.4.2 Beta diversity

Beta diversity is compares two samples and usually gives a number calculated to represent either similarity or difference between samples. This can be carried out in several ways, one useful approach is that of Bray-Curtis dissimilarity scores (Bray and Curtis, 1957). A dissimilarity score gives a number from 0 to 1, with a higher score indicating higher dissimilarity, and so fewer shared species within a sample. Two samples with the same composition would therefore score 0, and with no shared species would score 1. Bray-Curtis index of dissimilarity between samples is calculated using equation 2.9. In the equation i and j represent the two samples being compared, C_{ij} is the sum of the lesser counts for each species in common between both samples. S_i and S_j indicate the total number of species counted at in each sample.

$$BC_{ij} = 1 - \frac{2C_{ij}}{S_i + S_j}$$

Equation 2.9

Bray-Curtis indices can be plotted on to a matrix which is useful for distinguishing differences in shared species between samples. This could be very useful in assessing the transfer of species between biosolids and soils as shared species will be represented in the scores.

2.6.4.3 Taxonomic classification

Though Alpha and Beta diversity measures are very useful for statistically representing the diversity in and between samples, the taxonomic classification of microbial species in the samples is also required for a proper characterisation of the microbial populations present. Knowing which species are present allows for exploration of the reasons for any changes observed in abundance of those microbes based on previous research. Depending on the quality of the data collected sequences can potentially be characterised as far as to the species level of taxonomic rank. This is beneficial, but not essential, because as the level of specificity of taxonomic rank increases then its confidence of being accurately identified lowers. For this reason, classification to the phylum level is often used as it is a broader classification and can represent a larger part of the overall microbial community which has been identified with adequate confidence. Identification to phylum level therefore allows for useful visualisation of the relative abundances of phyla between samples across the whole microbial population on an appropriate scale.

Accurate taxonomic classification is vital to understanding any data collected from 16S rRNA gene sequencing. Many resources are available online for the purpose of taxonomic assignment, with open-source databases available containing extensive datasets of identified 16S rRNA sequences. These databases are constantly being updated and expanded, and any sequencing data gathered from in this project can be cross referenced with them. The main databases identified and regarded as reliable for taxonomic classification in metagenomics (Santamaria et al., 2012) are those of SILVA (Yilmaz et al., 2013; Glöckner et al., 2017), Ribosomal Database Project (RDP)(Cole et al., 2013) and Greengenes (DeSantis et al., 2006; McDonald et al., 2012). The accuracy of use of these databases will depend on the quality of sequence data processing carried out and on the breadth of variation identified within samples. For the processing of any 16S rRNA gene sequence data gathered the two main software packages available are USEARCH (Edgar, 2010) and MOTHUR (Schloss et al., 2009; Schloss, 2020). These will be explored when data becomes available, and as the process pipeline progresses decisions about the most appropriate software and taxonomic reference database will be made.

2.7 EXPERIMENTAL DESIGN

2.7.1 Field trials versus laboratory experiments

Many of the experiments which focused on N and P transformations in soil and water and the different redox reactions occurring utilised highly controlled laboratory experiments to isolate particular effects (Williams and Patrick, 1973; Boström and Pettersson, 1982; Moore and Reddy, 1994; Young and Ross, 2001; Amarawansha et al., 2015; Amarawansha et al., 2016; Van Nguyen and Maeda, 2016; Dharmakeerthi et al., 2019). These experiments provide valuable insights into the geochemical reactions that can occur in soils and water but lack key variables which may be present in real-world settings. For instance, crop growth is difficult to include in purged flask or small microcosm experiments but has an enormous influence on soil geochemistry and microbiology in agricultural soil rhizospheres (Bakker et al., 2013; McNear Jr., 2013; Iannucci et al., 2017). The soil ecosystem contains many interlinked factors which, when considered as part of a whole system, may behave differently than closely controlled laboratory experiments would suggest. The physical characteristics of the soil, the wide range of geochemical interactions, microbial activity and nutrient uptake, and the growth and influence of crops in the soil will all have an impact on the system. Understanding the individual factors of the system is important but understanding the behaviour of the whole system may be more valuable. This was put well by Sturz and Christie (2003) who said:

"The fractionation of the whole into manageable sub-components, with no integration, has often led to the development of disjointed concepts. To address this difficulty, soils, comprising of physical, chemical and biological components, need to be considered as a whole rather than as a sum of their separate parts."

Field trials carried out in the ecosystem itself can give a closer representation of the whole picture. However, measurement of important individual influencing factors can then be difficult due to the wide range of variables present and difficulty with establishing reliable control conditions (Unger et al., 2009b). This lack of control in field trials can mean it is difficult to target which variables are having the most significant impacts on the system, and often only end results can be observed. Field trials also take a long time to complete, relying on the growing season, and need to be run over a long time with multiple replicates to observe actual trends (Rasmussen et al., 1998). A well-developed baseline understanding of the most important concepts in isolation, coupled with a holistic approach to experimental design could help to bridge the gap between both concepts. This project will seek to accomplish this. Utilisation of microcosm experiments which contain a representative agricultural topsoil layer, can be applied with variable rates and types of biosolids, can contain a growing crop, can be flooded will allow for study of all those influences in a system.

Ensuring the control of key variables such as light input, water, temperature, and crop stress such as pests and disease, will be key to obtaining reliable results and control conditions. Any experiments must also allow for the precise measurement of sensitive chemical and microbial factors and be robust and repeatable.

2.7.2 Crop selection

As discussed, it is important in this project to consider cropping and plants in any experiments carried out, both for determining their influence on soil microbial and chemical conditions during flooding and to assess crop recovery from flooding and any biosolid influence on this. Low oxygen transfer to plant roots is the predominant form of stress for plants in flooded soils. As well as producing energy through photosynthesis plants require oxygen for energy production through respiration, so prolonged oxygen deprivation can lead to plant death (Banti et al., 2013; Shaw and Meyer, 2015). Any factors which improve early root development in plants and improve soil structure for improved oxygen diffusion and drainage, such as biosolid applications, could therefore be very important in plant flood survival. Identifying and selecting an appropriate crop to demonstrate these differences and represent a UK cropping system is therefore important to the success of this project.

In 2019 the total cropped area of the UK was 4.552 million hectares, with the three most important crops by area all arable crops: Wheat area was at 1.815 million hectares, barley at 1.166 million hectares (452,000 hectares winter sown, 715,000 hectares spring sown) and oilseed rape (OSR) at 529,000 hectares (525,000 hectares winter sown) (DEFRA, 2019). Carrying out any experiments using one of these crops would therefore be advantageous for improving the relevance and impact of any study carried out. For carrying out experiments then the reduced growth cycle of a spring crop may be beneficial which would make spring barley an optimal choice. A cereal crop would also be easier to cultivate and grow than oilseed rape, which requires sufficient soil depth for a deep taproot and has a much higher biomass per plant. However, the known responses of these crops to flooding needs to be understood before making any final selection. A study by Ploschuk et al. (2018) assessed the differences in flood response at different growth stages of wheat, barley and OSR. Wheat saw some yield loss but recovered well from both early late waterlogging during growth, attaining 71-86% of yield. Both barley and OSR were able to recover well from early waterlogging, attaining 79-85% yield, but both suffered under late waterlogging and achieved only 26-32% yield. Barley showed a better yield recovery than both wheat and oilseed rape to early flooding despite not faring as well as wheat to late flooding. Due to its importance as a UK crop, its ease of cultivation and its ability to recover from short-term flooding spring barley may present the best crop to use during any experiments.

2.7.3 Laboratory considerations

If establishing experiments in a laboratory then several considerations need to be made including light input, crop watering and temperature. Greenhouse experiments are often implemented for indoor, controlled crop trials. However, daylight hour variation throughout the year could add another variable to crop growth which is difficult to control for and provide repeatability of experiments in the timeframe of this project. Utilising a fixed light source in a growth chamber with controllable hours of light and dark would ensure repeatability at any time of the year. Laboratory experiments also have the benefit of not having variable rainfall as with field trials, but a watering regime will need to be devised which provides enough water for crop growth but does not leave soils oversaturated. Controlling the temperature of laboratory experiments will be difficult as it will be directly linked to the environmental conditions maintained within the lab, which may be different to those required for simulating field conditions. However, this could also be beneficial as it again provides repeatability of experiments at any time of the year. A higher temperature than field conditions would be expected in a laboratory, and this has several implications for any experiments. N mineralisation starts to occur more significantly in soil at around 4^0C due to increased microbial activity (AHDB, 2017) and crops planted will germinate quickly due to warmer soil temperatures. However, the conditions available in the laboratory could be compared to warm spring conditions which would fit with the life cycle of the spring barley crop selected. Germination is likely to occur immediately once seeds are planted and increased microbial activity would mean immediate decomposition of applied biosolids. If simulating a spring application and drilling in warm temperatures then this would also be the case, but laboratory conditions could accelerate these processes. Overwintering a crop in a greenhouse or incubator would be an option for temperature control to allow slower processes of biosolid breakdown to occur, but this would also significantly extend the time required to run an experiment.

Another benefit of using a spring barley crop is that it has a shorter spring growing season and so enough crop growth should occur in a relatively short amount of time. With this project exploring flooding caused by extreme weather, then an unseasonal heavy rainfall event resulting in a spring flood would fit this description and could be simulated in any experiments. Taking a spring barley crop to maturity if using growth chamber may be an issue and could require termination of trials before yield data can be obtained. However, terminating the trials before full crop maturity could have advantages such as reduced crop nutrient requirements. If crops were taken to full maturity with no nutrient additions other than a single biosolid application then any effects observed may be due to low crop nutrient supply rather than overall biosolid or flood effect. As discussed in section 2.1.1 the levels of P provided in biosolids are high and likely to adequately supply any barley crop

grown. However, biosolids generally have a low potassium (K) content (Tab.2.1), which is a major crop nutrient (AHDB, 2017). One option to combat this would be to apply an inorganic application of K to suit crop requirements, but this would be altering the soil chemistry and could interfere with other geochemical measurements and crop performance indicators. Relying on any residual K in a well-maintained arable soil, and any K added from biosolid applications, would therefore be preferable for maintaining controlled conditions. Crop N requirements also may not be fully met by biosolid applications alone, but this will be an important variable for assessing biosolid application differences and can also be minimised by not taking crops to maturity. Therefore, rather than a yield measurement as a final crop performance indicator it may be more beneficial to take crop dry weight biomass. This will reduce the length of time that the experiments need to run, remove the need for chemical or inorganic nutrient additions and has also been suggested to be the best indicator for comparative crop performance results (Ramdani et al., 2015).

2.7.4 Addressing research gaps

As highlighted throughout this chapter, biosolids are a high value crop nutrient source in agriculture which can act as a feed substrate for soil microbes and have potential for improving soil structure and flood resilience. The high N and P content of biosolids and their behaviour in soils, especially under flooded conditions, is complex. Research has been carried out into flooded soil and various organic inputs, however biosolids themselves have received little attention in these circumstances. Furthermore, many studies have focused on long-term flooding rather than acute flooding brought about by extreme weather events, so data on the rapidly changing, early stages of flooding and the effects of short-term floods in arable cropping systems is lacking. This project will focus on this area of short-term flooding of arable soils, monitoring water redox conditions, pH and oxygen to understand how the changing system will affect N and P release to floodwater. Crops will be established in any experiments, and their performance and influence on the flooded soil system monitored to understand field conditions and the potential impacts on agricultural production. Additionally, the emergence of NGS technologies has broadened the scope for further investigation into the characterisation of soil microbiomes. NGS can be utilised to explore the effects of soils amended with microbially-rich biosolids and the impact of flooding on these soil microbial populations.

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Chapter 3: METHODOLOGY

3.1 INTRODUCTION

A versatile experimental setup was required for this project which could satisfy the project aim and the wide range of objectives outlined in section 1.2. Crop performance, geochemical changes and microbial population changes all needed to be measured accurately within a system that closely resembled field conditions but allowed for the control of environmental variables. Any experiments carried would also need to be robust and repeatable. To satisfy these objectives a growth box experiment was developed which could contain a suitable volume of soil and biosolid for study and contain a growing crop. To control the growing conditions in a laboratory environment a growth chamber was also constructed. Details of the overall experimental design, setup and justification are presented in the following chapter. Descriptions of the measurement methods for individual factors which were studied are provided in their respective chapters.

3.2 MATERIALS COLLECTION AND CHARACTERISATION

3.2.1 Initial soil characterisation

A representative soil from a typical UK agricultural system was required for the experiments and the field 'Big Substation' at the University of Leeds Farm (53.869927,-1.329049) was selected. The field has been in arable and vegetable rotation for over 20 years and a full cropping history if the field is included in Appendix A.1. The field topsoil is classed as Harrogate Till formation overlying calcareous mudstone and dolomitic limestone, and was characterised as a Cambisol with a medium-strong silt loam texture containing some stone fragments deriving from the underlying bedrock.

The field was soil sampled prior to beginning any experiments and was sent for a full agricultural nutrient analysis at NRM laboratories to ensure that the soil had no nutrient deficiencies which could limit crop growth. This was a standard agricultural analysis carried out for assessment of soil major nutrient and trace element levels for supporting crop growth, and the results are reported as received in Table 3.1. The soil appeared to be in good condition for an arable soil with no apparent limiting factors to crop growth. The pH is in the range of a well limed or slightly calcareous agricultural soil which would not limit nutrient availability. The P levels are in the range of ADAS soil index 2, which is the correct range for cropping without any excess build up to run down. Biosolid applications can therefore be applied at maximum rates with no P restriction. Soil K is at ADAS soil index 1, indicating that it is low but not deficient and should not restrict crop growth during the

experiments. Crops not being taken through to maturity in the experiments also reduces the risk of any growth limitations due to low K. The SOM levels are at the low-end range for arable soils. However, this would make the soil a good candidate for organic applications and supports the use of biosolids to improve the production value of this soil. All other secondary and trace elements are in acceptable ranges for cereal growth.

Table 3.1- Nutrient analysis results of initial soil sampled from 'Big Substation' field on 27/03/2017. Analysis carried out and reported by NRM laboratories (reported 11/04/2017, report number: 55198-17). Units are presented as reported, conversion to mg kg^{-1} of soil was not possible as the sample weights used were not available.

Property	Analysis Method	Quantity	Unit
pH	1:2.5 in water	7.6	
P	Olsen	20	mg l^{-1}
K	Ammonium Nitrate Extractable	106	mg l^{-1}
Mg	Ammonium Nitrate Extractable	499	mg l^{-1}
Na	Ammonium Nitrate Extractable	8	mg l^{-1}
Ca	Ammonium Nitrate Extractable	1780	mg l^{-1}
Cu	Mehlich 3	6.9	mg l^{-1}
Zn	Mehlich 3	5.8	mg l^{-1}
Fe	Mehlich 3	175	mg l^{-1}
Mn	Mehlich 3	166	mg l^{-1}
Mo	Mehlich 3	0.5	mg l^{-1}
Co	Mehlich 3	0.8	mg l^{-1}
SO_3	Mehlich 3	39.6	mg l^{-1}
B	Hot water soluble	1.7	mg l^{-1}
Total N	DUMAS	0.16	% w/w
TOC	DUMAS	1.6	%
SOM	DUMAS	2.8	%

3.2.2 Soil collection

As the experiments were designed to simulate a working arable soil, soil collection for each experiment was undertaken at different points throughout the cropping year. Fertiliser applications throughout the year would then allow a variety of different geochemical conditions to be observed. Timing of soil collection was carried out to align with the duration of each experiment, when the field was accessible without causing damage to the crop, and to give a spread of conditions throughout the growing season. The soil collection dates for experiments 1-4 were 25-27/09/2017, 9-11/1/2018, 9-11/4/2018 and 15-17/10/2018 respectively. The field fertiliser application records for the cropping years 2017-18 (oilseed rape) and 2018-19 (winter wheat) are provided in Tables 3.2 and 3.3. The soil was recovered using either a core auger or Dutch auger to a depth of 15cm, filling buckets with approximately 20kg of soil in each. Each bucket comprised of a representative topsoil sample of the whole field area, sampled in a random pattern. Soil was preserved in its field-moist condition without sieving, to minimise disturbance of its micro-structure. Soils were analysed prior to each experiment for Olsen P, SOM, TKN and pH and these results are reported in Chapter 4

alongside other geochemical results. The influence of fertiliser additions to the field at different times can be observed in many of these experimental soil samples and are discussed further in Chapter 4.

Table 3.2- 'Big Substation' field fertiliser application record 2017-2018.

Product	Date	Rate/ha	Units	Nutrient	Nutrient kg/ha
Boron Headland	03/10/2017	0.49	L	B	0.07
Manganese Headland	03/10/2017	0.98	L	Mn	0.15
Boron Headland	13/10/2017	0.50	L	B	0.07
Epso CombiTop	13/10/2017	1.48	kg	MgO	0.19
				Mn	0.06
				SO3	0.50
				Zn	0.01
Origin TSP	08/11/2017	114.60	kg	P2O5	52.72
CF DoubleTop	24/02/2018	168.42	kg	N	45.47
				SO3	0.50
Origin MOP	24/02/2018	62.20	kg	K2O	36.72
Omex N26 + 5 (HH)	07/04/2018	357.87	L	N	119.10
				SO3	22.90
Omex N26 + 5 (HH)	16/04/2018	191.37	L	N	63.69
				SO3	12.25

Table 3.3- 'Big Substation' field fertiliser application record 2018-2019.

Product	Date	Rate/ha	Units	Nutrient	Nutrient kg/ha
Origin TSP	01/08/2018	133.80	kg	P2O5	63.67
Omex N20+ 12.5 (HH)	15/02/2019	286.30	L	N	71.92
				SO3	44.95
Origin MOP	22/02/2019	196.81	kg	K2O	117.72
Manganese Headland	01/04/2019	2.00	L	Mn	0.30
Headland Cereal Plus	01/04/2019	13.81	L	MgO	0.02
				Mn	0.05
				Zn	0.01
				Cu	0.02
Omex N26 + 5 (HH)	08/04/2019	354.34	L	N	117.56
				SO3	22.61
Headland Cereal Plus	23/04/2019	11.51	L	MgO	0.02
				Mn	0.04
				Zn	0.01
				Cu	0.02
Epso CombiTop	23/04/2019	1.58	kg	MgO	0.21
				Mn	0.06
				SO3	0.54
				Zn	0.02
Omex N26 + 5 (HH)	02/05/2019	158.03	L	N	52.43
				SO3	10.08
Epso CombiTop	24/05/2019	3.41	kg	MgO	0.44
				Mn	0.14
				SO3	1.16
				Zn	0.03
Mag Super 80	24/05/2019	1.54	L	MgO	0.20

3.2.3 Biosolids

Biosolids were collected from Esholt Water Treatment Works in West Yorkshire, UK. The biosolid was representative of a digested sewage cake and consisted of sewage sludge which had undergone thermal hydrolysis prior to anaerobic digestion. An initial biosolid sample was taken for characterisation prior to any experiments and sent to NRM laboratories for a full nutrient analysis. Table 3.4 displays the full results as reported from NRM.

Table 3.4 – Full chemical analysis results of the initial biosolid sample 'Esholt 0' as reported by NRM laboratories.

	Units	Esholt 0
Date sampled		06/04/2017
pH		8.25
DM	%	24.5
Total N	% w/w	4.87
Ammonium N	mg/kg	3486
Nitrate N	mg/kg	<10
P	mg/kg	24029
K	mg/kg	1150
Mg	mg/kg	2817
S	mg/kg	8446
Cu	mg/kg	227
Zn	mg/kg	667
Na	mg/kg	573
Ca	mg/kg	22637
Fe	mg/kg	36773
Mo	mg/kg	5.85
Mn	mg/kg	466
C	% w/w	34.4
Co	mg/kg	4.92
B	mg/kg	5.12
C:N Ratio		7.06

These results were then compared to the standard values presented in RB209 (AHDB, 2017) and to values found in a study by Dad et al. (2019) which presented a range of different biosolid types for comparison. These comparisons are shown in Table 3.5. The 'Esholt 0' sample was comparable in nature to the RB209 'Digested Cake' and the Dad et al. (2019) 'Dewatered MAD'. All three materials had similar dry solids content, though the total N content of the 'Dewatered MAD' was higher than the 'Digested Cake' and 'Esholt 0' samples, which were comparable. The NH_4^+-N content of the 'Dewatered MAD' was also higher than both other materials, with the 'Esholt 0' NH_4^+-N also lower than 'Digested Cake'. However, the lower NH_4^+ in 'Esholt 0' may have been due to ammonia loss during sample collection and transport to the NRM laboratory, despite efforts to ensure a quick sample turnaround, whereas the samples presented in the Dad et al. (2019) study are likely based on fresh weights. The RB209 NH_4^+ values are based on 15% of the total N content of the material rather than a set value, so comparison to this may not be entirely accurate.

Table 3.5 – Comparison of N and P contents of 'Esholt 0' biosolid sample with values for other biosolids found in the literature.

Source	Biosolids	DS	Total N	NH_4^+-N	NO_3^--N	Total P	Available P
		%	%	mg/kg DS	mg/kg DS	% DS	mg/kg DS
	Esholt 0	24.5	4.87	3486	<10	2.40	12014*
RB209 standards (AHDB, 2017)	Digested cake	25	4.40**	6600	–	1.92	9607*
	Thermally dried	95	4.21**	6316	–	1.84	9193*
	Lime-stabilised	25	3.40**	5100	–	1.48	7424*
	Composted	40	2.75**	4125	–	1.20	6004*
(Dad et al., 2019)	Dewatered MAD	26.7	5.70	8734	<0.01	2.32	1135***
	Thermally Dried MAD	87.5	4.15	967	<0.01	2.07	523***
	Thermally dried raw biosolids	85.6	4.10	254	7.49	1.61	183***
	Liquid MAD	1.98	1.47	0.77	–	2.35	8171***
	Lime Stabilized	39.7	1.03	506	<0.01	0.38	1527***
	Composted Biosolids	55.3	1.32	113	1073	0.61	394***
	Thermally Hydrolyzed MAD	2.24	1.65	0.67	–	1.92	1053***
	'*' = values determined as 50% of total P, as per RB209						
	'**'= values determined as 15% total N, as per RB209						
	'***' = values determined using Olsen's extraction						
	DS = Dry Solids, MAD = Mesophilic Anaerobically Digested.						

The total P content of 'Esholt 0' was higher than that of the 'Digested Cake' and 'Dewatered MAD', but was comparable. The crop available P of both the 'Esholt 0' and 'Digested Cake' were determined as per RB209 by assuming 50% of the contained total P would be available. However, in the 'Dewatered MAD' the available P was measured using Olsen extraction (Olsen et al., 1954), which is the method used to determine soil available P, and was found to be much lower. This difference could be due to an overestimation from the RB209 method, which is determined by what may become available in the soil on application rather than what is already available in the material. The full results of the analysis of 'Esholt 0' show that is has a high Fe content. This is typical in many biosolids, as Fe dosing is used as a method for P extraction in many wastewater treatment processes (Semblante et al., 2015; Korving et al., 2019; Prot et al., 2020). Addition of Fe salts to wastewater can form FeP compounds such as vivianite or strengite which are then insoluble in water and not crop available but make up much of the biosolid contained P content. In many cases more than half of the biosolid contained P can be in FeP forms (Zhang et al., 2019). This would suggest that a much lower proportion of the total P contained in the biosolid samples is available than the 50% figure suggested in RB209. The high Fe content of the Esholt biosolid sample could have important

implications for this project. If conditions in the flooded soils become highly reducing then this could lead to the release of much of the P which is bound in Fe minerals, the mechanisms of which were discussed in section 2.4.

Further samples of biosolids were collected for use immediately before the start of each experiment as they were required to be applied fresh. A sample of each was then sent immediately after collection to NRM for nutrient analysis, the results of which are presented in section 4.3.1. Each experiment was started as soon as possible after collection of the biosolids to minimise degradation of the fresh sample in storage. This meant that the nutrient analysis results for the biosolid were not available at the initiation of each experiment. Rates of biosolid application in each experiment therefore needed to be calculated without accurate nutrient values for that sample. To account for this, it was decided to use the RB209 nutrient figures as a benchmark for the biosolid applications rather than the initial 'Esholt 0' biosolid sample. The RB209 data was a more reliable source than the single initial sample from Esholt, as it was based on large empirical datasets and could therefore account for sample variation. The initial 'Esholt 0' digestate sample nutrient values were deemed to be close enough to the RB209 values to justify this. The 11kg total N/ha figure was therefore used when calculating the 250kg/ha maximum total N application rate for biosolids. Full nutrient analysis results for the biosolid applications were then retrospectively considered within the context of each experiment. The decision was also made to use a range of biosolid application rates in the experiments as a variable. This negated the problem of having completely accurate nutrient applications in each experiment as a full range was being studied. The maximum 250kg/ha total N, 22.7 t/ha digested cake application was still used as the benchmark from which other applications were based.

3.2.4 Biosolid-applied soil chemical characterisation

To further expand the characterisation of the soil and biosolids chemical analysis by X-ray fluorescence (XRF) was carried out on the biosolid-applied soils of Experiment 4. Samples were collected from soils in the growth boxes of Experiment 4, setup of which is detailed in section 3.4. Six boxes were included in the experiment, with two replicates of each treatment of unapplied control and 400g and 800g biosolid-applied . Samples were then combined into composite samples for each box to give an average representation of the chemical composition of the soil during the experiment. This included three samples from each of the three timings of pre-flood, post-flood and final, to give a total of nine soil samples in the composite.

The composite soil samples were milled to pass a 100µm sieve and placed in an oven at 105^{o}C until at constant weight. A 2g mass of each sample was then weighed into a crucible and placed into a furnace at 900^{o}C for 2 hours. The crucibles and samples were weighed again after cooling to determine the loss on ignition (LOI). A 0.7g mass of the ash of each sample and 6.7g of lithium borate flux were then weighed into a platinum gold crucible and thoroughly mixed. The crucible was then inserted into a Katanax® K1 Prime Fluxer and the oxides program run to produce a moulded fused bead. The fused bead was then analysed using a Rigaku ZSX Primus II instrument to quantitatively determine the oxide content of the samples. Results of the XRF analysis are displayed in Table 3.6. The values obtained for Sample 4, from the 800g (2) box, were determined to be anomalous due to a LOI % measurement much lower (~5% sample weight) than the other samples. Sample preparation was identical for all samples and no obvious errors were observed in the preparation process. The weight of the oxides in the samples was determined using the LOI % and so the subsequently analysed values were also affected by this discrepancy.

Application of biosolids showed an increase in the total P content of the soils as anticipated from the high P content of the biosolids. However, the main P sorbing element contents of Fe, Ca, Mn and Al did not increase in the soil with biosolid application, despite the high Ca and Fe content measured in the biosolids. This was due to the concentrations of Fe and Ca found in the control soils being similar to that of the biosolids, meaning there was no net increase in concentration of these elements from biosolid application. However, Much of the P contained in the biosolids could be sorbed as FeP or CaP minerals, meaning changes in Eh and pH in the soil system could lead to the release of SRP. The main PTEs regulated in biosolids of Zn, Ca, Cu, Pb, Ni, Cr and Hg did not appear to be increased above background soil levels after biosolid application, supporting that the selected biosolids were safe for land application in agriculture.

Table 3.6 - Experiment 4 soil XRF analysis results in full. XRF was carried out using a fused bead method. 800g(2) box results were anomalous due to a low LOI%.

Sample No.	Box biosolid application	Loss on ignition at 900°C (%)	Al (mg kg^{-1})	As (mg kg^{-1})	Ba (mg kg^{-1})	Ca (mg kg^{-1})	Co (mg kg^{-1})	Cr (mg kg^{-1})	Cu (mg kg^{-1})	Fe (mg kg^{-1})	Ga (mg kg^{-1})	K (mg kg^{-1})	Mg (mg kg^{-1})	Mn (mg kg^{-1})
3	0g (1)	19.08	3344.97		149.65	7272.44		25.66		2195.87		1058.11	4186.28	116.25
5	0g (2)	17.52	3584.94		60.90	6308.45		31.47		2287.99		1151.25	3657.35	115.24
2	400g (1)	19.26	3334.70	5.00	69.31	7127.28		30.45		2222.38		1064.08	4117.23	115.39
6	400g (2)	18.28	3533.21	5.07	84.10	6632.93		33.94	6.47	2290.64		1088.82	3800.57	115.63
1	800g (1)	18.30	3512.72	4.32	68.26	6419.44	5.33	29.56	5.91	2283.23		1082.68	3705.96	125.15
4	800g (2)	13.20	4061.24		78.41	3836.01		35.99	5.99	2485.51	4.17	1311.55	2380.54	98.82
			Na (mg kg^{-1})	Ni (mg kg^{-1})	P (mg kg^{-1})	Pt (mg kg^{-1})	Rb (mg kg^{-1})	Si (mg kg^{-1})	S (mg kg^{-1})	Sr (mg kg^{-1})	Th (mg kg^{-1})	Ti (mg kg^{-1})	Zn (mg kg^{-1})	Zr (mg kg^{-1})
3	0g (1)	19.08	282.20	8.64	82.40			23888.14	80.77	16.57		252.93	12.69	46.57
5	0g (2)	17.52	310.99		75.72	17.10		25411.11	88.74	14.88		285.07	9.24	50.04
2	400g (1)	19.26	254.75	6.84	85.76			24059.18	93.23	16.49		250.95	9.80	45.08
6	400g (2)	18.28	463.07	6.76	99.24		28.07	24639.78	98.11	16.91		266.42	12.45	50.19
1	800g (1)	18.30	286.65	9.98	121.19		28.62	24988.45	95.35	17.08		247.72	10.44	51.67
4	800g (2)	13.20*	326.64	6.44	116.83			29237.94	122.42	19.70	13.53	324.99	11.49	54.19

3.3 EQUIPMENT AND EXPERIMENTAL DESIGN

The experiments for this project required several microcosms which could house a growing barley crop for 10 weeks on a 15cm topsoil layer, be watertight and have controlled drainage, light and temperature. The experimental setup was also required to be compact enough to run as a laboratory bench experiment which could be monitored in detail and be easily set up and repeated. Oxidation-reduction potential (ORP), pH and dissolved oxygen were also monitored in real time during flooding.

3.3.1 Growth Boxes

Six clear plastic boxes with an area of 0.165m^2 (50 x 33 cm) and depth of 30cm were selected as the growth box containers. A hole was drilled at the base of each box to accommodate a 15mm to 22mm brass compression connector that was tightened with a rubber washer to create a watertight seal. On the inside of each box a 22mm plastic pipe with holes drilled every 30mm was attached along the length of the box to provide a drain for flood and overflow water. The internal drainage pipe was covered in a 3cm gravel layer to prevent blocking with soil and to mimic the function of an agricultural field drain. On the outside of the compression connector a short length of 15mm pipe was attached with a brass screw tap to control water drainage. The inside surface of the boxes were roughly sanded to prevent any water channelling when draining and to minimise soil particle loss. The outside of each box was spray painted black to a height of 18cm to prevent light infiltration into the 15cm soil layer and 3cm gravel drainage layer. Completed empty boxes are shown in Figure 3.1.

3.3.2 Growth Chambers

Growth chambers were constructed from steel box frame measuring 138cm (L) x 56cm (W) x 56cm (H). The frames were covered with Mylar foil which provided a non-flammable, anti-bacterial, lightweight, easy to clean, reflective and light-sealed solution. The front of the Mylar covers was made as a roll-back flap for access to the inside and a vent slit was made in the top of the cover to prevent heat and humidity build-up within the chambers. Two 54W LED grow lamps (Model: HY-55cm-18*3W-RB) were mounted to the top and back of each frame and were powered through timer switches on a 12 hour on/off cycle. The lamps delivered 65.3-80.4 µmol $m^{-2}s^{-1}$ of photoshynthetically active radiation (PAR) in the 400-700nm range, measured at the level of the soil surface. Details of the PAR exposure measured at the soil surface in each box, including a light map and calculations, can be found in Appendix A.2. The growth chambers were operated in a temperature-controlled laboratory at 20°C and each chamber was separately monitored internally by a temperature autologger during the experiments. Three growth boxes were placed inside each frame in random order for each experiment. Each box was positioned on top of a 20-litre spill tray

measuring 60x40cm to catch water lost in the event of any leaks or breakages. The completed growth box and growth chamber setup is shown in Figure 3.1.

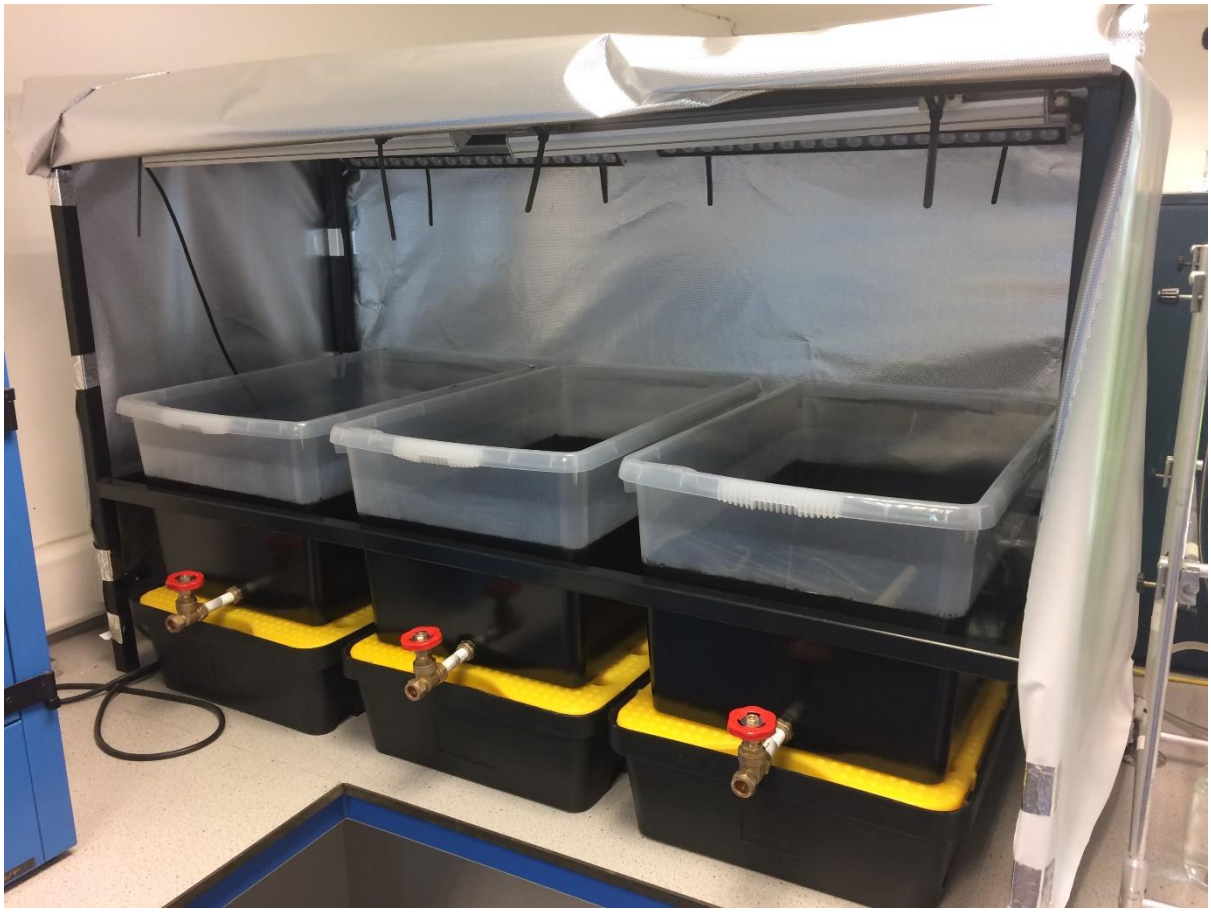

Figure 3.1- Empty growth boxes set up in growth chamber on top of spill trays

3.3.3 Redox electrodes

Platinum redox reference electrodes were constructed using 0.5mm 99.5% pure platinum wire cut into 1.5cm lengths. The platinum was wrapped in, and soldered to, a length of insulated copper wire. The copper wire and platinum were inserted into a 5ml plastic pipette with the bulb removed and the bottom of the copper wire protruding from the pipette tip. The solder joint was sealed into the end of the pipette with epoxy resin, leaving 1cm of platinum wire exposed. Holes were drilled into the growth boxes at 5cm and 10cm below the soil surface to accommodate the Pt electrodes. A wooden dowel was pushed through the drilled holes into the soil inside to create a void for insertion of the electrodes without damaging the delicate platinum wire ends. Once inserted the electrodes were sealed into the boxes with epoxy resin two days before flooding to create a watertight seal once dried. Measurements of ORP were taken by attaching a crocodile lead to the protruding copper wire on the Pt electrodes which was connected to a voltmeter (TENMA environmental

multimeter, P/N IN05691), to which was also connected an AgCl electrode to act as an electron donor through the soil solution. The AgCl electrode was a pH probe (Sentek pH electrode, P/N P11-DJ) with the internal reference electrode bypassed by connecting a pin to just the outer ring of the BNC connector and not the internal pin. This bypassing of the internal reference electrode allowed completion of the circuit through the soil solution to the Pt electrodes. Eighteen Pt electrodes were constructed. Twelve electrodes were mounted in the boxes, one was used for measuring surface water ORP and the remainder used as spares to replace any failing probes and for calibration tests. All electrodes were calibrated using a buffer solution (Hamilton ORP buffer +475mV ±5mV at 25°C, P/N 238322) to within ±2mV with temperature compensation. Before and after use all probes were thoroughly cleaned by dipping in 0.5M H_2SO_4 to remove impurities from the surface of the platinum, washing with acetone to remove any organic contaminants and rinsing several times with distilled water. Any probes which did not meet the calibration standard were carefully rubbed down with fine sandpaper to remove any heavier impurities and then washed again. Pt electrodes which still failed to meet the calibration standard were either dismantled and reconstructed or discarded. Figure 3.2 shows the equipment used for measuring the ORP.

Figure 3.2- (Left) A constructed Pt reference electrode; (Centre) multimeter set to measure DC voltage for measuring ORP; (Right) pH probe with BNC connector to act as AgCl electron donor electrode.

3.3.4 pH and dissolved oxygen probes

3.3.4.1 Standpipes

To measure pH and dissolved oxygen in surface water and porewater a low maintenance gel filled field pH electrode (Hach Intellical™ PHC101-05) and field optical dissolved oxygen probe (Hach Intellical™ LDO101-30) were used with a handheld digital meter (Hach HQ40D portable multimeter). For surface waters the probes were held under the water surface approximately 1cm from the soil surface until equilibrium was reached. For soil porewater 45mm diameter plastic standpipes were inserted to 5cm and 10cm depth in the soil before the start of flooding. The end of the standpipe was long enough to protrude above the water surface to prevent disturbance when inserting. The standpipes fitted the probes snugly inside and were held in place by the probe collars. The probes were each 16cm long from the collar and the standpipes were cut to length to hold the probe at the correct depth when it was fully inserted. The 5cm standpipe was constructed to extend deeper into the soil to prevent it tipping or becoming dislodged. Holes were drilled and openings cut in the standpipes to aid water mixing around the probe and prevent a contained water column from forming in the pipe. The pipes are displayed in Figure 3.3.

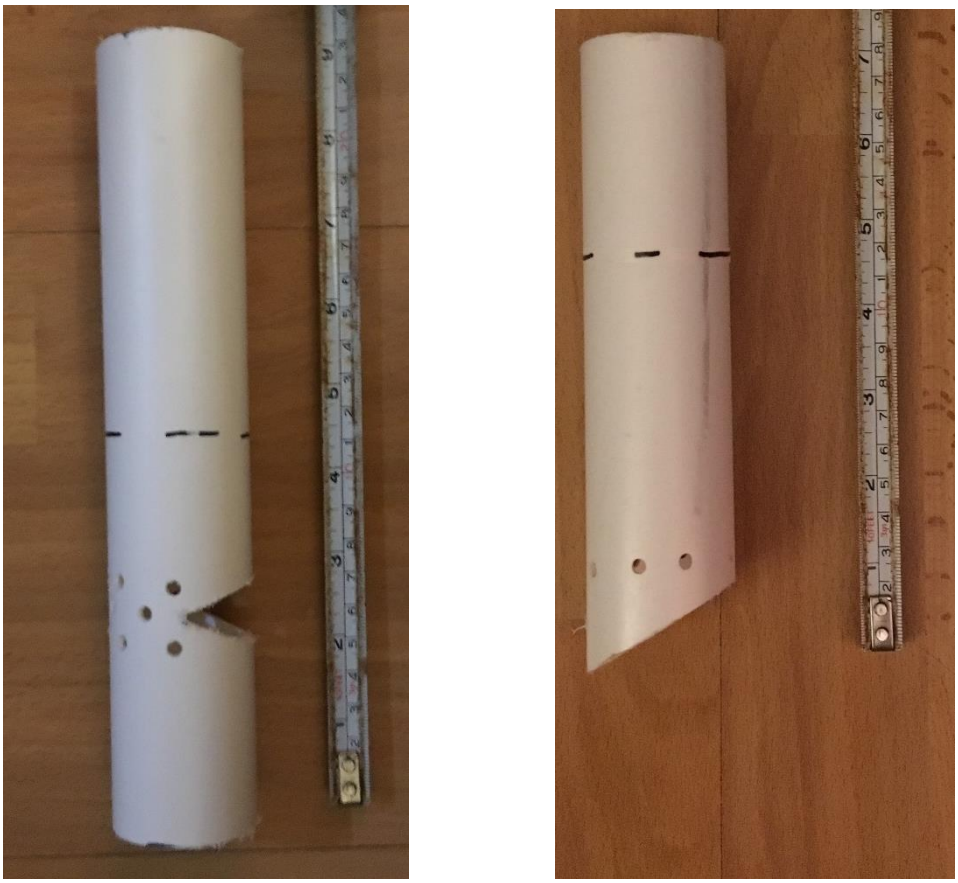

Figure 3.3- (Left) 5cm depth standpipe with the dotted line indicating the soil surface and a cut out section at the depth that the probe was inserted to measure; (Right) 10cm standpipe with dotted line indicating soil surface and the angled cut at the bottom where the probe was inserted to measure.

3.3.4.2 Oxygen spots

For Experiment 4 the method for measuring soil porewater dissolved oxygen was changed to avoid the problem of introducing oxygen into the system by insertion of the probe in the standpipes. Oxygen sensitive spots (PreSens Planar trace oxygen-sensitive spots, P/N SP-PSt6-YAU) were fixed to the centre of the bottom of 20cm long clear plastic tubes. These spots could be read via fibre optic cable through the clear plastic material using the PreSens Fibox 4 standalone oxygen meter. The tubes were inserted into the soil immediately before flooding to depths of 5cm and 10cm below the soil surface. To align the fibre optic cable to the spots at the bottom of the tubes a metal rod with spacers was used to guide the cable into each tube. This method allowed for accurate readings of soil porewater dissolved oxygen without disturbing the closed system. Surface water dissolved oxygen was measured as previously with the dipping probe. The oxygen spot tube design is shown in Figure 3.4.

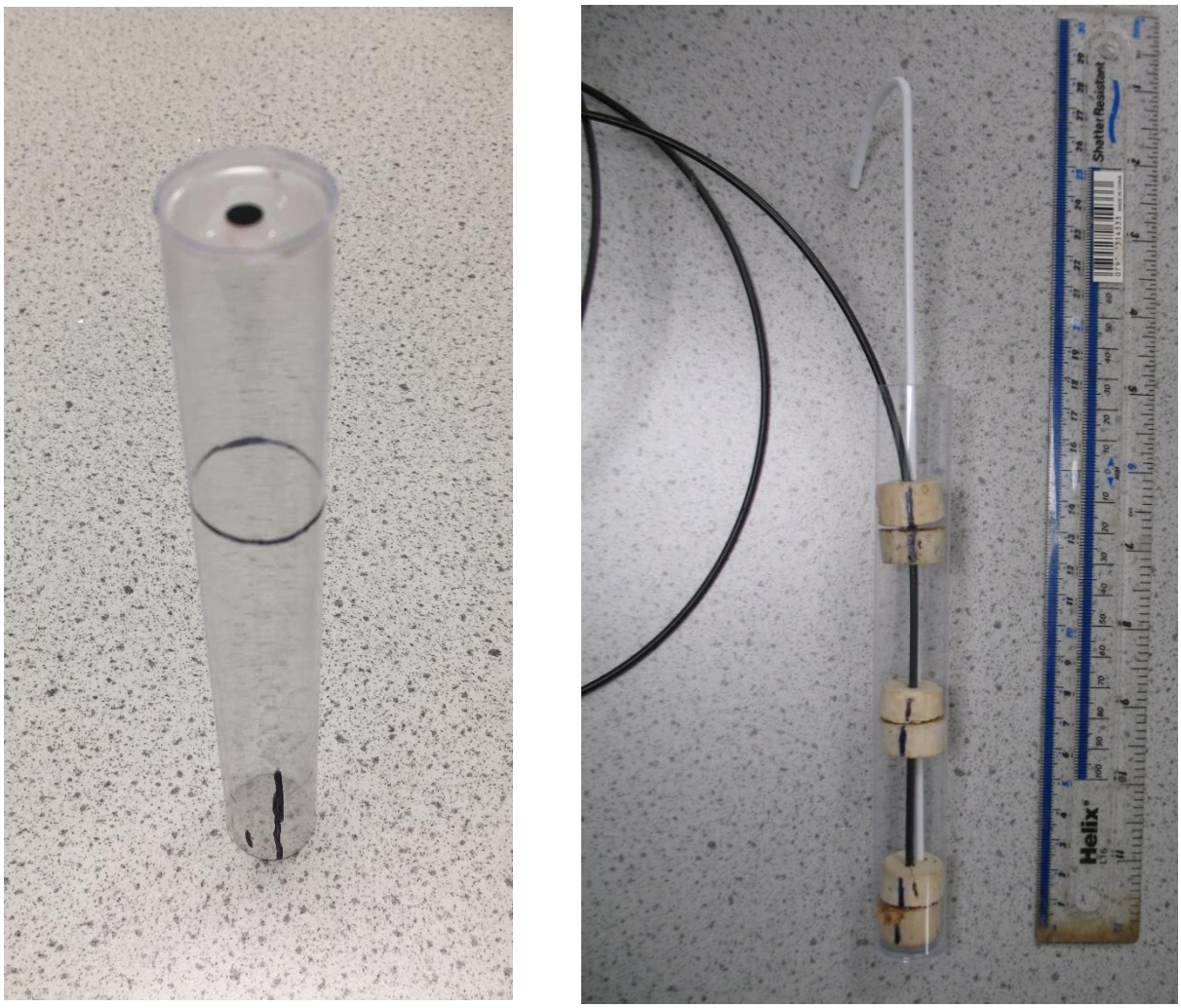

Figure 3.4 – (Left) Inverted plastic tube with oxygen spot attached, horizontal line to indicate soil surface level and vertical line to align fibre optic cable; (Right) Fibre optic cable mounted to metal rod and spacers inserted into tube, with vertical lines drawn on spacers to align with oxygen spot.

3.4 EXPERIMENT SETUP AND MAINTENANCE

3.4.1 Soil preparation and biosolid application

For each experiment soil was weighed out of the collection buckets and 40-45kg was initially added to each box. Any large clumps were removed, and the soil was mixed until 34kg of friable soil remained in each box at a depth of 15cm over the gravel drainage layer. Biosolids were applied at different rates to each box depending on the experiment, with at least one control box with no biosolid application in each experiment. All rates were based on the 250kg N/ha maximum organic fertiliser total N application, with the biosolid nutrient values being assumed as a digested cake, as given in RB209 (AHDB, 2017). As 6 boxes were available application rates ranging from control, ¼, ½, 1, 2 and 4 times the maximum were selected. The maximum total N application based on the box area of 0.165m^2 was therefore 373g of biosolid. Based on the range of rates to be studied this base application was adjusted to 400g to slightly over apply N. This ensured that the maximum N application was covered within the lower application rates for more detailed study and would reduce the risk of under-applying in case of a low N sample. Application rates of 0g, 100g, 200g, 400g, 800g and 1600g of biosolid were therefore used for the boxes in a random order. The biosolid was applied to the soil surface of each box and uniformly mixed into the whole soil with a handheld garden claw to simulate incorporation into a cultivated arable topsoil layer. In Experiment 4 the biosolid application rates were changed to 2x 0g, 2x 400g and 2x 800g to provide duplicate boxes to check the reproducibility of the experimental method. The random order of biosolid application for each experiment is presented in Table 3.7.

Table 3.7: Biosolid applications to boxes in each experiment.

	Growth chamber 1			**Growth chamber 2**		
Box	**1**	**2**	**3**	**4**	**5**	**6**
Experiment	**Biosolid application (g)**					
1	1600	800	100	200	400	0
2	100	400	200	0	1600	800
3	400	200	800	100	1600	0
4	800	400	0	800	0	400

3.4.2 Crop planting and establishment

Sixty seeds of untreated barley *(Hordeum Vulgare)* were selected for planting in each growth box, from a seed sample provided by GrainCo ltd. Seeds were selected which looked healthy, not small, broken or shrivelled to maximise chances of germination and reduce variation in seed quality between boxes. The seeds were then laid on the surface of the soil in each box in three rows of twenty, spaced evenly apart with 8cm between rows and between the outside of the box. The seeds were then pressed into the surface of the soil and covered with loose soil so they were approximately 1-2cm deep. Three litres of water was added to the surface of each box to ensure there was sufficient moisture for germination and to consolidate the surface soil. The LED growth lights in the growth chambers were then switched on to a 12-hour on, 12-hour off cycle and the mylar foil front of the growth chambers closed until maintenance and monitoring was required. The crops were then allowed to establish and grow for a period of 28-days in all experiments.

Experiment 4: A modified approach to planting and plant establishment was used in Experiment 4. Seeds were initially planted in the same way as previously stated, however after seven days a large difference in established plants was noted between treatments. For this experiment a uniform plant number was desired to remove any establishment difference effects from later measurements of other variables in the experiment. To counteract the uneven establishment the boxes with low plant numbers were supplemented with additional seeds. The number of established plants in each box was noted and if this number was below fifty then additional seeds were planted in gaps to supplement the existing plants up to fifty. The initial 28-day growth period then continued as normal.

3.4.3 Watering and leaks

The aim of the watering protocol was to maintain the soil at 'field-capacity' throughout the growth period, which is the point at which the soil is saturated but does not drain. This method ensures that sufficient water was provided for growth but soils did not become waterlogged and any differences in evaporation, crop water uptake and soil water holding capacity would be naturally accounted for. Watering was done twice a week on Monday and Thursday. First the drainage valve on the bottom of a box was opened and any drained water collected and measured. The amount of water collected was noted and then reapplied to the soil surface of the box. The difference up to one litre minus the drained water was then applied as distilled water. Each box therefore received a surface application of one litre of water at each watering with no excess water for draining. If no water was drained from the valve then one litre of distilled water was added to the soil surface of the box and left to settle for approximately ten minutes before repeating the draining process to ensure soil saturation.

Leaks in the boxes were rare but did occur. These were detected quickly during the first few days of watering as water visibly pooled in the spill trays and no water was drained from the box. In all cases the leak occurred at the joints around the drainage valve or the compression connector. To repair these leaks PTFE tape was wrapped tightly in several layers around the joint which stopped leaking during watering and remained watertight during the flood.

3.4.4 Temperature and lighting

As discussed in section 3.3 lights were run on a 12-hour on, 12-hour off cycle and temperatures were monitored using an autologger placed in each growth chamber. The daily light cycle timings, the dates each experiment ran, and the average temperatures and variations recorded during each experiment are presented below:

The light cycle in Experiment 1 ran from 07:00-19:00 for the duration of the experiment from 02/10/2017 to 07/12/2017. The mean temperature inside both growth chambers was 22°C (±2°C).

The light cycle in Experiment 2 ran from 07:00-19:00 for the duration of the experiment from 15/01/2018 to 22/03/2018. The mean temperature inside both growth chambers was 21.5°C (±2°C).

The light cycle in Experiment 3 ran from 07:00-19:00 for the duration of the experiment from 16/04/2018 to 04/06/2018. The mean temperature inside both growth chambers was 22.5°C (±3°C).

The light cycle in Experiment 4 ran from 08:00-20:00 for the duration of the experiment from 22/10/2018 to 19/12/2018. The mean temperature inside both growth chambers was 21°C (±1°C).

Graphs detailing the temperature monitoring measurements for each experiment can be found in Appendix A.3.

3.5 FLOOD SIMULATION

3.5.1 Flood setup and maintenance

After the 28-day crop growth period the boxes were flooded with 16-18 litres of distilled water to 5cm above the soil surface. The flood duration was ten days and care was taken to ensure minimal disturbance to the water in this time to maintain stagnant flood conditions. The water level was marked and maintained as needed using a syringe to carefully add to the surface water in the corner of each box to minimise mixing and the introduction of extra oxygen to the water. Measurements of the floodwater properties were taken during the flood and are described in section 4.

3.5.2 Flood draining and recovery

After the 10-day flood duration was complete the final measurements were taken and the flood was drained. Draining occurred by opening the valve at the bottom of each box and capturing the water in a 20-litre secondary container from which samples could be taken from the mixed total volume before disposal. This process was repeated for each of the six boxes. The soil and crop in the boxes were then left to recover from the flood for 10-28 days under the same conditions and watering protocol as in the initial growth stage. Details of differences in the recovery periods are described in Chapter 5.

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Chapter 4: GEOCHEMICAL RESPONSE OF BIOSOLID-AMENDED SOILS TO SHORT-TERM FLOODING

4.1 INTRODUCTION

Due to climate change, the growing occurrence of extreme weather events which can threaten crop production is an increasing possibility. In the UK and Western Europe the likeliness of increased annual rainfall, summer rainfall and flash flood events is predicted to worsen in coming years (Lenderink and van Meijgaard, 2008; Kendon et al., 2014). If measures are not taken to improve soil and crop resilience to flash flood events and waterlogging then this could pose a risk to sustainable food production. An option for improving soil flood resilience is to increase the soil organic matter (SOM), which is a known way to improve soil structure, drainage and crop yields and often considered a reliable indicator of soil fertility (Singh and Agrawal, 2008; Johnston et al., 2009; Hijbeek et al., 2017). One way to increase SOM is through applications of organic fertilisers such as manures, straws, composts or biosolids (digested sewage sludges), all of which can affect soil physical, chemical and biological properties in different ways (Haynes and Naidu, 1998; Fuentes et al., 2012; Kaleeem Abbasi et al., 2015).

Biosolids are potentially a very valuable organic input for farmers as the high levels of organic carbon I, nitrogen (N) and phosphorus (P) they contain mean they are an effective option for improving soil fertility and crop yields (Mantovi et al., 2005; Kathijotes et al., 2016). Application of biosolids to agricultural land is therefore a sustainable and economically attractive alternative to either incineration or landfill of water treatment waste, and around 78% of biosolids are recycled to agricultural land in the UK, totalling 3.6 million tonnes (Assured Biosolids Limited, 2019). Historically there has been concern about the use of biosolids on agricultural land due to potential problems with odour, contamination by heavy metals, threat to public health and environmental pollution (Sterritt and Lester, 1980). Modern treatment methods of sewage sludge such as anaerobic digestion and lime stabilisation have greatly reduced the risks associated with biosolid application in agriculture. Coupled with legislative controls these treatment methods now mean that biosolids are considered a safe and sustainable supplement to inorganic fertilisers in crop production (Smith, 2009b; Clarke and Smith, 2011; Al-Gheethi et al., 2018). The high N:P ratio in biosolids make them distinct from other organic fertiliser options, meaning larger amounts of P can be applied per application when based on total N content. This large P application offers farmers a valuable resource as a crop nutrient, but N and P are also the main drivers of eutrophication if they enter

surface waters (Sharpley et al., 2001; Withers and Haygarth, 2007; Selman and Greenhalgh, 2010b; Huang et al., 2017). If biosolids are utilised as a management option for improving soil flooding and waterlogging resilience then consideration must be given to the fate of N and P within the amendment under saturated soil conditions.

Loss of N and P from soils has been extensively studied due to concern around contamination of watercourses from agricultural applications. However, much of the focus has been towards the reduction of long-term P losses from leaching and erosion, with acute P losses from waterlogged soils and flash flooding receiving little attention. Upon inundation, conditions within the flooded soil system can change quickly as oxygen (O_2) is consumed by microbes and the oxidation-reduction potential (ORP) of the system becomes more reducing. The three main factors of O_2, NO_3^- and soil organic matter (SOM) strongly influence microbial respiration and ORP in wet soils (Gardiner and James, 2012). If microbial activity is increased from biosolid applications this could result in rapid consumption of O_2, leading to conditions in those soils becoming highly reducing more quickly. In addition to the added P from biosolids this drop in ORP could favour the release of soluble reactive phosphorus (SRP) from soil P minerals to soil porewater and overlying floodwater. As conditions become more reducing in the absence of O_2 and NO_3^- as electron acceptors supporting microbial respiration, solid phase Mn^{4+} and Fe^{3+} can be reduced (Patrick and Turner, 1968; Amarawansha et al., 2015). The reduction of Mn^{4+} and Fe^{3+} to soluble Mn^{2+} and Fe^{2+} can lead to the P associated with inorganic minerals such as reddingite ($Mn_3[PO_4]_2)\cdot 3H_2O$), hureaulite ($Mn_5H_2[PO_4]_4\cdot 4H_2O$), trivalent Mn phosphate ($MnPO_4\cdot 1.5H_2O$), strengite ($FePO_4 2H_2O$) and vivianite ($Fe_3[PO_4]_2\cdot 8H_2O$) being released as SRP (Boström and Pettersson, 1982; Moore and Reddy, 1994; Hupfer and Lewandowski, 2008; Van Nguyen and Maeda, 2016). An increased solubility of P from soil minerals, coupled with the P addition and NO_3^- release from a biosolid application, could therefore pose a significant eutrophication risk to surface waters.

Much of the previous research has focused on long-term floods such as those in soil systems which are seasonally flooded, wetlands, lake sediments or rice paddies (Moore and Reddy, 1994; Young and Ross, 2001; Zhang et al., 2003; Hupfer and Lewandowski, 2008; Unger et al., 2009b; Amarawansha et al., 2016; Dharmakeerthi et al., 2019), rather than soils in arable production at risk of unseasonal flooding. Studies have also favoured either very closely controlled laboratory flask experiments or large-scale field experiments. Closely controlled laboratory bench experiments lack many of the environmental factors which will significantly affect how an agricultural system behaves, such as the influence of crops and microbial community behaviour in the rhizosphere. Large field experiments can lack the control of variables such as rainfall, temperature and other environmental stressors which would be required to accurately measure many of the

biogeochemical changes occurring within the system. The objective of this study was to create an experimental microcosm which could be used to measure key geochemical factors such as SRP, NO_3^- and ORP in a closely controlled agricultural system. It was hypothesised that increased biosolid application will lead to an increase in floodwater NO_3^- and SRP. These increases may occur due to the creation of conditions favouring the reduction of inorganic P minerals by rapid depletion of O_2 and by direct application of organic N and P in biosolids.

4.2 METHODOLOGY

To study the geochemical changes occurring in biosolid-amended agricultural soils under short-term flooding conditions, growth chamber experiments were set up as described in the general methodology of Chapter 3. The following section outlines in greater detail the methodologies used specifically in relation to the geochemical measurements made.

4.2.1 Biosolids

Prior to each experiment approximately 10kg of fresh thermal hydrolysis digested sewage sludge was collected from Esholt Water Treatment Works in West Yorkshire, UK. Immediately after collection a sample of digestate was sent to NRM laboratories for analysis of major nutrients and trace elements as discussed in Section 3.2.3. The bulk of the sample was then refrigerated at 4°C and stored for 3 days before its application in the growth boxes at the start of each experiment.

4.2.2 Soils

4.2.2.1 Sample collection

Three initial soil subsamples were taken immediately after collection of the bulk of the soil for use in the experiments; these were representative of the whole soil and were frozen in storage for later analysis with other samples. Experimental soil samples were taken at three stages during the experiment, immediately pre-flood, immediately post-flood and a final sample at the end of the experiment after the recovery period. Three samples were taken from each box at each stage using a 2.5cm diameter core auger to a depth of 15cm. All samples from an experiment were frozen in storage ready for sample preparation and analysis after completion of the experiment.

Recovery periods: For Experiments 1 and 2 the recovery period after flooding was 28 days and the soil samples were taken after this time. In Experiment 3 the focus was primarily on the flood conditions and the recovery period was shortened to 10 days with no final soil sample being taken, just pre- and post-flood. In Experiment 4 the recovery period was 20 days to adhere to timings required for the microbiological sampling described in Chapter 6; soil samples were taken at the end of this period.

4.2.2.2 Sample preparation

Upon completion of an experiment and sample collection the soil samples were weighed, placed in individual open containers and allowed to thaw and air-dry for 48 hours. After this first 48-hour drying period the samples were partially ground to break up any larger clods and allowed to air-dry for a further 48-72 hours until fully dried. After drying each sample was weighed and ground to pass through a 2mm sieve, with any material not passing through the sieve being weighed and discarded. As small soil volumes would be needed in some analyses, approximately 50g of each sample was then ground again to pass through a 355µm sieve to improve sample mixing and representativeness. Samples were stored in a refrigerator until required for analysis.

Oven dry weight: To provide accurate analytical results on a by-weight basis air dried samples of all the soil samples from Experiment 4 were oven dried at 105°C until constant weight. The difference between the air-dried soils used for analysis and the averaged oven dry weight was then included in the results.

4.2.2.3 Soil analysis

4.2.2.3.1 Soil pH

Soil pH was measured in a 1:2.5 (v/v) suspension in water (World Agroforestry Centre, 2014). A 10g soil sample was placed in a 50ml centrifuge tube with 25ml distilled water and mixed on a shaker rack for 10 minutes. After mixing a dipping probe (Hach Intellical PHC201 pH electrode) was used to measure pH and the results recorded.

4.2.2.3.2 Olsen P

Soil available P was extracted using Olsen's reagent (extracting solution) (Olsen et al., 1954). All equipment used for determination of P was thoroughly washed with 1M hydrochloric acid (H_2O:HCl) before use. A 2500ml volume of extracting solution was prepared each time for use with a batch of 20 samples and 4 blanks. This was used within 4 hours of creation and kept at room temperature (20±1°C). A 105g mass of sodium hydrogen carbonate ($NaHCO_3$) was dissolved in 2000ml of distilled water using a magnetic stirrer and 12.5ml of 0.05% polyacrylamide solution was added. The reagent

was adjusted to pH 8.50±0.02 using 1M sodium hydroxide solution (NaOH) and made up to 2500ml. A 5g sample of each soil was weighed and placed in a 500ml polyethylene bottle. A 100ml volume of the extracting solution was then added to each of the bottles and they were placed on a shaker rack for 30 minutes at room temperature (20±1°C). Immediately after shaking the sample solutions were poured into 50ml centrifuge tubes and centrifuged at 1800g for 5 minutes. The sample solutions were then pipetted into clean 50ml collection tubes while avoiding the pellet and refrigerated for a maximum of 48 hours until required for analysis.

Available P was determined colorimetrically via spectrophotometry using the molybdenum blue method (Murphy and Riley, 1962). A 300ml volume of combined reagent was made up using 150ml 2.5M sulphuric acid (H_2SO_4), 15ml potassium antimonyl tartrate solution ($C_8H_{10}K_2O_{15}Sb_2$), 45ml ammonium molybdate solution ($(NH_4)_6Mo_7O_{24}$) and 90ml ascorbic acid solution($C_6H_8O_6$). The spectrophotometer (Thermo Scientific, BioMate 3 spectrophotometer) wavelength was set to 880nm and a calibration curve was determined using 8ml combined reagent and 50ml of diluted P solution at 0, 0.25, 0.5, 0.75, 1.0 and 1.25 mg P L^{-1} concentrations. A batch of 20 samples and 4 blanks could be analysed using the remaining combined reagent within its 4-hour stability time. A 16ml volume of soil sample solution and blanks were diluted to 50ml and 8ml combined reagent added. Ten minutes of standing time was allowed between the addition of the combined reagent and sample measurement on the spectrophotometer. Bubbles formed in the samples after addition of the combined reagent, but the sample cuvettes were tapped carefully to remove bubbles and prevent interference with the colorimetric readings before insertion into the spectrophotometer. The concentration of P in each diluted sample was then determined using its absorbance and the calibration curve. Soil Olsen mg P kg^{-1} was calculated using Equation 4.1.

$$Soil\ P\ (mg\ kg^{-1}) = \frac{\left(\left(Sample\ P\ (mg\ l^{-1}) \times \left(\frac{50}{16}\right)\right) - \left(Blank\ P\ (mg\ l^{-1}) \times \left(\frac{50}{16}\right)\right)\right) \times\ 100}{Soil\ sample\ weight\ (g)}$$

Equation 4.1

4.2.2.3.3 Total Kjeldahl Nitrogen

Soil total N was determined by the Total Kjeldahl Nitrogen (TKN) method. A 1g mass of soil was weighed into a digestion tube containing 50ml distilled water, one copper catalyst tablet (Fisher Scientific, 1g Na_2SO_4 and the equivalent of 0.1g $CuSO_4$, Fisher chemical K/0120/80) and several glass beads. A 10ml volume concentrated sulphuric acid (H_2SO_4) was added and the digestion tubes were boiled over a heating block (Buchi B-435 digestion unit) until the soil sample was fully digested. One

blank was run for every 5 samples digested. After digestion the samples were distilled (Buchi B324 Distillation Unit) into a 50ml indicating 2% (w/v) boric acid ($B(OH)_3$) solution. 10mM sulphuric acid was titrated into the boric acid solution until it changed from green back to purple and the amount of acid titrated recorded. Soil TKN (NH_3-N) was calculated using equation 4.2.

$$Soil\ N\ (mg\ kg^{-1}) = \frac{(V(Sample) - V(Blank)) \times 0.02 \times 14.007}{Soil\ sample\ weight\ (g) \times 1000}$$

Equation 4.2

V(Sample) = Volume (ml) 10mM H_2SO_4 titrated for sample.
V(Blank) = Volume (ml) 10mM H_2SO_4 titrated for blank.
0.02 = H_2SO_4 molar reaction factor × 0.01M concentration.
14.007 = Molecular weight of N.

4.2.2.3.4 Soil organic matter

SOM was determined using the Walkley-Black dichromate method (Walkley and Black, 1934). A 1.5g mass of soil was weighed into a conical flask. A 10ml volume of 0.167M potassium dichromate solution ($K_2Cr_2O_7$) and 20ml concentrated sulphuric acid was added to the flask, swirled thoroughly to ensure mixing and left to cool in a fume cupboard for 30 minutes. After cooling 200ml of distilled water was added to the mixture followed by 10ml of orthophosphoric acid and 1ml indicator (sodium diphenylamine sulphonate, $C_{12}H_{10}NNaO_3S$). Ferrous sulphate solution ($FeSO_4$) was then added to the mixture from a burette until it changed from dark blue to green and the volume of $FeSO_4$ used was recorded. Prior to the soil testing two blanks were run with the same procedure to standardise the ferrous sulphate solution. The standardisation was repeated for each batch of soil samples and whenever new ferrous sulphate solution was made up. The calculation to determine the volume of potassium dichromate (V) used to oxidise the organic matter is shown in Equation 4.3.

$$V = 10 \times \left(1 - \left(\frac{V(Sample)}{V(Standard)}\right)\right)$$

Equation 4.3

V(Sample) = Volume of ferrous sulphate solution titrated for sample (ml).
V(Standard) = Volume of ferrous sulphate solution used in standardisation test (ml).

SOM and total organic carbon (TOC) can then be calculated using Equations 4.4 and 4.5:

$$SOM\% = \frac{0.67\ V}{Soil\ sample\ weight\ (g)}$$

Equation 4.4

$$TOC\% = 0.56\ SOM\%$$

Equation 4.5

4.2.3 Floodwater

4.2.3.1 Sample collection

After the 10-day flood duration was complete 500ml of the surface water from each box was taken by syringe and retained in a sample bottle ready for analysis. The drainage valve at the base of each box was then opened and the whole volume of floodwater, consisting of all porewater and the remaining surface water, drained into a 20-litre holding container. The draining to a secondary container ensured mixing of the whole water volume and a 500ml sample was then taken from this. Both surface water and floodwater samples from each box were then immediately taken for analysis. All samples were filtered through Fisherbrand® MF200 filter paper (1.2µm pore size) prior to analysis.

Experiment 1: Samples in Experiment 1 were not filtered prior to analysis, subsequently the floodwater P measurements are not reliable and so are not presented in this chapter but can be found in Appendix C.1.

4.2.3.2 Floodwater analysis

For analysis of all floodwater and surface water samples the Hach® LCK cuvette test system was used with a Hach® DR3900 laboratory VIS spectrophotometer. The LCS349 kit was used for total phosphate and orthophosphate (0.01-0.5 mg PO_4-P L^{-1} range), the LCK304 kit was used for ammonium (0.015-2 mg NH_4-N L^{-1} range) and the LCK339 kit was used for NO^{3-} (0.23-13.5 mg NO_3-N L^{-1} range). All procedures for use of the cuvette test kits were carefully followed as per the instructions provided. High temperature digestions were performed in a Hach® HT200S high temperature thermostat when required. Any samples which were over the range of reliable measurement for the cuvette test kit were diluted as appropriate and repeated. Samples below the test range were reported as such.

4.2.4 Flood monitoring

4.2.4.1 Measurement timings

Dissolved oxygen (DO_2), pH, ORP and temperature were recorded at each measurement timing and all measurements were taken for one box before moving on to the next box. The first measurements for each experiment were taken 3 hours after the initiation of flooding to allow the system to hydraulically and chemically equilibrate. During the first 48 hours of each experiment measurements were taken at either 3-, 6- or 12-hour intervals to monitor changes in the early stages after flooding. Details of the precise timings are shown in the results. After the initial 48 hours measurements were then taken every 24 hours until the end of the flood.

Timing deviations: In Experiment 1 after the initial 48 hours of timed measurements the subsequent measurements were taken daily rather than every 24 hours. These timings were kept close to the time of the previous day to minimise deviation from a 24-hour period. However, drift in timings did occur and measurements were taken at different times each day, including during the dark cycle of the growth chamber lights. The measurement timings in Experiments 2-4 were strictly adhered to every 24 hours to eliminate any effects of variation in time of day.
In Experiment 3 no extra measurements were taken during the first 48 hours, with all measurements taken every 24 hours. Extra measurements during the first 48 hours were not deemed necessary as no extreme changes were observed in Experiment 2 during that time and no methods were changed between the experiments. Measurements were again recorded more frequently during the first 48 hours of Experiment 4 due to the introduction of the new DO_2 measurement method, as detailed in section 4.2.4.4.

4.2.4.2 Temperature

The temperature of surface water and porewater at 5cm and 10cm depth was recorded using the built-in temperature sensor of the pH dipping probe (Hach Intellical™ PHC101 Field Low Maintenance Gel Filled pH Electrode).

4.2.4.3 Floodwater pH

Surface water pH measurements were taken by carefully dipping a probe to 4cm below the water level, 1cm above the soil surface. The pH probe (Hach Intellical™ PHC101 Field Low Maintenance Gel Filled pH Electrode) was allowed to equilibrate for as long as needed for a stable reading. Three readings were taken from each box at random locations.

In Experiments 3 and 4 it was decided to take soil porewater pH measurements to help better understand soil ORP. To measure soil porewater pH the dipping probe used for surface water measurements was inserted into standpipes mounted at depths of 5cm and 10cm in the soil. The probe was allowed to equilibrate for as long as required for a stable reading. One measurement of each porewater depth in each box was taken.

4.2.4.4 Dissolved oxygen

4.2.4.4.1 Surface water

Surface water DO_2 measurements were taken in all experiments by carefully dipping an O_2 probe (Hach Intellical™ LDO101 Field Luminescent/Optical Dissolved Oxygen (DO) Sensor) to 4cm below water level, 1cm above the soil surface. The O_2 probe was allowed equilibrate for as long as needed

for a stable reading. Three readings were taken from the surface water in each box at random locations.

4.2.4.4.2 Standpipe oxygen method

In Experiment 3 it was decided to measure soil porewater DO_2 to better understand the ORP of the flooded soil system. For this the O_2 dipping probe used for surface water measurements was inserted into standpipes mounted in the soil at 5cm and 10cm depth. Care was taken when inserting the probe to minimize disturbance of the water in the pipe, however this could not be fully prevented. To minimize the water disturbance and possible introduction of O_2 to the porewater the probe was allowed 10 minutes to equilibrate. One measurement for each of the porewater depths in each box was taken.

4.2.4.4.3 Oxygen sensor spots

To solve the identified issue of introducing O_2 into the system through the dipping probe method a new method for porewater O_2 measurement was devised for Experiment 4. Oxygen-sensitive sensor spots (PreSens Oxygen Sensor Spot SP-PSt3-NAU) were attached to the bottom of clear plastic tubes which were inserted to depths of 5cm and 10cm below the soil surface. The sensors responded to DO_2 levels in the water, which could be read using a fibre-optic cable through a clear material. To align the fibre optic cable to the sensors at the bottom of the tubes a metal rod with spacers was used to guide the cable into each tube. This method allowed accurate readings of soil porewater DO_2 without disturbing the closed system. During the first 24 hours of measurements several of the tubes floated free of their place in the soil due to their light weight and the air space inside them. These tubes were filled with clean water to weigh them down in the soil without interfering with the fibre-optic measurements.

4.2.4.5 Oxidation reduction potential

4.2.4.5.1 Primary method

To measure ORP the AgCl electrode was inserted into the surface water of the box resting on the soil surface in the front left corner. A Pt electrode was inserted into the surface water to 4cm below the surface, 1cm above the soil, in the front right corner of the box. Pt electrodes for 5cm and 10m porewater measurements were permanently mounted in the boxes. To measure ORP the AgCl and Pt electrodes were connected via leads across a voltmeter. Immediately after connection to the voltmeter a stopwatch was started and readings were taken after 5, 10, 30, 60 and 120 seconds. To correct for the use of a AgCl electrode in the measurements all voltmeter readings were adjusted by +200mV give ORP results in Eh (Vepraskas and Cox, 2002). Each Pt electrode was connected in turn

and measured separately with the AgCl electrode remaining undisturbed in the surface water until moved to the next box. The surface water Pt electrode was also removed, cleaned and used for each box to eliminate any variation in measurements that may occur between different electrodes.

4.2.4.5.2 Standpipe ORP method

In Experiment 1 the AgCl electrode was inserted into standpipes in the soil. The standpipes were mounted at depths of 5cm and 10cm in the soil, 5cm away from and opposite their respective Pt electrodes. Measurements were not timed but were taken immediately after connection to the voltmeter and again after the electrodes had equilibrated. This method was to ensure that the ORP measured was a reliable representation of the porewater at the desired depth.

In Experiment 2 the standpipe method was repeated, but the 5, 10, 30, 60 and 120 second timings were used. In addition to the standpipe method the AgCl electrode was also measured from the surface to the 5cm and 10cm Pt electrodes and allowed to equilibrate to compare results. After a comparison of results to ensure reliability (Tab. 4.1) the primary method described above was subsequently used for Experiments 3 and 4, with the AgCl electrode at the soil surface.

Table 4.1 - Comparison of ORP results between AgCl electrode inserted to surface water and AgCl electrode inserted to standpipe. Pt electrodes remained fixed for both measurement methods. Figures are displayed as mV read from voltmeter without +200mV correction for Pt electrode to ORP.

			Hours															
Box	**Depth**	**Method**	**3**	**6**	**9**	**12**	**18**	**24**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
1	**5cm**	**Surface**	245	244	240	216	201	182	158	39	29	0	-2	-20	-30	-39	-45	-70
		Standpipe	241	231	225	206	188	166	143	32	9	0	-2	-9	-15	-23	-35	-44
	10cm	**Surface**	234	241	239	234	225	221	227	194	168	28	12	7	-13	-19	-16	-10
		Standpipe	238	236	232	227	218	213	214	188	147	23	6	2	-6	-7	-8	-6
2	**5cm**	**Surface**	299	291	278	286	269	260	233	102	24	-1	0	1	0	-6	0	-4
		Standpipe	294	285	277	272	259	239	216	83	12	0	0	0	0	-1	-1	-1
	10cm	**Surface**	214	212	211	214	195	186	169	43	-3	-5	-7	-14	-13	-60	-40	-31
		Standpipe	211	208	208	204	192	179	162	27	-1	-5	0	-9	-10	-22	-20	-22
3	**5cm**	**Surface**	Pt electrode failure															
		Standpipe																
	10cm	**Surface**	239	234	213	209	140	243	206	135	61	5	3	5	12	22	0	-9
		Standpipe	227	215	198	190	137	233	200	123	49	5	2	1	3	9	-2	-4
4	**5cm**	**Surface**	314	305	312	300	275	247	241	190	118	-9	-68	-128	-113	-85	-84	-76
		Standpipe	303	300	296	284	254	236	227	188	105	-4	-56	-88	-73	-58	-53	-49
	10cm	**Surface**	204	194	206	194	191	178	165	97	37	5	-1	-7	-17	-21	-34	-31
		Standpipe	192	183	183	181	171	163	154	86	24	1	0	-3	-7	-11	-15	-17
5	**5cm**	**Surface**	252	248	253	235	222	210	186	91	40	15	15	7	10	11	4	12
		Standpipe	249	245	241	231	217	193	174	67	26	12	6	5	3	4	2	3
	10cm	**Surface**	290	278	279	271	257	254	245	200	147	81	56	51	65	40	62	50
		Standpipe	283	274	271	262	257	244	237	197	129	70	52	43	43	35	37	32
6	**5cm**	**Surface**	Pt electrode failure															
		Standpipe																
	10cm	**Surface**	197	190	182	194	174	170	164	146	124	102	64	50	18	19	13	6
		Standpipe	181	176	166	175	165	157	150	139	117	98	58	36	14	10	5	2

4.2.5 Methods summary

As detailed in this section and in Chapter 3, the methods across each experiment were changed and refined as the project progressed. Table 4.2 summarises the experimental setup, conditions, measurements taken, and various methods used in each experiment.

Table 4.2- Summary of measurements taken and methods used in each experiment.

	Box biosolid application rates	**Soil sample timings**	**Soil field condition**	**Soil analysis**	**Flood surface water monitoring**	**Flood porewater monitoring**	**Floodwater analysis**	**Additional Analysis**
Experiment 1	0g, 100g, 200g, 400g, 800g, 1600g	Pre-flood, post-flood, final	Bare soil post-harvest	SOM, TKN, Olsen P, pH	DO_2, pH, ORP	ORP (standpipe)	NO_3^-, NH_4^+	
Experiment 2	0g, 100g, 200g, 400g, 800g, 1600g	Pre-flood, post-flood, final	Post mineral P fertilser application	SOM, TKN, Olsen P, pH	DO_2, pH, ORP	ORP (standpipe + surface)	NO_3^-, NH_4^+, total P, ortho-P	
Experiment 3	0g, 100g, 200g, 400g, 800g, 1600g	Pre-flood, post-flood	Post liquid N fertiliser application	SOM, TKN, Olsen P, pH	DO_2, pH, ORP	ORP (surface), pH (standpipe), DO_2 (standpipe)	NO_3^-, NH_4^+, total P	
Experiment 4	0g x2, 400g x2, 800g x2	Pre-flood, post-flood, final	Bare soil post-harvest	SOM, TKN, Olsen P, pH	DO_2, pH, ORP	ORP (surface), pH (standpipe), DO_2 (sensor spot)	NO3-, NH4+, total P, ortho-P	Bacterial characterisation

4.3 RESULTS

4.3.1 Biosolids

Table 4.2 shows a summary of the biosolid nutrient analysis provided by NRM Laboratories Ltd. on a dry matter basis. No trace elements exceeded normal ranges, although the typically high P, Ca and Fe content of the materials should be noted. Esholt 0 was the biosolid sample sent for initial characterisation, Esholt 1-4 were the samples used in Experiments 1-4, respectively.

Table 4.3- Major and micronutrient content of Esholt biosolids

		Values					
	Units	Esholt 0	Esholt 1	Esholt 2	Esholt 3	Esholt 4	Mean
Date sampled		06/04/2017	06/10/2017	16/01/2017	25/04/2018	19/10/2018	
pH		8.25	7.71	8	7.8	8.25	8.00
Dry Matter	%	24.5	27.7	25.6	27.7	26.4	26.4
Total N	% w/w	4.87	5	5.46	5.07	5.39	5.1
Ammonium N	mg/kg	3486	7260	7457	6596	7984	6557
Nitrate N	mg/kg	<10	<10	<10	<10	<10	<10
P	mg/kg	24029	22031	25565	24670	29975	25254
K	mg/kg	1150	1288	1143	1307	1270	1232
Mg	mg/kg	2817	2658	3070	4375	3405	3265
S	mg/kg	8446	9746	9114	9219	13004	9906
Cu	mg/kg	227	201	199	234	213	215
Zn	mg/kg	667	543	595	633	631	614
Na	mg/kg	573	688	786	699	797	709
Ca	mg/kg	22637	18535	24432	25344	23934	22976
Fe	mg/kg	36773	32793	40591	43099	47793	40210
Mo	mg/kg	5.85	4.75	5.51	5.91	7.91	5.99
Mn	mg/kg	466	782	546	495	622	582
C	% w/w	34.4	30.8	34.7	32.9	33.4	33.2
Co	mg/kg	4.92	6.36	6.08	6.88	11.5	7.15
B	mg/kg	5.12	6.93	9	6.61	11.8	7.89
C:N Ratio		7.06	6.16	6.36	6.49	6.20	6.52

Figure 4.1 compares the major nutrients found in the analysed biosolids from Esholt Water Treatment Works with the digested sludge cake values found in RB209. Both N and P levels in all Esholt biosolid samples were higher than estimated from the RB209 standard used to predict application rates. The proportion of N and P in the material was as expected, however as the applied rates to the boxes were based on total N content a slightly higher than expected N was applied. The target N application for the 100g, 200g, 400g, 800g and 1600g box biosolid amendments were intended as approximately 0.25, 0.5, 1, 2 and 4 times the maximum 250kg/ha N application. Based on the mean Esholt total N content, the rates applied in the experiments are approximately 0.3, 0.6, 1.25, 2.5 and 5 times the maximum field application. This deviation from the

target N application does not affect the validity or goals of the experiments, however it should be considered when interpreting results.

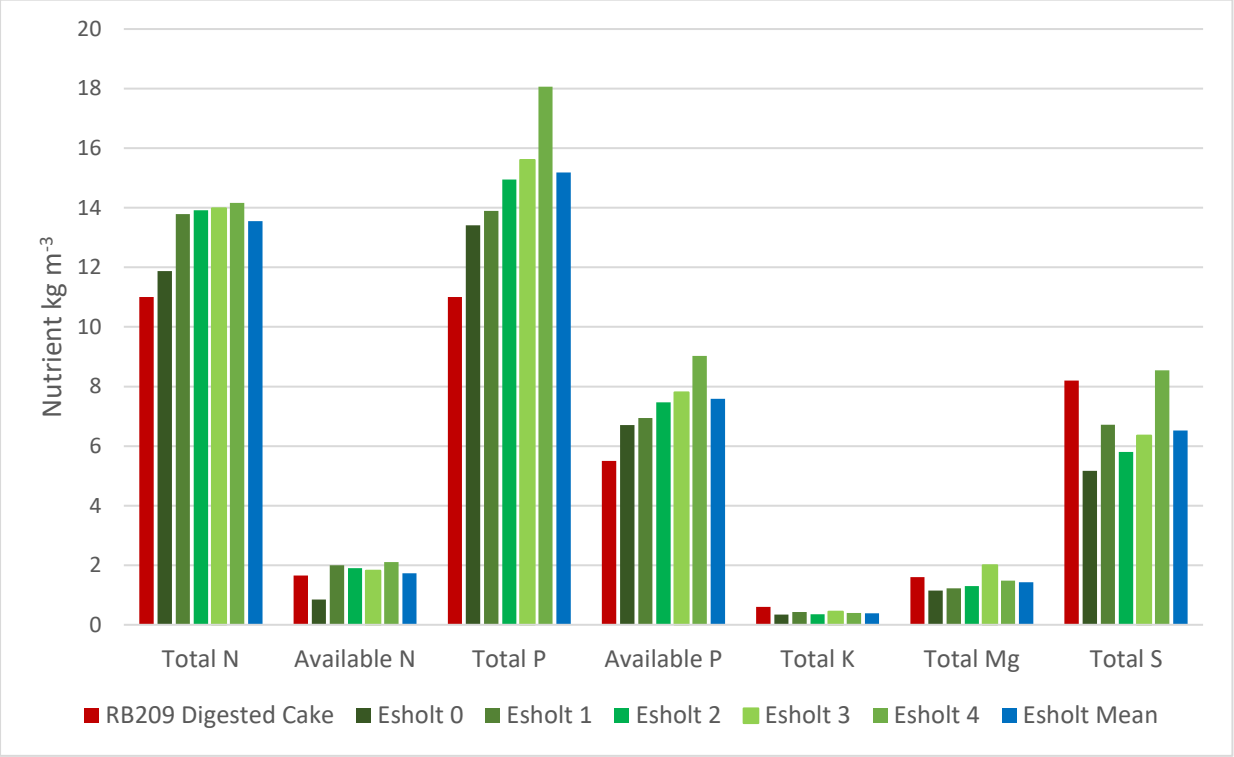

Figure 4.1- Kg nutrient per tonne Esholt biosolid compared with RB209 Digested Cake (AHDB, 2017). Available N is the sum of the ammonium N and nitrate N in the biosolids. Available P was calculated as 50% of total P as per RB209.

4.3.2 Soil analysis

The analysis data for SOM, TKN, Olsen P and pH of soils from each experiment are presented in the following section. 'Initial' samples were taken in triplicate from the field during collection. 0g biosolid-applied soils acted as the laboratory control but had experienced 28 days of crop growth before sampling at 'pre-flood' stage, so variation between Initial and Pre-flood 0g samples was from the effects induced during this growth period. All soil samples were taken in triplicate from each box at each sample timing of pre-flood, post-flood and final. Experiment 3 had no final soil sample results as they were not taken in that experiment, as explained section 4.2.2.1.

4.3.2.1 Soil Organic Matter

The SOM increased in proportion to the biosolid application across all experiments. There were no observable differences between sample timings in each box. Initial and control soils were very similar and ranged from 2.67-2.80% SOM on average, rising to 3.29-3.62% SOM in the 1600g application boxes. Full SOM numerical results can be found in Appendix B.2.

Figure 4.2- Soil organic matter for each experiment with samples pre-flood, post-flood and final at completion of the experiment. Error bars indicate standard deviation.

4.3.2.2 Soil Total Kjeldahl Nitrogen

Soil TKN increased with increased biosolid application in all experiments, with a slight decrease found from initial to control soils. The soil used in Experiment 3 had a liquid N application to the field 2 days prior to collection, though there was no increase in soil TKN levels above any other experiments. There was no observable difference between sample timings in any boxes. Sample variation was greater in high biosolid-applied soils, as indicated by the larger standard deviation. Full TKN numerical results can be found in Appendix B.3.

Figure 4.3- Soil TKN for each experiment with samples pre-flood, post-flood and final at completion of the experiment. Error bars indicate standard deviation.

4.3.2.3 Soil Olsen Phosphorus

Soil Olsen P increased from the initial soil to the control in all experiments. Control-400g soils then showed mixed results, with similar Olsen P levels in most cases. The 800g and 1600g biosolid applications showed a large increase over the control in all experiments but the extent of the increase compared with lower applied soils was variable. Higher soil Olsen P than other experiments can be observed in Experiment 2, where soils were sampled after a field mineral P fertiliser application. There was no observable difference between sample timings in the soils. Full soil Olsen P numerical results can be found in Appendix B.4.

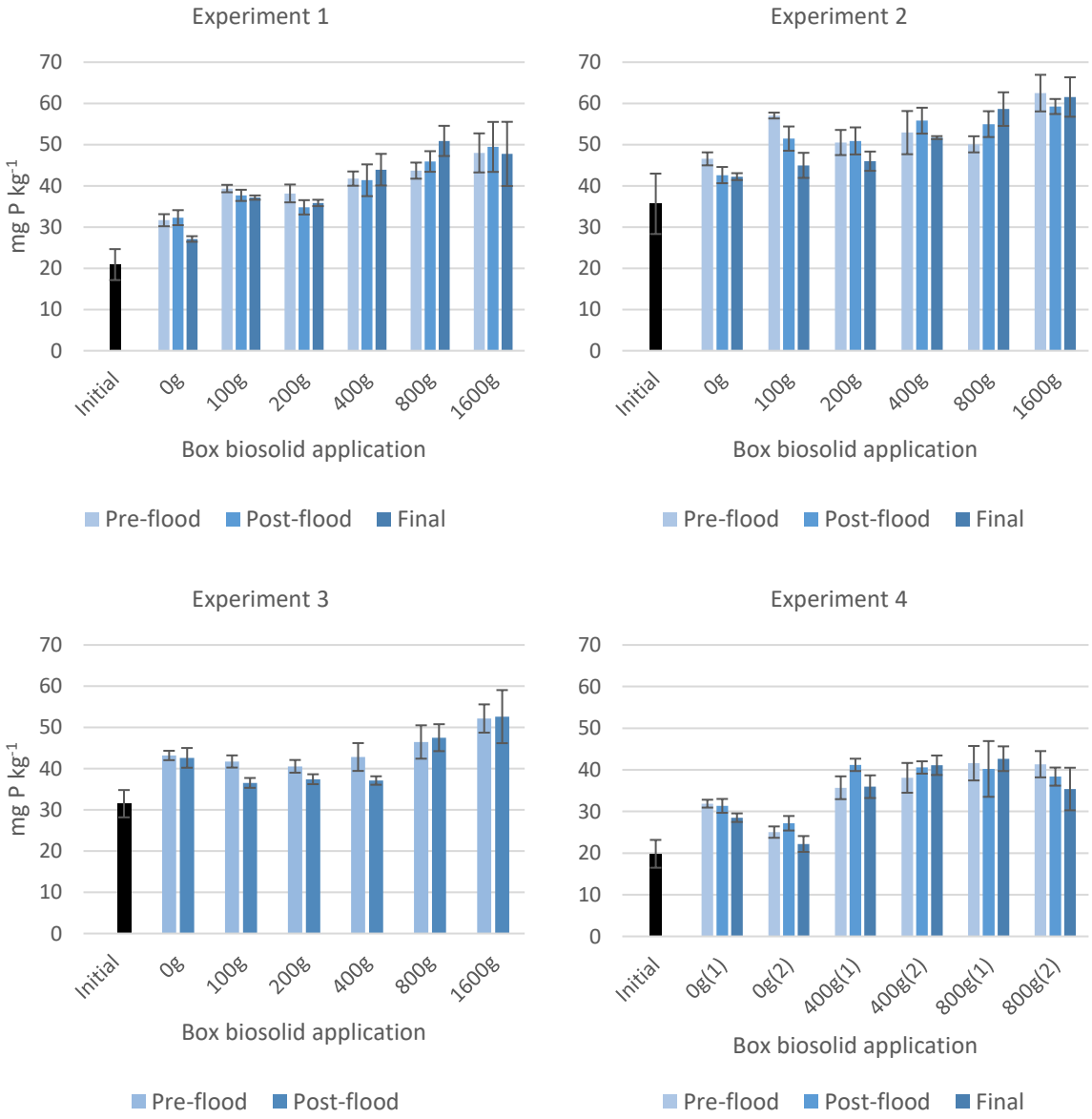

Figure 4.4- Soil Olsen P for each experiment with samples pre-flood, post-flood and final at completion of the experiment. Error bars indicate standard deviation.

4.3.2.4 Soil pH

Initial soils had a mean pH of 7.55. This increased slightly in the control box soils of each experiment, then decreased with increased biosolid application. After flooding the trend towards lower pH was still apparent. Results were more variable after the recovery period, with some boxes displaying a trend towards recovery of pH to pre-flood levels. Full soil pH numerical results can be found in Appendix B.5.

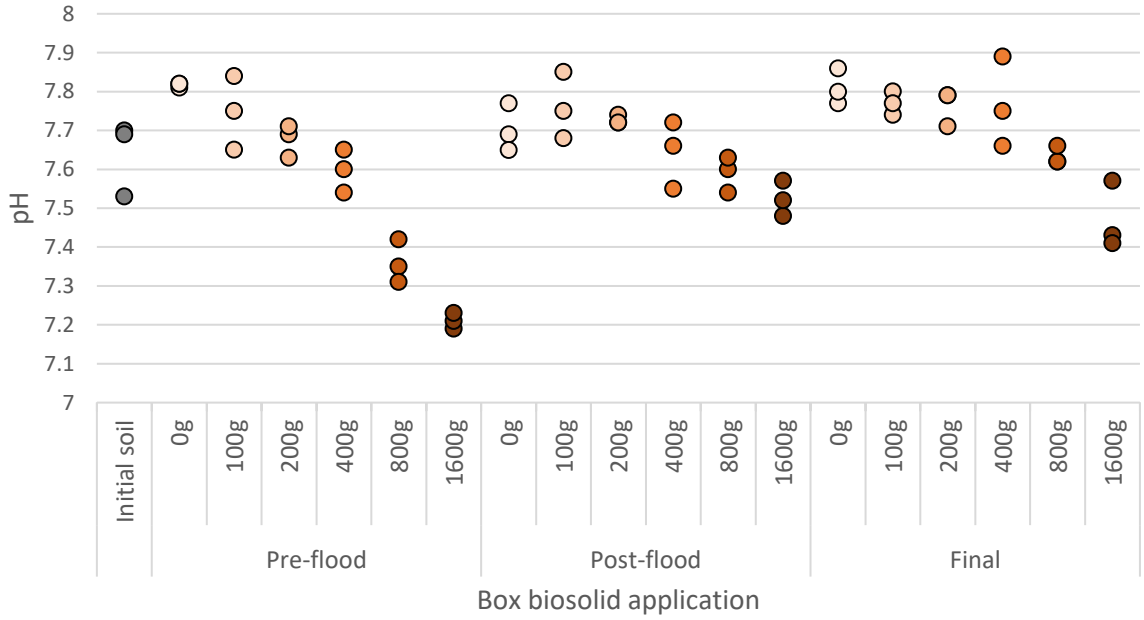

Figure 4.5- Experiment 1 soil pH. Each sample value is displayed individually. Samples are grouped by sample timing; pre-flood, post-flood and final.

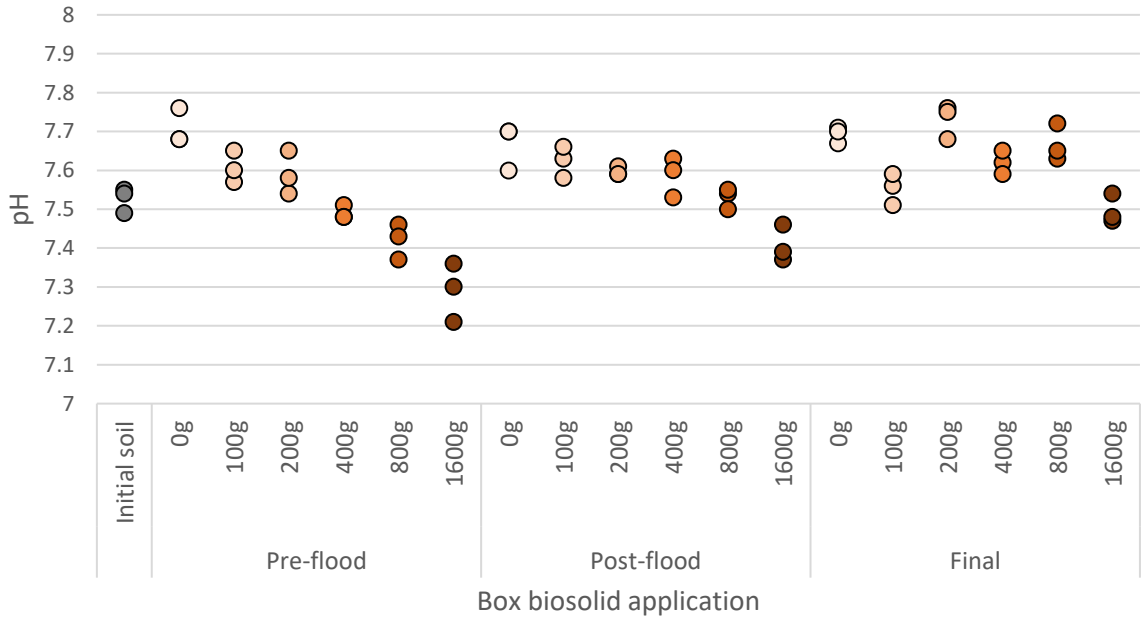

Figure 4.6- Experiment 2 soil pH. Each sample value is displayed individually. Samples are grouped by sample timing; pre-flood, post-flood and final.

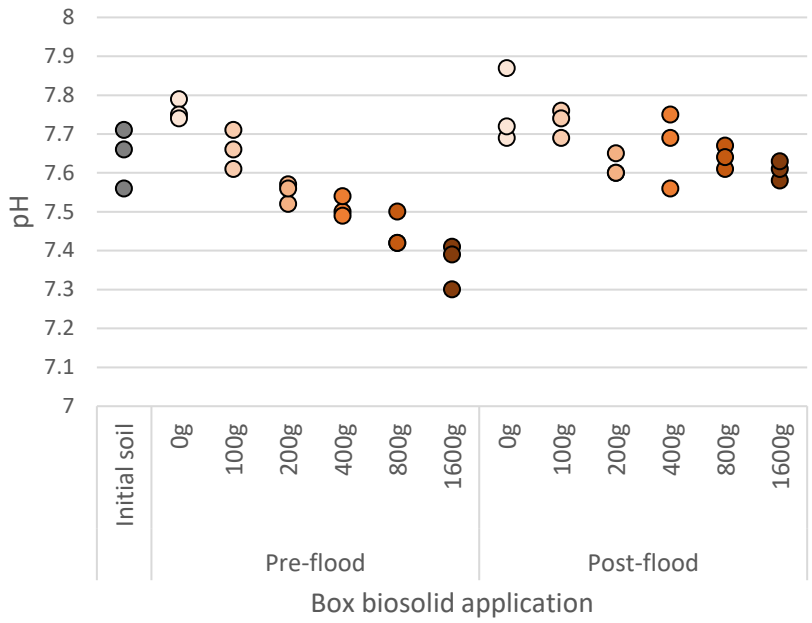

Figure 4.7- Experiment 3 soil pH. Each sample value is displayed individually. Samples are grouped by sample timing; pre-flood, post-flood and final.

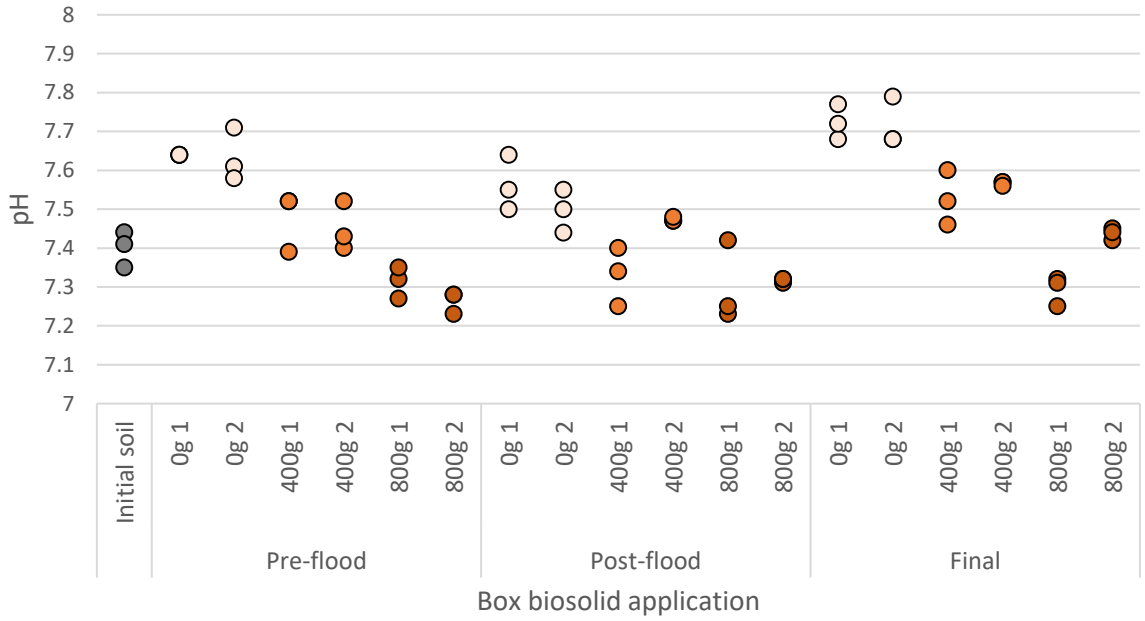

Figure 4.8- Experiment 4 soil pH. Each sample value is displayed individually. Samples are grouped by sample timing; pre-flood, post-flood and final.

4.3.3 Flood monitoring

The following results display the factors measured during the flood period. These are grouped by experiment to display the relationships between each factor. All graphs are displayed on an hourly timescale and cover the 10-day flood period. The first measurements were taken 3 hours after the initiation of the flood and the final measurements were taken immediately prior to flood draining. ORP measurements are available for all experiments at all depths of surface water, 5cm porewater and 10cm porewater. Experiments 1 and 2 have only surface water data available for pH and DO_2, as explained in section 4.2.4. Experiments 3 and 4 have complete ORP, pH and DO_2 data for all depths. Full numerical data detailing all the results presented in this section are available in Appendix C.

4.3.3.1 Temperature

The average atmospheric temperatures recorded within the growth chambers for each experiment were presented in section 3.4.4 when discussing growth chamber setup and maintenance. During flood monitoring, when taking DO_2 and pH measurements the probes used automatically adjusted readings based on the temperature of the water. ORP measurements are not adjusted based on temperature, but temperatures do need to be reported, and were measured alongside the ORP in Experiments 2-4. Water temperatures ranged between 19^{o}C and 21^{o}C in Experiment 2, between 20^{o}C and 24^{o}C in Experiment 3 and between 19^{o}C and 21^{o}C in Experiment 4. No differences were observed between surface water or porewater at either depth. The full temperature data for each experiment is recorded alongside the detailed ORP data in Appendix C.

4.3.3.2 Experiment 1 surface water

The surface water pH and DO_2 in Experiment 1 increased gradually over the duration of the flood. A drop in both DO_2 and pH occurred on day 6 (156 hours) when measurements were taken during the night cycle of the growth chambers, annotated as 'dark' on the graphs. Surface water Eh remained between 400 and 500mV throughout the flood, corresponding with the high DO_2 value.

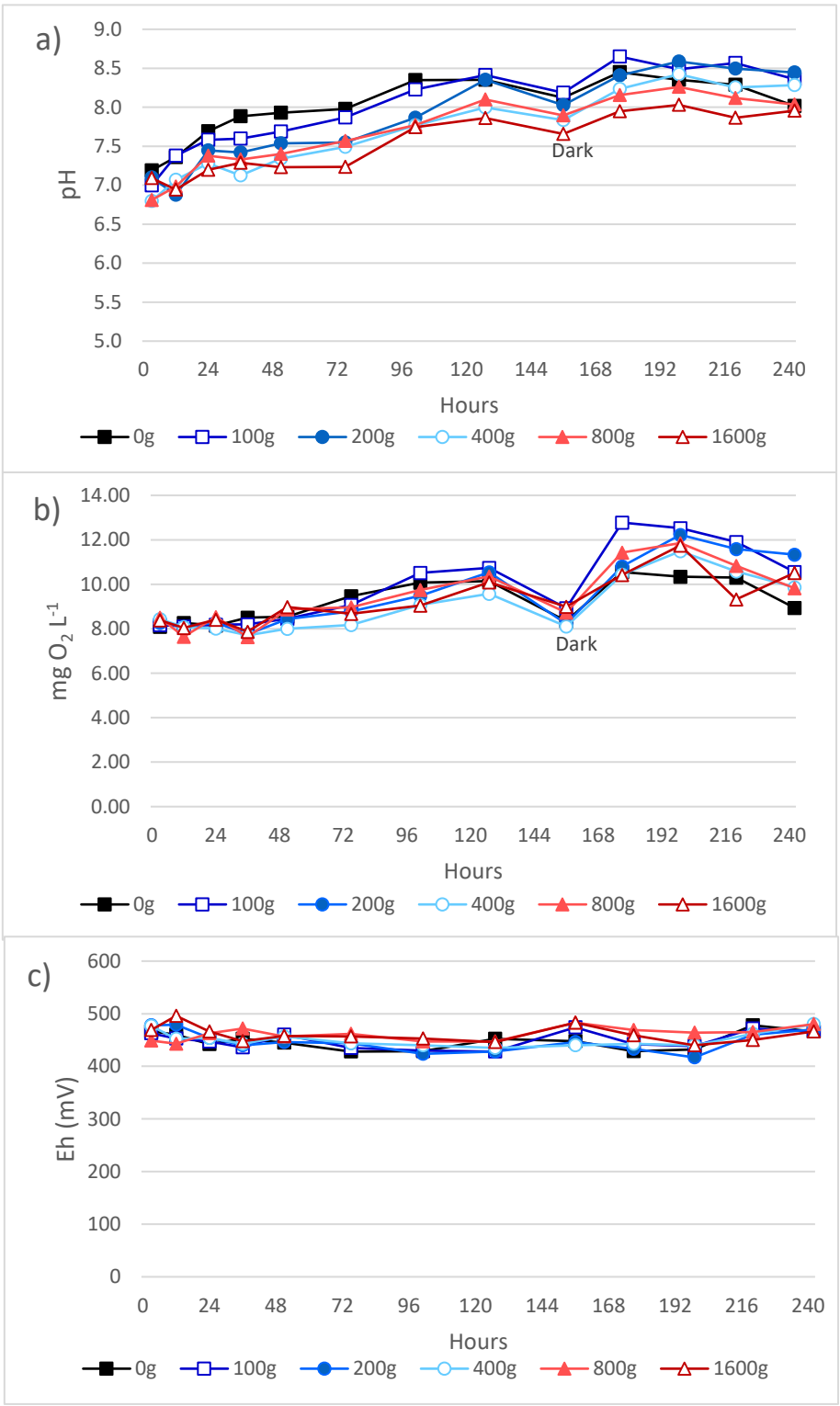

Figure 4.9- Experiment 1 flood surface water measurements: a) pH; b) Dissolved oxygen; c) Redox potential.

4.3.3.3 Experiment 1 porewater

ORP dropped immediately in all boxes at both 5cm and 10cm porewater depths. The Eh decreased to a point of 200mV over 5 days (120 hours) in all boxes and then remained there for the duration of the flood in all but the control box. The control box Eh dropped below 200mV at both 5cm and 10cm depths but was more pronounced in the 5cm porewater; neither values became highly reducing during the flood.

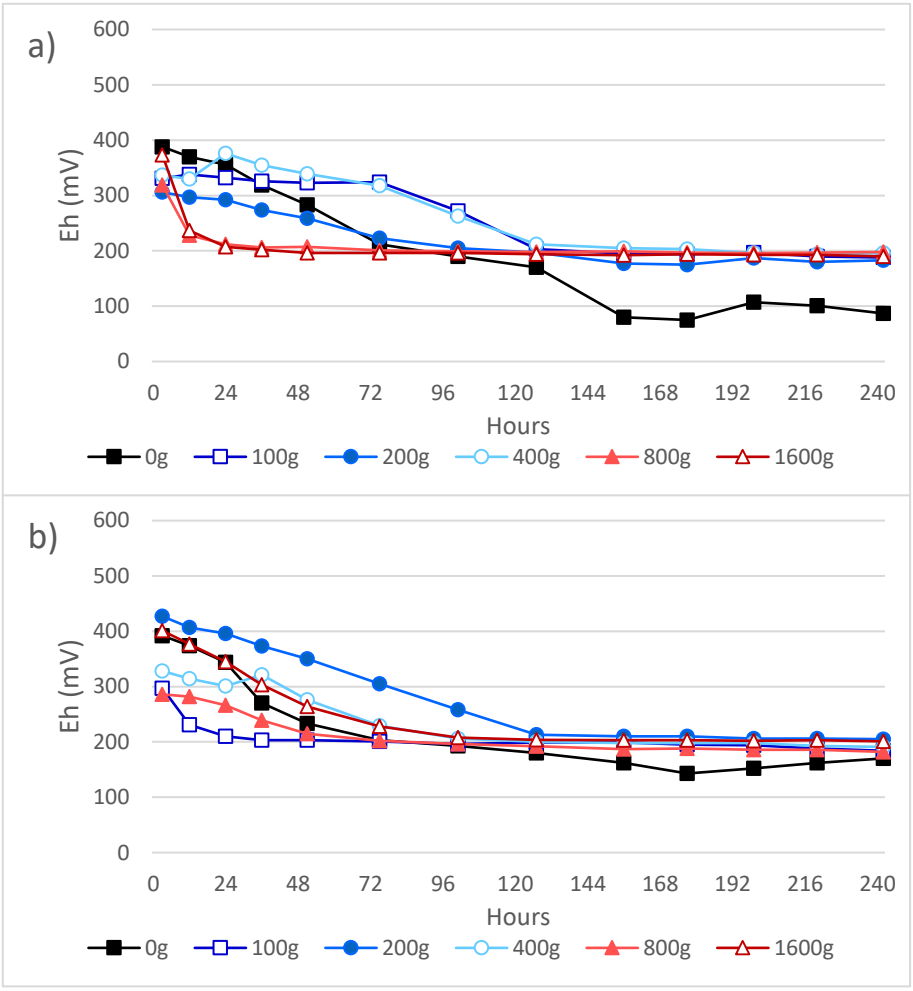

Figure 4.10- Experiment 1 flood porewater redox potential measurements: Redox potential at a) 5cm and b) 10cm depth below the soil surface.

4.3.3.4 Experiment 2 surface water

Surface water pH and DO_2 for Experiment 2 increased slowly over 9 days of the flood before starting to decrease just prior to flood draining. Surface water Eh remained between +400mV and +600mV in all boxes throughout the flood. No trend was observed between biosolid treatments in any measurements. However, the 200g box where algae growth was not present on the surface water had a lower DO_2 content than the other boxes.

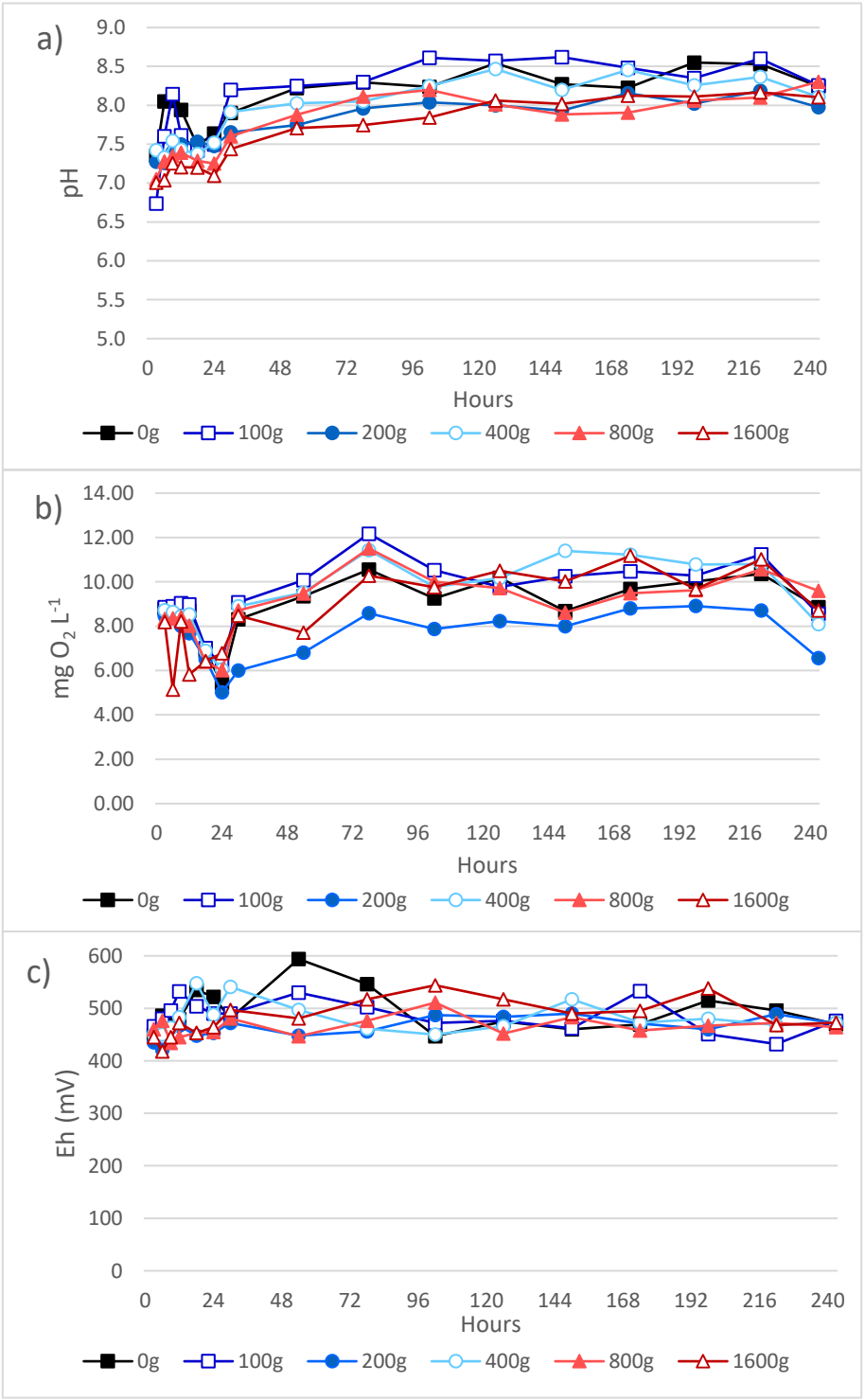

Figure 4.11- Experiment 2 flood surface water measurements: a) pH; b) Dissolved oxygen; c) Redox potential.

4.3.3.5 Experiment 2 porewater

The Eh in the 5cm depth porewater of Experiment 2 dropped to 200mV over a 4-day period in all boxes. After 4 days the control box Eh continued to drop below 200mV and stabilised around 120mV. The Eh in the 100g biosolid applied box also dropped below 200mV after 6 days and continued to drop for the remainder of the flood. In 10cm porewaters the Eh in most boxes dropped to around 200mV after 4 days, with the 800g box taking longer at around 7 days and the 1600g box maintaining a higher Eh than other boxes during the flood.

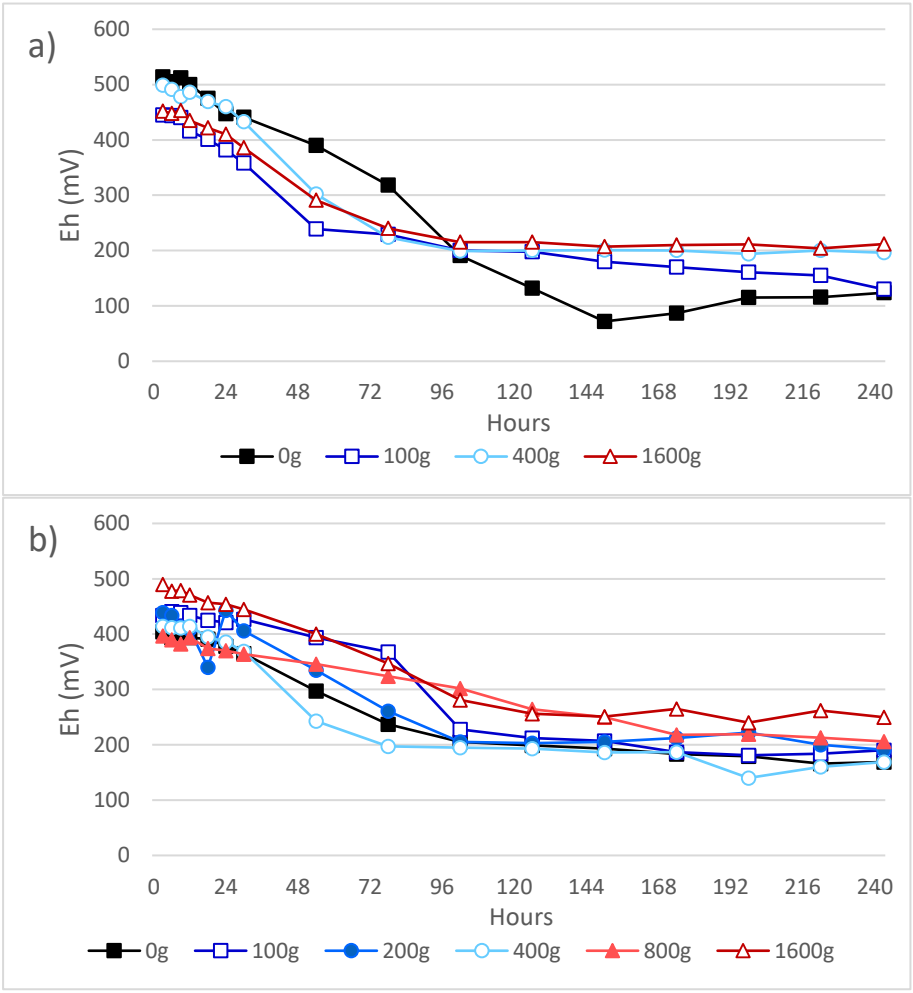

Figure 4.12- Experiment 2 porewater redox measurements: Redox potential at a) 5cm and b) 10cm depth below the soil surface. The 200g and 800g box Pt electrodes at 5cm failed and no results are included.

4.3.3.6 Experiment 3 surface water

Both pH and DO_2 in Experiment 3 surface waters increased for the first 5 days of the flood before decreasing. DO_2 remained high in all boxes during the flood and was reflected by the surface water Eh remaining between 450mV and 550mv for most of the flood. The reason for dip observed in pH in the 200g and 400g applied boxes on day 5 was not clear but may be an error with the probe.

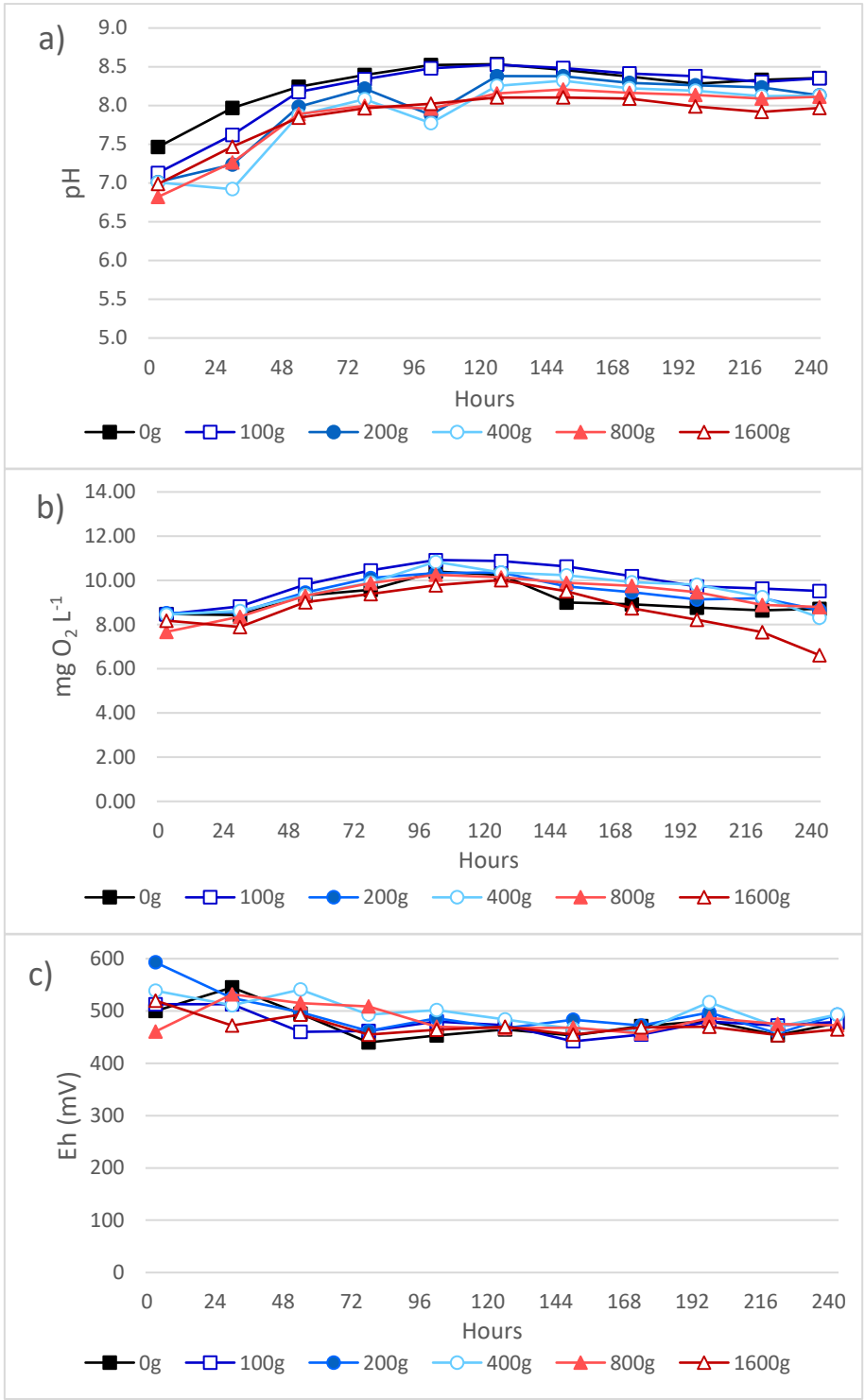

Figure 4.13- Experiment 3 flood surface water measurements: a) pH; b) Dissolved oxygen; c) Redox potential.

4.3.3.7 Experiment 3 5cm porewater

In Experiment 3 the soil porewater pH at 5cm depth rose over time and was lower than that of the surface water. DO_2 measured by the standpipe method initially dropped steeply in all boxes and then stabilised between 4 and 8mg L^{-1}, except in the 100g box where it dropped close to 0mg L^{-1} and remained there. This was likely due to a problem with the method used. The Eh dropped to 200mV in all boxes over 4 days with Eh in the control box continuing to drop until the end of the flood.

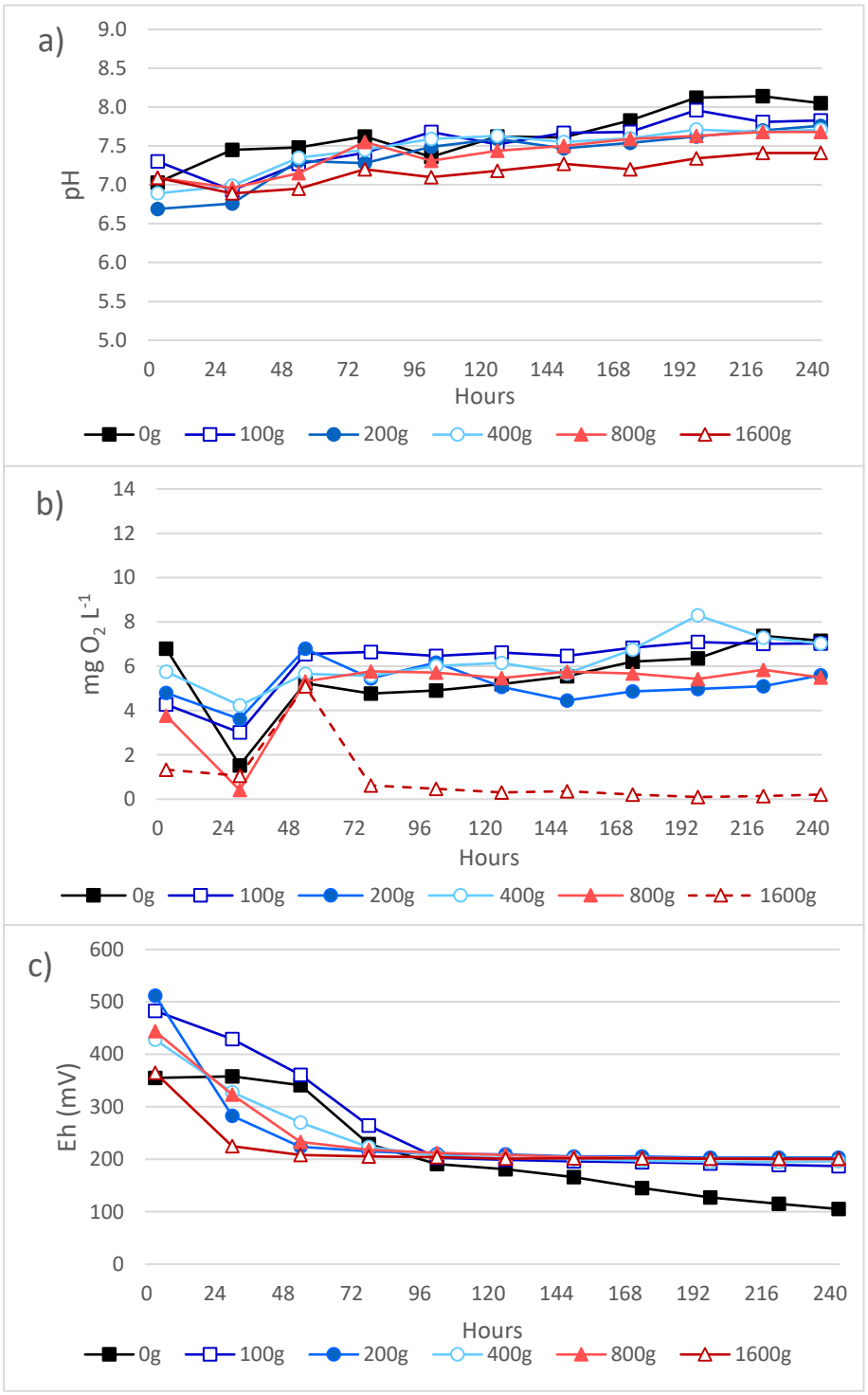

Figure 4.14- Experiment 3 porewater measurements at 5cm depth: a) pH; b) Dissolved oxygen (standpipe method), dotted lines indicate probable measurement errors; c) Redox potential.

4.3.3.8 Experiment 3 10cm porewater

In Experiment 3 the pH at 10cm depth rose during the flood and was lower than that of the surface water and 5cm porewater. DO_2 measured by the standpipe method dropped towards 0mg L^{-1} and remained there for the duration of the flood, except in the 800g box where it was between 3 and 5mg L^{-1}, likely due to problems with the method used. The Eh dropped to approximately 200mV in all boxes over 5 days and remained there until the end of the flood.

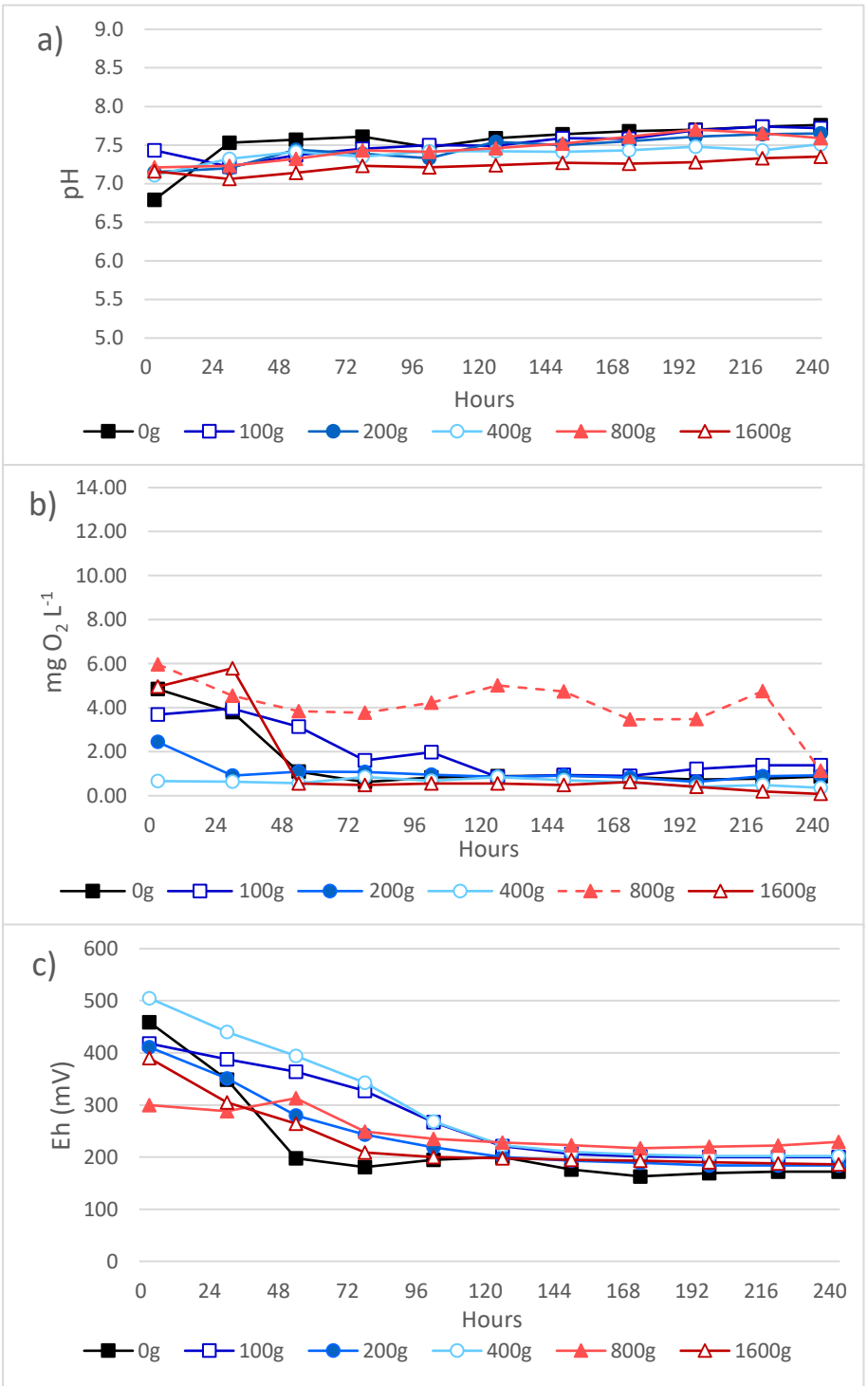

Figure 4.15- Experiment 3 porewater measurements at 10cm depths: a) pH; b) Dissolved oxygen (standpipe method), dotted lines indicate probable measurement errors; c) Redox potential.

4.3.3.9 Experiment 4 surface water

A clearer separation of differences in pH was observed in Experiment 4 due to use of duplicate application rates, with higher biosolid applications leading to lower pH in surface waters. DO_2 again remained high in surface water and was reflected by a high Eh which remained between 480mV and 600mV during the experiment.

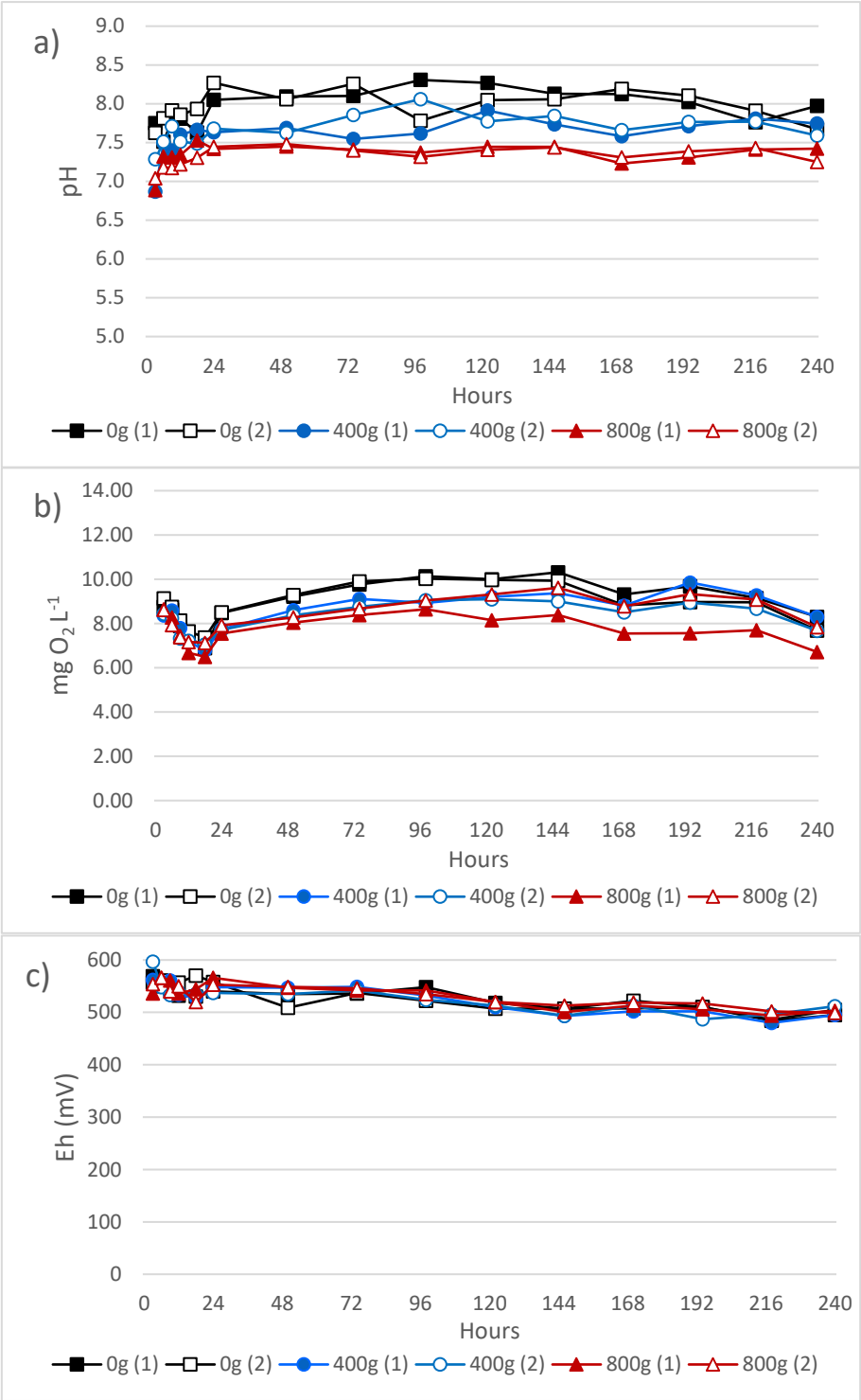

Figure 4.16- Experiment 4 surface water measurements: a) pH; b) Dissolved oxygen; c) Redox potential

4.3.3.10 Experiment 4 5cm porewater

The pH in Experiment 4 5cm depth porewater rose over time and was lower than the surface water pH. DO_2 measured using the oxygen sensor spot method immediately decreased to 0mg L^{-1} in all boxes for the duration of the flood, measured using the oxygen sensor spot method. The ORP dropped to 200mV over 5 days in all boxes and remained there in all except the control box, where it decreased further and stabilised between 100mV and 200mV.

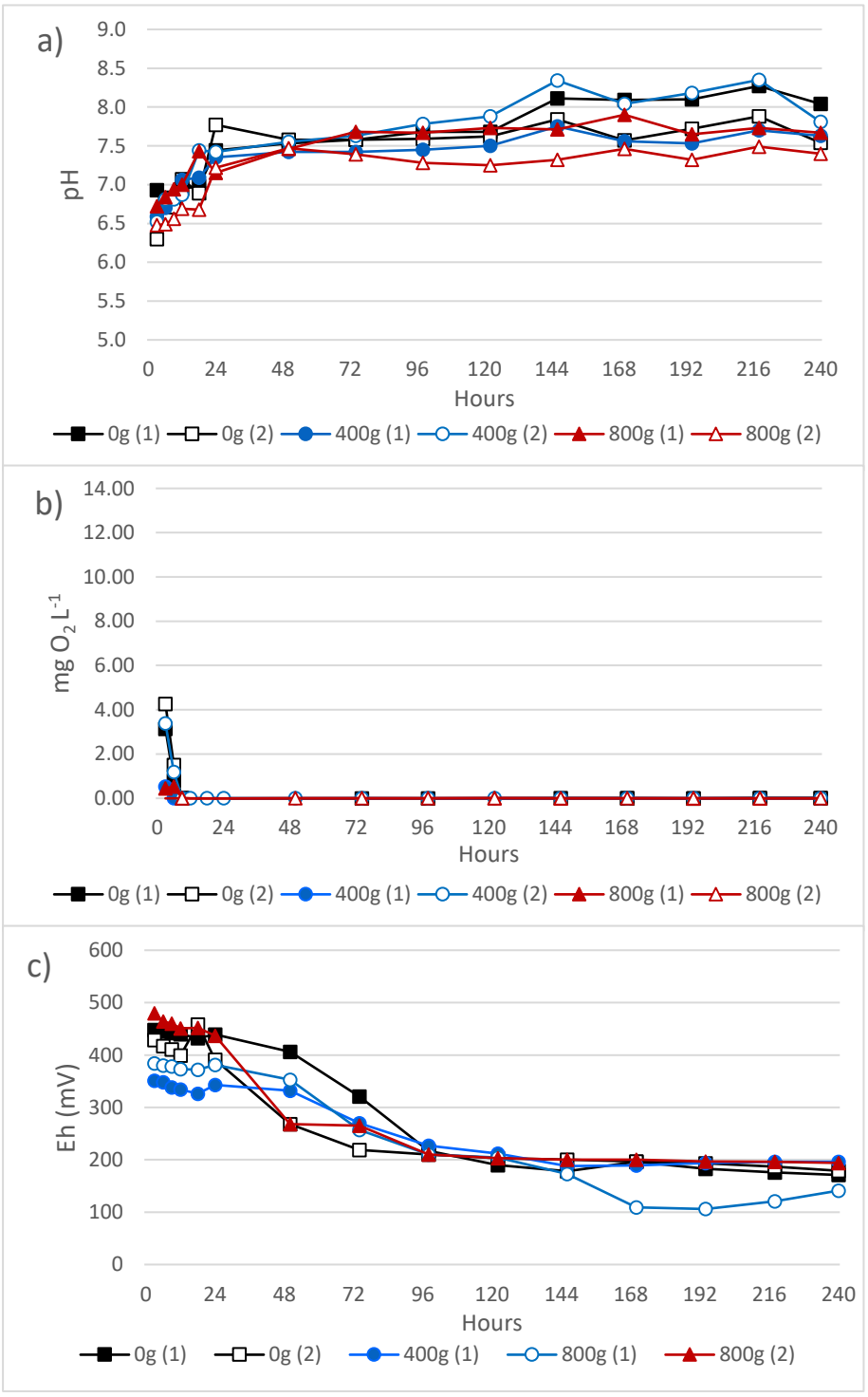

Figure 4.17- Experiment 4 porewater measurements at 5cm depth: a) pH; b) Dissolved oxygen (oxygen spot method); c) Redox potential. The 400g (2) Pt redox electrode failed and results are not included.

4.3.3.11 Experiment 4 10cm porewater

The pH in the 10cm depth porewater in Experiment 4 rose initially and then stabilised between 7.0 and 7.5 in all boxes with no differences observed relating to biosolid application. The DO_2 dropped immediately to 0mg L^{-1} for the duration of the flood in all experiments, measured using the oxygen sensor spot method. The ORP in the 10cm porewater dropped to 200mV in all boxes over 4 days and remained there until the end of the flood.

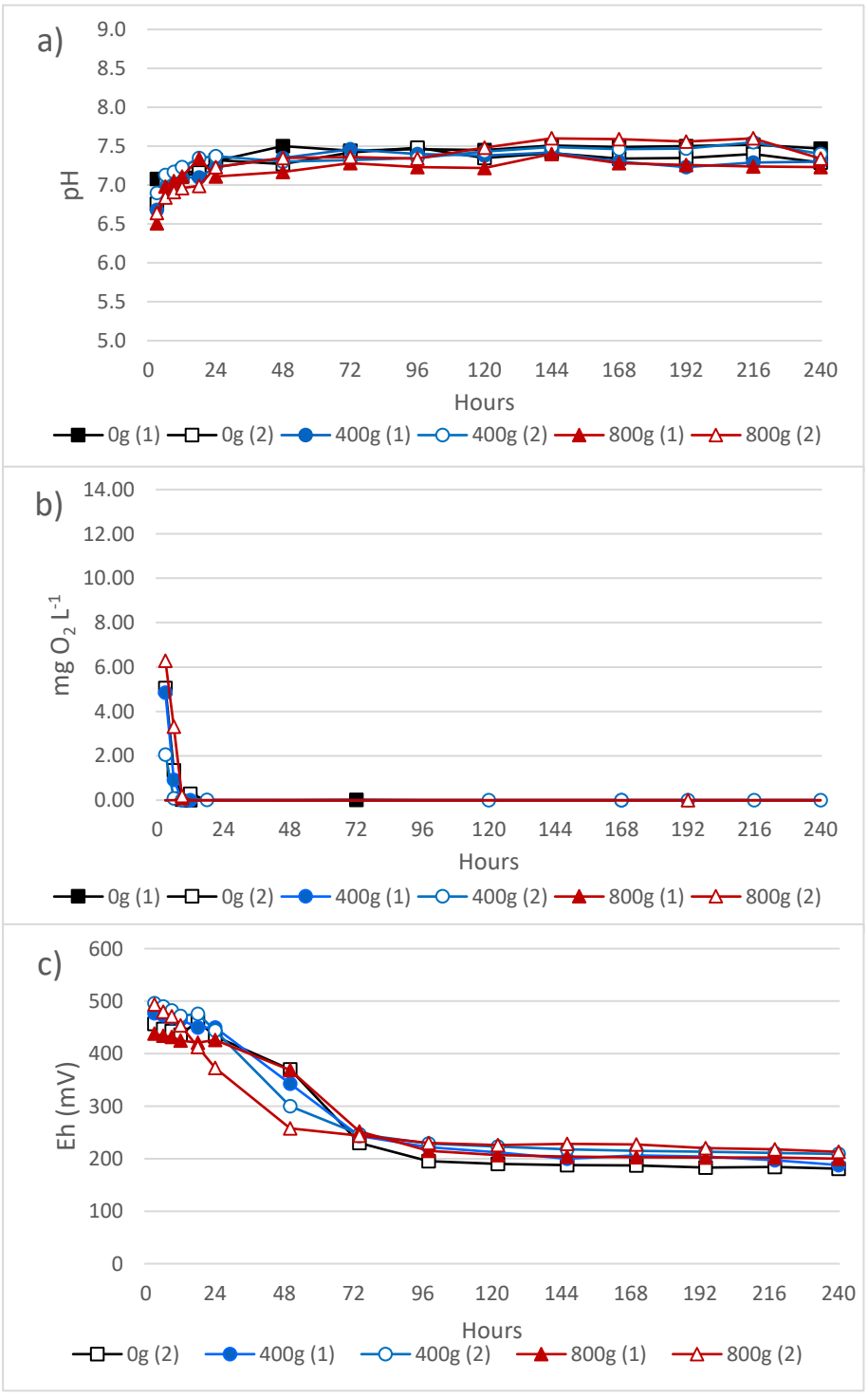

Figure 4.18- Experiment 4 porewater measurements at 10cm depth: a) pH; b) Dissolved oxygen (oxygen spot method); c) Redox potential. The 0g (1) Pt redox electrode failed and no results are included.

4.3.4 Floodwater analysis

The analysis of the final floodwater collected from each experiment is presented in this section. 'Surface water' indicates samples taken from water overlying the soil surface. 'Floodwater' indicates the whole drained volume of the box, including surface water and soil porewater. Data is presented by experiment to better present the relationships between factors.

4.3.4.1 Experiment 1

Water samples in Experiment 1 were not filtered prior to analysis (an error addressed in subsequent experiments). NO_3^- and ammonium (NH_4^+) are soluble in water whereas phosphate (PO_4^{3-}) can be contained in significant amounts on soil particles. For this reason, the NO_3^- and NH_4^+ results are presented in Figure 4.19, but the total phosphate results are excluded as unreliable. However, while the soluble N contents provide a useful comparison with the other experiments the lack of filtering still needs to be considered. Only total floodwater measurements were taken in Experiment 1, with no separate surface water samples. NO_3^- in the floodwater showed a marked increase in the >800g biosolid applied boxes and NH_4^+ showed a small increase in proportion to biosolid application, but only accounted for a very small portion of total soluble N compared with NO_3^-.

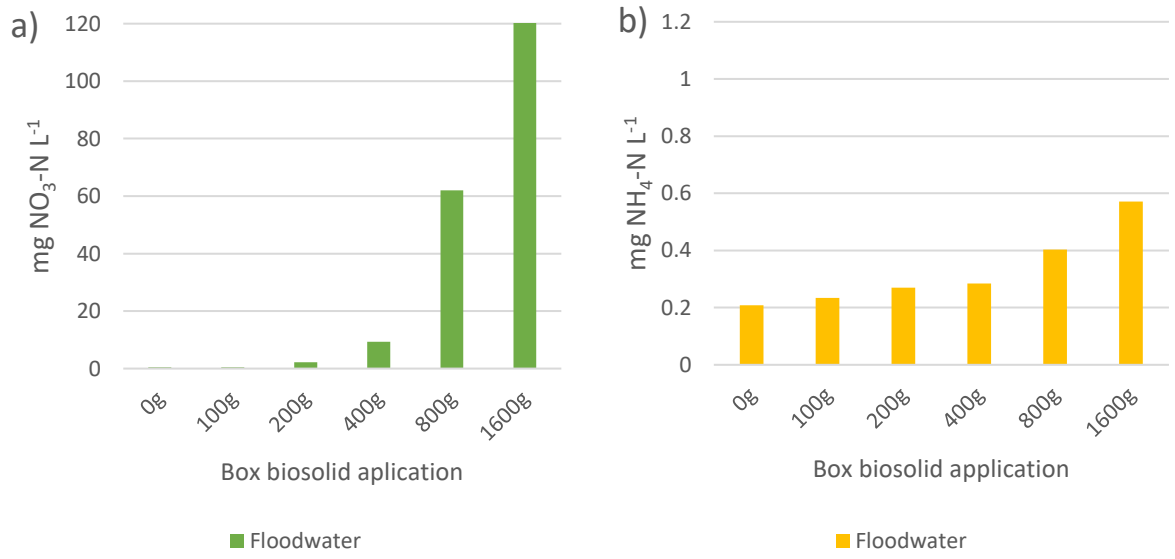

Figure 4.19- Experiment 1 floodwater nitrogen content: a) Nitrate; b) Ammonium.

4.3.4.2 Experiment 2

SRP levels in Experiment 2 were the highest in the three experiments, with most total phosphate present in the form of orthophosphate. Surface water phosphate was lower than floodwater in all but the 200g box, which was the only box observed with no surface water algae growth. Higher P levels may have been observed in these floodwaters due to the application of a mineral P fertiliser application to the soils prior to collection from the field, providing more SRP to the soil available pool. NO_3^- was present in quantities >1mg L^{-1} in the >400g applied boxes and then increased with biosolid application. NH_4^+ was a small fraction compared with NO_3^- and there was no trend with biosolid application except for a large increase in the 1600g applied box.

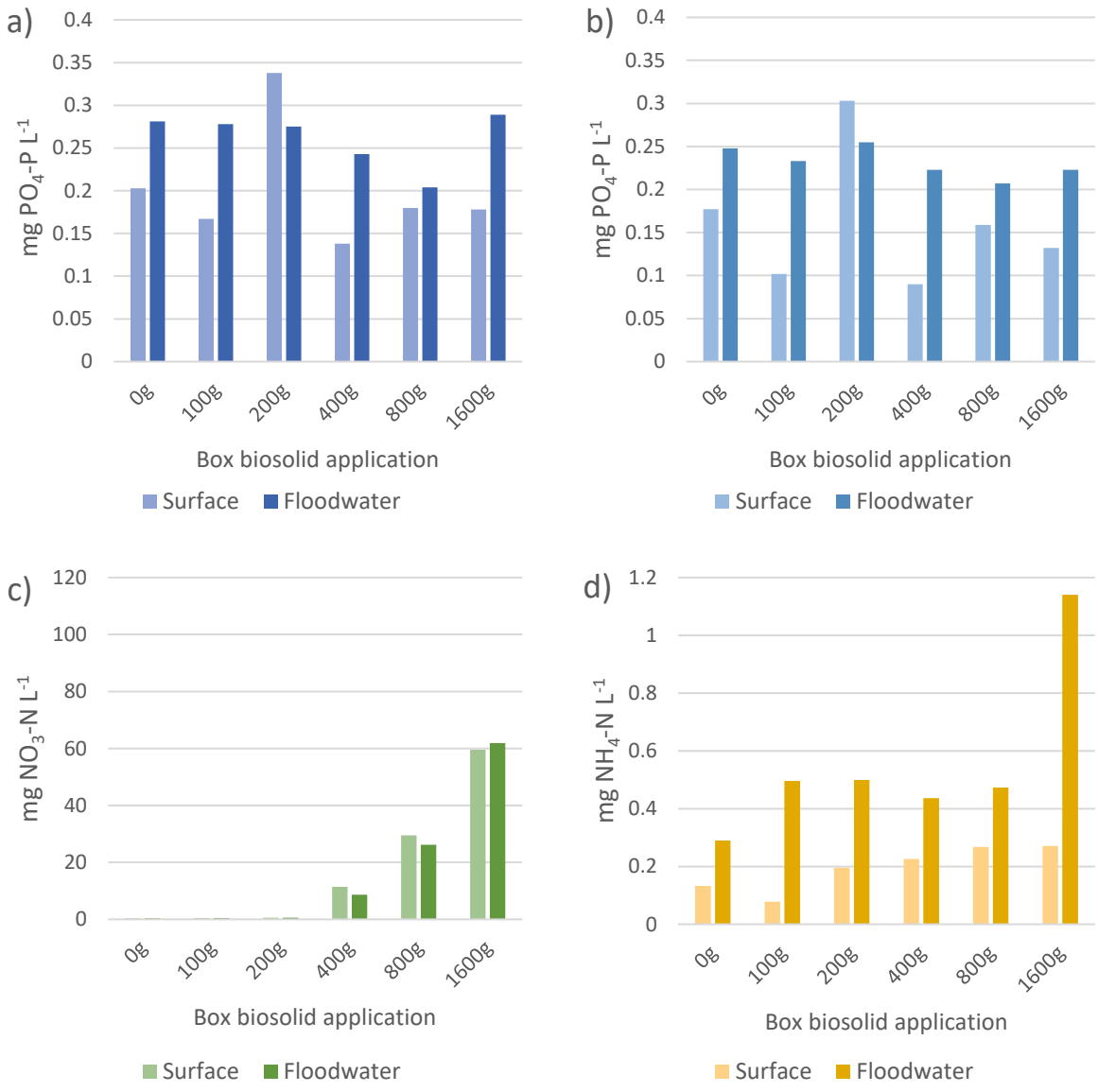

Figure 4.20- Experiment 2 floodwater nitrate and phosphate measurements: a) Total phosphate; b) Orthophosphate; c) Nitrate; d) Ammonium.

4.3.4.3 Experiment 3

Soluble phosphorus in Experiment 3 surface water was lower than in floodwater, with no trend observed based on biosolid application. Orthophosphate was not measured in this experiment. NO_3^- levels had the highest recorded of any experiment in Experiment 3 for all treatments. The NO_3^- present in control soils could be attributed to the liquid N fertiliser application to the field 3 days prior to soil collection for this experiment and may have inflated the results of other treatments. There was a large increase from the control to the 100g applied box and then an increase in proportion to biosolid application. NO_3^- was similar in floodwater and surface water. NH_4-N was fractional compared to NO_3-N and increased with biosolid application.

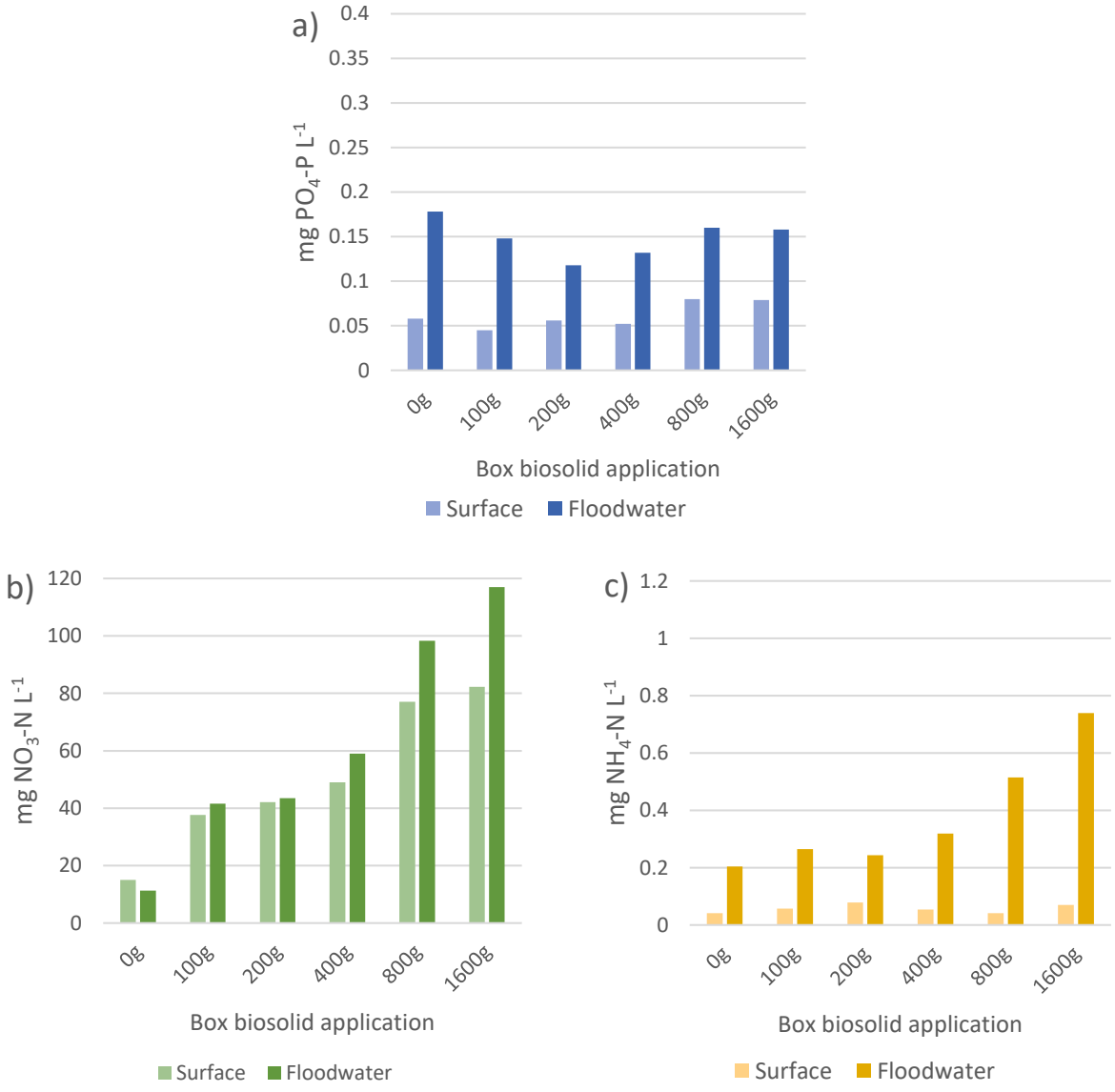

Figure 4.23- Experiment 3 floodwater phosphate and nitrogen: a) Total phosphate; b) Nitrate; c) Ammonium.

4.3.4.4 Experiment 4

There was no trend in SRP with increasing biosolid application. Surface water phosphate was lower than in the floodwater and orthophosphate accounted for most of the total phosphorus. NO_3^- was low in most boxes, showing a slight increase in the 800g (1) box but a very large increase in the 800g (2) box which was considerably higher than any other treatment. NH_4^+ was a fraction of the NO_3^- and a small upwards trend was observed with increased biosolid application.

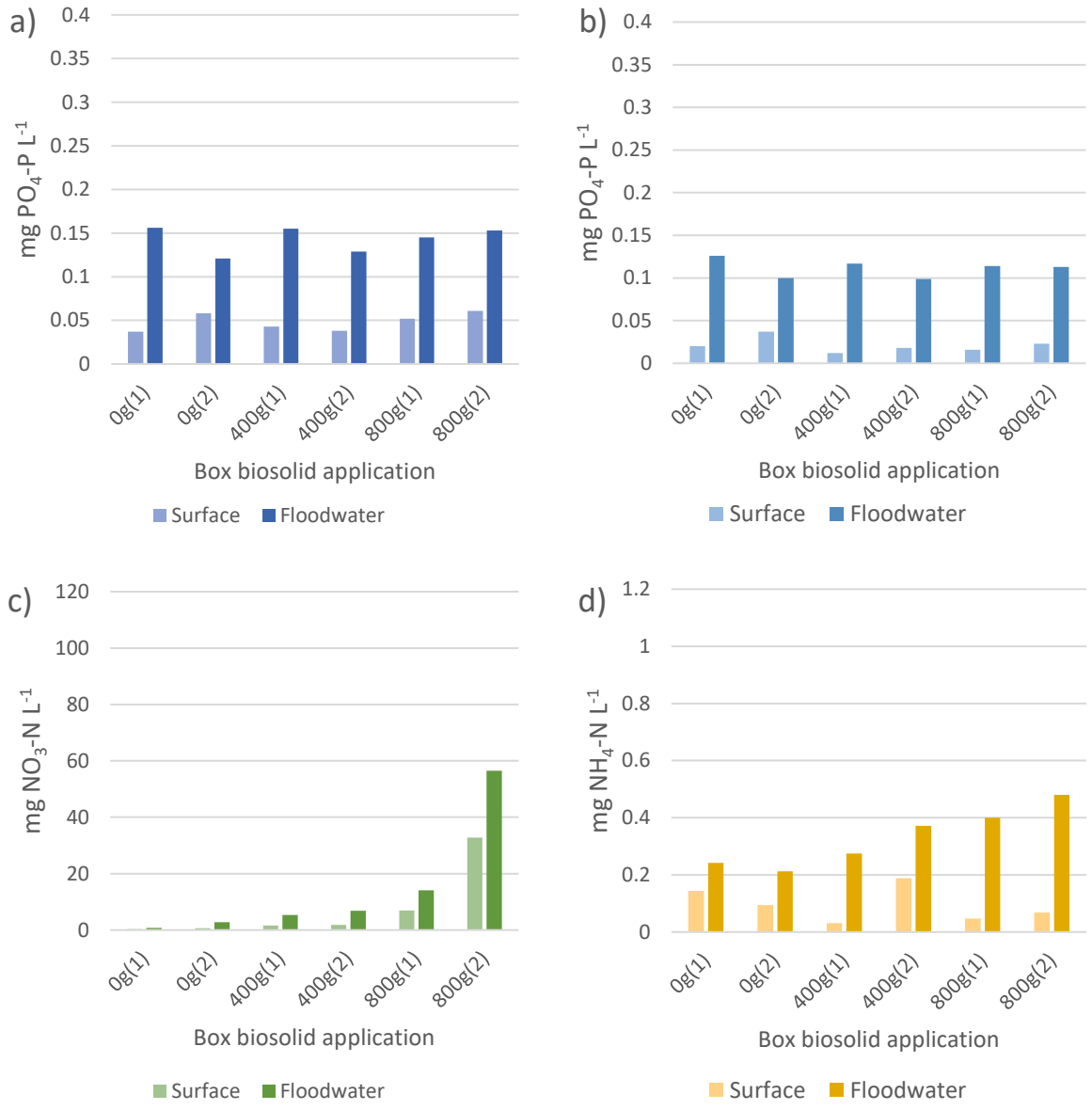

Figure 4.24- Experiment 4 floodwater phosphate and nitrogen: a) Total phosphate; b) Orthophosphate; c) Nitrate; d) Ammonium

4.4 DISCUSSION

4.4.1 Initial soil and biosolid characteristics

The physical characteristics and nutrient content of the biosolid collected for all experiments was very consistent (Table 4.2). Total N content had a standard deviation from the mean of just 0.98% in the biosolids applied to the experiments (Esholt 1- Esholt 4) and total P had a deviation from the mean of 9.81%. Differences in the applied nutrient from biosolids across experiments was therefore minimal.

Based on the analysis and recommended nutrient levels provided by NRM laboratories and found in RB209 (AHDB, 2017), the initial soil had no macro- or micro-nutrient deficiencies which would restrict crop growth during the experiments. The optimal soil pH for barley growth is 6.50 in mineral soils (AHDB, 2017), so the initial soil was slightly calcareous, with a pH in the range of 7.40-7.64. Although this range is higher than optimal it is still typical of a well limed or calcareous arable soil and should not provide any direct limitation on crop growth. However, the higher pH can alter the solubility of phosphate minerals and the availability of phosphorus to plants, which must be considered. Initial SOM levels were slightly below optimal at 2.8%. An SOM content of 3.4% has been suggested as a value to maintain soils above, but fresh organic additions can allow soil productivity to be maintained below this level (Loveland and Webb, 2003). The SOM level of the selected field would therefore be an excellent target for organic additions such as biosolids to improve SOM and maintain crop productivity.

Differences in the time of year when soil was collected caused variation in initial soil available P (Fig. 4.4), from mean $\pm$ 1 SD values of 19.83 $\pm$ 3.34 mg P kg^{-1} (Experiment 4) to 35.64 $\pm$ 7.33 mg P kg^{-1} (Experiment 2). This variation was expected due to the sample timings selected, with soil taken from a working arable field which received fertiliser applications throughout the growing season. The higher Olsen P levels in Experiment 2 can be attributed to the phosphate application (Triple Superphosphate ($Ca(H_2PO_4)_2.H_2O$)) in November 2017 (Table 3.2), with elevated P still being present in the soil during collection in January during the soil collection due to low uptake from the overwintering crop. Experiment 3 also showed slightly elevated soil P levels when collected in April 2018, likely due to residual soil P from the same November application, but with more P having been taken up by the growing crop. Soils for experiment 1 and 4 were both collected after harvest when the next crop had been freshly drilled, meaning soil P levels were back to a baseline level. This assumes that the fertiliser P applied was calculated based on the crop requirement and all applied P was taken up by the crop and exported during harvest. The initial soil Olsen P levels from Experiments 1 and 4 support this, as they were very similar at mean $\pm$ 1 SD values 20.89 $\pm$ 3.76 mg P

kg^{-1} and 19.83 $\pm$ 3.34 mg P kg^{-1} respectively, having collection dates in October 2017 and October 2018. The higher soluble P found in the floodwater of Experiment 2 can also be accounted for by the proximity of the $Ca(H_2PO_4)_2$ fertiliser application, which would contribute heavily to the soluble P pool in the soil. The floodwater of Experiment 2 having such high soluble P may indicate that there is a greater threat of P loss to watercourses from mineral P fertiliser additions than from biosolid P additions. This will be discussed further later in this section.

Soil TKN levels did not vary as much as Olsen P levels between initial soils (Fig. 4.3), ranging between a mean $\pm$ 1 SD of 1769 $\pm$ 78 mg N kg^{-1} (Experiment 2) to 2041 $\pm$ 89 mg N kg^{-1} (Experiment 1). However, the floodwater in Experiment 3 had a much greater concentration of NO_3-N than in other experiments. The control box in Experiment 3, which received no fresh N addition through biosolids, contained 11.30 mg l^{-1} NO_3-N in the floodwater compared with 0.40 mg l^{-1} in Experiment 2 and 0.74 mg l^{-1} and 2.8 mg l^{-1} in the Experiment 4 controls. A liquid N fertiliser application of 118 kg ha^{-1} N was applied to the crop in Big Substation field in April 2018, 3 days prior to the soil collection for Experiment 3. Though this N application did not appear to affect the TKN of the soils in experiment 3 it was all applied in readily crop available soluble forms as NO_3^-, NH_4^+ and CH_4N_2O. Any residual fertiliser N left in the soil may therefore have had a large effect on the soluble N content without greatly altering total soil N content. This may also indicate that NO_3^- leaching is of higher risk from mineral N fertilisers than biosolid applied N. This will be discussed later in this section.

4.4.2 Biosolid application effects on soil

Biosolid applications affected SOM and TKN in soils, with both increasing in proportion to biosolid application (Figs. 4.2 and 4.3). Both SOM and TKN were reduced from initial soils to pre-flood soils. From initial to pre-flood control soils there was a mean change of -0.06% SOM and -34 mg kg^{-1} TKN, and from initial to 100g biosolid-applied soil there was a small mean change of +0.03% SOM and +19 mg kg^{-1} TKN. The reduction in SOM and TKN from initial soil to control is likely due to increased microbial and crop activity during the initial 28-day growing period. Increased microbial activity from the warmer conditions in the laboratory than in the field could have led to increased microbial respiration and decomposition of SOM. Mineralisation of organic N could then also occur, releasing NH_4^+ which could then be nitrified to NO_3^- for plant uptake, with small potential losses by volatilisation and denitrification also occurring. The mean SOM and TKN values in the 100g applied boxes were slightly higher than in initial soils, indicating that the biosolid addition provided enough nutrients to support crop growth and maintain soil nutrient levels over the 28-day growth period. When considering the mean values of all soil samples compared with the control in each experiment there was a clear correlation between SOM, TKN and biosolid application. Each 100g of biosolid

applied to soils amounted to an increase of 48 ± 7 mg kg^{-1} TKN and 0.07 ± 0.02 % SOM over control soils, which had mean contents of 1858 mg kg^{-1} TKN and 2.77% SOM.

Olsen P was not as closely correlated to biosolid application as TKN and SOM. A significant increase was observed between initial soils and all experimental soils, including the control boxes despite no addition of P. The increase in available P is likely due to increased microbial activity and P mineralisation in the soil during the initial growth stage stimulated by increased temperatures which has been observed in previous studies (Magdoff and Weil, 2004). The elevated Olsen P levels in control soils had a mean increase across all sample timings of 6.41 mg P kg^{-1} above initial soils, with a mean of 27.22 mg P kg^{-1}. The increase of Olsen P from biosolid application, assessed by comparing biosolid applied soils with the control, showed a mean increase of 9.37, 7.70 and 8.87 mg P kg^{-1} across the 100g, 200g and 400g applied boxes, respectively. These changes indicate that temperature-induced activity had a large impact on P availability and was almost as significant as the effect of biosolid additions at lower rates.

The 100g, 200g and 400g applications showed little difference between them in terms of Olsen P, despite the differences in P addition from biosolids. The 100g-applied boxes also had a higher Olsen P on average than the 200g and 400g boxes, despite the lower total P addition. This indicates that microbial mineralisation of P from the organic fraction could be a larger contributor to Olsen P than the quantity of available P added in biosolids. Mineralisation of existing organic P in the SOM to the available pool must be occurring in addition to the added available P, which could be an indication of the 'priming effect' (PE). The PE can occur when microbial activity is boosted beyond a sustainable level by fresh OM addition, leading to the consumption of both the fresh OM and some of the native SOM (Fontaine et al., 2003; Thiessen et al., 2013; Chowdhury et al., 2014; Mehnaz et al., 2019). If microbial populations are increased beyond a point that can be sustained in the long-term then this can lead to nutrient mineralisation upon their death. The lower application rate boxes could be showing the range at which PE occurs in these soils, with the organic addition boosting microbial populations but dying off and releasing their contained P when resources are quickly depleted. The 400g-1600g biosolid applied boxes displayed a more linear increase from the control soil Olsen P, increasing in proportion to the biosolid application, with no apparent PE occurring over the time frame studied. The possibility of the Olsen P results being due to sample variation from uneven biosolid distribution is possible, but unlikely. Sample variation was apparent at higher application rates which displayed a larger standard deviation, but the lower application rates in question were more closely grouped. The trend indicating a PE was also observed to some extent in all three experiments in which it was measured. In Experiment 1 the 100g box had higher Olsen P than the 200g box, in Experiment 2 the 100g box was higher than both the 200g and 400g boxes and in

Experiment 3 the control was higher than the 100g-400g boxes which all had similar Olsen P. These results indicate that soil Olsen P is as dependant on mineralisation from microbial activity, either from organic input or higher temperatures, as it is from direct P application from biosolids.

The measured soil pH levels also support the hypothesis that increased microbial activity by biosolid addition significantly affects soil geochemistry. Biosolids had a higher pH (7–7 - 8.3) than initial soils (7–4 - 7.7) although increased biosolid application led to lower pH in all pre-flood soils. There was a mean pH decrease across experiments from 7.7 in control soils to 7.3 in 1600g biosolid applied boxes. This may be attributed to increased respiration of microorganisms in the soil from increased biosolid application and increased oxidation of C, N and S compounds leading to acidification (McBride et al., 1994). For example, the released CO_2 from microbial respiration can create weak, soluble organic acids (Equation 4.6), the biological oxidation of S can cause the formation of sulphuric acid (Equation 4.7), and the process of nitrification leads to the release of free H^+ ions which acidify the soil (Equation 4.8).

$$CO_2 + H_2O \rightarrow H_2CO_3 \text{ (carbonic acid)}$$

Equation 4.6

$$2S + 3O_2 + 2H_2O \rightarrow 2H_2SO_4 \text{ (sulphuric acid)}$$

Equation 4.7

$$NH_4 + 2O_2 \rightarrow NO_3^- + H_2O + 2H^+$$

Equation 4.8

4.4.3 The impact of flooding on soil nutrient and organic matter content

Flooding had no measurable effect on soil TKN, Olsen P or SOM contents (Figs. 4.2-4.4). While N and P losses were measured in the floodwater, these were negligible in terms of overall soil content and changes were not detected in the soil. The measured SOM levels remaining unchanged over the course of the experiment would indicate that negligible amounts were lost to microbial respiration. However, the soil acidification discussed in section 4.4.2 would suggest that microbial respiration did occur, and so there was likely some SOC loss and mineralisation of N and P from decomposition of organic compounds. It is therefore probable that small changes did occur in soil TKN, Olsen P and SOM contents, but the analysis methods and sampling protocol used were not sensitive enough to detect these small changes within the experiment.

Soil pH levels did indicate a flooding effect. As discussed in section 4.4.2, biosolid application lowered soil pH proportional to the rate of application. In post-flood soil samples this pH trend was reduced, with higher biosolid-applied soils rising closer to those of the control soil equilibrium level. This change could be due to the suspension of microbial aerobic respiration during the flood, leading

to a reduction in the acidification processes associated with it, as discussed in section 4.4.2. Rising pH in floodwaters potentially caused by denitrification of NO_3^-, which reduces acidity due to a net consumption of H^+ ions in the system (equation 4.9), could also have raised soil pH. Denitrification is expected to be higher in floodwaters with higher NO_3^- content due to its increased availability, and so corresponds to the raising of pH in biosolid-applied soils.

$$2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O$$

Equation 4.9

Final soils showed a recovery in pH back towards pre-flood soils in Experiments 1 and 4 (Figs. 4.5 and 4.8), with acidity increasing slightly again in higher rate biosolid-applied soils. This could indicate renewed microbial respiration and oxidation of C, N and S compounds (equations 4.6-4.8) once soils had become aerated again after flooding. However, this recovery was not observed in Experiment 2 (Fig. 4.6), where final soils showed no trend relating to biosolid application, and final soils were not analysed in Experiment 3 (Fig. 4.7).

4.4.4 Floodwater dissolved oxygen, pH and Eh

It was found that increased biosolid application increased the acidity of soils, and floodwater pH reflects the same trend. Porewaters showed lower pH as soil depth increased, which could be due to higher CO_2 levels in the anaerobic environment at depth causing increased partial pressures of CO_2 which reacts with water to form carbonic acid (Equation 4.6). This explanation has previously been attributed to the lowering of pH in calcareous soils (Ponnamperuma et al., 1966). Higher pH at shallower depths could be due to the diffusion of oxygenated surface water into shallow porewater limiting anaerobic conditions and reducing the acidification effect. No floodwater dropped below pH 7.0 and pH rose during the flood before levelling off in all surface waters and porewaters (Figs. 4.9-4.18). Similar trends have been observed in other studies (Dharmakeerthi et al., 2019). The rising pH is probably due to the effect of denitrification in floodwaters (Equation 4.9), with the effect lessened as NO_3 is depleted. This slowing of denitrification coupled with increasingly reducing conditions over the course of the flood, leading to greater acidity from CO_2 accumulation, could explain the levelling off of the floodwater pH. Furthermore, most microbially-mediated anaerobic respiration reactions in calcareous soils will lower pH, due to metal ions made soluble during reduction being able to precipitate as carbonates, hydroxides and sulphides. For instance, if Mn^{4+} and Fe^{3+} are reduced to Mn^{2+} and Fe^{2+} as the flood progresses then they can precipitate as carbonates, as shown in equations 4.10 and 4.11, generating protons which counteract the pH rise (McBride *et al.* 1994).

$$Fe^{2+} + H_2CO_3 \rightarrow FeCO_3 + 2H^+$$

Equation 4.10

$$Mn^{2+} + H_2CO_3 \rightarrow MnCO_3 + 2H^+$$

Equation 4.11

A deviation from the observed trend of rising pH occurred in Experiment 1 surface waters (Fig. 4.9) when measurements were taken after the lights were switched off on day 7 of the flood. A lowering of pH of around 0.25 units below what was expected in the trend occurred at this time, as well as a drop in the DO_2 content of approximately 2.0 mg O_2 l^{-1} from the long-term trend. These drops after the lights were switched off are attributed to differences in plant and algae photosynthesis and respiration between day and night cycles affecting floodwater geochemical conditions. The suspension of photosynthesis releasing less O_2 to water and the acidifying effects of CO_2 release from respiration (Equation 4.6) could account for the differences observed. Similar differences from expected trends were observed when taking more frequent measurements during the first 24 hours in Experiments 2 and 4 (Figs. 4.11 and 4.16) at different times during the day/night cycle. Though this observation was of interest is was outside the bounds of this project for further investigation. Measurement timings in future experiments were more closely controlled to be during daylight hours to ensure more accurate results.

Surface waters in all experiments remained highly oxygenated during the flood period and this was reflected in the Eh measurements, which were maintained above 400mV. Surface water DO_2 rose and began to drop off again at different points across experiments. This drop was attributed to the stress from flooding on the crop, causing photosynthesis to decrease. This would then lead to less O_2 being released into the surface water and can explain the differences measured. More support for photosynthesisers maintaining surface water DO_2 at higher levels comes from the anomalous result of the 200g applied box in experiment 2 (Fig 4.11). In this box algae did not grow on the water's surface for an unknown reason and the surface water DO_2 was between 1.09 and 2.70 mg l^{-1} lower than the mean DO_2 measured across the other boxes of the experiment. This clearly demonstrates the influence that algae had on surface water DO_2.

Porewater DO_2 measured by the standpipe method in Experiment 3 was unreliable due to the introduction of DO_2 with probe insertion, the mixing of oxygenated water in the standpipe and the risk of a standing water column forming in the pipe which would be separated from the actual porewater. The results in Experiment 3 from the standpipes were therefore disregarded, with the oxygen spot method used in Experiment 4 proving more reliable. Oxygen spot sensors showed O_2 becoming depleted in all soils within the first 9 hours (Figs. 4.17 and 4.18). Errors due to some of the

spot mounted tubes becoming dislodged influenced several results and the actual time for O_2 depletion was predicted to be even quicker than this, within the first 3-6 hours. No differences were observed between biosolid applied or control soils. These results indicate that microbial respiration consumed all available O_2 in the porewater more quickly than can be detected by the measurement frequency selected, but that this also made differences negligible in terms of impact on flood conditions. The results gathered from the oxygen spot method were deemed to be accurate and fitted with the Eh observations for the experiment. Similar Eh trends were observed in Experiments 1-3 and DO_2 is therefore assumed to have behaved similarly to Experiment 4 in response to flooding, with the porewater DO_2 becoming depleted in the first few hours.

4.4.5 Nitrogen behaviour in floodwater

Dissolved N in floodwater and surface water was predominantly found as NO_3^- in most boxes, with NH_4-N accounting for only a small fraction of the total dissolved N content (Figs. 4.19-4.22). An exception to this was where total dissolved N content was very low (<1.1 mg N l^{-1}) in which case NO_3-N and NH_4-N occurred in similar quantities at roughly a 1:1 ratio. Increased water total N concentrations therefore came predominantly from increased water NO_3^- concentration. The NH_4-N concentrations only rose above 0.5 mg l^{-1} in waters where NO_3-N exceeded 60 mg l^{-1}. The high NO_3^- and low NH_4^+ content in floodwaters are a good indication that nitrifying bacteria are active in these soils, leading to the oxidation of NH_4^+ to NO_3^- (nitrification) under oxidised conditions. The acidification of biosolid-applied soils as discussed in section 4.4.2 would also support this, as nitrification can contribute to soil acidity (equation 4.8). Significant reduction of NO_3^- to NH_4^+ by dissimilatory NO_3^- reduction to NH_4^+ (DNRA) does not appear to have occurred at any point during the experiments. This is indicated by the low NH_4-N levels measured and the ORP remaining above +100mV Eh in all porewaters (Figs. 4.9-4.18), with more highly reducing conditions required for DNRA to occur at high levels (Yin et al., 2002; Rütting et al., 2011). This is typical of flooded soil systems that do not become highly reducing (Unger et al., 2009b; Amarawansha et al., 2015; Dharmakeerthi et al., 2019). Some NH_4^+ may also have been immobilised into SOM by soil microbes, which could further contribute to explaining the low NH_4^+ measured. The NH_4^+ which was detected was concentrated more highly in floodwaters than surface waters, which is likely due to the action microbial mineralisation of organic N in the soil. Decomposition and N mineralisation would be localised to the SOM and so would result in the main concentrations of NH_4^+ appearing in soil porewater before being oxidised to NO_3^-. The small amounts of NH_4^+ that were found in surface waters are likely the result of diffusion from the porewater.

As previously discussed in section 4.4.1 the soils collected for the experiments were done so throughout the growing season in a working arable field, and as such were subject to fertiliser

applications. This allowed for the comparison of fertiliser N and P applications with N and P provided through biosolids. In the floodwaters of Experiment 3 the NO_3^- content was higher in all boxes than those observed in other experiments (Fig. 4.21), with even the control box floodwater in this experiment showing 11.3 mg NO_3^- L^{-1} compared to <1mg NO_3^- L^{-1} in most other experiment control box floodwaters. All biosolid-applied box floodwaters in Experiment 3 also showed higher NO_3^- content than those of other experiments. This increase in soluble N was despite no apparent increase in soil TKN content in Experiment 3 soils over those of other experiments. This suggests that the soluble liquid fertiliser N added shortly before soil collection may still have been present in the soil and acting as a readily available crop N source. This additional fertiliser N could have provided what was required for crop growth, with addition NO_3^- released to the soil through nitrification processes then building in excess. This excess NO_3^- may then be what was detected in the floodwaters of Experiment 3, with any denitrification happening over the 10-day flood not sufficient enough to see all this NO_3^- lost.

A small difference between floodwater and surface water NO_3^- concentrations was observed at higher biosolid application rates, with higher concentrations in floodwaters than surface waters in Experiments 3 and 4 (Figs 4.21 and 4.22). Differences in surface water N and floodwater N are attributed to algae uptake from surface water, as algae growth was present on the surface of waters in all experiments, although not measured. A sharp increase of NO_3-N can be observed in boxes with a 400g biosolid application and above, which was the application that represented the maximum field application rate. Low applications of biosolid at 100g and 200g tended to be comparable to the control boxes. This lack of increase at lower rates would suggest that soil microbes, crop and algae are buffering the amount of soluble N released to water at these levels through direct uptake, immobilisation or use in other metabolic processes. However, once a critical threshold is reached then the ability for the system to buffer excess soluble N is exceeded and the amount present in waters increases thereafter. The buffering capacity of the soil-crop system varies across each experiment and so a definitive point at which this effect occurs cannot be determined. Soils at, or under, the 400g application rate exhibited soluble N concentrations well below the 50mg N l^{-1} threshold determined by NVZ legislation. These experiments would therefore support the currently permitted maximum field application rate for minimising soluble N release from soils to surface waters. The exception to this was Experiment 3 where the synthetic fertiliser N application was made in the field prior to soil collection causing elevated floodwater NO_3^-. These results would indicate that applications of soluble fertiliser N could pose a greater risk of NO_3^- leaching to watercourses than the N supplied by biosolids alone, which becomes more slowly soluble by mineralisation over time.

ORP was lower in porewaters with very low NO_3^- content (<1 mg N l^{-1}). In the 5cm depth porewaters of the control soils in Experiments 1, 2 and 3 the Eh consistently dropped below +200mV. In almost all biosolid-applied boxes in these experiments the Eh remained at the +200mV threshold for the duration of the 10-day flood. This +200mV Eh threshold has previously been found in porewaters and directly linked to the presence of NO_3^- (Bailey and Beauchamp, 1971). A similar threshold was found in an environmental study by Reddy and Delaune (2008), with an Eh of +300mV maintained in wetlands when NO_3^- was present between 25-50 mg l^{-1}. An Eh of +200mV has been identified as the threshold at which denitrification occurs and becomes the more dominant biological process for controlling soil N chemistry, with nitrification dominating at higher Eh (Bell, 1969; Kralova et al., 1992; Chatterjee and Saha, 2018). Evidence shows that in soil systems with an Eh of +200mV NO_3^- reduction occurs, and it becomes the terminal electron acceptor (TEA) in the absence of O_2. This Eh will be maintained until all of the NO_3^- in the system is used (Grant and Long, 1981), at which point the Eh will decrease to more reducing conditions and use the next available electron acceptor.

A decrease below +200mV Eh occurred in most control boxes at 5cm soil depth but was also observed in the 5cm depth porewater of the 100g applied box in Experiment 2 (Figs 4.10, 4.12, 4.14, 4.15). Floodwater NO_3^- levels were particularly low in Experiment 2, with the 100g and 200g boxes being comparable to the control, and almost negligible in presence (Fig. 4.12). The 100g porewater showed a trend of Eh decreasing below +200mV about one day after the control soil. Unfortunately, in the 200g applied box in Experiment 2 the Pt electrode at 5cm depth failed, but it is assumed a similar gradient of lowering Eh would occur. The trend of Eh decreasing below +200mV observed in the control and 100g applied boxes of Experiment 2 supports the hypothesis that the presence of NO_3^- maintains the Eh value. As denitrification occurs it depletes the NO_3^- in the floodwater, which is lost to the atmosphere as N_2O or N_2 (equation 4.9). In biosolid-applied soils the Eh is therefore maintained for longer as there is a larger amount of NO_3^- in the soil, either applied directly or produced through nitrification. This trend was observed in all porewater from Experiments 1-3. The control boxes in Experiment 4 did not show any lowering of Eh below +200mV despite low floodwater NO_3^- concentrations (Fig. 4.22). The 800g (2) box 5cm porewater in Experiment 4 did show a decrease in Eh below +200mV, although this is suspected to be Pt electrode poisoning which was observed on several occasions in the experiments. In this case there may have been native soil NO_3^- which was present but depleted by denitrification soon before the flood was terminated. This would mean that the NO_3^- appeared low in drained floodwater but the Eh changes were not detected.

The NO_3^- content in Experiment 3 floodwater was higher than the other experiments across all boxes due to the fertiliser N application to the field prior to sampling (Fig. 4.21). The Eh still decreased

below +200mV in the control box despite having a NO_3-N content of 11.30 mg l^{-1}. This indicates that it may not solely be the presence of NO_3^- that is holding Eh above the +200mV threshold but that it is also linked to the biosolid application. This could be due to other chemical influences added with the biosolids, such as the biosolid Fe content addition potentially leading to Fe^{3+} acting as an electron acceptor alongside NO_3^- to some degree. Alternatively, or additionally, there could be a biological influence on the ORP. Biological mineralisation and nitrification of organic N through to NO_3^-, and plant uptake, could be altering the redox chemistry (pH and Eh) in the system prior to flooding, with these effects not present by the simple addition of N already in NO_3^- form. The distribution of NO_3^- throughout the soil profile may also be different when it is provided by fertiliser N as opposed to continuous nitrification from SOM. NO_3^- from nitrification could be present in higher concentrations in soil pores which would maintain the ORP locally. The measurement methods used in these experiments are not accurate enough to deduce the distribution of NO_3^- in the soil porewater or its change over time, having only been taken at the end point of the experiments. However, higher NO_3^- in the surface water than the whole floodwater of Experiment 3 suggests that NO_3^- in the porewater is lower than the 11.30 mg l^{-1} measured, but the precise NO_3^- concentration at which Eh begins to drop below +200mV cannot be determined. The possibility of microbial nitrification maintaining Eh is also supported by the Eh measurements in the 10cm porewaters which did not show a trend of decreasing below +200mV to the same degree as 5cm depth porewater. This could be since microbial biomass tends to be more concentrated in surface soils due to the presence of crop roots and residues, the increased availability of O_2 and increased water availability from rainfall (Alvarez et al., 1998; Murphy et al., 1998; Franzluebbers, 2002). The evidence therefore indicates that increased porewater NO_3^- due to enhanced nitrification from biosolid applications is governing the Eh of the soil biogeochemical system.

4.4.6 Mechanisms controlling phosphorus release to porewater

Orthophosphate in floodwater was proportional to floodwater total P content in the experiments in which it was measured. Orthophosphate accounted for 89.2 ± 7.6% of total P in Experiment 2 floodwaters and 78.0 ± 3.0% of total P in Experiment 4 floodwaters. Orthophosphate in surface water accounted for a slightly lower proportion of total P than in floodwater at 77.6 ± 11.5% of total P in Experiment 2. In Experiment 4 the surface water orthophosphate and total P levels were below the detection limits of the Hach® LCK test kit used and could not be reliably reported. Variation in the standard deviations is also unreliable as it was taken using just one measurement from each box. However, the conclusion that orthophosphate accounts for the majority of total P in floodwaters is consistent with what is expected in a flooded soil. The orthophosphate found in higher proportions in floodwater indicates that higher concentrations are present in porewater than surface water. This

is expected in a soil system where the SRP would be contained in the soil solution where conditions were more reducing, and microbial P mineralisation and sorption were most concentrated.

No differences were observed in the total P content of floodwater between any biosolid treatments. Control soils and all rates of biosolid-applied soils displayed similar levels of floodwater total P. This finding is contrary to what was expected, which was an increasing trend of SRP release to floodwater in proportion to the soil Olsen P which increased with biosolid application (Fig. 4.4). However, this assumption was based on previous studies where increased SRP from higher P soils occurred once conditions became reducing in those systems (Amarawansha et al., 2016; Dharmakeerthi et al., 2019). In the experiments in this project none of the flooded boxes became highly reducing. The boxes with the lowest measured Eh were the control soils, as previously discussed regarding floodwater NO_3^- in section 4.4.5. This lower Eh may be the reason that the SRP released from control soils was similar to biosolid-applied soils despite having no P addition through biosolids. Control soils appear to be releasing a larger proportion of the P bound within the soil than the biosolid-applied soils.

The release of SRP from inorganic P minerals as conditions become more reducing is key to understanding the soil geochemical system. Electron acceptors are usually reduced as ORP decline in the order: O_2, NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-} (McBride et al., 1994; Gardiner and James, 2012). In the absence of O_2 and NO_3^- as electron acceptors in the control box soils reductive dissolution of Mn^{4+} and Fe^{3+} phosphates can in principle occur. Total soil Mn was very small compared to Fe when measured by XRF (Table 3.6), as is typical in most soils (McBride et al., 1994). Though XRF is not a definitive measure of reactive forms of Mn and Fe in the soil it is an indicator of the theoretical potential P sorption ratio of each. The total Mn content of all the soils in Experiment 4 was 0.115-0.125g kg^{-1} (Table 3.6), compared with Fe which accounted for a much larger proportion of soil mineral content at 2.196-2.291g kg^{-1}. This could indicate there was a larger capacity for P to be adsorbed to Fe^{3+} compounds than to Mn^{4+}. Release of SRP from the reduction of Mn^{4+} in this case would be small compared to reduction of Fe^{3+} compounds. From the biosolid analysis results (Table 4.3) it is also known that the biosolids contained a high Fe content alongside the P content. This is likely due to Fe dosing for P extraction during wastewater treatment, as discussed in section 3.2.3, meaning that much of the P found in biosolids may be Fe bound. This would mean conditions leading to the reduction of Fe^{3+} could potentially lead to very large amounts of SRP being released from biosolids in the soil to floodwater. Conditions did not become reducing enough in these experiments to lead to widespread reduction of Fe^{3+}, but some may have occurred in localised areas of the soil. Mn^{4+} reduction to Mn^{2+} may be occurring in all soils (equation 4.12), as this process can happen as high as +300mV at pH 7 (McBride et al., 1994), and has been previously demonstrated at

around +200mV in flooded soil systems ranging from pH 7.3-8.4 (Amarawansha et al., 2015). All boxes in all experiments reached an Eh of +200mV during the flood. It is reasonable to assume therefore that P bound in Mn^{4+} minerals was released as SRP and this could explain why no significant difference was observed in floodwater total P measurements between soils.

$$Mn_3(PO_4)_4 + 4H^+ + 2e^- \rightarrow Mn_3(PO_4)_2 + 2H_2PO_4^-$$

Equation 4.12

$$FePO_4 + 2H^+ + e^- \rightarrow Fe^{2+} + H_2PO_4^-$$

Equation 4.13

Further lowering of soil porewater Eh below +200mV, as was observed in control soils, could lead to the mobilisation of Fe^{2+} by microbial anaerobic respiration using Fe^{3+}, leading to the liberation of its associated P (equation 4.13). In other studied soil systems, microbially-mediated redox reactions caused by microbial dissimilative metabolism can lead to Fe^{3+} reduction occurring at +100mV (McBride et al., 1994; Petruzzelli et al., 2005). Total P in all the control box floodwaters was consistently among the highest total P value measured between boxes in all experiments (although no significant difference was observed). In the 5cm porewater of Experiments 1-3 (Figs. 4.10, 4.12 and 4.14) the Eh approached or dropped below +100mV. In these control boxes Fe^{3+} may begin to be reduced and release PO_4^{3-} from dissolving ferric phosphates. This could explain why control boxes had consistently high total P measured in floodwaters, above many of the biosolid-applied soils, despite no addition of P. Control boxes in which Eh approaches +100mV towards the end of the 10-day flood also suggests that a further drop of Eh to more reducing conditions may have been imminent. This could have led to more significant loss of P from reduced Fe minerals, exaggerated further in biosolid-applied soils if a large proportion of the P contained within the biosolids was Fe bound.

The flood was terminated before the long-term effects of continued lowering in Eh could be observed. However, a similar study by Dharmakeerthi et al. (2019) found that continued lowering of Eh below +200mV in a long-term flooded agricultural system led to increased loss of SRP due to reduction of Fe^{3+}. The maintenance of the +200mV threshold in that study was attributed to gypsum applications to soils. However, the soils were also pre-treated with pig slurry prior to the experiment and NO_3^- was not measured during the experiment. A change in soil conditions brought on by the application of gypsum, which favoured microbial nitrification of soil N, may be a reason for the maintenance of Eh in those soils, rather than the gypsum application directly. NO_3^- may therefore be the controlling factor in those experiments and as it is depleted Fe^{3+} becomes the main electron acceptor for anaerobic respiration, releasing associated P to floodwater. Biosolid

applications also delay the decrease of Eh below +200mV, likely due to the presence of NO_3^- in the system from biogenic mineralisation of organic N as discussed in section 4.4.5. Biosolids could therefore delay the onset of reduction of ferric phosphate minerals in the soil over time and so reduce the long-term risk of SRP loss to floodwater.

Additional to SRP release from biosolids, Experiment 2 utilised soils which had been applied with a mineral P fertiliser prior to collection from the field. The floodwaters of Experiment 2 all displayed higher P content than was found in floodwaters of the other experiments (Fig. 4.20). The Initial soil Olsen P levels in Experiment 2 were also higher than in other experiments (Fig 4.4) showing the higher available P in the soil from this application. The mineral P fertiliser (Triple Superphosphate, $Ca(H_2PO_4)_2.H_2O$) application was relatively small at 53kg ha^{-1} P_2O_5 compared to the biosolid P application in the 400g biosolid-applied box which was 268kg ha^{-1} P_2O_5. Despite this difference no biosolid applied showed SRP release compared to the baseline control levels found in the Experiment 2 floodwaters. This would indicate that biosolid applied P is far less water soluble than mineral P fertiliser additions and poses less of a risk of loss to watercourses in the event of flood, even when biosolid P is applied in hugely excess amounts. This is dependent on floodwater conditions not becoming reducing enough to induce Fe^{3+} reduction to Fe^{2+} leading to the release of its associated P as SRP.

4.4.7 Mechanisms controlling phosphorus release to surface water

Surface water SRP was lower than porewater SRP in most cases. A possible reason for this was that surface waters remained highly oxygenated throughout the flood and may have acted as a barrier to SRP release from soil porewater. As discussed in section 4.4.6, PO_4^{3-} can be released from reduced Fe^{3+} oxyhydroxides and ferric phosphate in the porewater (equation 4.13). Fe^{2+} is soluble in the porewater but as it migrates into the soil surface layer, which remains oxygenated by the overlying surface water, it oxidises back to Fe^{3+}. In a reverse of equation 4.13, PO_4^{3-} in the soil surface layer can then precipitate with Fe^{3+} to form ferric phosphate or adsorb to ferric oxides and hydroxides, forming insoluble Fe^{3+} minerals. This process prevents the release of SRP from reduced soils to oxygenated surface waters. This mechanism is well established and has been found in several studies (Boström and Pettersson, 1982; Moore and Reddy, 1994; Young and Ross, 2001; Hupfer and Lewandowski, 2008; Van Nguyen and Maeda, 2016). However, this mechanism relies on porewater which is in a highly reducing state and has a large pool of Fe^{2+} and its liberated associated P. The porewaters in these experiments likely did not become reducing enough to support Fe^{3+} reduction, although they did have lower Eh values than surface waters. As discussed previously in section 4.4.4, the 200g biosolid-applied box of Experiment 2 (Fig. 4.20) had higher surface water P than floodwater, in contrast to all other surface waters in all experiments. This box had no algae growth

on the surface water for an unknown reason. This anomalous result could be indicative of the fate of surface water SRP which was not apparent in other boxes. Based on the SRP behaviour in this box, and the understanding that porewater was not reducing enough to create an oxidation-reduction threshold to act as a barrier for P release, it would seem algae uptake is controlling SRP release to surface water. The control of surface water SRP by algae, in addition to the oxygenated surface layer barrier has also been suggested in previous studies to contribute to the regulation of surface water SRP (Moore and Reddy 1994). This deduction highlights the risk of eutrophication to watercourses from SRP released from agricultural soils. However, the actual scale of SRP release to surface waters cannot be determined from this series of experiments due to the algae uptake. Algae growth was not measured and so differences between boxes and possible P content of the algae cannot be assessed. All that can be determined is that there were no differences between the SRP which remained in solution in surface waters, but surface water SRP was likely controlled by algae growth.

4.5 CONCLUSIONS

Biosolid applications increased soil SOM, TKN and Olsen P. However, small applications of biosolid below the maximum prescribed field rate may display a priming effect in Olsen P levels. Olsen P increased above the expected trend at lower biosolid applications which was determined to be from increased mineralisation from organic P by microbial activity beyond what the soil resources could maintain. The 10-day flood had no measurable effect on SOM, TKN or Olsen P quantities in soil, indicating no long-term effect on soil nutrient content. Differences in pH caused by biosolid influences on microbial activity were reduced post-flood, suggesting a reduction in microbial respiration which then recovered over time. All surface waters remained highly oxygenated throughout the flood. No differences were observed in O_2 levels in the porewater between control and biosolid-applied soils, with depletion in the first few hours after flooding in all soils. Eh values in porewater reflected this rapid O_2 depletion and decreased over time. They were then likely controlled by alternate TEAs in the soil, such as NO_3^-.

Soil microbial communities were likely shifting from aerobic to anaerobic populations due to the quick depletion of O_2 in the early stages of flooding. The Eh then decreased over time due to the lack of O_2 as an electron acceptor, causing Mn^{4+} to become reduced to Mn^{2+} with the release of associated P. The mineralisation of NO_3^- from biosolids then provides an electron acceptor to support anaerobic respiration, so the Eh becomes poised at NO_3^- reduction through denitrification in biosolid-applied boxes. In control soils, where N mineralisation was limited due to the lack of a fresh

organic input, Eh continued to decrease. As this continued decrease in Eh occurred it potentially caused Fe^{3+} to become the primary electron acceptor in these reduced systems, which could lead to the liberation of Fe^{3+} associated P in the longer-term. Biosolids could therefore be demonstrating a mechanism of self-limitation of SRP release under flooded conditions, with its NO_3^- content delaying the release of its P content to floodwater. These conclusions apply to short-term flooding where porewater conditions did not become highly reducing and floodwater was drained while crops were able to survive. The high Fe content of biosolids would potentially indicate that most of its P content was Fe associated, thereby preventing its release as SRP in conditions which did not lead to reduction of Fe^{3+} to Fe^{2+}. In the short-term, biosolids appear to have no adverse effect on increased SRP release to floodwater over unapplied control soils. Larger biosolid applications can prevent reducing conditions from occurring for longer periods of time due to increased potential for NO_3^- mineralisation. However, controlling the risk of NO_3^- pollution from leaching to watercourses still limits the application rate of biosolids which is practical. Current legislation restricting the application of biosolids based on total N content, due to the threat of NO_3^- leaching to watercourses, appears to be the correct approach.

4.6 References

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Chapter 5: CROP RESPONSE TO SHORT-TERM FLOODING ON BIOSOLID-AMENDED SOILS

5.1 INTRODUCTION

Water inundation of soils can occur in various forms throughout the growing season, with short term waterlogging, flash flooding and saturation from high water tables all possible symptoms of extreme rainfall. Flooding from extreme rainfall events has become more common in recent years, with major floods in the UK in 2012, 2014 and 2019 (Met Office, 2019). The 2014 floods alone were estimated to have caused £7.2m worth of damage to arable farming in England (ADAS, 2014b). Of all arable land in the UK cereals cover the largest area, accounting for 71% of the total arable crop area at 3.2m hectares, consisting mainly of wheat at 1.8m ha and barley at 1.2m ha (DEFRA, 2019). Effective management strategies for improving crop survival to short-term flooding should therefore be explored.

The predominant cause of plant stress during water inundation is from low oxygen transfer to roots in inundated soil, as plants require oxygen for energy production through respiration, and prolonged oxygen deprivation can ultimately lead to plant death (Banti et al., 2013; Shaw and Meyer, 2015). Wheat has been shown to be more resilient than barley to flood stress, with barley showing a significant reduction in root growth when flooded in early growth stages and a severe reduction in root and shoot performance when flooded in late growth stages (Ploschuk et al., 2018). However, barley also displayed an impressive ability to recover from short-term flooding in early growth stages, meaning it could be more useful to study than wheat when assessing flood impact and recovery. Factors which improve early root development in plants and improve soil structure for improved oxygen diffusion and drainage could be vital to improved barley flood resilience. Higher levels of soil organic matter (SOM) have been shown to improve soil structure and drainage, as well as allowing cereal crops to reach higher attainable yields (Singh and Agrawal, 2008; Johnston et al., 2009; Hijbeek et al., 2017). Improving SOM levels may therefore be a viable option for improving cereal crop flood survival.

Several options are available for improving SOM in arable cropping, however many of these require long term management changes to be effective such as cover crops, grass ley rotations and reduced tillage (Reeves, 1997; Ding et al., 2006; Koch and Stockfisch, 2006; Nascente et al., 2013). These options may not be an available in some situations and so for more immediate SOM improvements organic matter inputs such as manures, straws, composts and biosolids could offer a solution. The

effect that many of these inputs has on crop and soil health has been explored in previous studies (Pant et al., 2002; Unger et al., 2010; Amarawansha et al., 2016), however biosolids have not received much attention in this area. Biosolids are particularly interesting as they contain many trace elements which are key to crop growth and have a higher N:P ratio than other organic inputs which are major plant nutrients. P in soils tends to be tightly bound to soil particles and not available to plants in the soil solution in large quantities. Low P uptake can limit plant growth as P is a vital nutrient in living cells and plays an important role in plant energy storage and transfer (Soffe, 2003; Shen et al., 2011). The large influx of P from a biosolid application at the time of crop establishment could improve early crop growth and make plants more resilient to flood conditions throughout the growing season. Combined with the addition of N and micronutrients to further enhance crop health, and improved soil structure from increased SOM, biosolid applications could be a sustainable flood damage mitigation strategy.

Using the methodology outlined in Chapter 3 this project will use growth box experiments to assess plant growth and recovery from a short-term flood. Much is already understood about the effects of different soil treatments on long-term flooded soil conditions (Unger et al., 2009b; Amarawansha et al., 2016; Dharmakeerthi et al., 2019). However, the length of those experiments does not allow for the study of crop damage and recovery, as crops did not survive the flood length. When crop damage has been explored in short-term floods the investigations instead focus on differences in plant performance between controls and floods (Leyshon and Sheard, 1974; Drew and Sisworo, 1977; Brisson et al., 2002; Ploschuk et al., 2018), rather than the potential for damage remediation from different soil inputs. The experiments of this project, described in the following chapter, will explore what differences biosolid applications have on crop establishment and early growth, and whether there is any subsequent effect on crop stress, recovery and performance after a 10-day flood event.

5.2 METHOD

The following section describes the methodologies used regarding measuring crop performance and recovery in the experiments of this project. Broader details of the general experimental setup, crop planting and flooding parameters can be found in chapter 3.

5.2.1 Initial growth measurements

After planting, a 28-day period was allowed for plants to grow undisturbed. During this time the establishment and growth of the plants was monitored. During the first week of establishment no measurements were taken as the plants were still emerging. After 10 days plant counts were taken for each box and each plant was measured individually from the base of the soil to the tip of its flag leaf. These measurements were then repeated weekly on days 17 and 24 of initial growth directly before watering.

5.2.2 Flood monitoring and recovery

Following the 28-day initial growth period a final plant count was made and a photograph taken of each box. The 10-day stagnant flood was then initiated and carried out with no crop measurements taken during this time. Immediately prior to draining of the floodwater a photograph was taken of each box. After draining of the floodwater another photograph was taken of the drained boxes for comparison to the pre-flood condition. No post-flood plant measurements were taken as it was difficult to identify which plants were still alive and which might be dead or dying from the stress of the flood. Environmental conditions and watering protocol during the recovery period were kept the same as in the initial growth period. The length of the recovery time before termination varied by experiment: In Experiments 1 and 2 a 28-day recovery period was allowed from the end of the flood to properly assess any crop differences. In Experiment 3, where the focus was primarily on flood analysis, the recovery period was 10 days to reduce total experiment run time but allow for dying plants to be distinguished from living. In Experiment 4 the recovery period was 20 days to adhere to timings required for the microbiological sampling detailed in chapter 6.

5.2.3 Final crop performance measurements

5.2.3.1 Dry weight biomass

After the recovery period the plants from each box were carefully dug up with care taken to preserve the root structures. Once the plants had been removed from the soil they were washed to remove any clinging soil particles and laid flat on a tray corresponding to each box. The plants were photographed and then the trays were placed in a drying oven for 72 hours at 72^{o}C. After drying the plants were removed from the oven and roots and shoots were weighed separately.

5.2.3.2 ImageJ analysis

During the drying process of Experiment 2 two of the plant samples from the 0g and 1600g (Boxes 4 and 5) were lost due to equipment failure. The samples from the remaining four boxes were measured without incident. To recover a full set of data from this experiment the photographs taken prior to drying were used to estimate the weights of the lost samples for comparison to the known samples. The software ImageJ (Rueden et al., 2017) was used to adjust colour thresholds and isolate the plant area in each of the 6 photos. The background was deleted from the image and then any area which was isolated but was not identified as plant was manually deleted. The total pixel area of the plants was then measured and recorded. Following this the root area was manually deleted from each image and the shoot area measured from the remaining plant image, shown in Figure 5.1. The root area was determined by subtracting the shoot area from the total area. All original plant photographs used for this analysis are included in Appendix D.

Figure 5.1- ImageJ analysis of Experiment 2, Box 5 plants with the total plant area highlighted (left) and the shoot area after the root area had been cut from the image (right).

To calculate the dry weight biomass (DWB) of the plants in each image the total pixel areas of root and shoot were divided by the actual weight in grams of the known samples. This then gave a pixel weight for each image of the root and shoot, accounting for density differences in each material. The known pixel weights were then averaged to give a mean weight per pixel. To account for overlap of plants in the images only samples with >30 plants were used to determine the mean, with

box 1 results excluded under this criterion. The calculated mean pixel weight was then used to give an estimated pixel weight for the two unknown samples. Multiplying the estimated pixel weight by the measured number of pixels for root and shoot in each image gave an estimated DWB in grams for the lost samples.

It should be noted that the photographs were not initially taken for image analysis and so were not laid out in a manner which would provide highly accurate results from this form of analysis. The results gathered however did roughly match with visual assessments of the plants and the predicted performance of each crop from their observed growth throughout the experiment. The results are therefore adequate as a proportional comparison between boxes in this experiment.

5.3 RESULTS

5.3.1 Watering observations

During all experiments the infiltration of water applied to the surface of the boxes was observed but not quantified. Water applied to the surface of the 0g rate control boxes had a noticeably slower infiltration rate than all biosolid applied boxes, with water often pooling on the surface for approximately one minute. This effect was most noticeable in the early stages of the experiment before plants had become established. Later, as plants became more established, infiltration rates appeared to improve. Even in the very low rate biosolid applied boxes of 100g and 200g water infiltration appeared markedly better than in the control boxes. Higher application rates of biosolids (>400g per box) showed very quick water infiltration to the surface at all stages of the experiment. All boxes received the same mixing and cultivation regardless of whether they were biosolid applied or not.

5.3.2 Establishment

Table 5.1 details the plant establishment from 60 seeds after the first 10 days in Experiments 1-3. In Experiment 4 a second round of seed planting was carried out after 7 days due to uneven establishment. The details of the number of seeds added and the final establishment after 14 days also are included.

Table 5.1- Plant establishment after 10 days from 60 planted seeds.

Experiment 1						
Biosolid application (g)	0	100	200	400	800	1600
Box number	6	3	4	5	2	1
Plants	40	52	54	57	58	58
Experiment 2						
Biosolid application (g)	0	100	200	400	800	1600
Box number	4	1	3	2	6	5
Plants	50	49	49	53	49	51
Experiment 3						
Biosolid application (g)	0	100	200	400	800	1600
Box number	6	4	2	1	3	5
Plants	49	52	50	50	53	50
Experiment 4						
Biosolid application (g)	0	0	400	400	800	800
Box number	3	5	2	6	1	4
Plants (7 days)	20	16	29	30	41	48
Added seeds	30	34	21	20	9	2
Plants (14 days)	29	27	50	46	55	52

5.3.3 Initial plant growth

The following box plots (Figs. 5.2 and 5.3) display the plant height measurements taken from stem base to flag leaf tip of the plants during the initial growth period. The measurements are displayed by experiment with measurement timings at 10 days (week 2), 17 days (week 3) and 24 days (week 4).

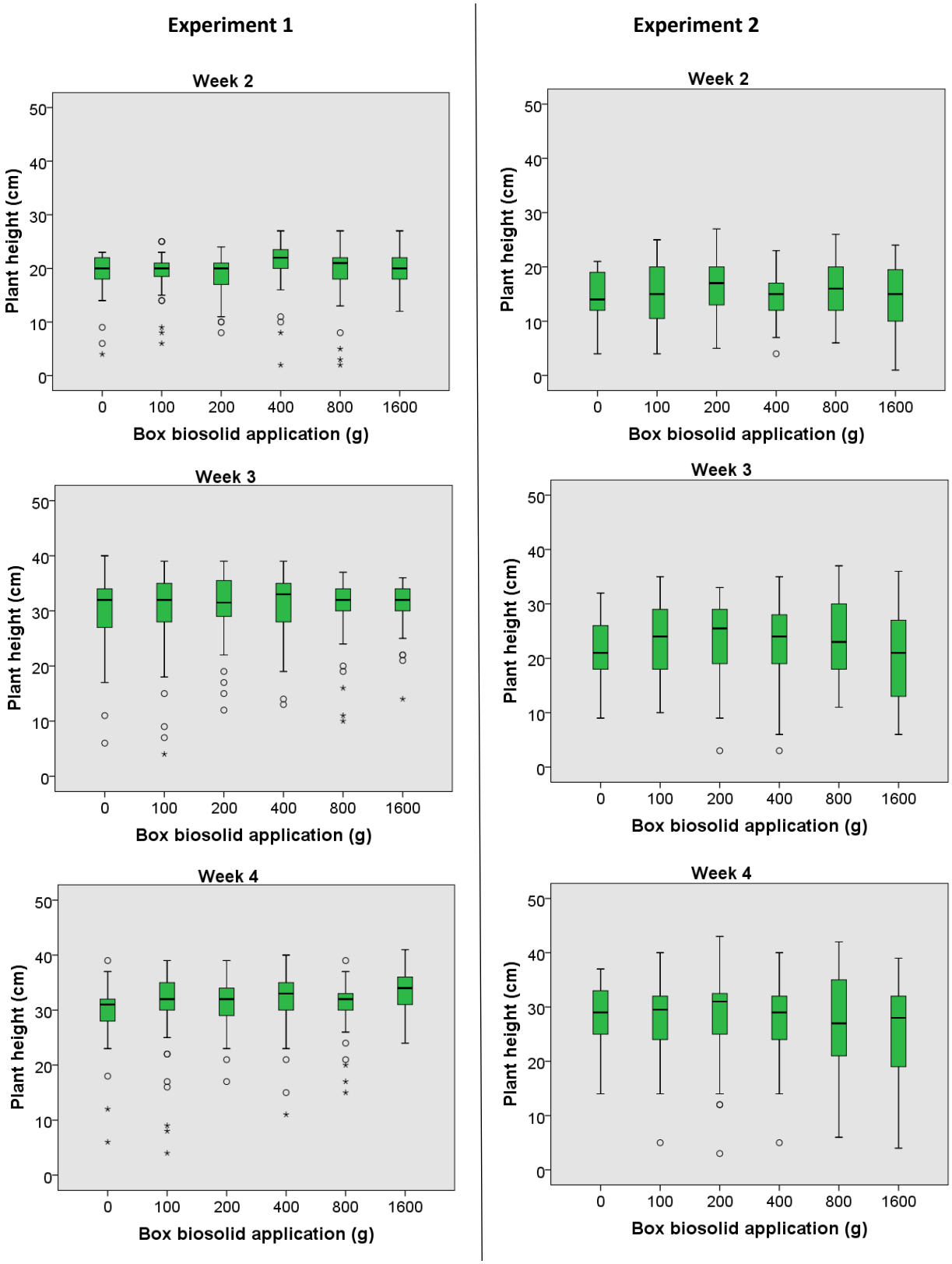


Figure 5.2- Experiment 1 and 2 plant height measurements by box biosolid application, showing population distribution based on individual height measurements of each plant. Outliers (1.5-3 times the interquartile range) are presented as a circle (o) and extreme outliers (>3 times interquartile range) are presented as an asterisk (*).

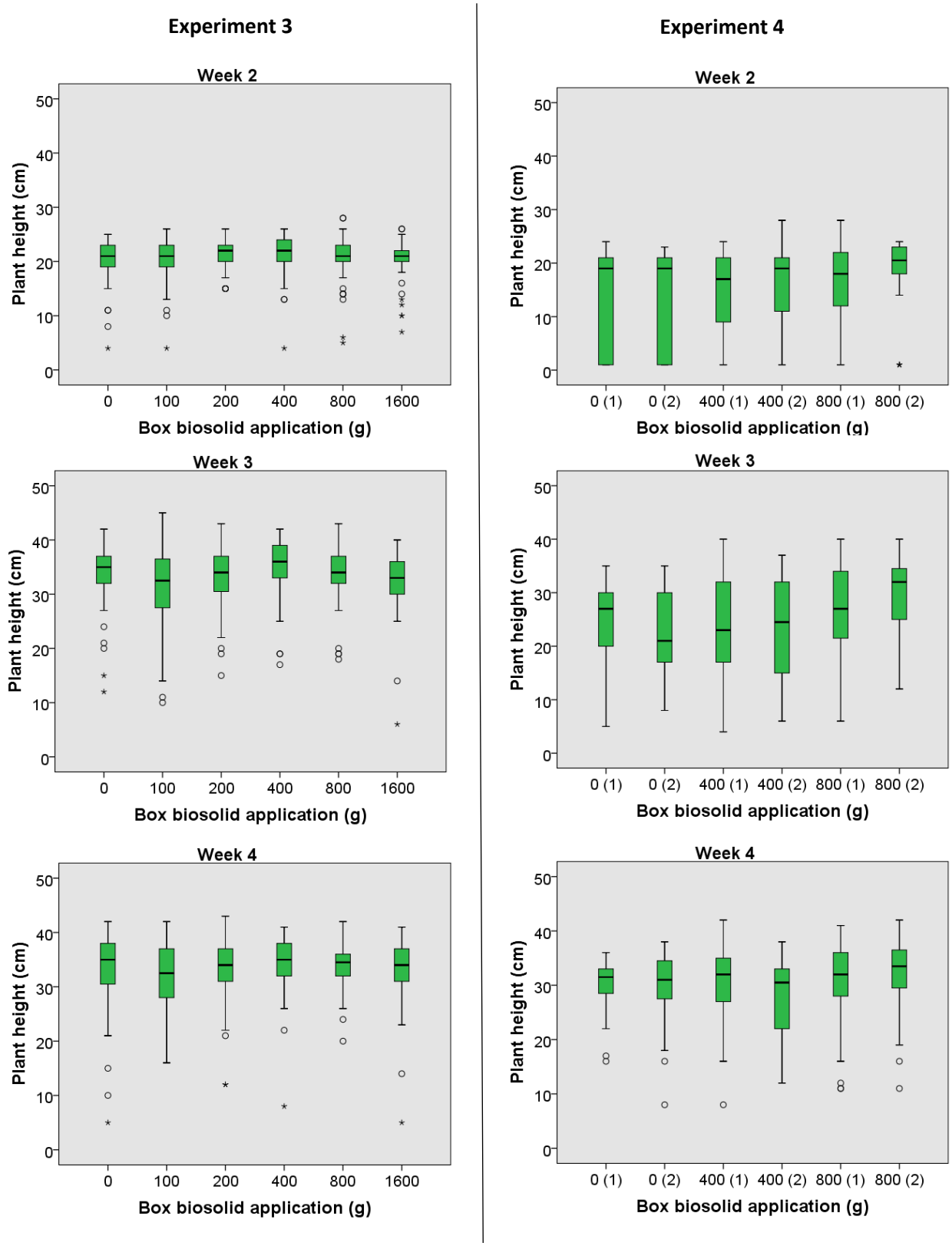


Figure 5.3- Experiment 3 and 4 plant height measurements by box biosolid application, showing population distribution based on individual height measurements of each plant. Outliers (1.5-3 times the interquartile range) are presented as a circle (o) and extreme outliers (>3 times interquartile range) are presented as an asterisk (*).

5.3.4 Growth box observations

The photographs in Figures 5.4-5.11 show the state of the growth boxes at the end of the flood period and then again at the end of the recovery period. The photographs taken at the end of the flood show most plants looking healthy and alive and are also a good representation of how the plants in each box appeared pre-flood. The photographs of the boxes at the end of the recovery period show how crop die-off occurred from the stress of the flood. In those photographs the dead plants clearly appear withered and dead as opposed to the still living green plants. Subsequent measurements of crop survival and the selection of plants to include in DWB were based on these visual assessments.

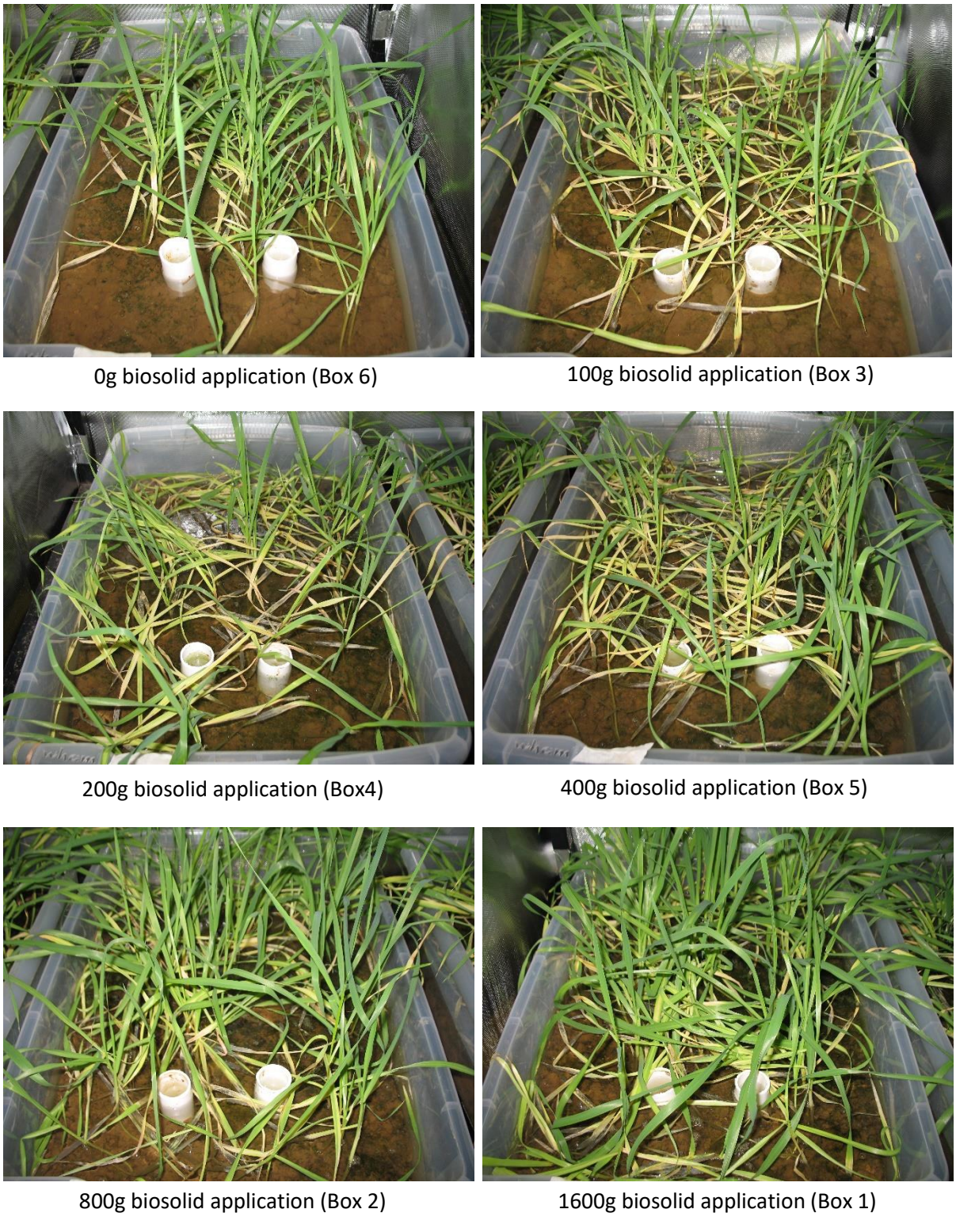


Figure 5.4- Experiment 1 growth boxes on day 10 of the flood, immediately prior to draining of the floodwater.

0g biosolid application (Box 6)

100g biosolid application (Box 3)

200g biosolid application (Box4)

400g biosolid application (Box 5)

800g biosolid application (Box 2)

1600g biosolid application (Box 1)

Figure 5.5- Experiment 1 growth boxes after 28-day flood recovery period, immediately prior to destructive sampling.

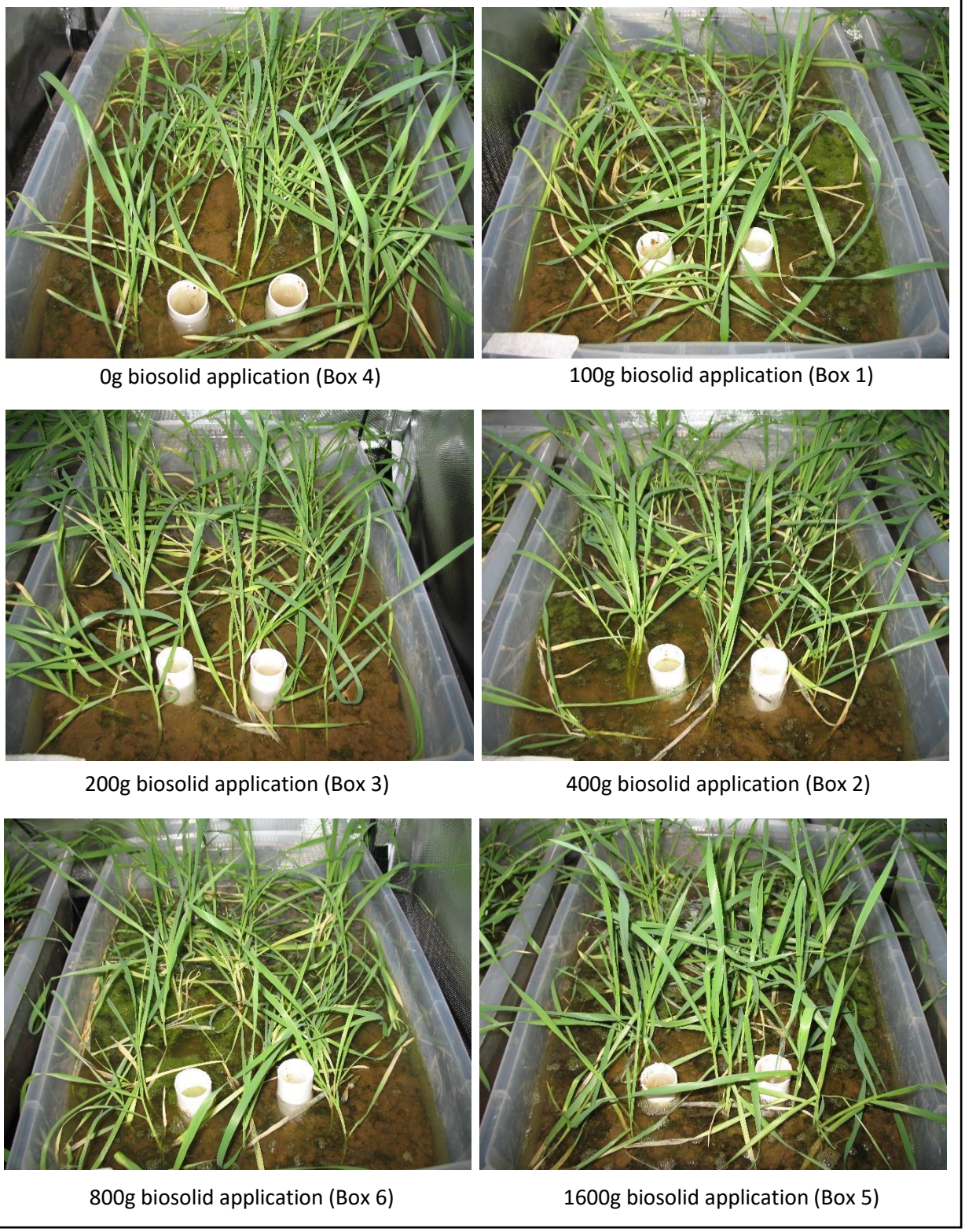


Figure 5.6- Experiment 2 growth boxes on day 10 of the flood, immediately prior to draining of the floodwater.

0g biosolid application (Box 4)

100g biosolid application (Box 1)

200g biosolid application (Box 3)

400g biosolid application (Box 2)

800g biosolid application (Box 6)

1600g biosolid application (Box 5)

Figure 5.7- Experiment 2 growth boxes after 28-day flood recovery period, immediately prior to destructive sampling.

0g biosolid application (Box 6)

100g biosolid application (Box 4)

200g biosolid application (Box 2)

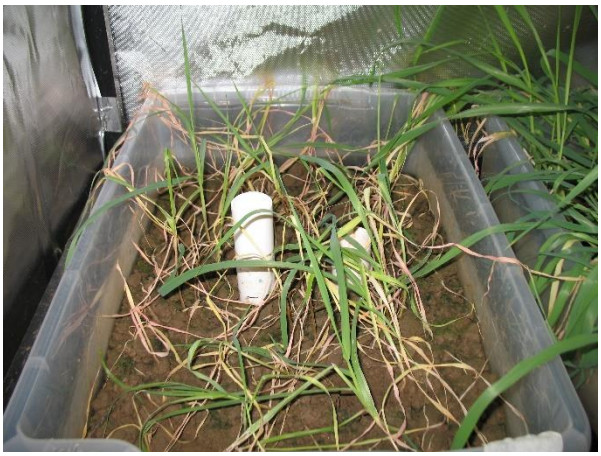
400g biosolid application (Box 1)

800g biosolid application (Box 3)

1600g biosolid application (Box 5)

Figure 5.8- Experiment 3 growth boxes on day 10 of the flood, immediately prior to draining of the floodwater.

0g biosolid application (Box 6)



100g biosolid application (Box 4)

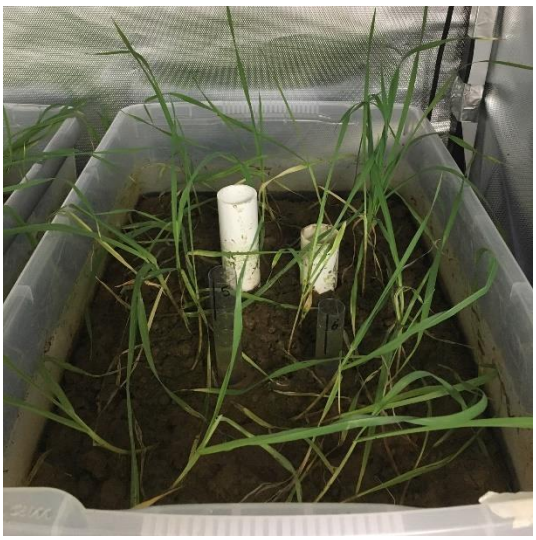
200g biosolid application (Box 2)

400g biosolid application (Box 1)

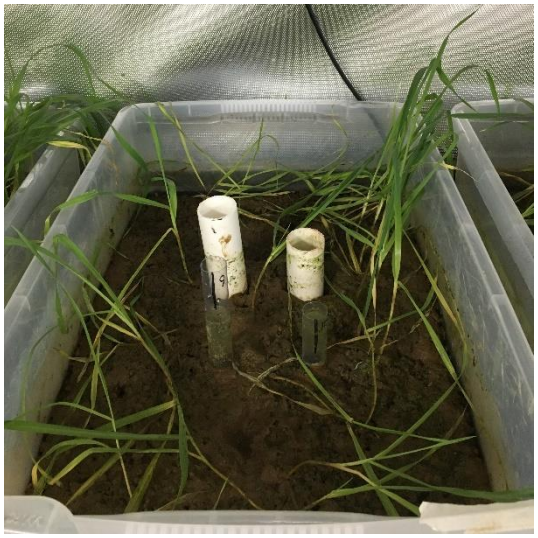
800g biosolid application (Box 3)

1600g biosolid application (Box 5)

Figure 5.9- Experiment 3 growth boxes after 10-day recovery period, immediately prior to destructive sampling.

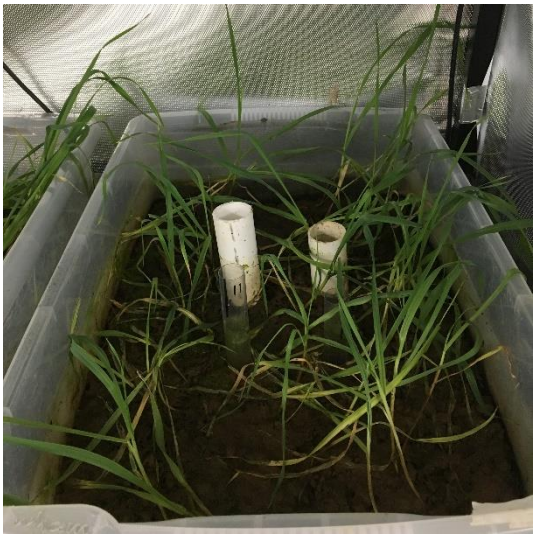


0g (1) biosolid application (Box 3)



0g (2) biosolid application (Box 5)

400g (1) biosolid application (Box 2)



400g (2) biosolid application (Box 6)

800g (1) biosolid application (Box 1)

800g (2) biosolid application (Box 4)

Figure 5.10- Experiment 4 growth boxes immediately after 10-day draining of the floodwater.

0g (1) biosolid application (Box 3)

0g (2) biosolid application (Box 5)

400g (1) biosolid application (Box 2)

400g (2) biosolid application (Box 6)

800g (1) biosolid application (Box 1)

800g (2) biosolid application (Box 4)

Figure 5.11- Experiment 4 growth boxes after 20-day recovery period, immediately prior to destructive sampling.

5.3.5 Flood survival

Table 5.2 shows the data for flood survival, which was the number of still living plants at the conclusion of each experiment after the recovery growth period. Dead plants were determined based on their withered, yellow look as can clearly be distinguished from the still living green plants in Figures 5.5, 5.7, 5.9 and 5.11.

Table 5.2 - Survival of plants after 10-day flood by box biosolid application rate.

Experiment 1					
Box	Rate	Original Plants	Surviving Plants	Plant Death	Survival %
6	0g	39	33	6	84.6
3	100g	50	21	29	42.0
4	200g	52	22	30	42.3
5	400g	54	14	40	25.9
2	800g	58	38	20	65.5
1	1600g	57	25	32	43.9
Experiment 2					
Box	Rate	Original Plants	Surviving Plants	Plant Death	Survival %
4	0g	56	40	16	71.4
1	100g	46	22	24	47.8
3	200g	52	44	8	84.6
2	400g	50	43	7	86.0
6	800g	50	32	18	64.0
5	1600g	52	39	13	75.0
Experiment 3					
Box	Rate	Original Plants	Surviving Plants	Plant Death	Survival %
6	0g	52	36	16	69.2
4	100g	50	24	26	48.0
2	200g	51	43	8	84.3
1	400g	54	40	14	74.1
3	800g	54	50	4	92.6
5	1600g	49	48	1	98.0
Experiment 4					
Box	Rate	Original Plants	Surviving Plants	Plant Death	Survival %
3	0g (1)	28	28	0	100.0
5	0g (2)	27	17	10	63.0
2	400g (1)	49	24	25	49.0
6	400g (2)	42	26	16	61.9
1	800g (1)	53	36	17	67.9
4	800g (2)	52	43	9	82.7

5.3.6 Dry weight biomass

Figure 5.12 presents the DWB of plants after destructive sampling at the conclusion of each experiment. The recovery time after flooding differed by experiment and so the results should be considered principally as a comparison across boxes within each experiment rather than between experiments. The results correspond to the visual crop performance observations shown in section 5.3.5 and the survival percentages shown in section 5.3.4. The average DWB per plant in each box, found by dividing total DWB by the number of surviving plants, is shown in Figure 5.13. Per plant DWB allows for the visualisation of average individual plant performance without differences in establishment and survival rates distorting the data.

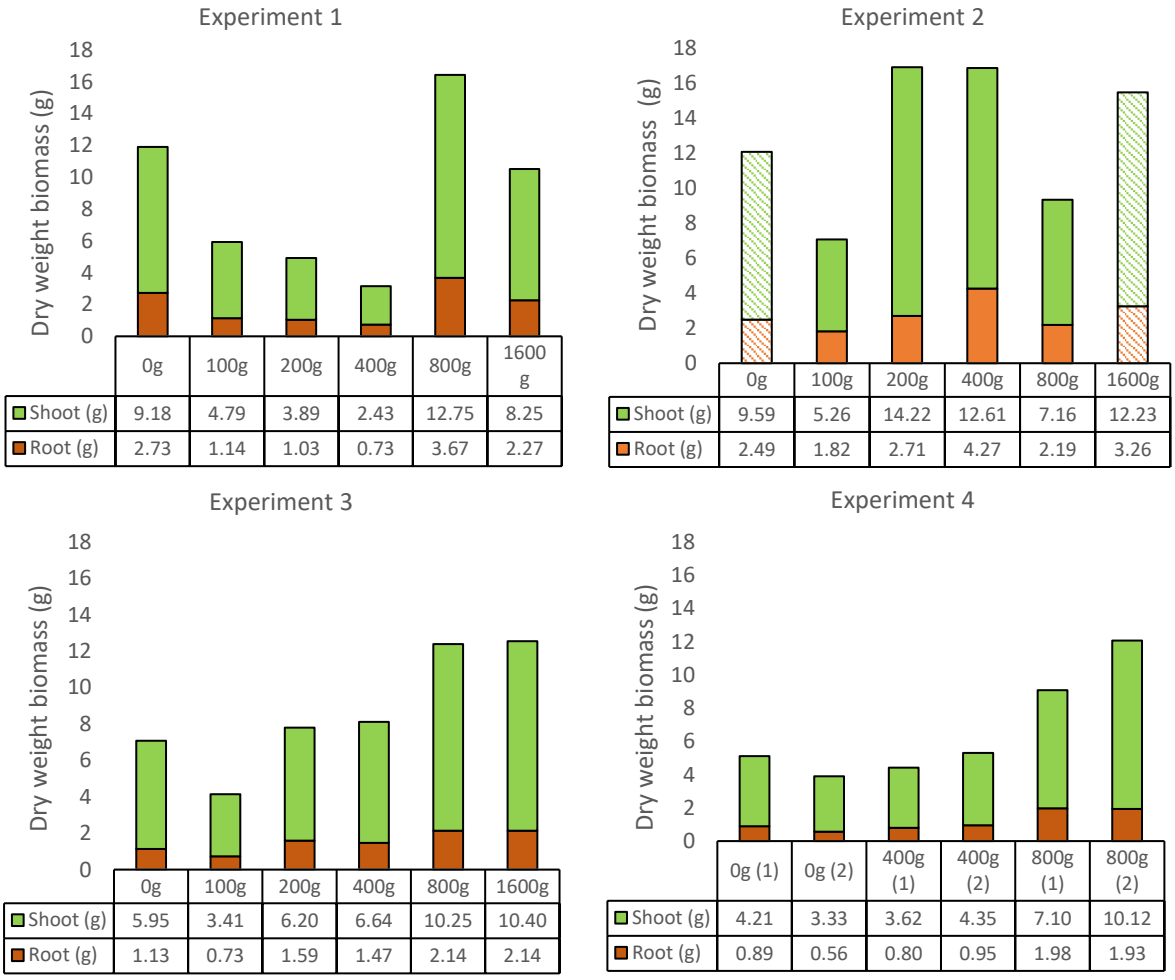


Figure 5.12- Total plant dry weight biomass of roots and shoots by box biosolid application for each experiment. Data from experiment 2 contains estimated dry weight from ImageJ analysis and is highlighted with a shaded fill.

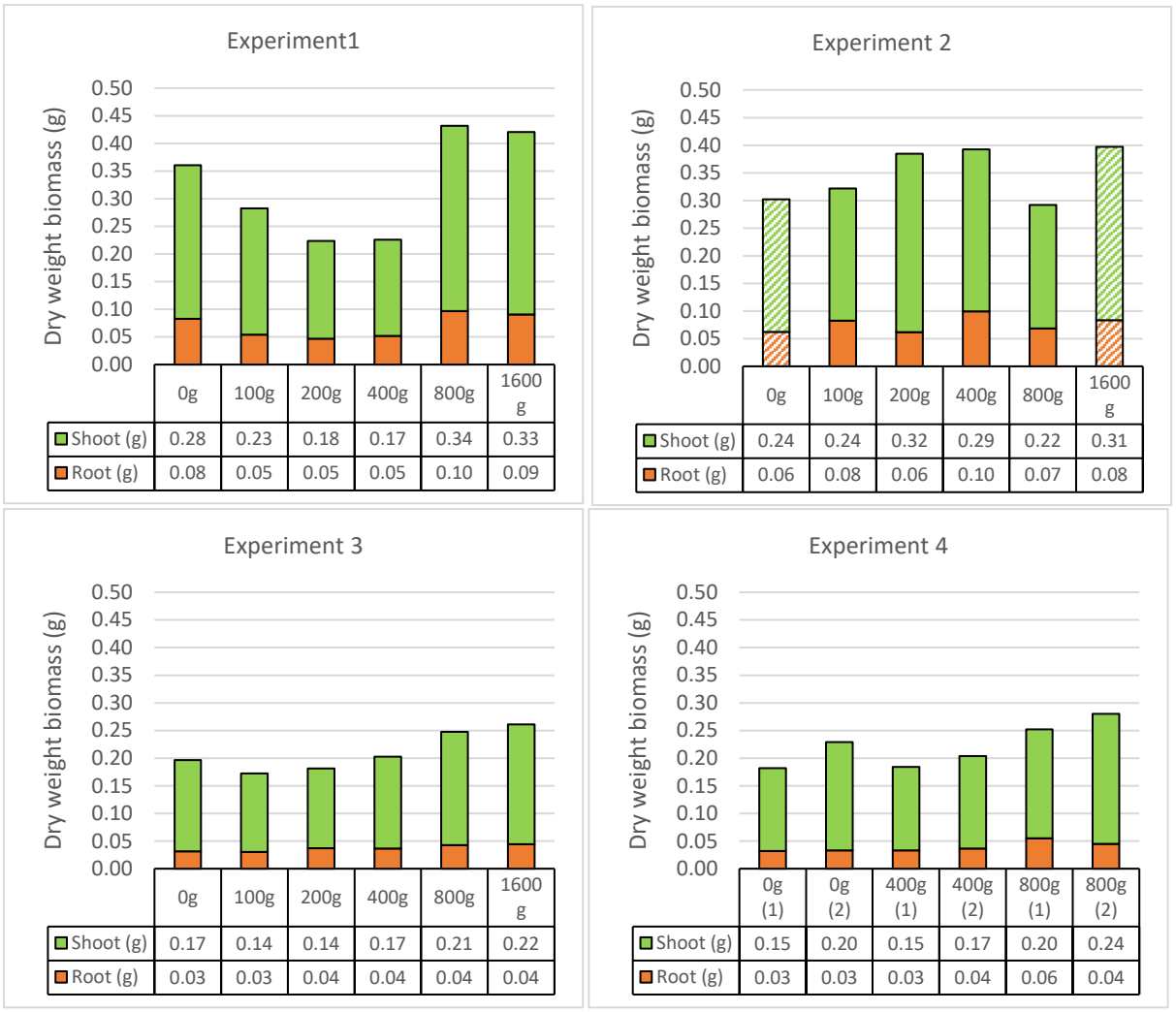


Figure 5.13 - Mean dry weight biomass of roots and shoots per plant by box biosolid application for each experiment. Data from experiment 2 contains estimated dry weight from ImageJ analysis and is highlighted with a shaded fill.

5.4 DISCUSSION

5.4.1 Biosolid impact on plant establishment

A slight trend in increased establishment with increased biosolid application was observed in experiment 1, which showed a gradual increase from 52 established plants in the 100g applied box to 58 plants in each of the 800 and 1600g applied boxes. In subsequent experiments increased rate of biosolid application did not appear to improve crop establishment in most cases. However, control soils in experiments 1 and 4 had notably lower establishment than all biosolid-applied soils across experiments. 40 plants established in experiment 1, and 29 and 27 plants established in the experiment 4 controls. This was compared to a 51-plant average across all other boxes. Seed germination is based on temperature being high enough (above 0-5^{o}C) and the presence of water and oxygen (Soffe, 2003). Differences in germination were therefore unlikely to be due to these factors, as temperatures were maintained above 20^{o}C in all experiments and all boxes received the same watering regime to maintain field capacity in soils. The differences in crop establishment between control and biosolid-applied soils in experiments 1 and 4 were possibly due to structural differences leading to difficulties with coleoptile emergence. As mentioned in section 5.3.1, differences in water infiltration into the soil surface of the control soils was slower than that of biosolid-applied soil and the high silt content of the soil led to capping in places. Capping was observed as a crust forming on the soil surface, with gaps between soil aggregates becoming obscured and filled by silt particles. This capped soil crust is more difficult for coleoptiles to break through (GRDC, 2018) and could lead to the death of newly germinated seedlings before emergence. A wet, capped soil may also have had restricted oxygen flow in the soil structure and prevented germination in some seeds. The soils for experiments 1 and 4 were also both collected in October, shortly after field cultivations had occurred. Though soils were sampled from the field by auger and thoroughly mixed in all boxes before seed planting microstructural differences may still have been present. The structure of the soil may have been degraded by cultivation in the short term making those soils more susceptible to capping. Experiment 2 and 3 soils which were sampled later in the growing season and may have had a more stable soil microstructure than the freshly cultivated soils. This could explain why establishment differences were not observed in experiments 2 and 3. In soils with degraded structure, or in soil conditions where establishment is poor, biosolid applications appear to counteract this negative effect, even at low application rates. This is supported by the good establishment observed in all biosolid applied soils, including the 100g applied boxes, over the poorly established control soils. However, the predominant reason for this effect cannot be clearly understood with the limited data collected in this project.

The quality of the seeds used in the experiments may have also played an important part in the crop establishment across experiments. A high-quality seed barley was sourced for the experiments and to minimise potential impact of deterioration seeds were visually assessed for defects prior to planting and subsamples were established in separate pots two weeks before the experiments to ensure seeds were still viable. The same seed stock was used throughout all experiments for two years. Although seeds were refrigerated in an air-tight container for storage some deterioration may have occurred. After the first two experiments seed germination trials were carried out in pots under the same conditions as the experiments to compare the existing seeds to a newly acquired stock. Five seeds of each original seed and new were planted in three pots of soil and the original seed outperformed the new, still having an average germination >90%. Therefore, the use of the original seed stock was continued for subsequent experiments. Despite these precautions small differences in seed quality may still have occurred across experiments. Deteriorated seed embryos have been associated with slower germination and seedling emergence, and reduced root and shoot growth (Harrison, 1977). The slight trend observed which correlated to biosolid application in experiment 1 may have been due to the freshness of the seeds used. Deterioration of seed quality in subsequent experiments may have masked potential small biosolid-induced differences. Seedlings from weaker seed embryos that germinated in experiment 1, which could establish due to increased nutrient availability in biosolid-applied soils, may have deteriorated beyond being able to successfully establish in later experiments. This lack of weaker seeds establishing, with only the healthier seeds able to grow, could have led to the more even crop establishment observed across boxes in later experiments. Further deterioration of the seeds may also account for the difficulty in achieving even crop establishment across boxes in experiment 4. The control and 400g boxes all required supplementation of more than 20 extra seeds after 7 days to try to achieve an average established plant count comparable to previous experiments. In the 400g applied boxes the supplemented plant counts were close the 51-plant target, but in control boxes the plant counts were still below 30, the lowest of any boxes across all experiments. This was a high enough plant count to continue with the experiment successfully, but the poorer establishment was notable.

5.4.2 Plant growth during initial 28-day period

No significant differences in plant heights between biosolid treatments were observed at any stage of the initial growth in any of the experiments. The low end of the distribution in the two control boxes in experiment 4 showed an increase in interquartile range but not in overall distribution. This difference was however due to the second round of seed planting to even out plant counts. The greater number of short plants at the low end of the distribution was due to the greater proportion of new seedlings when the measurements were taken, rather than any differences between crop

performances. This lack of any difference was expected as barley plant height is mainly determined by variety and season rather than nutritional differences in the soil, unless conditions are very poor (AHDB, 2018). Plant height was therefore not affected by biosolid application at any point of the initial 28-day growth period of these experiments.

5.4.3 Plant flood survival

Survival percentage of plants after flooding varied from as low as 26% up to 100% across all soils, with no consistent discernible trend in variation. The control box plants in experiment 1 survived the flood well with 84% of the pre-flood plants still living post-flood. Biosolid-applied soils in experiment 1 by comparison had only a 26-44% survival in most boxes, with a moderate 65% of plants surviving in the 800g box. This led to an early theory that biosolid application may be hindering flood survival, as plants provided with more abundant nutrients in the soil did not grow as large a root structure. A smaller root structure may then have led to difficulty sourcing oxygen from the soil, leading to greater crop stress. Higher biosolid applications (800g-1600g) then appeared to be producing very healthy plants which also survived flooding well due to an abundance of nutrients. However, flood survival results from experiments 2 and 3 did not support this presumption as many of the biosolid-applied boxes outperformed the control boxes, with some low rates also outperforming high rates of biosolid application. In experiment 4 flood survival was again highest on average in the control boxes with 100% and 63%, with moderate performance in higher rate biosolid-applied soils (800g). The similarities between experiment 1 and experiment 4 control boxes were discussed in section 5.4.1, with both having lower established plant numbers than all other boxes. This variation in establishment was speculated to be due to differences in germination in poorer soils and this could also account for crop survival. If only the strongest seeds germinated in the poorer, unamended soils of the control boxes then the established plant would be hardier and therefore more resilient to flood stress. Weaker seedlings which established in biosolid-applied soils with the aid of increased soil nutrients and SOM may have been more susceptible to flood stress and die off post-flood. If this is the case then biosolid application does not appear to have improved flood survival in the barley crop based on the data from the limited plant numbers in these experiments. Plant counts may be affecting flood survival, but it is more likely that establishment of strong plants is the main contributor to flood resilience.

Total crop DWB appeared to accurately reflect the total crop growth observations made in all boxes across experiments (Fig. 5.12). Due to differences in crop establishment and flood survival rate the total DWB of each box is not a good indicator of plant performance however, so an average DWB per plant was calculated to correct for these factors (Fig. 5.13). There was a trend towards increased per plant biomass with increased biosolid application, and high rate biosolid-applied soils (800g-1600g)

in experiments 1, 3 and 4 outperformed all other boxes. The average per plant DWB in 100g boxes in experiments 1, 3 and 4 was 0.15g, with 200g and 400g at 0.20g, but with 800g and 1600g boxes ahead with 0.30g and 0.34g respectively. Per-plant DWB and biosolid application was found to significantly correlate in the biosolid-applied boxes in these experiments with Spearman's $r = 0.61$, $p = 0.02$. When including experiment 2 data the 800g and 1600g applied crops still showed higher DWB per plant than 100g-400g applied plants which averaged 0.24-0.26g per plant, with 800g at 0.30g and 1600g at 0.36g. However, this relationship across all experiments only approached significance with $r = 0.42$, $p = 0.073$. Control boxes in all experiments appeared to follow a separate trend to biosolid-applied boxes. In experiments 1, 3 and 4 the controls outperformed most 100g-400g applied boxes, with an average 0.24g per plant DWB, but this was possibly due to the aforementioned establishment differences in section 5.4.1, with only strong plants successfully establishing on poorer soils. Experiment 2 did not display any trend observed in the other experiments, with a high average DWB per plant across all boxes.

The high plant DWB in experiment 2 and in high rate biosolid-applied soils appeared to correlate closely with the measured Olsen available P in soils (Fig.4.4). Experiment 2 soils had higher Olsen P than other experiments due to a prior mineral P application as discussed in section 4.4.1, and high rate biosolid-applied soils also had higher P from the biosolid added. The stimulation of root development by P and its importance in energy transfer and nitrogen use efficiency in plants does mean that higher available P could result in higher plant biomass and better flood resilience during the experiments. Exploratory data analysis was carried out with plant biomasses and mean measured values per box of Olsen P, TKN and SOM to assess whether they had any significant relationship. Soil Olsen P was found to significantly correlate with plant biomass across all experiments, Spearman's $r = 0.57$, $p = 0.004$. SOM ($r = 0.35$, $p = 0.097$) and TKN ($r = 0.32$, $p = 0.130$) were not significantly related to plant biomass. However, as discussed in section 4.4.2 TKN and SOM were more closely related to biosolid application rate than Olsen P. These results would indicate that soil Olsen P, either from biosolid application or present in the soil from mineral applications, is influencing crop DWB more than the combined factors of the biosolid application. However, the extent of this influence cannot be fully determined from the collected data.

5.5 CONCLUSIONS

Biosolid applications appeared to improve crop establishment in poorer soils where structure was degraded. The increased SOM from applied biosolids leading to better water infiltration and reduced capping in silty soils may be the reason for this. Even very low biosolid applications (100g) provided enough impact to remedy low crop establishment found in control soils. Further improvements to crop performance including flood survival and DWB were confounded by possible differences in seed quality and establishment so could not be accurately determined. However, DWB of plants was significantly correlated to soil Olsen P. This improvement in performance was present regardless of where soil P stocks originated from, whether already present in the soil or deposited through biosolid application. High soil P from high biosolid applications (800g-1600g) did appear to consistently improve crop DWB over the course of the experiments, including the 10-day flooding period. From these results it can be suggested that biosolid could offer a solution to remedy soils with poor establishment of barley, and in cases where soil available P is limited can aid in maintaining crop health through a short-term flood.

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Chapter 6: MICROBIOLOGICAL RESPONSE OF BIOSOLID-AMENDED SOILS TO SHORT-TERM FLOODING

6.1 INTRODUCTION

Biosolid application to arable soils and its response to short-term flooding has several important effects on the geochemical processes and crop responses as discussed in previous chapters. One area which requires extra focus for understanding the entire flooded biosolid-applied soil system is the microbial response of soil bacterial communities to both the application of biosolid and the subsequent flood event. The metabolisms of soil bacteria are wide ranging and control many soil processes, such as the form and transport of nitrogen and phosphorus, the decomposition of SOM, and oxidation-reduction reactions in soil and water. Understanding the response of the microbial communities is therefore essential to understanding the mechanisms governing the soil environment.

Soils and biosolids are highly dissimilar materials both chemically and microbiologically, hailing from very different environments. Biosolids, as a sewage sludge derived digestate, have bacterial communities resulting from the extreme environment of elevated temperatures and restricted O_2 within anaerobic digesters. This environment is designed to digest organic matter (OM) and to kill pathogenic bacteria from the original sewage sludge input, though concern has recently been raised about the effectiveness of this process (Zhao and Liu, 2019). The bacterial communities of soils on the other hand can vary greatly depending on climate, management history and a wide range of other environmental factors. Different moisture and temperature ranges can alter the size and proportions of dominant bacterial species present and their level of activity (Magdoff and Weil, 2004; Lu et al., 2018). Cropping or vegetation history can alter which species are present or favoured in soils, for instance through symbiotic relationships with plant roots (Buckley et al., 2006; Harman and Uphoff, 2019; Li et al., 2019). Application of different organic inputs can alter which decomposers dominate depending on the source and form of the OM (Möller, 2015; Wang et al., 2015; Xun et al., 2018). The activity of bacteria involved in the nitrogen cycle can be affected the forms of N present and the environmental conditions (Möller, 2015; Han et al., 2018; Norton and Ouyang, 2019). Contamination of soil can also lead to the reduction of microbial diversity due to toxicity or can lead to the presence of specialised bacteria which can metabolise trace or toxic elements (Chien et al., 2008; Feng et al., 2018). Many factors can therefore have a significant effect on soil microbial populations. The introduction of biosolid bacteria to soil bacteria communities

could have a range of effects depending on the characterisation of both materials and the environment in which they exist.

The introduction of biosolid as a nutrient-rich organic input to the soil could act as an energy source to native soil bacterial communities and allow the proportional increase of bacteria which favour the added nutrients. It could also introduce new bacteria in the material and provide the nutrients and environment needed for those bacterial species to survive in soils. The additional factor of flooding will also change soil environmental conditions leading to further changes. Flooding would normally result in a restriction to the metabolisms of many soil bacterial species, which mainly rely on respiration, due to limitations in O_2 availability from water inundation (Ponnamperuma, 1984; Magdoff and Weil, 2004). Any additional biosolid bacteria which may have persisted in the soil after application could then favour these flooded soil conditions and be able to multiply if the soils become anaerobic. Soil conditions would then more closely resemble the anaerobic digester environment from which biosolid bacteria originated and allow them to thrive. If any pathogenic bacteria survive the anaerobic digestion process and remain viable in biosolids then their possible multiplication and transfer to wider environments through floodwaters could be a cause for concern. The introduction of new non-pathogenic species from the anaerobic digestion process to new environments could also have unknown consequences if they upset the equilibrium of existing environmental microbial communities. The potential for such effects therefore needs to be explored.

The accurate and detailed characterisation of the microbial communities of both biosolid and soil is required for fully assessing and understanding the impacts of biosolid application and flooding. Quantification methods or identification of microbial metabolisms such as phospholipid-fatty-acid analysis, basal respiration rates, respiratory responses or enzyme activity levels, while valid methods, will not allow the detailed identification of microbial population required to gain a detailed picture of the impacts on the soil system of biosolid application and flooding. Next generation sequencing (NGS) techniques can offer a solution to this, using the information contained within the hypervariable 16s rRNA gene of bacterial DNA to determine its identity (Sanschagrin and Yergeau, 2014). The recent availability and fast advancement of high-throughput NGS technologies means that more in-depth and broader ranging microbial characterisation data is now continually being explored (Levy and Myers, 2016). NGS produces a large amount of DNA data from samples and would allow for the identification of a high proportion of the microbiological population of soil samples. The high data volume gained from NGS on soil samples allows for detailed bacterial diversity metrics to be gained and statistical analyses to be run to further understand changes to bacterial populations. Alpha diversity metrics, that is the population diversity within samples, can be

obtained using Hill numbers adapted for the high volume of data from NGS analysis (Hill, 1973; Chao et al., 2010; Kang et al., 2016). Various Beta diversity metrics exist which can determine diversity between samples and present multidimensional data from a range of variables. The relative abundance of different bacteria can be identified and assessed using a taxonomic reference database to gain a broad overview of the microbial communities present in the samples. Further investigation can then be carried out to identify different individual bacterial species and determine their influence in the wider soil environment. NGS could therefore allow for a detailed picture of bacterial communities in the soils to be built and show the detailed impacts of biosolid application and flooding on those communities assessed.

A wide range of previous studies utilising NGS have been carried out relating to the soil microbiome and the impact of various effects, including biosolid applications and water inundation in various forms. Recent studies show soil bacterial communities can include the dominant phyla of *Acidobacteria, Proteobacteria, Chloroflexi, Verrucomicrobia, Planctomycetes, Bacteroidetes* and *Actinobacteria* across different environments (Miyashita, 2015; Zeng et al., 2016; Hou et al., 2017; Feng et al., 2018; King and Henry, 2019). In submerged anaerobic soils such as river sediments, rice paddies or wetlands the dominant phyla appeared to shift towards increased *Proteobacteria* abundance, with *Acidobacteria, Bacteroidetes, Chloroflexi* and *Planctomycetes* also appearing as dominant phyla, as well as some cases of *Firmicutes* (Breidenbach and Conrad, 2015; Pittol et al., 2018; Vidal Dura et al., 2018; Huang et al., 2019). Seasonally flooded or intermittently flooded soils have varied results depending of flood type and duration but in general demonstrated little change to the relative abundances of taxonomic groups, showing soil communities may be resilient to flooding (Randle-Boggis et al., 2018; King and Henry, 2019). Anaerobic digester bacteria and their resultant digestate contained the dominant phyla of *Firmicutes*, *Bacteroidetes* and in some cases *Synergistetes* (Sun et al., 2015a; Li et al., 2016; Liu et al., 2016; Jiang et al., 2019). Interestingly, *Firmicutes* were present in both digestate and in some river sediments, hinting they could survive in flooded soils. The differences in bacterial composition between soils and biosolids are clear, even when only observing populations at the phylum level. Few studies have looked at applications of digestate to soils but in those that have results show that the main influence from the digestate input appears to be from the nutrient addition. Furthermore, native soil bacteria still dominate, are very resilient to change and return to equilibrium over time (Sapp et al., 2015; Podmirseg et al., 2019). Further study is needed into the effects on agricultural soil bacterial populations from biosolid application. Additionally, the coupled effect of flooding and biosolid application could lead to conditions favouring any bacteria which persist in the soil from the biosolids. This potential threat to soils and surface waters needs urgently addressing.

6.2 MATERIALS AND METHODS

DNA sampling for characterisation of soil and biosolid bacterial communities took place in experiment 4 which was tailored towards gaining the most reliable results possible for microbial characterisation. Growth boxes were set up using control boxes, 400g and 800g biosolid applications in duplicate. This box setup allowed for double the number of samples to be taken for each application and insured against any problems which might occur during the experiment or analysis. The recovery time after flooding in experiment 4 was set to 20 days to show microbial population recovery over a time period double the length of the 10-day flood. This 20-day recovery was as opposed to the usual 28-day recovery in experiments 1 and 2 which focused on an equal growth and recovery period for the crop.

6.2.1 Sampling

Before the initiation of the experiment samples of the initial soil and biosolid were taken. To provide an accurate representation of each fresh material the samples were taken randomly from various locations within the material immediately after collection. A sterile spatula was used to collect 5 samples of each the soil and biosolid and place them into a 2ml centrifuge tube. The tubes were then placed inside secondary containment and stored at -20^{o}C for later analysis.

Samples for microbial analysis of the growth box soils were taken at the same three stages that the soil samples were taken for geochemical analysis: Immediately pre-flood, immediately post-flood and final samples at the end of the recovery period. The holes created in the soil by the 2.5cm diameter core auger when collecting samples for the soil nutrient analysis were used to access the entire 15cm depth soil profile in the boxes. Disposable, sterile spreading loops were used to scrape small amounts of soil from the inside of the holes left by the auger. The samples were then placed in 2ml centrifuge tubes which were put inside secondary containment and frozen at -20^{o}C for later analysis. Three samples were collected from each box at each sampling stage in this way.

6.2.2 Extraction

Microbial genomic DNA was extracted from the soil and biosolid samples (~0.25 g) using a DNeasy PowerSoil Kit (QIAGEN Ltd, Product ID: 12888-100). To extract DNA fragments in the size range of 3kb ~ 20kb electrophoresis was carried out using a 1% agarose 1x Tris-borate-EDTA (TBE) gel stained with ethidium bromide (EtBr) for viewing under UV light. During the electrophoresis 25 volts was passed through the agarose gel for 20 minutes, then 50 volts was run through the gel for 60 minutes and finally 30 volts for 30 minutes. The gel containing the desired DNA was cut out and was extracted using a QIAquick gel extraction kit (QIAGEN Ltd, Product ID: 28704), with final elution carried out using a 1/10th strength elution buffer. DNA concentration was quantified using a Qubit™

dsDNA HS Assay Kit (Invitrogen, cat no.: Q32854) and a Qubit® 2.0 Fluorometer (Invitrogen, cat no.: Q32866). The manufacturer's instructions for each kit were followed precisely unless otherwise stated.

20µl of the extracted DNA samples in aqueous solutions ranging in concentration from 0.8 to 35.4 ng/µl were sent to the University of Liverpool Centre for Genomic Research (CGR) for paired-end (2x250bp) sequencing on the Illumina MiSeq platform. Illumina adapters and barcodes were attached to the DNA fragments in a 2-step PCR amplification to target the hyper-variable V4 region of the 16s rRNA gene of bacteria as per Caporaso et al. (2011). The target specific part of the primer sequences used were the 515F (FWD:GTGYCAGCMGCCGCGGTAA) (Parada et al., 2016) and 806R (REV:GGACTACNVGGGTWTCTAAT) (Apprill et al., 2015).

6.2.3 Data processing

After sequencing the raw Fastq files were trimmed by the CGR for the presence of Illumina adapter sequences using Cutadapt version 1.2.1 (Martin, 2011). The option -O 3 was used, so the 3' end of any reads which match the adapter sequence for 3 bp or more were trimmed. The reads were further trimmed using Sickle version 1.200 (Joshi and Fass, 2011) with a minimum window quality score of 20. Reads shorter than 20bp after trimming were removed.

The UPARSE pipeline (Edgar, 2013) within the USEARCH software package (version 11) (Edgar, 2010) was used for further downstream analysis of the paired-end reads. First, overlapping paired-end reads were merged together using the *fastq_mergepairs* command. Next the primer sequences were stripped, and the reads were truncated to 250bp using the *fastx_truncate* command. The *fastx_truncate* command performs the dual function of discarding any paired-end reads which were shorter than the target length and truncating the remaining reads to the 250bp length desired. Any partial reads were therefore discarded, and any longer, poorly merged reads were shortened to the target 250bp of the V4 region of the 16s rRNA. The low quartile, median and high quartile lengths of the untruncated reads were all 253bp. However, 250bp was selected as the length of truncation to preserve reads which may have been missing only a few characters but were still valid. Only 0.2% of reads were discarded due to insufficient length, which was a very low and acceptable level of loss. After truncating the samples were quality filtered using the *fastq_filter* command with an expected error of 1.0. Samples were then de-replicated and relabelled using the *fastx_uniques* command and all reads were pooled. Clustering and chimera filtering of reads was then carried out simultaneously using the *cluster_otus* command, with a minimum abundance of 2 reads used to eliminate singletons and a sequence identity threshold of 97% used to define operational taxonomic units (OTUs).

An OTU table was generated using the *otutab* command, mapping the previously filtered reads to the OTUs. Filtered reads were used rather than unfiltered merged reads as >96% passed through to the filtering stage, meaning a very high proportion of reads were maintained and were known to be high quality. The discarded reads from truncating and filtering were assumed to be low quality or misreads and so were not mapped to the OTU table. The *sintax* command was applied to the OTUs for taxonomical assignment using the RDP 16s rRNA training database v16, with the *-sintax_cutoff* option set to 0.8 for a confidence cut off of 80%. However, this database provided a high number of unassigned OTUs (>20%) and so a larger reference database was deemed necessary. The *sintax* command with a *-sintax_cutoff* option set to 0.8 was then used within the VSEARCH software package (Rognes et al., 2016) to process the larger SILVA 16s rRNA database v123 and define taxonomies. >99% of OTUs were successfully defined, with most of the defined taxonomies matching what was found in the RDP training set definitions. The OTUs which were previously unassigned using the RDP database were defined with high confidence using the SILVA database to taxonomies not included in the RDP training set. A review of the taxonomies which were newly defined, coupled with the high degree of confidence with which they were defined, led to the conclusion that the SILVA taxonomy definitions were accurate and could be reliably used going forward. After successful definition of taxonomies any OTUs which did not have a confidence value of at least 0.7 at bacterial phylum level or any which were classified as *Archaea* were discarded and not included in the diversity or statistical analysis.

6.2.4 Statistical analysis

Hill numbers (D_q) were used to determine bacterial diversity within all samples (Hill, 1973). Hill numbers allow for the proportional representation of diversity within samples by weighting taxa by abundance. This method compensates for differences in sample size and accounts for rare taxa by differentially weighing them, therefore allowing comparison of diversity between samples. The approach of using Hill numbers has been shown as a reliable estimation of diversity for NGS bacterial communities (Chao et al., 2010; Kang et al., 2016). The command *alpha_div* was used in the USEARCH software on the OTU table to provide alpha diversity metrics for all samples. The OTU richness (D_0), common OTUs (D_1, equivalent to the exponential of Shannon entropy) and dominant OTUs (D_2, equivalent the inverse of Simpson concentration) were used to characterise the samples.

Beta diversity metrics were obtained using the *beta_div* command in USEARCH to determine the relative differences between individual samples. Non-metric Multidimensional Scaling (NMDS) analysis was then carried out using RStudio (version 1.2.5001)(RStudio Team, 2019) and the 'vegan' package (Oksanen et al., 2013) to graphically represent the dissimilarity of bacterial samples in a 2-dimensional space using the pairwise Bray-Curtis distances. The full OTU table containing the

abundance of all OTUs in all soil and biosolid samples was input as a matrix into RStudio for NMDS analysis. After an initial run to generate an NMDS plot and with the Bray-Curtis data gained from the USEARCH *beta_div* command it was apparent that the biosolid samples were too dissimilar to the soils to be represented on the same plot without losing the ability to view differences between soil samples. The OTU table was then uploaded with the biosolid sample data omitted and NMDS analysis carried out on only the 59 soil samples to view differences between biosolid applications and flooding effects on the soils.

To investigate the relationship between soil properties and microbial communities Spearman's rank correlation was carried out on soil phyla abundances and the values of soil TKN, Olsen P, SOM and pH from all soils. The soil in each box was randomly sampled separately for geochemistry and microbial analysis, so there was no way to directly compare individual samples. Therefore, mean values for the geochemical factors and microbial populations for each box were used for the Spearman's rank correlation analysis.

6.3 RESULTS

6.3.1 Sample DNA yield

The DNA yield was calculated for each sample based on the Qubit quantification of DNA concentration in the samples, the final extractant solution volume (30 µl) and the weight of the sample used for extraction (Table 6.1). Biosolid samples had a much higher DNA yield than soil samples with a mean of 237.07µg g^{-1}. Soil DNA yield varied considerably across samples but 800g boxes yielded higher than other applications with a mean of 51.43µg g^{-1}, compared to 28.63µg g^{-1} in 0g control boxes and 28.19µg g^{-1} in 400g applied boxes. No trends in DNA yield were observed based on sample timing. All boxes yielded higher DNA concentrations than initial soils which had a mean yield of 16.74µg g^{-1}. DNA recovery was not limited by the DNeasy PowerSoil Kit or QIAquick gel extraction kit, which had maximum filter DNA recovery capacities of 20µg and 10µg respectively. The highest total DNA volume in any of the samples was calculated as being 1.06µg.

Table 6.1 - Extracted DNA yield of all soil and biosolid samples.

Samples	DNA yield (µg DNA/g soil or biosolid)				
Biosolid	259.74	256.67	304.20	225.78	138.96
Initial Soil	10.88	17.33	11.59	35.70	8.20
Pre 0g (1)	23.46	24.11	10.42		
Pre 0g (2)	21.51	53.14	33.95		
Pre 400g (1)	6.77	34.07	27.61		
Pre 400g (2)	33.43	16.46	19.36		
Pre 800g (1)	50.61	22.61	34.29		
Pre 800g (2)	55.03	44.97	33.24		
Post 0g (1)	51.72	25.77	59.63		
Post 0g (2)	30.93	25.72	24.81		
Post 400g (1)	18.95	18.42	23.57		
Post 400g (2)	39.70	71.75	23.02		
Post 800g (1)	52.93	33.97	24.30		
Post 800g (2)	75.67	76.84	60.92		
Final 0g (1)	7.82	9.96	51.12		
Final 0g (2)	8.00	9.22	44.09		
Final 400g (1)	17.55	58.10	34.34		
Final 400g (2)	29.06	14.46	20.83		
Final 800g (1)	52.79	82.66	33.16		
Final 800g (2)	26.84	84.23	80.61		

6.3.2 DNA sequencing results

The Illumina MiSeq sequencing yielded between 44,924 and 364,401 reads per sample with a mean of 167,421 and a total of 10.7m reads across all samples. Approximately 10.3m reads passed through the quality control and filtering pipeline from the original reads, giving a recovery >96%. These sequences were clustered using a >97% similarity sequence identity and allocated to 4,744 OTUs. When assigning OTUs to taxonomic groups >92% were mapped successfully, preserving 9.5m of the filtered reads. The sequences per sample mapped to OTUs were between 39,738 and 324,696 reads per sample with a mean of 145,416 reads. The sequence count distribution per sample for the filtered reads is illustrated in Fig 6.1 alongside the original Illumina MiSeq sequence sample distribution. Of the reads which were allocated to OTUs 155,130 were classified as *Archaea* (1.63%) and 5,448 were not classified to the bacterial phylum level with a confidence of at least 0.7 (0.42%). These OTUs were discarded and excluded from further analysis.

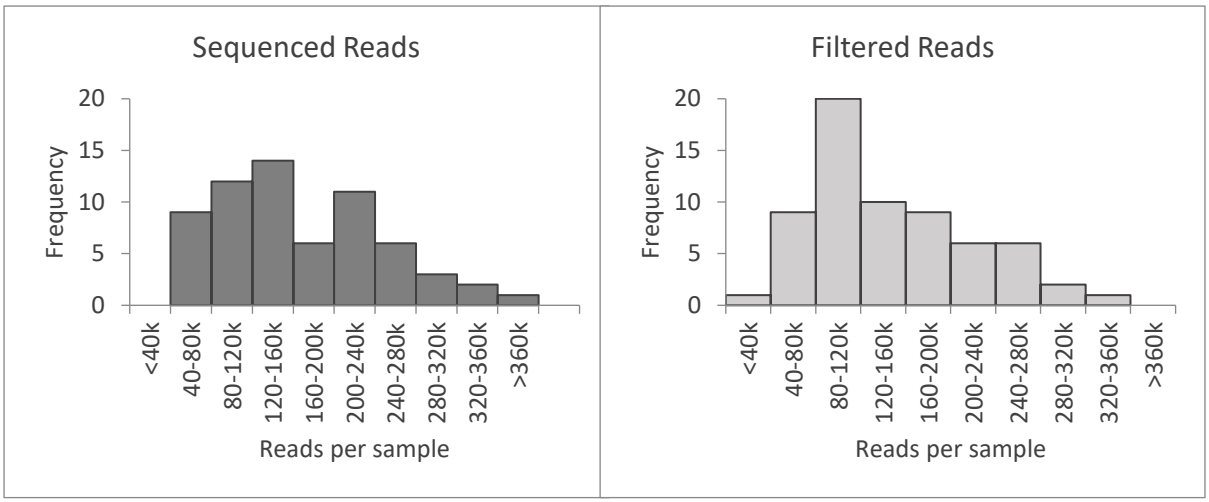

Figure 6.1 - Histograms displaying the distribution of total sequenced read length and total read length reads after the filtering pipeline in all samples.

Despite the large spread in number of reads yielded from each sample all samples were sufficient in size for downstream analysis. Rarefying reads for analysis was an option for normalising the data to a base count of the smallest sample size. However, this would discard a very large amount of data to satisfy the sample with lowest number of reads and has been questioned as an inefficient approach for microbiome data processing (McMurdie and Holmes, 2014). Therefore, to account for this variation in sample size Hill numbers were used as opposed to Shannon and Simpson indices directly.

6.3.3 Bacterial community composition and diversity

Across the soil samples the 20 most abundant of the 4,548 bacterial OTUs accounted for >20% of sequences, and >50% of total sequences were contained within the top 136 most abundant OTUs. The biosolid samples were composed of 527 OTUs with the top 3 most abundant OTUs accounting for >24% of all sequences and >50% of total sequences contained within the 13 most abundant OTUs. OTUs were characterised into 38 different phylum or groups using the USEARCH software and the SILVA (v123) database. Figure 6.2 illustrates the mean Alpha diversity differences between sample groups, with biosolid sample diversity being considerably less than all soil samples. Soil samples showed only very small differences in diversity measures at D_0, D_1 or D_2 regardless of biosolid application or flooding. A slight trend was observed based on biosolid application, with the mean D_0 results for the three time points showing an increase from 2730 species in 0g control soils to 2884 and 2923 in 400g and 800g biosolid-applied soils respectively. D_1 saw an increase from 638 species in control soils to 690 and 664 in the 400g soils and 800g soils and D_2 saw an increase from 212 species in control soils to 230 and 221 in the 400g and 800g soils. This may indicate a slightly

greater species diversity in biosolid-applied soils than in untreated soils. The lower D_0 richness score observed in the 'Final 0g' samples is reflective of the lower DNA recovery in four of the six samples than in samples from other groups.

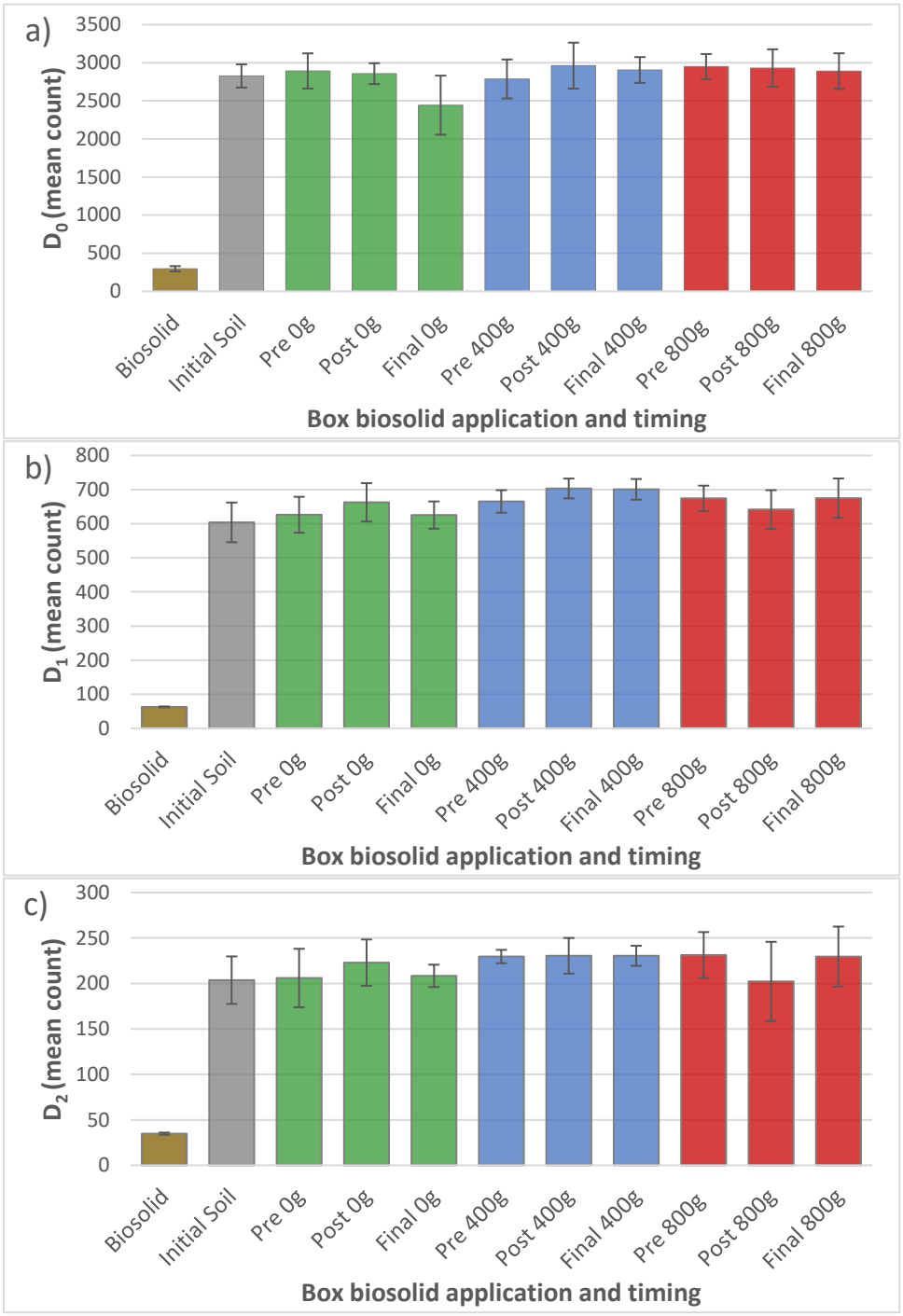

Figure 6.2 - Mean microbial community Alpha diversity within samples measured using Hill numbers. Error bars indicate standard deviation. Biosolid and initial soil results are based on the five (5) samples of each material. Box applications are based on six (6) samples from both boxes containing the respective biosolid application and at the stated sample timing (pre-flood, post-flood or final): a) Richness of species (D_0), b) Common species (D_1), c) Dominant species (D_2).

On average >51% of all soil reads comprised of the two phyla *Acidobacteria* (28.40 ± 3.57%) and *Proteobacteria* (22.87 ± 4.14 %) which were barely present in biosolids, with both accounting for just 0.62% of reads. *Chloroflexi, Planctomycetes* and *Actinobacteria* were then the next most abundant phyla in soils and along with *Acidobacteria* and *Proteobacteria* accounted for >78% of all soil taxa. Biosolid phyla were dominated by *Firmicutes* (37.60 ± 0.74%) followed by *Bacteroidetes* (13.16 ± 0.67%), *Synergistetes* (12.92 ± 0.95%), *Saccharibacteria* (6.75 ± 1.24%) and *Atribacteria* (6.60 ± 0.35%), together accounting for >77% of biosolids taxa. A summary of the mean proportional bacterial phyla across all samples is displayed in Fig. 6.3. Full results of the relative abundances of bacterial phyla in each sample are included in Appendix E.1.

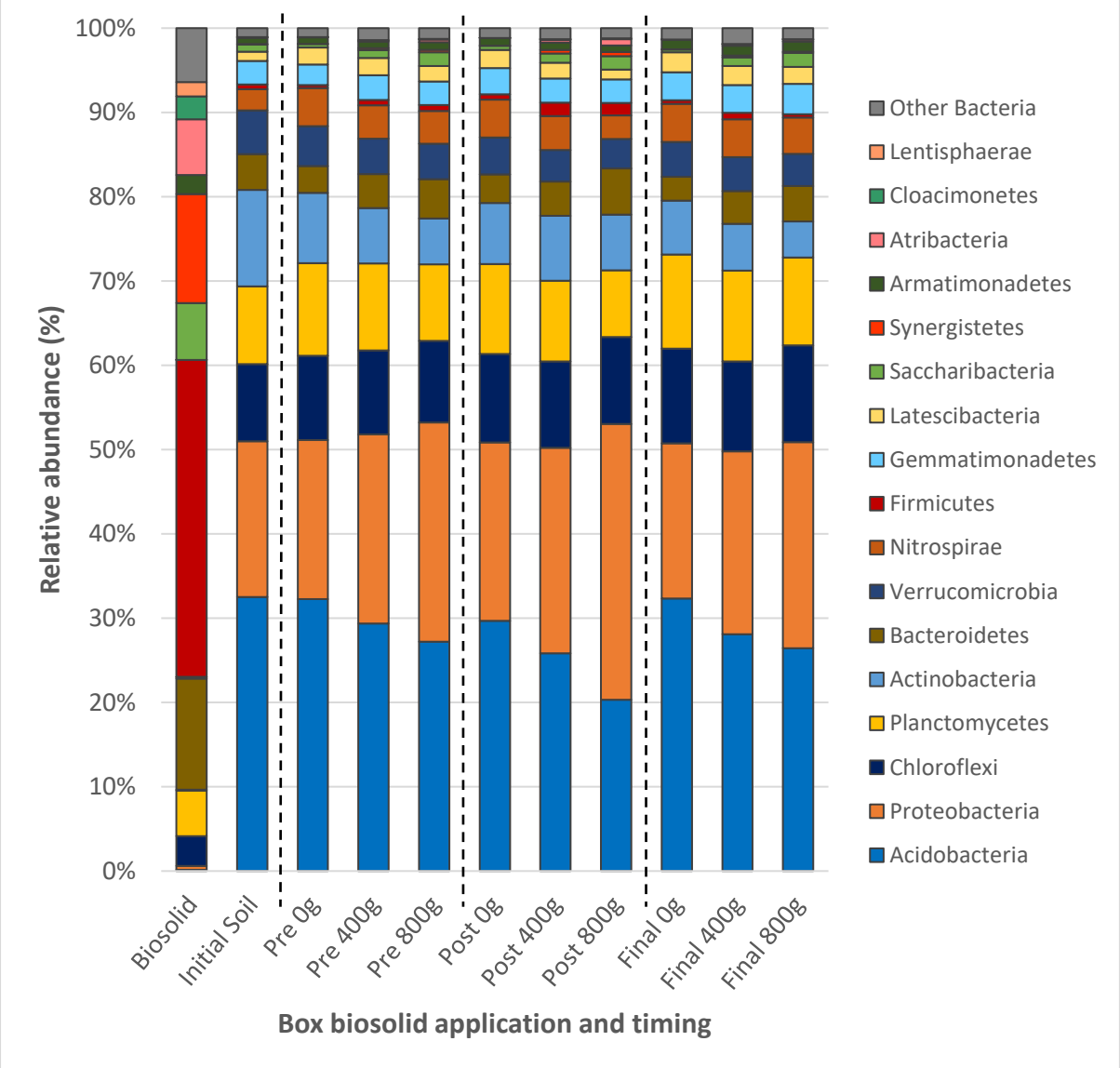

Figure 6.3 - Average taxonomical composition of samples. Biosolid and initial soil are the mean results from the five (5) samples of each material. Timing and biosolid application rate results represent the mean of the six (6) samples from both boxes treated at each rate from each sampling timing. Taxonomies are ordered bottom to top by mean abundance across all samples. Taxonomies with <1% abundance in any sample after averaging are grouped with 'Other Bacteria' alongside unassigned phyla.

6.3.4 Changes in soil phyla

Several phyla changed in proportional abundance in the soil samples dependent on biosolid application. Most notable was the relative changes of *Acidobacteria* and *Proteobacteria*, with the proportion of *Acidobacteria* decreasing with increased biosolid application and *Proteobacteria* increasing. This relationship can be seen in Fig. 6.3 which shows that the total combined abundance of both phylum in the soil changes very little but that there is change proportional to each other. Pre-flood and final soils had very similar proportions of both phyla, whereas post-flood soils saw the proportional trend between them exaggerated. *Acidobacteria* showed a mean decrease in proportion in pre-flood and final soils of -11.09% from 0g to 400g and -17.00% from 0g to 800g, in post-flood soils this trend was exaggerated to -12.98% from 0g to 400g and -31.52% from 0g to 800g soils. *Proteobacteria* showed a reversed trend with a mean proportional increase in pre-flood and final soils of +18.31% from 0g to 400g and +35.30% from 0g to 800g, in post-flood soils this was an increase of +15.18% from 0g to 400g and +54.52% from 0g to 800g. The proportional relationship between *Acidobacteria* and *Proteobacteria* is highlighted in Fig. 6.4 which displays just the relative abundance of the two phyla only. From Fig. 6.4 the biggest trend in proportional change in post-flood soils can clearly be observed, with the 'Post 800g' soils being the only case where the abundance of *Proteobacteria* exceeds that of *Acidobacteria.*

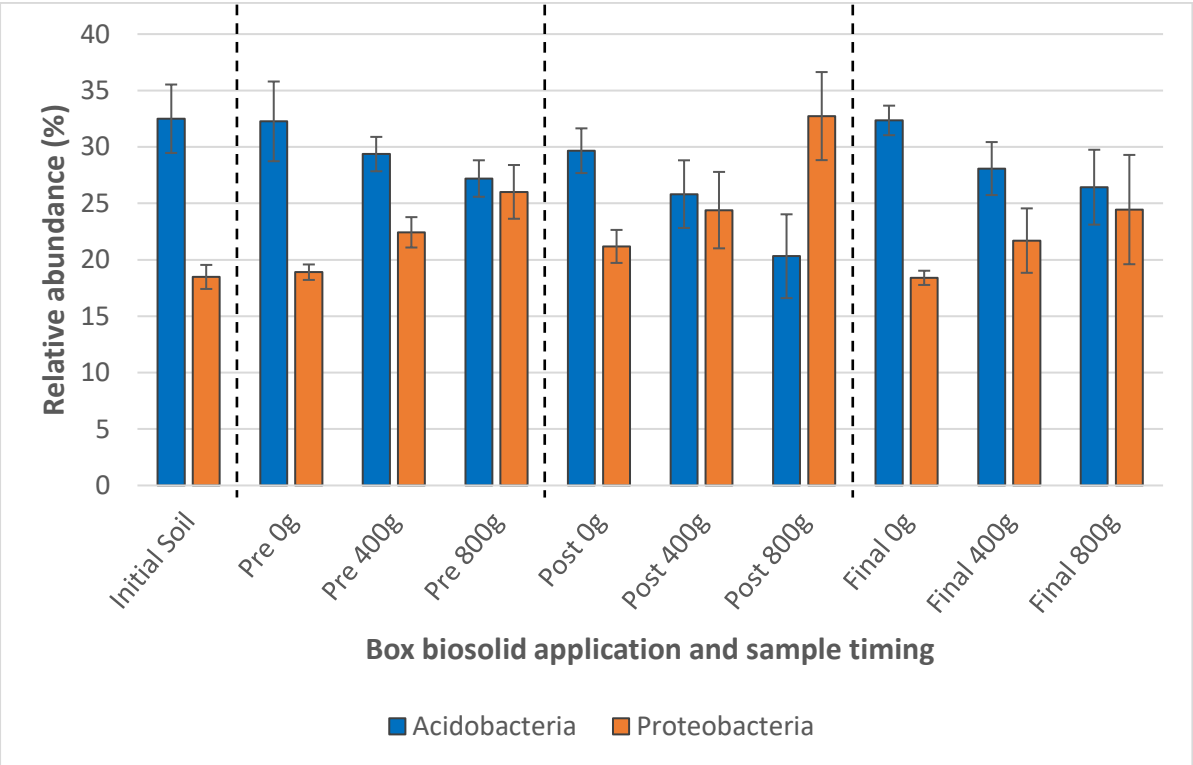

Figure 6.4 – Highlight of mean relative abundances of Acidobacteria and Proteobacteria from soil samples in Figure 6.3. Initial soil results are from the mean of five (5) samples. Timing and biosolid application rate results represent the mean of six (6) samples from both boxes treated at that rate at each of the sample timings pre-flood, post-flood and final. Error bars indicate standard deviation.

Further investigation of the OTU table showed that the *Acidobacteria* OTUs in the top 20% of abundance across all samples generally followed the trend indicated by the relative abundance changes, decreasing with increased biosolid application. Seven of the eleven OTUs in the top 20% of reads across all samples belonged to the order 'Subgroup 6', three to 'Subgroup 4' and one to 'Subgroup 7' with >98% confidence. In the Proteobacteria populations the first and third most abundant OTUs were defined at the family level as 'Xanthomonadaceae', class *Gammaproteobacteria*, with 100% confidence. The second and fourth most abundant of the *Proteobacteria* OTUs which were also present in the top 20% of reads belonged to the families 'Comamonadaceae' and 'Alcaligenaceae' in the class of *Betaproteobacteria*. Total read counts of individual OTUs are unreliable indicators of relative population changes due to the differences in total reads per sample. However, read counts were still useful for indicating whether an OTU was present or not in a sample. For instance, if an OTU was not present in control soils but was highly abundant in biosolids and appeared in biosolid-applied soils it may indicate a transfer of species from biosolids to soils. Using this method, the changes in relative abundance of both *Acidobacteria* and *Proteobacteria* OTUs does not appear to be related to the introduction of any new species from the biosolid populations but is from changes in abundance of the native species.

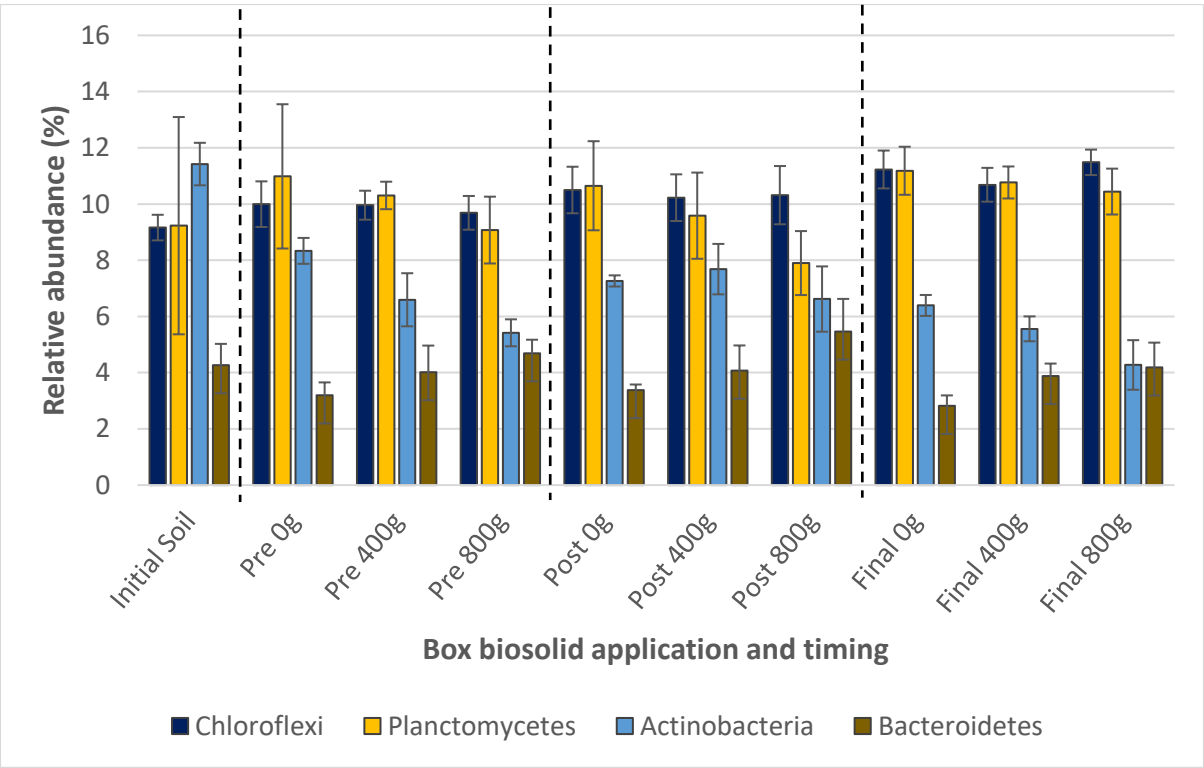

Figure 6.5 – Highlight of mean relative abundances of Chloroflexi, Planctomycetes, Actinobacteria and Bacteroidetes phyla from soil samples in Figure 6.3. Initial soil results are from the mean of five (5) samples. Timing and biosolid application rate results represent the mean of six (6) samples from both boxes treated at that rate at each of the sample timings pre-flood, post-flood and final. Error bars indicate standard deviation.

Chloroflexi, Planctomycetes and *Bacteroidetes* all appeared in both biosolids and soils with >2.5% mean abundance in all samples and are shown in more detail in Figure 6.5. *Chloroflexi* and *Planctomycetes* appeared in greater relative abundance in soils than in biosolids, with *Chloroflexi* at a mean of 10.32% in soils and 3.52% in biosolids, and *Planctomycetes* at 10.01% in soils and 5.42% in biosolids. *Chloroflexi* showed no trend based on biosolid application despite biosolid additions containing populations of the phylum. Further investigation of the OTU table showed the 33 most abundant *Chloroflexi* OTUs in all samples appearing almost exclusively in soil samples and only negligibly in biosolid samples. The most abundant *Chloroflexi* OTU in the biosolid samples, defined as 'Anaerolineaceae' at the family level, appeared in small amounts in biosolid-applied soils but not in control soils. This indicates there may be some, albeit small, transfer of biosolid-specific species within the *Chloroflexi* phylum to soils. However, many of the *Chloroflexi* OTUs defined as 'Anaerolineaceae' in the soils were found to be native soil populations, and these vastly outnumbered the biosolid-introduced 'Anaerolineaceae' OTUs.

Planctomycetes showed a slight decreasing trend in overall relative abundance with increased biosolid application, with an average -6.63% change from 0g to 400g applied soils and -16.61% from 0g to 800g applied soils. The *Planctomycetes* populations responded similarly to those of the *Chloroflexi*, with minimal transfer of species from biosolid to soil populations within the phylum. Inspection of the OTU table showed the largest *Planctomycetes* OTU in the biosolid samples, which was the third most abundant overall biosolid OTU, had only a very small transfer to soils. This OTU was defined as the genus 'p-1088-a5 gut group' with 100% confidence. The six most abundant *Planctomycetes* OTUs in soils within the top 50% of total reads appeared only in soil samples and negligibly in biosolids, showing they were native to the soil. Three of these OTUs were defined in the 'OM190' class and three in the order 'WD2101 soil group' of the 'Phycisphaerae' class, both of which are typical of soil *Planctomycetes.*

Bacteroidetes appear in biosolids with a relative abundance of 13.16% compared to the mean 4.00% appearing in soils. *Bacteroidetes* showed a trend of increasing in relative abundance in soils reflecting biosolid application, with a mean increase of +28.03% from 0g control soils to 400g applied soils and an increase of +52.41% from 0g to 800g applied soils. However, the four most abundant *Bacteroidetes* OTUs appeared to be native soil species and were identified at the class level as 'Cytophagia', 'Sphingobacteriia' and 'Flavobacteria'. The most abundant *Bacteroidetes* OTU present in the biosolid samples was identified in the class 'VadinHA17' and had negligible presence in control soils but was present in biosolid-applied soil samples. Of the top four OTUs which were most abundant in biosolids all showed transfer to soil, with two in the class 'VadinHA17' and two in 'Bacteroidia'. The increase in relative abundance of Bacteroidetes in biosolid-applied soils is

principally due to an increase in native populations but there is also evidence for the transfer of some species from biosolids and these species persist over the three sampling timings.

Actinobacteria were present with only 0.10% mean relative abundance in biosolids but at 6.96% in soils as the fifth most abundant phylum (Fig. 6.5). *Actinobacteria* showed a decrease in relative abundance with increased biosolid application in pre-flood and final soils, with a -16.95% change from 0g to 400g and -34.05% from 0g to 800g in those soils, but this effect was not present in post-flood soils. The most noticeable difference in *Actinobacteria* relative abundances was the change from initial soils to the experimental soils. In initial soils collected from the field *Actinobacteria* appeared at a mean of 11.42% whereas pre-flood control soils they appeared only as 8.33% relative abundance. This would indicate that the change in conditions from field to laboratory was having a significant effect on the populations in this phylum. Across all experimental soils the *Actinobacteria* relative abundance remained lower than that observed in initial soils, with a mean of 6.46%.

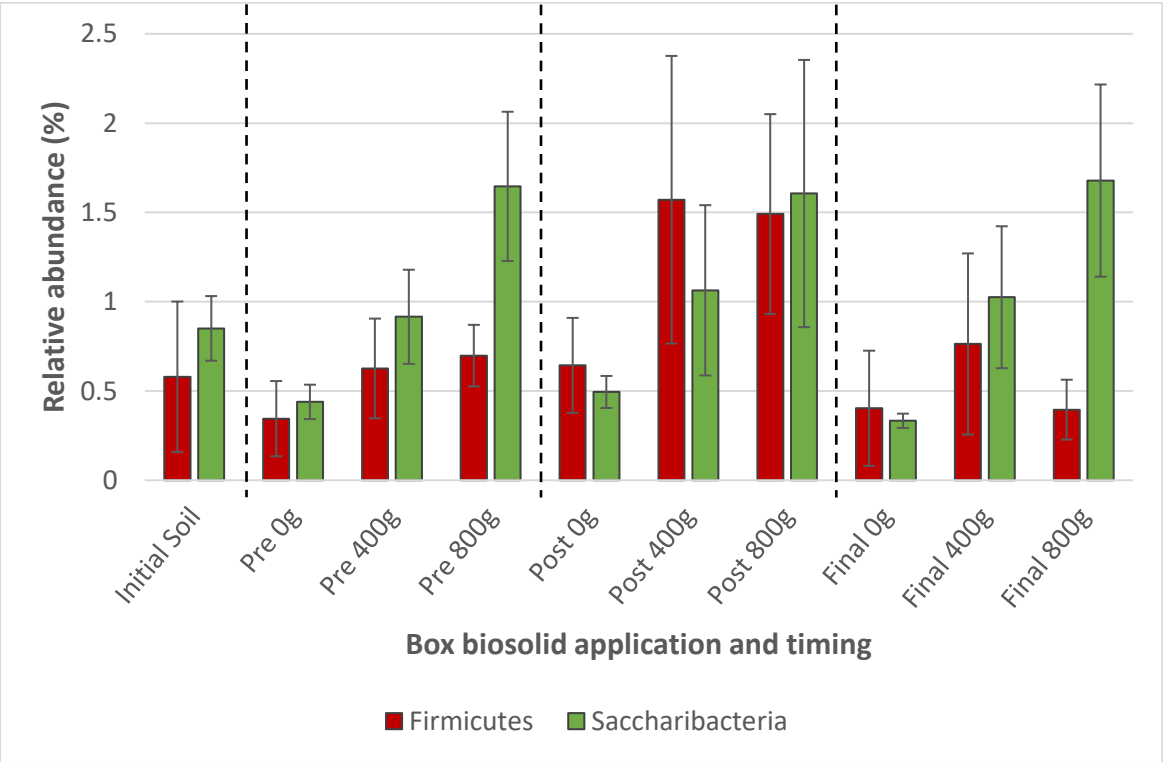

Figure 6.6 – Highlight of mean relative abundances of Firmicutes and Saccharibacteria from soil samples in Figure 6.3. Initial soil results are from the mean of five (5) samples. Timing and biosolid application rate results represent the mean of six (6) samples from both boxes treated at that rate at each of the sample timings pre-flood, post-flood and final. Error bars indicate standard deviation.

Saccharibacteria appear in biosolids with a mean relative abundance of 6.75% but appeared very little in untreated soils. However, there was a large increase in *Saccharibacteria* abundance in soils with increased biosolid application. Figure 6.6 presents the relative abundances of *Saccharibacteria* in detail. In 0g control soils there was a mean relative abundance of 0.42%, this rose to 1.00% in 400g applied soils and 1.64% in 800g applied soils, showing a considerable increase based on biosolid application, though no significant influence from flooding was observed. The most abundant OTU of *Saccharibacteria,* which was not defined beyond phylum level, appeared negligibly in control soils but did appear in biosolid-applied soils and with a high abundance in biosolids. This was the only *Saccharibacteria* OTU to appear in the top 50% of total soil sample abundance but was the 79th most abundant species in all soils, putting it well within the D_2 dominant species for those samples it was present. This indicates a significant change to the soil population brought about by direct introduction of a new species from the biosolid application rather than an increase in any native species from nutrient additions.

Firmicutes relative abundance did not significantly increase in soils based on biosolid application. Despite *Firmicutes* being the most dominant phylum in biosolids (37.60 ± 0.74% relative abundance) the relative abundance in pre-flood biosolid-applied soils was low and comparable to that of control soils (Fig. 6.6). However, there was a small transfer of *Firmicutes* species found from biosolids to soils upon inspection of the OTU table. The most abundant *Firmicutes* OTU, defined in the 'Clostridia' class and as 'Sedimentibacter' at the genus level, was negligibly present in control soils but was present in small amounts in biosolid-applied soils. Despite this small species transfer the relative abundance of *Firmicutes* in all pre-flood soils remained similar. However, in post-flood soils the relative abundance of *Firmicutes* in biosolid-applied soils was greatly increased to a relative abundance of 1.53%, compared to an average of 0.55% in all other soils (Fig 6.6). This effect being observed only in flooded biosolid-applied soils and not in flooded control soils suggests a coupled effect of biosolid application and flooding is responsible for the induced changes. It is not possible to reliably tell whether this change was due to increases in native soil *Firmicutes* species or those introduced through biosolids. In final soil samples after the flood recovery the *Firmicutes* relative abundances returned to that of pre-flood soils, showing that any increased abundance from flooding and biosolid application was acute and did not persist once soils returned to field conditions.

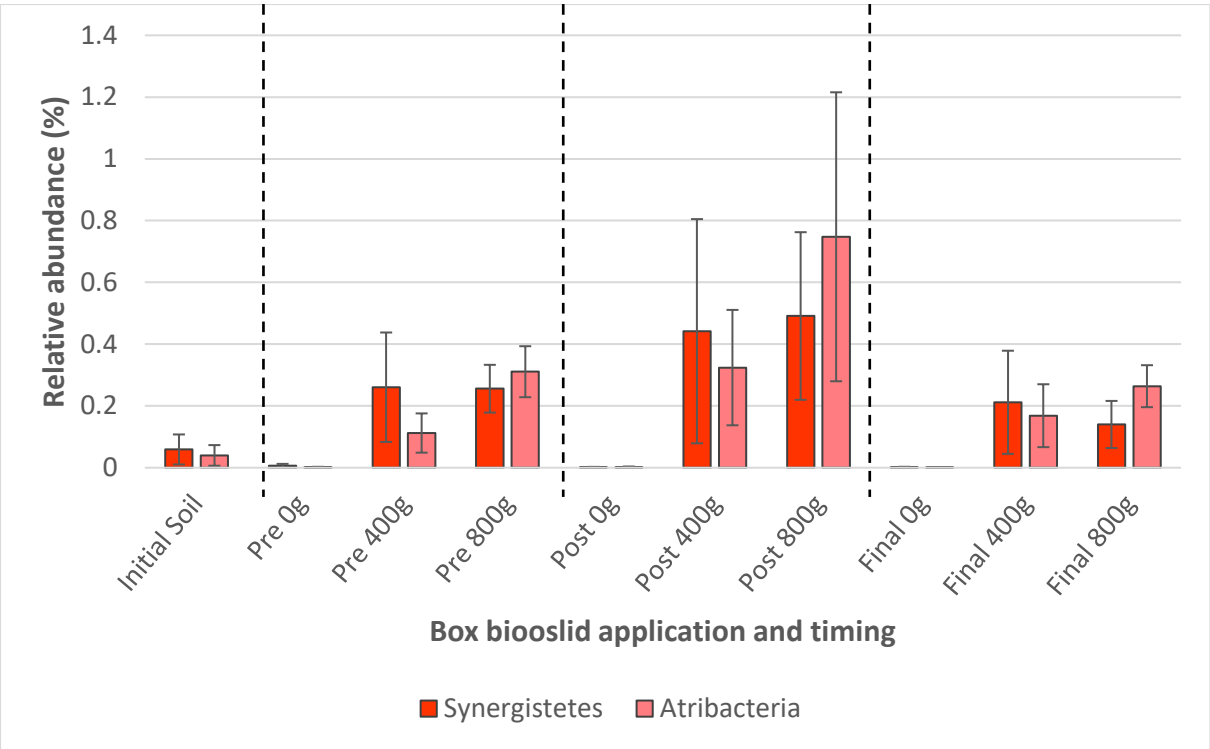

Figure 6.7 – Highlight of mean relative abundances of Synergistetes and Atribacteria from soils samples in Figure 6.3. Initial soil results are from the mean of five (5) samples. Timing and biosolid application rate results represent the mean of six (6) samples from both boxes treated at that rate at each of the sample timings pre-flood, post-flood and final. Error bars indicate standard deviation.

Synergistetes and *Atribacteria* were low abundance phyla in the soil but demonstrated important changes based on the experimental conditions. Both phyla had negligible presence in control soils but did appear in biosolid applied soils and saw large proportional increases in relative abundance after flooding (Fig 6.7). *Synergistetes* increased from a negligible relative abundance in control soils to 0.22% in pre-flood and final soils and to 0.47% in post-flood soils (+115%), highlighting the flood effect. *Synergistetes* were highly abundant in biosolids, accounting for 5 of the 35 D_2 dominant species in biosolid samples. Those species were also present in the D_1 dominant species of biosolid-applied soil samples but did not appear in control soils. This reinforces what is reflected in the relative abundances, that almost all the *Synergistetes* presence in the soil was introduced by biosolid applications. Despite this, only a minor effect was observed based on biosolid application rate, with the main effect appearing to be whether biosolid was applied or not, rather than how much was applied.

Atribacteria showed a biosolid application rate trend, increasing from no presence in control soils to 0.14% in 400g applied and 0.29% in 800g applied soils across pre-flood and final samplings. In post-flood soils the relative abundance of *Atribacteria* was increased further above that of pre-flood and

final soils to 0.32% (+130% increase) in 400g applied soils and 0.75% in 800g (+160% increase), indicating a large flood effect. The most abundant *Atribacteria* OTU ranked it within the D_2 dominant species (161st) across all experimental soil samples and was the 5th most abundant OTU in biosolid samples. As with *Saccharibacteria* this indicates that increases in the relative abundance of *Synergistetes* and *Atribacteria* came from additions of new microbial populations from the biosolids, rather than a change in native soil populations. Curiously both phyla were present in very small amounts in some initial soils but did not persist under laboratory conditions in control soils.

Of the other phyla identified in the *Cloacimonetes* and *Lentisphaerae* phyla displayed similar trends to *Synergistetes* and *Atribacteria* but did not appear in significant amounts, with relative abundances >0.1% in all soil samples. *Verrucomicrobia, Gemmatimonadetes, Latescibacteria* and *Armatimonadetes* did appear in significant amounts in soil samples but no trends were observed related to biosolid application or flood effect and so they were not discussed in detail. *Nitrospirae* are discussed further in section 6.3.5 below.

6.3.5 Nitrifying and denitrifying bacteria

Nitrogen behaviour in soils and floodwaters is a central focus of this project, and the geochemical reactions influencing N were explored in chapter 4. Nitrification and denitrification were highlighted as particularly important processes. Therefore, identifying bacteria with nitrifying and denitrifying metabolisms is important as it directly relates to the analysis and conclusions discussed in chapter 4. The metabolic capability of denitrification is present in a very wide range of genera and species and are estimated to typically account for approximately 10-15% of bacterial populations in soils (Robertson and Groffman, 2015). For instance, within the *Proteobacteria* phylum many species are facultative anaerobes, meaning they can switch from aerobic to anaerobic respiration as O_2 is depleted and reduce alternative electron acceptors such as NO_3^- (Marín, 2014). Identification of specific denitrifying bacteria is usually carried out by targeting and sequencing specific genes responsible for denitrification within the bacterial populations (Wang et al., 2017). As these genes were not targeted in the sequencing carried out in this project it is difficult to identify individual species which may be important for denitrification in the systems studied. However, as discussed in Chapter 4, the geochemical conditions which were achieved in the floodwaters of the experiments favour denitrification. The identification of the individual species which carry out this function is secondary to the activity itself (Philippot, 2005). Therefore, due to the widespread presence of this function in soil bacterial communities it can be assumed that denitrifiers are present and active.

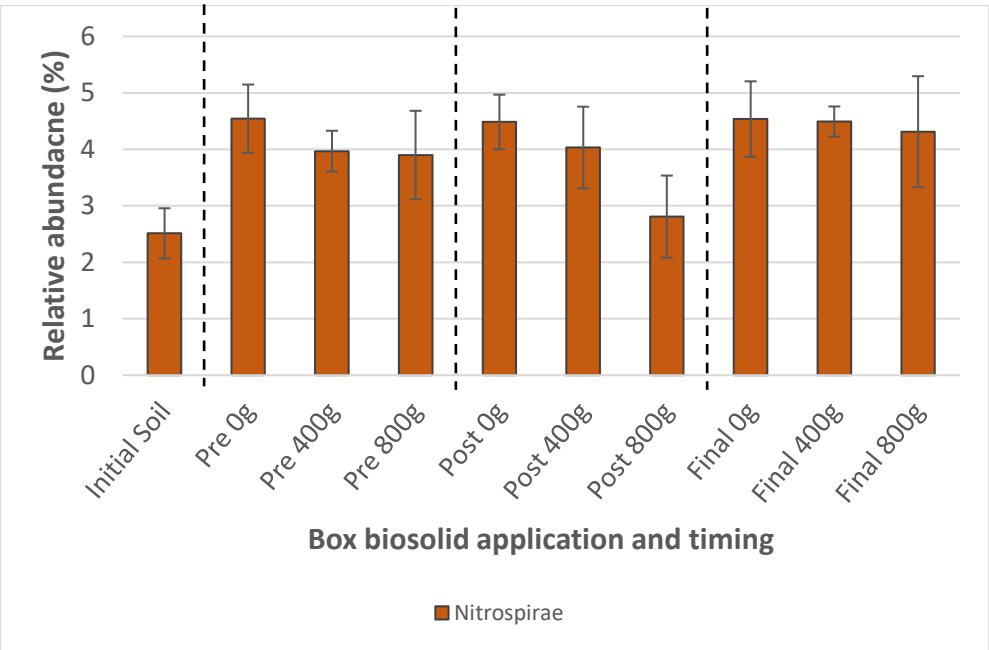

Figure 6.8 - Highlight of mean relative abundance of Nitrospirae from soils samples in Figure 6.3. Initial soil results are from the mean of five (5) samples. Timing and biosolid application rate results represent the mean of six (6) samples from both boxes treated at that rate at each of the sample timings pre-flood, post-flood and final. Error bars indicate standard deviation.

Nitrifying bacteria in soils belong to a narrower taxonomic group in the environment and so can more easily be identified than denitrifying bacteria by exploring the OTU table for certain phyla and genera. The *Nitrospirae* phylum contains nitrite-oxidising bacteria and several species within the *Proteobacteria* phylum are ammonia- and nitrite-oxidizing bacteria. *Nitrospirae* did not show any strong trend relating directly to biosolid application or flooding until both factors were combined (Fig. 6.8). In post-flood soils there was a negative trend of decreasing *Nitrospirae* relative abundance with increased biosolid application, with a significant decrease from control to 800g applied soils. This trend in relative abundance returned to pre-flood levels after recovery in final soils. There was also a large increase observed from initial soils to pre-flood experimental soils. Analysis of the OTU table showed that the second most abundant OTU across all soils belonged to the *Nitrospirae* phylum and there were seven OTUs ranked within the D_2 dominant species. All these OTUs were defined to the order level as 'Nitrospirales' which contains the important nitrifying bacterial genus 'Nitrospira'. A mean abundance count of these OTUs in 400g biosolid applied soils at all sample timings saw a small decrease of -2.15% from control soils, but in 800g soils the decrease in abundance was much greater at -20.02% from controls. This change reflected the negative trend observed in the relative abundances with increased biosolid application.

For identification of nitrifiers within the *Proteobacteria* phylum a search of the OTU table was carried out for OTUs which could be in the genera 'Nitrosomonas' or 'Nitrosospira' of the order 'Nitrosomonadales', the genus 'Nitrosococcus' of the order 'Chromatiales', and the 'Nitrobacter' genus in the order 'Rhizobiales'. The OTU ranked as 42nd most abundant in soil samples, the eleventh most abundant *Proteobacteria* OTU, was defined to the family level as 'Nitrosomonadaceae' (which contains the genera 'Nitrosomonas' and 'Nitrosospira') in the 'Nitrosomonadales' order and was the most abundant OTU found in all the searched groups. Five 'Nitrosomonadales' OTUs in total were identified within the D_2 dominant species of the soils. The mean change in abundance across these OTUs based on biosolid application was relatively stable with a small decrease of -4.09% from control to 400g applied soils and an increase of +8.82% from control to 800g soils. The mean relative abundance across all soil samples for OTUs defined to the order level as 'Nitrosomonadales' was 1.24%, with some unidentified OTUs in the 'Betaproteobacteria' class which could add to this number. These figures show that 'Nitrosomonadales' order of bacteria were a significant population in the soil and could mean significant populations of the 'Nitrosomonas' and 'Nitrosospira' genera are present. For 'Nitrosococcus' there was only one OTU found just within the D_2 dominant species, but this was only defined with 5% confidence after the phylum level so could not be reliably identified. Several OTUs were found in the D_2 dominant species range belonging to the 'Rhizobiales' order. However, only one was found, defined as an unidentified 'Bradyrhizobium' at genus level, which could be 'Nitrobacter'. These results indicate that the main populations of nitrifying bacteria in these soils probably belong to the *Nitrospirae* phylum or the 'Nitrosomonadales' order of *Proteobacteria*.

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6.3.6 OTU table highlights

To highlight the transfer of bacterial species from biosolids to soils summaries from the main OTU table are presented in Tables 6.2 and 6.3. Table 6.2 shows the top 13 OTUs from the biosolid samples which accounted for >50% of total read abundance. Table 6.3 displays a selection of the top 750 ranked OTUs in experimental soils which had high abundance in biosolid samples and negligible presence in control soils but appeared in biosolid-applied soils. The species of these OTUs therefore appear to have been introduced to soils directly from biosolids. The OTUs displayed all belong in the D_2 dominant species and D_1 common species of the soil bacterial populations. Overlap can be observed in both tables, with the most highly abundant biosolid OTUs often also appearing in biosolid-applied soils. A copy of the full OTU table with all sample data is included in the supplement 'Microbiology data supplement', and further details are available in Appendix E.2.1.

Table 6.2 – Highlight of top OTUs found in biosolids ranked in order of total abundance. The OTUs shown accounted for >50% of total reads in biosolid samples. The mean read count for each sample group is displayed, as well as the relative abundance of each OTU and its classification.

	Biosolid samples		Box biosolid application (reads)									
OTU ID	OTU Rank	Relative abundance (%)	0g	400g	800g	Initial Soil (reads)	Biosolid (reads)	Phylum	Class	Order	Family	Genus
OTU237	1	7.623	2	124	79	64	12110	Firmicutes	Clostridia	Clostridiales	Family_XI	Sedimentibacter
OTU236	2	6.575	2	399	522	64	10445	Saccharibacteria	Unknown_Class	Unknown_Order	Unknown_Family	Ca.Saccharimonas
OTU225	3	5.163	0	88	74	66	8202	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	p-1088-a5_gut_group
OTU388	4	4.907	2	164	130	41	7795	Synergistetes	Synergistia	Synergistales	Synergistaceae	
OTU338	5	4.756	1	202	327	42	7556	Atribacteria	Atribac._Incertae_Sedis	Unknown_Order	Unknown_Family	Ca.Caldatribacterium
OTU592	6	3.553	1	70	37	25	5645	Unassigned				
OTU492	7	3.508	2	302	462	37	5573	Bacteroidetes	Bacteroidetes_vadinHA17			
OTU370	8	3.413	1	161	95	43	5422	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Rikenellaceae_RC9_gut_group
OTU347	9	3.172	1	19	11	40	5040	Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	Syntrophomonas
OTU1311	10	2.357	1	70	51	15	3744	Synergistetes	Synergistia	Synergistales	Synergistaceae	Anaerobaculum
OTU540	11	2.041	0	33	36	25	3242	Cloacimonetes	Cloac._Incertae_Sedis	Unknown_Order	Unknown_Family	Ca.Cloacamonas
OTU1019	12	1.982	1	74	53	12	3149	Synergistetes	Synergistia	Synergistales	Synergistaceae	
OTU1067	13	1.860	1	69	43	22	2956	Synergistetes	Synergistia	Synergistales	Synergistaceae	

Table 6.3 - Summary of OTU table containing all OTUs which featured in the top 750 most abundant OTUs across experimental soil samples with no or negligible presence in control soils. This table highlights the OTUs which were introduced by biosolid application in the D_2 dominant species and D_1 common species of the soils. Mean read count of each sample group is displayed, as well as the sequence's relative abundance in the soils, and its classification

	Experimental soil samples		Box biosolid application (reads)									
OTU ID	OTU Rank	Relative abundance (%)	0g	400g	800g	Initial Soil (reads)	Biosolid (reads)	Phylum	Class	Order	Family	Genus
OTU236	79	0.215	2	399	522	64	10445	Saccharibacteria	Unknown_Class	Unknown_Order	Unknown_Family	Ca.Saccharimonas
OTU492	105	0.178	2	302	462	37	5573	Bacteroidetes	Bacteroidetes_vadinHA17			
OTU338	161	0.123	1	202	327	42	7556	Atribacteria	Atribac._Incertae_Sedis	Unknown_Order	Unknown_Family	Ca.Caldatribacterium
OTU779	241	0.084	1	139	222	16	2640	Bacteroidetes	Bacteroidetes_vadinHA17			
OTU388	290	0.069	2	164	130	41	7795	Synergistetes	Synergistia	Synergistales	Synergistaceae	
OTU370	328	0.060	1	161	95	43	5422	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Rikenellaceae_RC9_gut_group
OTU565	393	0.051	0	81	140	28	2577	Atribacteria	Atribac._Incertae_Sedis	Unknown_Order	Unknown_Family	Ca.Caldatribacterium
OTU237	418	0.048	2	124	79	64	12110	Firmicutes	Clostridia	Clostridiales	Family_XI	Sedimentibacter
OTU469	479	0.039	0	90	77	34	2723	Firmicutes	Clostridia	Clostridiales	Christensenellaceae	Christensenellaceae_R-7_group
OTU225	494	0.038	0	88	74	66	8202	Planctomycetes	Planctomycetacia	Planctomycetales	Planctomycetaceae	p-1088-a5_gut_group
OTU905	592	0.031	1	77	54	21	2702	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Petrimonas
OTU1019	606	0.030	1	74	53	12	3149	Synergistetes	Synergistia	Synergistales	Synergistaceae	
OTU1311	631	0.028	1	70	51	15	3744	Synergistetes	Synergistia	Synergistales	Synergistaceae	Anaerobaculum
OTU1067	670	0.026	1	69	43	22	2956	Synergistetes	Synergistia	Synergistales	Synergistaceae	
OTU592	701	0.025	1	70	37	25	5645	Unassigned				
OTU429	731	0.024	1	47	55	31	2293	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Fastidiosipila

6.3.7 Dissimilarity of bacterial communities between samples

All soil samples had a taxonomically similar composition to each other but were highly dissimilar to biosolid samples. Bray-Curtis dissimilarity scores indicate how many shared species a sample has with another sample. The mean Bray-Curtis dissimilarity (Tab. 6.4) between biosolid and 0g applied soils was 0.998, with a score of 0 indicating samples are the same and 1 indicating that samples do not share any species. For biosolids compared to 400g applied soil samples the score was 0.978 and for 800g it was 0.975. This indicates there is some carryover from biosolid applications to soil taxonomic composition, but that soil taxonomy is largely unchanged and is still highly dissimilar to biosolids. These Bray-Curtis scores demonstrate why it was not possible to include biosolids on the NMDS analysis and plot (Fig. 6.9), as the difference in scale obscured the more closely related soil sample data. The mean Bray-Curtis score of soil samples within the same test group was 0.309 which represents the mean replicate variation. The mean Bray-Curtis score for all soil comparisons was 0.397, which shows there was some variation between groups, but this was modest, and soils were not highly dissimilar to each other. A copy of the full table detailing Bray-Curtis scores between all individual samples is included in the supplement 'Microbiology data supplement', and further details are available in Appendix E.2.2.

Table 6.4 - Average Bray-Curtis dissimilarity scores. Results displayed are the mean scores for the comparison of samples within one group with each sample in a second group. A score of 1 indicates that samples do not share any species and a score of 0 indicates the samples are the same. The diagonal therefore represents the comparison of all samples within its own group, including the 0 score for each individual sample's comparison with itself. The table is colour coded with red showing more dissimilarity and green indicating less dissimilarity between samples.

	Biosolid	Initial	Pre 0g	Post 0g	Final 0g	Pre 400g	Post 400g	Final 400g	Pre 800g	Post 800g	Final 800g
Biosolid	0.196	0.992	0.999	0.998	0.999	0.983	0.970	0.979	0.975	0.966	0.985
Initial	0.992	0.330	0.388	0.395	0.494	0.375	0.457	0.443	0.417	0.516	0.457
Pre 0g	0.999	0.388	0.276	0.342	0.453	0.315	0.420	0.370	0.378	0.503	0.397
Post 0g	0.998	0.395	0.342	0.230	0.405	0.294	0.370	0.364	0.339	0.462	0.365
Final 0g	0.999	0.494	0.453	0.405	0.406	0.434	0.461	0.453	0.451	0.526	0.460
Pre 400g	0.983	0.375	0.315	0.294	0.434	0.213	0.365	0.341	0.298	0.440	0.341
Post 400g	0.970	0.457	0.420	0.370	0.461	0.365	0.366	0.403	0.376	0.445	0.405
Final 400g	0.979	0.443	0.370	0.364	0.453	0.341	0.403	0.315	0.372	0.462	0.367
Pre 800g	0.975	0.417	0.378	0.339	0.451	0.298	0.376	0.372	0.255	0.407	0.339
Post 800g	0.966	0.516	0.503	0.462	0.526	0.440	0.445	0.462	0.407	0.384	0.443
Final 800g	0.985	0.457	0.397	0.365	0.460	0.341	0.405	0.367	0.339	0.443	0.311

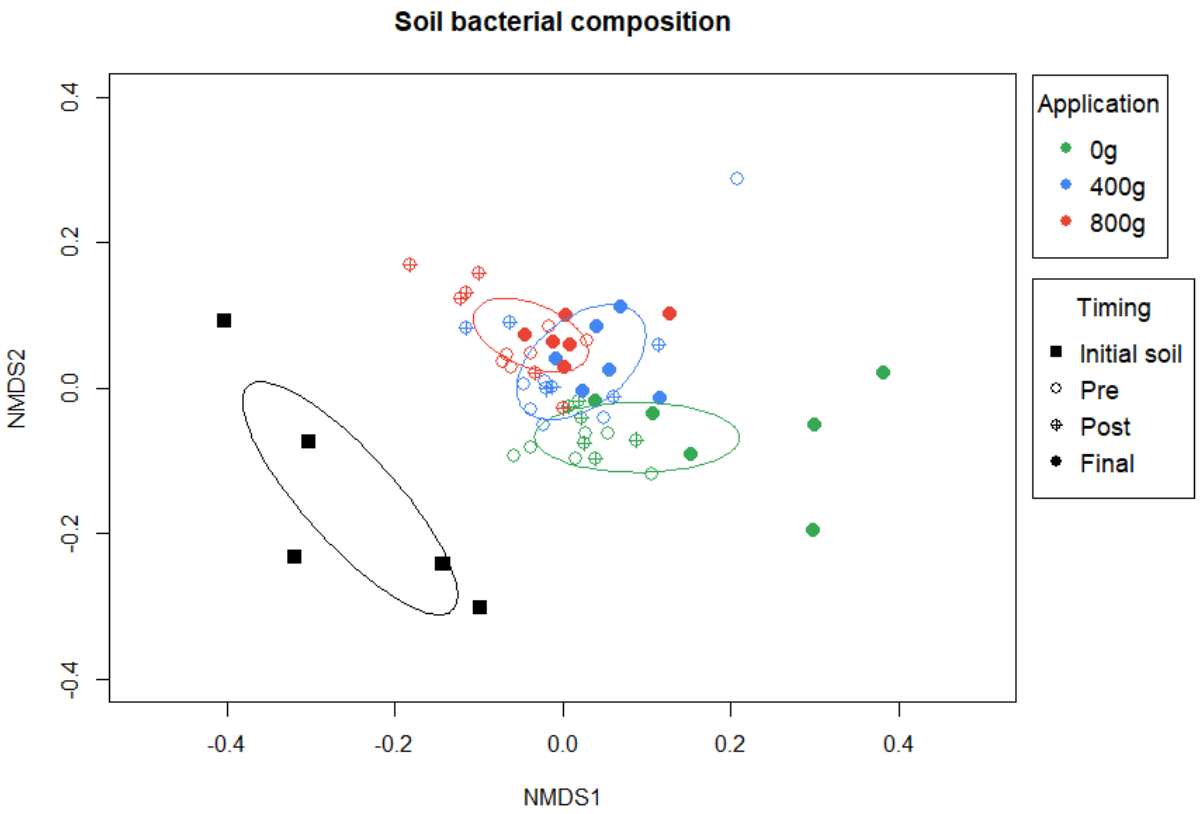

Figure 6.9 – Non-metric Multidimensional Scaling (NMDS) plot showing dissimilarities between soil bacterial community samples determined by Bray-Curtis distances (k = 2, stress = 0.162). Points are coloured to represent biosolid application to boxes, shape fills represent the different sample timings of initial soils, pre-flood, post-flood and final. Ellipses represent the standard deviation of the points belonging to initial soils and biosolid applied soils at all timings and are coloured according to application.

The NMDS plot (Fig. 6.9) clearly displays the Bray-Curtis dissimilarity distances between samples. Interestingly, Initial soils appear to be more dissimilar to experimental soils, including control soils, than just biosolid-applied soils. This would indicate that the crop growth process and laboratory experimental conditions are affecting microbial populations. Though the difference between initial soils and experimental soils appears to be larger than the difference between control soils and biosolid-applied soils it is important to note that the significance of the difference cannot be determined from Bray-Curtis scores alone. Bray-Curtis determines dissimilarity based on the number of shared species but does not consider the importance of those species within the samples. Introduction of a small number of new species into the D_2 dominant species of the biosolid-applied soils which are not present in the control soils could have a large impact on the microbial populations without impacting Bray-Curtis scores. Alternatively, many low abundance species dying off may not have a great impact on overall soil bacterial populations but may disproportionately affect Bray-Curtis scores. It is therefore important that this data is used in conjunction with other

diversity measures such as Hill numbers to determine the significance of bacterial population changes. Nevertheless, 400g and 800g biosolid-applied soil shared more overlap on the NMDS plot than the control soils. This would indicate that they are sharing several bacterial species with each other that are not present in control soils, possibly introduced from the biosolids. Biosolids were highly dissimilar to soils and were not included in Figure 6.9 so that soil dissimilarity could more clearly be displayed. Figure 6.10 displays an NMDS plot with biosolids included and highlights their dissimilarity and the distortion to the NMDS scale upon their inclusion.

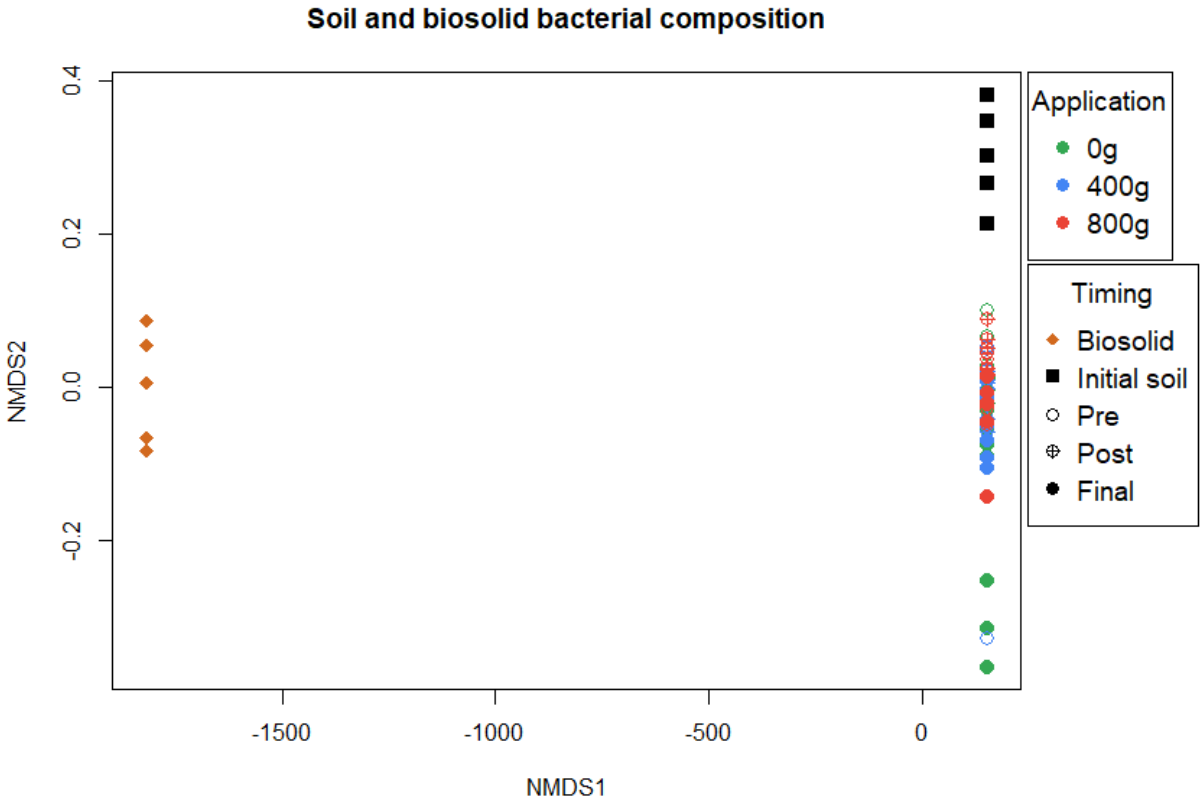

Figure 6.10 - Non-metric Multidimensional Scaling (NMDS) plot showing dissimilarities between soil and biosolid bacterial communities determined by Bray-Curtis distances (k = 2, stress < 0.001). The NMDS analysis was run as in Figure 6.9 but with the addition of the biosolid samples. This plot demonstrates the large dissimilarity between soil and biosolid bacterial populations and highlights why the soil only NMDS plot was used in Figure 6.9.

6.3.8 Microbial phylum correlations with soil properties

Table 6.5 - Spearman's rank correlations between relative abundances of soil bacterial phyla and soil properties. Phyla are in order of relative abundance across all soil and biosolid samples. An asterisk (*) indicates a significant correlation at p<0.05, a double asterisk (**) indicates a significant correlation at p<0.01, a triple asterisk (***) indicates a significant correlation <0.001.

	TKN	Olsen P	SOM	pH
Acidobacteria	-0.730***	-0.767***	-0.723***	0.656**
Proteobacteria	0.802***	0.821***	0.858***	-0.781***
Chloroflexi	-0.018	-0.096	-0.140	0.225
Planctomycetes	-0.565*	-0.439	-0.700**	0.774***
Actinobacteria	-0.407	-0.381	-0.381	0.119
Bacteroidetes	0.811***	0.725***	0.825***	-0.846***
Verrucomicrobia	-0.565*	-0.502*	-0.519*	0.391
Nitrospirae	-0.401	-0.248	-0.381	0.573*
Firmicutes	0.418	0.518*	0.425	-0.449
Gemmatimonadetes	-0.019	-0.123	-0.123	0.267
Latescibacteria	-0.372	-0.279	-0.456	0.647**
Saccharibacteria	0.849***	0.716***	0.879***	-0.823***
Synergistetes	0.721***	0.714***	0.728***	-0.649**
Armatimonadetes	0.005	0.025	-0.070	0.284
Atribacteria	0.860***	0.746***	0.842***	-0.818***
Hydrogenedentes	0.318	0.295	0.168	0.063
Cloacimonetes	0.795***	0.735***	0.775***	-0.768***
Cyanobacteria	0.114	0.105	0.142	-0.168
Lentisphaerae	0.709***	0.663**	0.782***	-0.740***

Table 6.5 presents the Spearman's correlation data between the abundance of different microbial phyla and the soil properties measured and explored in chapter 4. Many phyla within the soil correlate significantly with several of the soil geochemical factors. Soil TKN, Olsen P, SOM and pH all had trends related to biosolid application, so correlations may reflect the overall impact of the biosolid application on the soil rather than the importance of individual factors. Other factors within the biosolid application may also have influenced soil phyla but were not measured in this project. The correlations presented are useful as a statistical indicator of whether biosolid application did or did not have an impact on microbial populations and support the trends in relative abundances described in section 6.3.4.

6.4 DISCUSSION

6.4.1 Soil microbial biomass

Biosolids contain a high nutrient and organic matter content which could directly impact soil microbial populations by boosting the numbers of bacteria which utilise those resources. However, no direct quantification of microbial population biomass was conducted in this experiment. The DNA sequencing carried out indicates the abundance of bacterial populations relative to each other and the diversity of the populations, but reliable conclusions on microbial biomass cannot be drawn from those results. DNA yields may allow an indication of microbial biomass from the density of microbial DNA recovered from the samples. However, the inconsistency of the soil material and any variability in the sampling and laboratory analysis means that DNA yields alone are not a reliable indicator of microbial biomass differences between soil samples. Yet, DNA yield differences between soils and biosolids can give an indication of increased microbial biomass in soils from biosolid applications. Biosolids contained a much greater microbial DNA yield per gram of material than soil, having approximately 8x the yield of the mean DNA recovery from control box soils. When considering total volume of bacterial DNA in the 34kg of soil in each box then biosolid additions would account for an addition of ~8% bacterial DNA to 400g applied boxes and ~15% to 800g applied boxes. Additionally, DNA recovery was higher from 800g biosolid applied soils than from control and 400g applied soils. Control soils had a mean DNA recovery of 28.63µg DNA/g and 400g applied soils 28.19µg DNA/g, whereas 800g applied soil had 51.43µg DNA/g. The lack of difference between control and 400g could be due to replicate variation, but the larger yields from 800g applied soils could indicate a higher microbial biomass in the samples for DNA recovery.

The SOM content measurements discussed in chapter 4 could also be indicators of the potential microbial biomass in soils. SOM indicates SOC content which provides a source of C for microbial respiration. SOC usually comprises of 1-4% microbial C (Sparling, 1992) and SOC content is the limiting factor for microbial biomass over other soil nutrients such as N and P (Heuck et al., 2015). As found in chapter 4 SOM increases with biosolid application in all cases (Fig. 4.2). In experiment 4, which is the focus of this chapter, SOM contents equated to an increase from 2.81% in control soils to 3.04% in 400g and 3.42% in 800g biosolid applied soils. This indicates that the higher biosolid-applied soils in the experiment could have a larger microbial biomass based on SOC as a controlling factor. These differences in potential microbial biomass affect how changes in the microbial relative abundance from increased biosolid application to soil should be considered. For instance, increasing *Proteobacteria* and decreasing *Acidobacteria* relative abundance could be from an increase in *Proteobacteria* and a decrease in, maintenance of or lesser increase in *Acidobacteria*. The

decreasing relative abundance of a phylum does not necessarily reflect a decrease in biomass of that phylum in the sample. This does not reduce the validity of the changes to microbial phyla relative abundance but needs to be considered when evaluating results.

6.4.2 Soil changes from field to laboratory

When transferring soil samples from field to laboratory care was taken to preserve soils in their field state to prevent any degradation of soil and microbial community structure. Despite this, the NMDS analysis (Fig. 6.9) would appear to indicate that initial soils are more dissimilar to laboratory soils than laboratory soils were to each other after biosolid application. However, it is important to remember that the NMDS analysis is based on Bray-Curtis scores which give equal prominence to all bacterial species in the sample, whether they are rare or dominant. The differences observed could be due to influences on bacterial species from several experimental factors such as increased temperature in the laboratory, growth of the barley crop, the watering and light regime or other unknown factors. However, investigation of the OTU table shows some evidence that the difference in shared species from initial to experimental soils may have been from anaerobic bacteria which were lost when soils were aerated during cultivation of the soil on setup of the experiments. This is evidenced in Table 6.3, where all the OTUs shown had negligible or no presence in control soils but did have a small presence in initial soils and in biosolid-applied soils. These bacteria were also highly abundant in biosolids, which indicates that they favour anaerobic conditions and may have some direct transfer to the soils from biosolid bacterial populations, as well as a small native presence. Despite the aeration of all soils on setup biosolid-applied soils may have contained small pockets of biosolid which had high abundances of these anaerobes, a high nutrient content, and may have had localised anaerobic conditions, allowing them to survive. These changes alone would not account for the difference observed between initial and experimental soils, just between control and biosolid-applied soils. However, these results could suggest a trend in other bacterial species outside the D_1 common species, and lower abundance anaerobic bacteria OTUs which did not have high presence in biosolids may have been lost from the soils entirely upon aeration. If these bacterial populations were not bolstered by populations from the biosolids and completely died off then this could account for the differences observed between initial and experimental soils. The anaerobes which did persist after biosolid application may also therefore be due to the direct introduction of populations from biosolids rather than an increase in the existing native bacteria.

Despite these difference between initial and experimental soils the scale at which they are represented on the NMDS plot may over-amplify the actual scale of the differences on overall bacterial population change. For instance, The NMDS plot including the biosolid samples in Figure 6.10, and the Bray-Curtis matrix in Table 6.4, show that soils are very highly dissimilar to biosolids

but are not dissimilar to each other. The Hill numbers presented in Figure 6.2 also suggest that initial soils had similar diversity to experimental soils. Furthermore, the relative abundances of soil bacterial phyla presented in Figure 6.3 show the similarity of soil samples across all treatments. Therefore, though there was a difference observed in shared species, as presented in the NMDS and Bray-Curtis scores, it is likely that they were outside the D_1 common species of the soils and therefore only had a small influence on overall bacterial populations. Using the relative abundances of bacterial phyla in soils (Fig. 6.3) is a better method for observing the total shift in bacterial populations.

Initial soils were especially close to pre-flood control soils when considering the relative abundances of the most dominant phyla of *Acidobacteria, Proteobacteria, Chloroflexi and Planctomycetes.* However, there were some differences observed in less abundant phyla. The most noticeable difference was in the proportion *Actinobacteria* which appeared at a mean of 11.42% in initial soils from the field but only 8.33% in pre-flood control soils in the laboratory. *Actinobacteria* are common of terrestrial soils and are important in the decomposition of plant OM in the soil such as cellulose, polysaccharides, protein fats, organic acids and more (Ranjani et al., 2016). The precise reason for this decrease in relative abundance of *Actinobacteria* from initial to experimental soils is unknown. A potential reason could be the breakdown of plant material in the soil over the 28-day growth period leading to an exhaustion of *Actinobacteria's* favoured nutrient source. Another possibility is that *Actinobacteria* failed to respond as quickly as other phyla in the soil to increased temperatures or other conditions, and so were outcompeted and became relatively less abundant. *Nitrospirae* saw the opposite change to *Actinobacteria* with an increase in relative abundance from initial soils to pre-flood experimental soils. This may be accounted for by the higher temperature in the laboratory, with the activity of nitrifying bacteria well linked to increased soil temperatures (Chen et al., 2018; Lu et al., 2018). Another difference noted when analysing the relative abundances of soil phyla was the presence of small numbers of *Synergistetes* and *Atribacteria* present in initial soils but not control soils. The presence of these phyla, even in small quantities, could reflect that a wider variety of environmental conditions were available for microbes under field conditions. The loss of many of these *Synergistetes* and *Atribacteria* species could also go a substantial way to explaining the differences observed in the NMDS analysis.

6.4.3 Biosolid influence on soil microbial communities

6.4.3.1 Direct introduction of bacterial species from biosolids to soils

As discussed in section 6.4.1 biosolids contained a much greater microbial DNA yield per gram of material than soil and likely increase the soil microbial biomass substantially, with an increase of up to ~15% indicated by soil DNA yields. The bacterial diversity within the biosolid samples compared to that of the soil samples was also much lower, indicating fewer, but more prominent bacterial species in biosolids than in soils. The persistence of biosolid-applied bacterial populations was not clearly observed in any relative abundance changes to soil bacterial phyla. This would indicate that the majority of biosolid-introduced bacteria were not able to survive in the soil during the 28-day initial crop growth period of the experiments. This difference was expected as the biosolids originated from sewage sludge which had undergone anaerobic digestion under high temperature and O_2 deprived conditions. The soils on the other hand came from the natural environment of an arable field where a diverse range of organisms were present, and a variety of environmental conditions were possible. Another possible reason for this lack of difference is that although biosolid DNA yields were high it is unknown how many of the microbial cells were alive and viable, or dead but with the rRNA gene not yet degraded. Regardless of the reason, the relative abundances of phyla in biosolid-applied soils closely resembled initial and control soils throughout the experiment, rather than showing any shift closer to biosolid samples (Fig. 6.3). The Bray-Curtis dissimilarity scores (Tab. 6.4) and the NMDS analysis (Fig. 6.9) also support this as they showed very large differences between biosolid and soil bacterial populations, indicating very few shared species between them. However, despite the apparent lack of change induced by the introduction of biosolid bacterial populations to the soil from phyla relative abundances, investigation of the OTU table and diversity indices attested to the contrary.

A small trend of increase was observed in D_2 and D_1 Hill numbers with increased biosolid application (Fig. 6.2) and though this appeared not to be statistically significant, any introduction of new bacteria into a population's D_2 dominant species would be an important change. Closer inspection of the OTU table was therefore carried out to further assess these changes. Table 6.2 displays the 13 most abundant OTUs found in the biosolid samples, accounting for over 50% of all biosolid reads, and all were found in biosolid-applied soils but not in control soils. This indicates that these species may have been directly introduced into the soil through biosolid application and persisted there in the long term. However, these species also had a small presence in initial soils, which would indicate that they were already native species which were bolstered by the biosolid application as discussed in section 6.4.2. Judging by the lack of presence in control soils, the low abundance in initial soils and the very high abundance of these bacteria in biosolids then this could suggest that the

introduction of the bacteria from biosolids was the main influencing factor. Some combination of both introduced bacteria and the nutrients they needed to survive from the biosolids was likely inducing the changes in these biosolid-applied soil OTUs, but the changes induced were important. Three of the most abundant biosolid OTUs appeared in the D_2 dominant species of experimental soil bacterial populations when averaged across all soils (Tab 6.3). Considering this averaging also included control soils, then the actual rank of these OTUs in biosolid-applied soils was likely even higher than indicated in Table 6.3.

The species introduced from biosolids to the soil D_2 dominant species belonged to the *Saccharibacteria, Bacteroidetes* and *Atribacteria* phyla. All three phyla demonstrated a positive trend associated with biosolid application shown by significant correlations to soil nutrient contents (Tab.6.5). This trend was particularly important in *Atribacteria* and *Saccharibacteria*. *Atribacteria* had no presence in control soils and so the increasing trend in biosolid-applied soils was likely mostly influenced by the biosolid introduced bacterial species. *Saccharibacteria* was present in control soils but with a mean relative abundance of less than 0.5%, which then more than doubled in 400g biosolid applied soils and almost quadrupled in 800g applied soils. Coupled with the evidence from the OTU table which saw specific OTUs present only in biosolid-applied soils and not controls this would indicate a very high proportion of the *Saccharibacteria* increase was from biosolid-introduced bacterial species. *Synergistetes* also saw increases in relative abundance correlated to biosolid application and had no presence in control soils like *Atribacteria*, showing all increase was most likely from biosolid-introduced species. Although no *Synergistetes* appeared in the D_2 dominant species of soils four of the top ranked biosolid OTUs belonged to the phylum and showed persistence in biosolid-applied soils at lower abundances (Tab. 6.2). The changes to the *Saccharibacteria, Synergistetes* and *Atribacteria* relative abundances in soils therefore appear to almost be entirely from the introduction of new species from biosolids.

Though *Saccharibacteria, Bacteroidetes* and *Atribacteria* were the most prominent soil bacterial phyla to demonstrate trends resulting from the introduction of new species from biosolids other less abundant phyla had species which also showed transfer. When considering the large relative abundance differences between soil and biosolid bacterial populations a very evident contrast is in *Firmicutes*. This was the dominant phylum in biosolid samples at 37.60% relative abundance, but only made up 0.56% of pre-flood soil samples with little effect from biosolid application. However, investigation of the OTU table showed that there was a transfer of *Firmicutes* species directly from biosolids to soils. The two most abundant *Firmicutes* OTUs in biosolids, classed as belonging to the 'Sedimentibacter' and 'Syntrophomonas' genera, were found in biosolid-amended soils, but were not present in control soil and had only a very small presence in initial soils. Table 6.2 clearly

highlights this transfer of *Firmicutes* species from biosolids to soils, alongside species from other phyla, and shows that this transfer is widespread across biosolid-applied soil bacterial populations. Despite the persistence of these species, the overall bacterial population composition of biosolid-applied soils still closely resembled control and initial soils. There was no major shift in soil bacterial populations towards those of biosolid populations as indicated by the Bray-Curtis and NMDS analyses, and the overall relative abundances displayed in Figure 6.3. The majority of the biosolid species introduced to the soil appear to have been out competed by the native soil bacterial populations and prevented from dominating under normal conditions.

6.4.3.2 Bacterial response to nutrient additions from biosolids

As well as the impact from direct introduction of bacteria from biosolids the large nutrient increase from the biosolid application also contributed to relative abundance changes in many native species. Many phyla within the soil correlate significantly with soil geochemical factors of TKN, Olsen P, SOM and pH results described in chapter 4 (Tab. 6.5). However, the soil geochemical factors were not independent, and all had a trend related to the rate of biosolid application. This means that even with a significant correlation to the measured nutrients the main influencing factors cannot be isolated from this data alone. Alongside the measured nutrients there was a wide range of micronutrients introduced and changes to the soil structure which were not quantified but were caused by biosolid application. Still, significant correlation across all geochemical factors is useful for indicating which bacterial phyla in the soil were influenced by biosolid application and which were not, rather than which individual factor or combination of factors was responsible for the response. More detailed exploration of the potential functions of different phyla needs to be discussed to better understand any of the significant relationships shown.

Acidobacteria and *Proteobacteria* were the dominant phyla in the soil regardless of biosolid application, accounting for between 49.78% and 53.22% of total phyla. The proportion of *Acidobacteria* to *Proteobacteria* did change with increased biosolid application, with *Acidobacteria* decreasing in relative abundance and *Proteobacteria* increasing (Figs. 6.3 and 6.4). Both phyla contain a significant amount of morphological, physiological and metabolic diversity but general trends in their behaviour in soil can still be observed using the broad classification at phylum level (Gupta, 2000; Fierer et al., 2007; Spain et al., 2009; Kielak et al., 2016). The decreased abundance of the *Acidobacteria* phylum with increased biosolids would fit with previous studies which have found that *Acidobacteria* are more suited to soils with poor nutrient availability due to their slower metabolisms, and many can also favour more acidic conditions (Fierer et al., 2007; Ward et al., 2009; Sun et al., 2015a; Kielak et al., 2016). Conversely, *Proteobacteria* have been shown to favour more nutrient-rich soils or soil rhizospheres where nutrients and SOM were more highly concentrated

(Fierer et al., 2007; Sun et al., 2015a; Zeng et al., 2016). Biosolid application both added soil nutrients and raised soil pH as discussed in chapter 4 (Figs. 4.2-4.8). This would mean that biosolid-applied soils were less favourable for *Acidobacteria* and led to them being outcompeted by *Proteobacteria* which favoured the nutrient-rich soils. This relationship is also supported by the Spearman's rank correlations presented in Table 6.5, with *Acidobacteria* showing a highly significant negative correlation with increased soil nutrients (TKN, SOM and Olsen P) and a positive correlation to soil pH, and *Proteobacteria* having a highly significant positive correlation to soil nutrients and negative correlation with pH. *Proteobacteria* would therefore appear to resemble r-strategists in their lifecycle, using available resources and multiplying quickly to capitalise on them. Whereas *Acidobacteria* more closely resemble K-strategists, investing in long-term development but not reacting quickly to environmental changes.

The six most abundant *Proteobacteria* OTUs, which accounted for 4.09% of all soil reads, belonged to the classes 'Betaproteobacteria' and 'Gammaproteobacteria'. Both of these classes have been shown in previous study to have a positive relationship with SOC (Fierer et al., 2007; Li et al., 2017). The overall increase in relative abundance of *Proteobacteria* also does not appear to be related to the introduction of any new bacteria from the biosolid populations. *Proteobacteria* were barely present in biosolids and those that were appear only in very small abundance in biosolid-applied soil samples and were negligible in comparison to native soil *Proteobacteria*. The increased abundance of *Proteobacteria* therefore appears to be entirely due to the response to the nutrient additions brought about in soils by the application of biosolids. This conclusion was supported by further investigation of the OTU table, where several OTUs saw very large increases in abundance in biosolid-applied soils but were not introduced from biosolid populations and had very low abundances in control soils, indicating a nutrient effect.

6.4.3.3 Combined influence of soil nutrients and directly introduced biosolid bacteria

Several phyla showed that they may be influenced both my introduction of new species from biosolid application and the accompanying nutrient addition. *Bacteroidetes* are typical of gut microbiomes and anaerobic digesters but are also widely distributed in the environment (Miyashita, 2015; Liu et al., 2016; Wang et al., 2019) and many species were native to the soil in high abundance before biosolid application. The relative abundance of *Bacteroidetes* in the soils was bolstered proportionally from the addition of biosolids and this difference was maintained across all pre-flood, post-flood and final soils. The overall increase of *Bacteroidetes* in biosolid-applied soils appeared to be from both an increase in native soil species from the increased nutrient input and some introduction of new species directly from the biosolids. *Bacteroidetes* are linked to the breakdown and digestion of SOM and have been found to positively correlate with SOC mineralisation rates in

previous studies (Fierer et al., 2007). This was supported by the highly significant correlation between *Bacteroidetes* abundance and soil nutrients including SOM in this study (Tab. 6.5). Investigation of the OTU table showed that native soil *Bacteroidetes* species dominated all soil populations including biosolid-applied. Some highly abundant species of *Bacteroidetes* in the biosolids did transfer to the soil and these belonged to the classes 'VadinHA17' and 'Bacteroidia' (Tab 6.2), with the 'VadinHA17' present in the D_2 dominant species of the soils and 'Bacteroidia' in the D_1 common species (Tab. 6.3). Both of these bacterial classes are typical of anaerobic digesters (Li et al., 2016; Liu et al., 2016). No significant change in *Bacteroidetes* relative abundance was seen over the course of the experiment caused by flooding or sample timing. However, over a longer time frame, as the increased SOM content from biosolid application is depleted, biosolid-applied soil populations may return to an equilibrium similar to the control soils.

6.4.3.4 Other bacterial responses

Chloroflexi and *Planctomycetes* were both present in biosolids and as native populations in the soil with >10% mean relative abundance. *Chloroflexi* relative abundances in soils appeared to remain unaffected by biosolid applications and flooding. *Planctomycetes* had a slightly negative response to both biosolid application and flooding and showed a significant negative correlation to soil nutrient levels (Tab. 6.5). The *Chloroflexi* bacteria added to soil from biosolids shared commonalities with the native soil populations, with a lot of the family 'Anaerolineaceae' in both. The native populations far outweighed the added biosolid bacteria in terms of abundance and remained very stable and resilient, indicating the native *Chloroflexi* population was well established and versatile. The negative response of *Planctomycetes* populations may indicate they are less versatile and resilient in the soil and were outcompeted by other organisms for the resources available through biosolid application and by more adaptable bacteria during flooding. The decrease in the *Planctomycetes* phylum, despite it also being present significantly in biosolids, appears to be due to a greater difference between the species present in biosolids and soil. As with *Chloroflexi,* which had distinct species between biosolids and soils, the most abundant OTU of biosolid *Planctomycetes* was a 'p-1088-a5 gut group' gut digestive bacteria (Tab. 6.2) which will have favoured the digester environment (Sun et al., 2015b). Little of this OTU persisted in soils despite its very high abundance in biosolids, though the OTU did still appear as the sole *Planctomycetes* species transferred from biosolids to the D_1 common species of soils (Tab. 6.3). In soils the most abundant *Planctomycetes* OTU was instead of the order 'WD2101 soil group' typically found in soil environments (Ivanova et al., 2016). *Actinobacteria* also appeared to decrease in abundance with increased biosolid application, though this was found to be not significantly correlated to the measured soil nutrients. However, the lack of significant correlation may be due to a flood effect confounding the data, as

the decreasing trend did not appear in post-flood soils, unlike in *Planctomycetes* where the effects were exaggerated by flooding. As discussed in section 6.4.2 the relative abundance decrease in *Actinobacteria* with biosolid application may be down to competition for resources. *Actinobacteria* favour plant material for their metabolism, the presence of different forms of SOC from biosolid application may therefore be favouring other decomposers. *Actinobacteria* were also not present in biosolids so there was no direct transfer of any populations to soils.

Other phyla in the soil not yet discussed includes the *Verrucomicrobia* phylum, which appeared with a mean relative abundance of 4.19% in soil samples so had a substantial presence, but no obvious trend was observed from either biosolid application or flooding effect. Nevertheless, *Verrucomicrobia* were found to have a significant negative correlation to soil nutrients (Tab. 6.5) which suggests a biosolid effect. However, this change appears to be minor compared to other phyla with minimal influence on overall bacterial populations. Several other phyla were also only present in biosolid-applied soils, such as *Cloacimonetes* and *Lentisphaerae,* but had very low abundances which were insignificant compared to the wider microbial populations. Despite the many trends and changes observed across all soil phyla the relative abundances of phyla within soil samples shows they are still very similar in terms of overall populations and dominant phyla.

6.4.4 Flood impact on biosolid-amended soils and recovery

As with biosolid addition flooding did not dramatically alter soil populations. Post-flood soils still closely resembled pre-flood soils in terms of relative abundances of the dominant phyla (Fig. 6.3), but some differences were observed. The largest flood induced change was in the most abundant phyla of *Acidobacteria* and *Proteobacteria* which also saw changes based on biosolid application as discussed in section 6.4.3. The relative abundance of *Acidobacteria* decreased and *Proteobacteria* increased in response to increased biosolid application, and this trend was exaggerated with the addition of the flooding effect. Post-flood 800g biosolid applied soils were the only samples in which the mean relative abundance of *Proteobacteria* exceeded that of *Acidobacteria,* and 400g biosolid applied soils had no observable difference between the phyla. This increased relative abundance of *Proteobacteria* may be due to it containing more diversity and therefore being more resilient to change than *Acidobacteria,* as has previously been suggested (Faoro et al., 2010; Miyashita, 2015). Also, *Proteobacteria* may be more suited to lower O_2 and submerged soil conditions, with the phylum known to contain several species that are facultative anaerobes (Marín, 2014). This is supported by its high abundance in many river sediments and the low abundance of *Acidobacteria* in those same environments (Vidal Dura et al., 2018; Huang et al., 2019; Zhang et al., 2019).

Another influence on soil bacteria from flooding was in those phyla which saw species introduced directly from biosolid-application, such as the *Firmicutes* phylum which saw large increases in relative abundance after flooding in only biosolid-applied soils (Fig. 6.6). This would indicate a coupled effect of biosolid application and flooding being responsible for the increase, likely due to biosolid-introduced anaerobic species. Most *Firmicutes* are anaerobic and are typically found in gut microbiomes, anaerobic digestate and anaerobic soils (Sun et al., 2015a; Liu et al., 2016; Jiang et al., 2019). The two most abundant *Firmicutes* OTUs in biosolid samples belonged to the genera 'Sedimentibacter' and 'Syntrophomonas' and are known anaerobes (McInerney et al., 1981; Imachi et al., 2016). These two OTUs were found only in biosolids and biosolid-applied soils and had no presence in control soils. However, the three most abundant OTUs across all experimental soils were native species, belonging to the families 'Bacillaceae' and 'Gracilibacteraceae' and appearing in the soil D_1 common species. These native species had varied abundances across samples, but some had very little to no presence in pre-flood soils. Upon flooding soil porewaters all became anoxic in a very short time (Figs. 4.17 & 4.18) and will have allowed the anaerobic *Firmicutes* to flourish. The increase from flooding appeared in both native and biosolid-introduced species, however biosolid-applied soils saw a much larger increase. The large change observed in *Firmicutes* relative abundance in only post-flood biosolid-applied soils would suggest that it is mainly biosolid-introduced species which are responsible. Some native species increase may also have been a contributing factor, with dormant native anaerobic bacteria able to capitalise on the nutrient addition of the biosolids to increase in abundance during flooding. After the 20-day recovery period the *Firmicutes* populations returned to pre-flood levels in all soils as they became aerated again. This would suggest the flooding effect is short term. However, the increase in relative abundance of native as well as introduced species during the flood would indicate that *Firmicutes* populations can remain dormant for a long time in soils until anaerobic conditions allow them to grow. Biosolid-introduced species may therefore remain in the soil long after application until favourable conditions become available. Despite these important changes the *Firmicutes* effect on overall soil populations was still small in terms of total relative abundance, but its behaviour could be indicative of the response of other phyla or species with similar metabolisms.

Similar responses were seen from bacteria in the *Synergistetes* and *Atribacteria* phyla which were not present in control soils but were present in biosolid-applied soil and saw a boost in relative abundance in post-flood soils. Both *Synergistetes* and *Atribacteria* are associated with anaerobic environments (Jumas-Bilak and Marchandin, 2014; Nobu et al., 2016) and were therefore likely able to capitalise on the anaerobic flood conditions. *Saccharibacteria* saw an increase in abundance with biosolid application and were also present in biosolid samples. The relative abundance of

Saccharibacteria remained stable throughout the experiment, with no flood effect observed. This would indicate that *Saccharibacteria* did not capitalise on any anaerobic conditions created by the flood even if many of the species were introduced from the anaerobic biosolid environment. *Saccharibacteria* is a widespread environmental phylum and is well known to persist in many environments including soils, bogs and activated sewage sludge (Ferrari et al., 2014). The environmental changes caused by flooding may therefore not have been extreme enough to illicit a large change to the species abundance in this phylum, which was resilient to a range of environments. The *Saccharibacteria* population therefore appears to have just been maintained during flooding, while the relative abundances of more sensitive phyla changed around it.

The phyla in all final soils after the 20-day recovery period from flooding returned to similar relative abundances to pre-flood soils. This shows that the changes induced by short-term flooding were also short-term effects. However, the increase in abundance of biosolid-specific species during flooding could present a threat to watercourses if flooded biosolid-applied soils allowed them a pathway into surface waters. Increased abundance during short-term flooding may allow those populations to persist for long enough to be transported to more favourable environments for their reproduction such as lake or river sediments. The extent of this transport, or the potential threat of any specific bacteria to wider environments, cannot be commented on within the bounds of this project. Longer-term flooding may also lead to greater extents of those effects but was again not explored in this project.

6.4.5 Response of nitrifying bacteria to biosolid application and flooding

The response of nitrifying bacteria was of importance to this project as the nitrogen cycle in soils has important effects on the soil geochemical, crop and microbiological responses, as discussed in section 2.3. NH_3/NH_4^+ is first mineralised from organic N through ammonification by decomposer microbes. Nitrification then occurs in a two-step process, with the first step being the oxidation of NH_4^+ to NO_2^- and then the oxidation of NO_2^- to NO_3^- by different nitrifying bacteria (Prosser, 1990). In soils ammonia-oxidizing bacteria (AOB) and ammonia-oxidising archaea (AOA) are responsible for the first stage and nitrite oxidising bacteria (NOB) for the second stage. Though AOA and AOB are both important in many soils for NH_4^+ oxidation AOA are more present in acidic and low nutrient soils, whereas AOB favour higher nutrient alkaline and neutral pH soils (Shen et al., 2012). Within the parameters of this experiment, using calcareous soils from a farmed arable field, AOA are considered less likely to be influential than AOB. Though archaea were not studied in this experiment, the primers used for targeting of the v4 region of the 16s rRNA gene of the bacteria are also effective for targeting archaea (Caporaso et al., 2011). Therefore, to support the assumption that AOA were less prominent in these soils than AOB a search for the presence of AOA was carried

out on the archaeal OTUs discarded from the main bacterial OTU table. Only a very low abundance of AOA was identified, confirming the focus for NH_4^+ oxidation should be on the AOB.

Soil AOB typically belong to the 'Nitrosomonas' or 'Nitrosospira' genera of the 'Nitrosomonadales' order of *Proteobacteria* (Norton and Ouyang, 2019). The NH_4^+ oxidising step is the limiting factor of the nitrification process, as without this there is no source of NO_2^- for further oxidation to NO_3^-. In this experiment the largest occurrence of AOB identified in soils was the 'Nitrosomonadales' order which had a significant mean relative abundance of 1.24% identified to a high confidence, including several OTUs in the D_2 dominant species of soil samples. The NOB in soils generally belong to the 'Nitrobacter' genus of the *Proteobacteria* phylum or 'Nitrospira' of the *Nitrospirae* phylum (Han et al., 2018; Norton and Ouyang, 2019). The largest populations of NOB were identified as the *Nitrospirae* phylum, which had a mean relative abundance of 4.12% across all experimental soil samples (Fig. 6.8). Some members of 'Nitrospira' have also been recently found which are able to catalyse both nitrification steps and have been called 'complete ammonia oxidisers' (comammox) (Daims and Wagner, 2018). 'Nitrobacter' was not identified in any of the soil samples of these experiments. Some 'Nitrobacter' may be present as unidentified bacteria OTUs, but their abundance will still have been very small compared to the *Nitrospirae* NOB population.

In the experiments of this project all floodwaters had very low NH_4^+ levels. This would indicate that the rate of mineralisation of NH_4^+ from organic N is not exceeding the ability of AOB to oxidise the NH_4^+ to NO_2^- in soils, and large NH_4^+ pools were not able to accumulate. It could also indicate that immobilisation of NH_4^+ to organic N was active in soil microbes and kept soil NH_4^+ balanced. Based on the bacteria present in these soils the accumulation of NO_2^- was not expected to occur, due to the NH_4^+ step being the limiting factor of nitrification. This is supported by the findings of a previous study by Yao and Peng (2017) which found very similar relative abundances to this experiment of both 'Nitrosomonadales' (1.27%) and 'Nitrospira' (4.02%) as the primary AOB and NOB populations. In that study the average metabolic rates of NOB were found to be between 1.08 and 2.00 times greater than AOB, and no large NO_2^- pools could be formed in the system as it was immediately oxidised to NO_3^-. The NOB populations would therefore appear to be surviving at a low range of their metabolic capability. This could explain why little increase was seen in *Nitrospirae* populations with increased biosolid application (Fig. 6.8). Even with increased N mineralisation and AOB activity in biosolid-applied soils the resultant increase in NO_2^- would still be within the metabolic range of existing NOB populations, without excess for long term population growth.

The reduction in relative abundance of *Nitrospirae* in post-flood biosolid-applied soils (Fig. 6.8) may be due to the relative abundance increases in other phyla. With *Nitrospirae* relying on O_2 presence

to oxidise NO_2^-, or NH_4^+ if comammox species, then their metabolisms will have been limited during the flood with little opportunity for growth. However, anaerobic bacteria in other phyla will have been able to grow in population size. In biosolid-applied soils this effect may have been exaggerated, with the microbial biomass of anaerobes able to increase even more due to the increased nutrient availability. The decrease observed in the relative abundance of the mostly aerobic *Nitrospirae* may therefore be due to those bacteria becoming dormant under anaerobic conditions and other, more adaptable phyla increasing in abundance around them. For instance, *Proteobacteria* saw large increases in relative abundance in post-flood, biosolid-applied soils. This could be a large contributor to the decreasing relative abundance of any aerobic bacteria, which may have become dormant during flooding but may not have reduced in total abundance. After the recovery period in final soil samples, as with other phyla, the *Nitrospirae* populations returned to similar relative abundances as pre-flood soils.

6.4.6 Nitrate reduction

As discussed in section 4.4.5 NO_3^- presence in floodwater acting as a terminal electron acceptor is maintaining the ORP above highly reducing conditions. As NO_3^- is lost from floodwater the ORP will begin to drop to more reducing levels. However, over the 10-day flood of the experiments only a small drop in Eh was observed due to this (Figs. 4.10-4.18) in control soils which saw their lower NO_3^- content depleted more quickly. The anoxic conditions brought on by the flood likely then limited the nitrification process in soil by preventing the oxidation of NH_4^+ to NO_2^- and then NO_3^-, preventing the floodwater NO_3^- pool being renewed as it was depleted. As the Eh was maintained above highly reducing conditions then NO_3^- is not expected to have been reduced to NH_4^+ in large amounts by DNRA, rather it is lost through denitrification. This is supported by the low levels of NH_4^+ present in all floodwaters and the high Eh levels. Further NH_4^+ accumulation in floodwater does not appear to have been able to occur due to the lack of mineralisation from organic N, with SOM decomposition slowed during flooding (Ponnamperuma, 1984; Magdoff and Weil, 2004). Anaerobic NH_4^+ oxidation (anammox), which is the conversion of NH_4^+ and NO_2^- to N_2 and water, is also not likely to have occurred in large amounts due to the high Eh in floodwaters. Anammox does not usually occur at high levels in agricultural soils and is generally found in marine sediments, freshwater marshes, rivers, lakes, peat bogs and other environments with limited O_2 and reducing conditions (Long et al., 2012). The OTU table was further explored to identify if any known anammox species were present within the *Planctomycetes* phylum where they are known to occur, but none could be identified based on genera previously identified in the literature (Shehzad et al., 2016; Zhou et al., 2017). This does not necessarily mean that there were no anammox microorganisms present, but that they were not detected by the analysis carried out. Denitrification on the other hand is a widespread

ability in many soil bacteria including several species identified in this experiment. Therefore, with the conditions present in the floodwaters of these experiments, the main form of NO_3^- reduction and loss occurring is expected to be from denitrification.

6.5 Conclusions

6.5.1 Biosolid applications to soil

In hypotheses 3 it was predicted that the nutrient content of a biosolid application would increase the relative abundance of species which favoured biosolids as a nutrient source. It was also predicted that the bacterial content of the overall soil would not change with biosolid application due to the extreme differences between the two environments. The difference between the biosolid and soil bacterial populations was apparent from the relative abundances of bacterial phyla found in both sets of samples. Biosolid and all soils had very different bacterial compositions, as highlighted in Figure 6.11. The scale of this difference was also highlighted in the Bray-Curtis dissimilarity scores (Tab. 6.4) and the NMDS analyses of the samples (Figs. 6.9 and 6.10). These both showed biosolid bacterial populations shared very few similar bacterial species with soils.

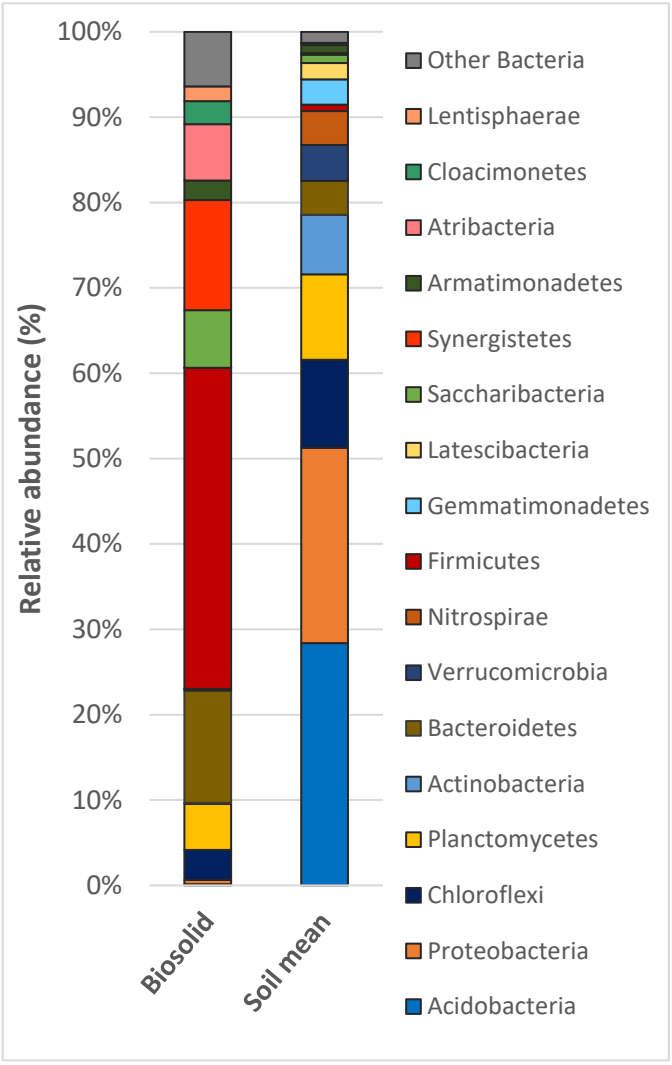

Figure 6.11 - Biosolid and Mean Soil bacterial phyla relative abundances. Phyla are presented in order of total abundance across all samples from bottom to top.

In soils a proportional response was observed in several bacterial phyla based on the nutrient additions through biosolid application (Fig. 6.3). *Proteobacteria, Bacteroidetes, Saccharibacteria, Synergistetes, Atribacteria, Cloacimonetes* and *Lentisphaerae* all had significant positive correlations to biosolid applications, increasing with soil TKN, SOM and Olsen P (Tab. 6.5). *Acidobacteria* and *Verrucomicrobia* had significant negative correlations to increasing soil nutrients from biosolid

applications. Changes in relative abundance in the most abundant phyla of the soil, which included *Acidobacteria, Proteobacteria* and *Bacteroidetes,* appeared to mostly be caused by changes to the native soil species of those populations from the increased nutrient content of biosolid-applied soils. *Bacteroidetes* did however also see increase due to bacteria directly introduced from biosolid applications. Other significant soil phyla with lower abundances also increased with biosolid application, namely *Saccharibacteria, Synergistetes* and *Atribacteria*. Investigation of the individual species of these phyla showed that most increase in their relative abundance came from the introduction of new species from biosolids rather than the nutrient inputs. *Atribacteria* and *Synergistetes* in fact had no presence in control soils, but were present in biosolid-applied soils, showing that the entirety of their population was introduced through biosolids. Furthermore, OTUs from *Saccharibacteria, Bacteroidetes* and *Atribacteria* which were not present in control soils appeared in the D_2 dominant species of biosolid-applied soils. Other species from *Synergistetes* and *Firmicutes* appeared prominently in biosolid-applied soil's D_1 common species which were not present in control soils. Table 6.6 below provides a highlight of all OTUs in the D_2 dominant species of the soils which increased from very low abundances in control soils to high abundances in biosolid-applied soils, indicating a large biosolid effect. The transfer of *Saccharibacteria, Bacteroidetes* and *Atribacteria* species can clearly be observed, as well as the abundance increase in native *Proteobacteria* species.

Table 6.6 - Highlight of OTU table showing OTUs from the top 250 ranked experimental soil OTUs (D_2) which had a mean abundance lower than 50 reads in control soils. These OTUs indicate species which were greatly affected by biosolid application, either by introduction of new species or an increase in native species. Mean reads across each sample group are displayed as well as the rank of the OTU across all experimental soils, the mean relative abundance in soils and the phylum classification.

	Experimental soil samples		Box biosolid application (reads)					
OTU ID	OTU Rank	Relative abundance (%)	0g	400g	800g	Initial Soil (reads)	Biosolid (reads)	Phylum
OTU4019	8	0.955	3	1841	2265	1	1	Proteobacteria
OTU4337	50	0.293	0	382	879	1	0	Proteobacteria
OTU1119	61	0.264	49	338	750	23	1	Proteobacteria
OTU474	77	0.219	29	287	627	63	0	Proteobacteria
OTU236	79	0.215	2	399	522	64	10445	Saccharibacteria
OTU2717	86	0.207	14	224	653	2	0	Proteobacteria
OTU492	105	0.178	2	302	462	37	5573	Bacteroidetes
OTU3554	115	0.164	21	173	511	59	0	Proteobacteria
OTU1013	120	0.159	20	149	516	12	0	Proteobacteria
OTU3014	125	0.153	24	344	290	3	0	Proteobacteria
OTU338	161	0.123	1	202	327	42	7556	Atribacteria
OTU779	241	0.084	1	139	222	16	2640	Bacteroidetes

Assessment of Hill numbers for all soil samples showed only a small increase in bacterial diversity throughout the experiment at D_1 and D_2 from increased biosolid application (Fig. 6.2). However, though these diversity increases appeared to not be significant, the introduction of bacteria to the soil D_2 dominant species of biosolid-applied soils indicates that these changes were important to overall bacterial populations. NMDS analysis also supported this difference between control and biosolid-applied soil populations, showing the groups as largely separate from each other, but with a small overlap (Fig. 6.9). The effect of biosolid application to soils was also visible from Bray-Curtis scores (Tab. 6.4) which showed slightly less dissimilarity between biosolid-applied soils and biosolids than between control soils and biosolids, indicating an increase in shared bacteria. The data gathered therefore suggests small but important changes to soil bacterial populations from biosolid applications.

6.5.2 Flood effect on biosolid-applied soils

Hypothesis 4 predicted that microbial populations would change under flooded conditions proportional to biosolid application rate. This hypothesis was linked to the assumption that biosolid application would increase microbial activity in soil, accelerate O_2 consumption in floodwaters and therefore create anaerobic conditions which would in turn alter the soil microbial populations. Soil porewater O_2 was depleted quickly during flooding, as discussed in chapter 4, which could favour anaerobic bacteria or those which were more adaptable to environmental changes. In the most abundant soil phyla of *Acidobacteria* and *Proteobacteria* flooding saw an increase in effects already observed due to biosolid application, with the relative abundance of *Proteobacteria* increasing further and *Acidobacteria* decreasing further. This change was attributed to *Proteobacteria* favouring both higher nutrient conditions and water inundated soils, likely due to the large diversity and versatility within the phylum. *Acidobacteria* which favoured lower nutrient soils and were not resilient to flooding therefore decreased under both conditions. The changes in relative abundance of these phyla caused by the coupled effect of biosolid application and flooding is

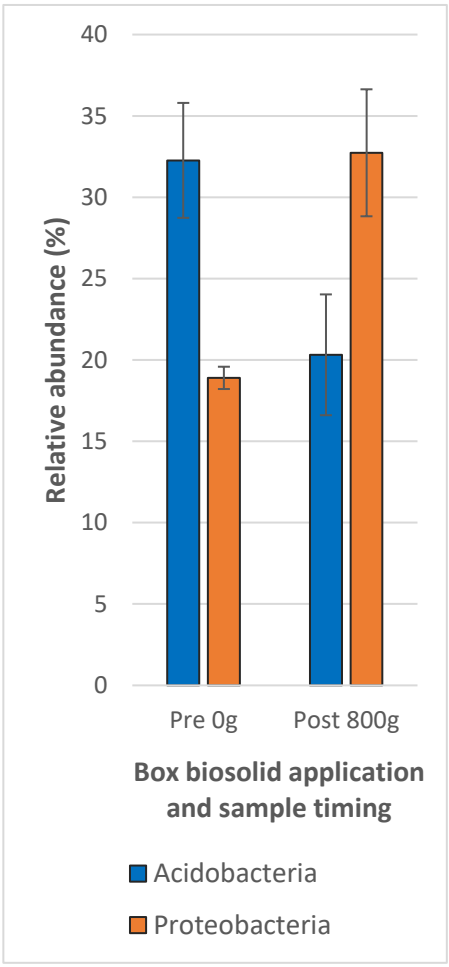

Figure 6.12 – Differences in mean relative abundance of Acidobacteria and Proteobacteria between pre-flood control soils and post-flood 800g biosolid-applied soils. Error bars indicate standard deviation.

emphasised in Figure 6.12. Effects on the *Nitrospirae* phylum were also observed, with a decreasing relative abundance based on increased biosolid application under flooded conditions. The decrease in abundance of these nitrifying bacteria could be from stress caused from limitation of their metabolisms through the restriction of NO_2^- from AOB and limited O_2 diffusion from surface waters. However, the rate of total nitrification in the experiments appeared to be limited by the activity of soil AOB, namely the 'Nitrosomonadales' order of bacteria in the *Proteobacteria* phylum.

The proportional changes in *Acidobacteria, Proteobacteria* and *Nitrospirae* were the most substantial changes observed within the dominant soil populations. However, effects on less abundant phyla in the soil reflected some other important changes. Several phyla including *Firmicutes, Synergistetes* and *Atribacteria* all increased in abundance under flooded conditions in biosolid applied soils. The population increase in these phyla was important as it did not occur in control soils, meaning that the increase in abundance was only from species introduced from biosolid application. These species likely favoured the anaerobic conditions of the digestion process and so could increase in population in the newly anaerobic conditions of flooded soil porewaters. This response could be of concern if any of the species present were pathogenic and could be transferred to the wider environment through transport in floodwater. The response observed in this experiment was also from a short-term flood where anaerobic conditions were short lived. The increase in abundance of biosolid-introduced bacteria in soils may be exaggerated in long-term floods where anaerobic conditions persist for longer periods. Further investigation would be needed to expand on these concerns.

Overall, the investigation into the microbiological changes of biosolid application and short-term flooding in the project yielded a variety of results. Biosolid applications appear to have a small but important effect on soil bacterial populations. Changes are induced by both the response of native soil bacteria to the input of nutrients and the introduction of small numbers of new bacteria directly from the biosolids. Flooding exaggerated many of the changes induced, including allowing for anaerobic bacteria from biosolid applications to multiply under anaerobic conditions. The exploration of the coupling effects of biosolid application and flooding were noteworthy and could open possible new avenues for investigation in the future.

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Chapter 7: GENERAL DISCUSSION

The previous chapters focused on exploring the geochemical, crop response and microbiological factors of the experiments in detail, but discussion on how each of these factors interacted was limited. This chapter seeks to more closely discuss and explore these three areas in more detail to better understand the wider soil-water-crop-microbe system. The important results will be highlighted and summarised, and the overarching themes of the project will be explored.

7.1 PRE-FLOOD

Initial soils which were collected from the field provided a baseline for each experiment dependent on the field fertiliser applications prior to collection. Experiment 2 had received a fertiliser P application prior to collection, and this was reflected in the higher soil Olsen P measured in those soils. This was also apparent to a lesser degree in the soils of Experiment 3 which were collected later in the same season. Experiment 3 soils had also received a liquid N application to the field prior to soil collection, and while this was not reflected by an increase in soil TKN levels a residue of soluble N was likely present, and this was reflected later by higher NO_3^- levels in Experiment 3 floodwaters. Transfer of soils from field to laboratory for experiments had no effect on soil TKN or SOM contents. However, soil Olsen P increased, and pH rose from Initial soils to pre-flood control soils in all experiments. This was likely due to aeration of the soils during mixing in the experimental setup, temperature increase in the laboratory from the field, and crop growth effects. Greater mineralisation of P from organic matter due to increased microbial activity during the initial growth period likely contributed to the increased crop available P reflected by the higher soil Olsen P values. Changes to the bacterial populations were reflected in the Bray-Curtis scores (Tab. 6.4) and NMDS analysis (Fig. 6.9) which showed initial soils were more dissimilar to experimental soils than experimental soils were to each other, even with biosolid amendment considered. Investigation of the OTU table pointed towards the loss of anaerobic bacteria from initial to experimental soils being responsible for the population dissimilarity observed, supporting that aeration of the soils may have been the main cause of the diversity changes. Any lowered diversity appeared to be from rare bacterial species in the soil rather than common or dominant species, as the overall bacterial populations and the relative abundances of bacterial phyla in initial and experimental soils were still very similar.

Biosolid applications to the soils increased soil TKN, Olsen P and SOM, as expected due to the high N, P and organic C content of biosolids. Soil pH was lower in soils with higher biosolid applications and

this was likely due to increased nitrification and respiration in those soils, from increased N and organic C contents, creating acidifying effects. This was supported by the bacterial characterisation which showed increased relative abundance in the *Proteobacteria* phylum in biosolid-applied soils proportional the biosolid application. *Proteobacteria* were one of the two most abundant phyla observed in all soils alongside *Acidobacteria. Proteobacteria* is a diverse phylum containing many species which consume SOM, and demonstrated the characteristics of ecological r-strategists, capitalising on increased nutrient availability from biosolids to increase in abundance more quickly than other phyla. It can therefore be assumed that the increased *Proteobacteria* relative abundance would lead to increased SOM consumption through microbial respiration, and to greater N mineralisation, subsequently leading to increased nitrification activity. The effects of these changes are then reflected in the lowered soil pH which was witnessed. Differences observed in P availability, with higher Olsen P in low biosolid application rate soils, may also be explained by this increase in r-strategist bacteria in the soil. If SOM was broken down at an unsustainable rate due to stimulation of microbes from low biosolid applications then organic P may be released back to the soil as SRP when these microbes die off, due to exhausting the available SOC for sustaining their metabolisms. This is the so-called 'priming effect' and would explain the higher soil Olsen P measured in low biosolid application soils, leading to more plant available P in these soils in the short-term.

Crop establishment differences were only observed in the control boxes of Experiments 1 and 4, which saw fewer plants establish. These were the soils collected during the autumn and which had not had any fresh fertiliser applications prior to collection. The Olsen P of these soils was measured lower than in Experiments 2 and 3, likely due to the P fertiliser application prior to Experiment 2 soils being collected, and the increased P still being present in Experiment 3 soils collected during the same growing season. The higher crop available P appeared to mitigate poor plant establishment, with biosolid applications also mitigating poor establishment effects at any application rate. Improved soil TKN, Olsen P, SOM, soil structure and other plant nutrients contained in the biosolids also likely contributed towards improved crop establishment, but it is difficult to isolate individual effects. Despite the establishment differences crop development did not appear to be affected further during the initial growing period, with crop height measurement taken showing no differences between treatments. However, crop dry weight biomass measurements taken later in the experiment after flood recovery show that high rates of biosolid application may have improved plant size and resilience beyond what could be discerned from plant height measurements alone.

7.2 FLOODING

Upon initiation of the 10-day flood soil porewaters quickly became oxygen depleted, as determined through the DO_2 levels measured using oxygen sensor spots in Experiment 4, and by the immediately lowering soil porewater Eh in all experiments. In contrast, all flood surface waters remained oxygenated throughout the duration of the experiments. Despite evidence in the bacterial characterisation of increased microbial respiration in biosolid-amended soils no differences were observed in the rate of oxygen depletion between control or biosolid-applied soil porewaters. The rate of O_2 depletion in all soils was too rapid to detect differences with the methods used and was therefore likely negligible. Eh in soil porewaters began to drop immediately, but its response was not as extreme as observed with the O_2 depletion. Eh took several days to reach an equilibrium value, which in most cases was +200mV, with the exception of several control box porewaters which continued to slowly drop below this threshold. The slowly lowering Eh was likely due to the depletion of trace amounts of O_2 in porewaters, after which the ORP became poised at NO_3^- reduction, which was identified as the +200mV threshold. In control soils where no N addition had been made through biosolids NO_3^- became reduced quickly through denitrification and the Eh continued to drop below +200mV in these systems. However, some soils saw a maintenance of the +200mV threshold even where NO_3^- was very low, and the control soil of Experiment 3 saw a drop below +200mV despite having an NO_3^- presence measured in floodwaters. This could suggest other factors contribute to the maintenance of the +200mV threshold, not simply the presence of NO_3^- as an electron acceptor, but another factor relating to biosolid application. One possible explanation is that the Eh is also coupled to the reduction of Fe^{3+}, with high Fe content identified in the biosolid analysis results (Tab. 4.3). Whichever the reason, the presence of alternative electron acceptors in the system maintained a +200mV threshold in most biosolid-applied soils. This prevented highly reducing conditions from occurring in those soil porewaters and therefore prevented the further reduction of Fe^{3+} to Fe^{2+} in those systems.

This maintenance of ORP above highly reducing conditions was important to this project, which sought to identify the risk of P loss to floodwater. Had ORP conditions reached a level which led to the reduction of Fe^{3+} in the system then any P sorbed in FeP minerals will have been released as SRP to floodwater. However, the moderate reducing conditions which occurred in all soils are expected to have reduced solid phase Mn^{4+} and led to the release of its P associated with this phase, though this was likely small. Drained floodwater results from the experiments showed that SRP release to floodwater was not influenced by biosolid application, despite biosolids containing very high amounts of P. This indicates that the P contained in biosolids is tightly bound and not readily soluble. The high Fe content of the biosolids used could indicate that a large proportion of the P

contained is Fe bound, and therefore only slowly soluble in soils unless highly reducing conditions occur in the soil, leading to the release of this Fe associated P. The soils used contained a similar concentration of Fe as biosolids, as indicated in the XRF analysis (Tab. 3.6), meaning there is likely also a large pool of Fe bound P in the soil. Though biosolid-applied soil porewaters did not become reducing enough to lead to Fe^{3+} reduction, had the flood been longer and led to the depletion of NO_3^-, which was likely maintaining the ORP threshold, then this may lead to very large amounts of SRP in floodwater. The lowering of Eh below the +200mV threshold may have shown the beginnings of this, with P measured in several of the control soil floodwaters being higher than many of the biosolid-applied soils. Though this is difficult to support with the limited floodwater analysis data gathered. If this is the case however, then control soils with low N contents may pose a greater risk to watercourses from release of their native soil P than biosolid-applied soils. In biosolid-applied soils the N addition from the biosolids could delay the onset of highly reducing conditions, with the high biosolid Fe content preventing the loss of SRP under only mildly reducing conditions. This would indicate that biosolid-applied P could be a low-risk method for applying P to soils at risk of flooding. The comparison to mineral fertiliser P afforded by the soils of Experiment 2 which had a fertiliser P addition prior to collection also supports this. The floodwaters of Experiment 2 had the highest total P contents of any experiment and these did not correlate to biosolid addition. Instead, they appeared to be linked to the P which was applied in the fertiliser in a soluble form. This demonstrated the higher stability of biosolid contained P over fertiliser P in flooded conditions and the lower risk of biosolids for causing P pollution, even when applied closer to the time of the flood.

As with floodwater P, surface water P also showed no correlation to biosolid application. Surface water P was measured lower than floodwater P in all but one case, which was an anomaly caused by lack of algae growth on that box. This would indicate that algae controls the release of P to overlying surface waters. The lack of strong redox threshold between the surface and porewater would support this, as porewater conditions did not become reducing enough to create such a threshold. This means P release to surface waters was being controlled by another mechanism, in this case algae uptake. Floodwater NH_4^+ levels were very low in all floodwaters, as was to be expected in an aerated soil system prior to flooding where nitrification would occur. The occurrence of nitrification was also supported by the high abundances of nitrifying bacteria identified in all soils, including high abundances of bacteria in the phylum *Nitrospirae*. Conditions then did not become reducing enough during the flood to see any NO_3^- reduction to NH_4^+. Denitrification does appear to have occurred in floodwaters, as indicated by the rising pH over time in all surface and porewaters. Additionally, low NO_3^- in several low-rate biosolid applied soils, despite a known N addition, indicates depletion over time, and denitrification is a widespread function in environmental microbial

populations, so is likely to have occurred. NO_3^- contents of floodwaters then appeared to show a heightened presence in the 400g and above biosolid-applied soils after the 10-day flood. This would support current legislation which limits applications to this maximum field application rate.

In the soil bacterial populations during the flood the *Proteobacteria* phylum further increased in relative abundance in addition to the increases observed due to biosolid application. This again highlights the *Proteobacteria* phylum's ability to quickly adapt to and capitalise on changing environmental conditions more than other bacterial phyla. In contrast, the *Acidobacteria* phylum decreased in relative abundance in the same soils, showing it could not compete at the same level as *Proteobacteria.* Several other phyla increased in relative abundance from during the flood, including *Firmicutes, Synergistetes* and *Atribacteria*. These increases were seen only in biosolid-applied soils, with several species from the three phyla found to be present only in those soils. This is believed to be due to those species being anaerobes present in biosolids, then transferred to soils upon biosolid application, or present in the soil in small numbers and able to increase in abundance due to the substrate provided by biosolids. Upon flooding these species were then been able to flourish in the anaerobic soil conditions created by the flood, with anoxic conditions shown to be induced quickly in floodwaters as previously discussed.

7.3 POST-FLOOD RECOVERY

Varied timeframes for recovery were allowed in each experiment after draining of the flood, but all allowed time for the determination of plant survival after flooding. Results across experiments were mixed, but the control boxes of Experiments 1 and 4, which had lower established plants, had the highest survival rates. This is probably explained by less competition for resources due to lower plant numbers and that only strong seedlings had established in those soils, so were better equipped to deal with the stress caused by the flood. The only other consistent trend was that the 100g biosolid-applied boxes in all three experiments where it was conducted (Experiments 1-3) had consistently poor flood survival rates. This may be due to sample variation but could also reflect the low nutrient application, which helped the plants establish but could not sustain crop growth, leading to weaker plants which were more susceptible to flood stress and death. Plant flood survival in all other boxes in the experiments showed no trend based on biosolid application. Plant dry weight biomass measurements showed that 800g and 1600g biosolid-applied boxes consistently performed better than other boxes and had larger plants. This was likely due to the high nutrient content supplied to those soils, resulting in increased nutrient uptake by the crop and stronger growth. However, these 800g and 1600g biosolid application rates exceeded the maximum permissible field application rates. The dry weight biomasses in other boxes were inconsistent, but

there were suggestions that control soil plants outperformed low-rate biosolid-applied soils in Experiments 1, 3 and 4. This trend was again attributed to poor establishment and nutrient supply, leading to only stronger plants surviving the flood and continuing to grow well post-flood with less competition. Plant establishment at the beginning of the experiment therefore had an effect on all plant measurements during the experiments.

Bacterial populations post-flood appeared to recover to pre-flood levels, with no lasting changes induced by the flood. Changes in relative abundance of the two main phyla of *Acidobacteria* and *Proteobacteria*, decreasing and increasing during the flood respectively, returned to pre-flood ratios. The influences of the biosolid nutrient addition, increasing the relative abundance of *Proteobacteria* in biosolid-applied soils, remained after recovery. This therefore appears to be a longer-term effect induced by the biosolid nutrient addition, and soil TKN, Olsen P and SOM remained elevated from biosolid addition throughout the experiments, with no flood or time effect observed. The less abundant bacterial phyla identified as anaerobes which increased during flooding in biosolid-applied soils, namely *Firmicutes, Synergistetes* and *Atribacteria*, also returned to pre-flood levels after the 20-day recovery period. This shows that, though these species were able to increase in relative abundance when conditions were favourable, these were not lasting effects. Any bacteria introduced to the soil from biosolids are not likely to outcompete native populations under stable soil conditions. However, over a longer-term flood these species may continue to increase in abundance and could be transferred to other, more favourable conditions in the wider environment where they could persist. Over a long-enough time frame under normal, aerated soil conditions however, these species may not persist in the environment at all.

7.4 LIMITATIONS

The main limitation of this project was the lack of statistical analysis of the crop and geochemical results. This limitation was identified early in the project due to the limited space available for controlled laboratory experiments. To compensate for this issue and to gather a broad range of data about the system the gradient of treatment conditions (biosolid application) was decided on. This was coupled with repeatable experiments, with frequent measurements and a range of analysis carried out throughout each. The nature in which the experiments evolved, with varying basal soil properties from different fertiliser regimes in the field, and the control soils yielding the most interesting results from the flood monitoring measurements, meant that such results could not be reliably statistically compared. An option to standardise soils across all four experiments would have been to incubate a large volume of soil at the beginning for use throughout the project. However, this was impractical, and would have had potential consequences such as changes in microbial

activity and composition, resulting in a soil which was not representative of a living field soil. As the microbial response was integral to this project the incubation option was deemed unacceptable. Running six boxes with three control replicates and three biosolid-applied replicates may have offered an option for statistical comparison, but the range of data gathered would have been much narrower and many of the effects observed and understood may have been missed. The ideal solution would have been to run one large experiment with multiple replicates at the same time. However, due to the area required for controlled laboratory conditions and the range of analysis carried out this methodology was beyond the scope of the project. While the lack of statistical analysis was a limitation and meant visual assessment of data from each experiment was required instead, it did also allow for extensive qualitative analysis. Several findings and conclusions of this research result from the inherent variation between boxes and experiments, rather than in spite of them.

Another limitation which was identified at the start was the relatively high temperature in the laboratory, above that which would be expected under field conditions. However, this limitation existed due to the benefits of being able to closely control the light and water conditions for crop growth, to eliminate any threat of disease and pest damage, and to allow repeatability of the experiment year-round. A greenhouse experiment was considered, but the conditions were more variable than the laboratory and the logistics of monitoring and sample analysis would have been more difficult. A cooler temperature-controlled laboratory space was not available. Temperature-controlled growth chambers were not available and were outside the financial limitations of the project, considering the resources needed for the wide range of analysis undertaken. Therefore, the decision was made to carry out the experiments in a slightly warmer laboratory due to the benefits outweighing the negatives. The higher temperature may have increased microbial activity in the soil above that observed in the field and some geochemical reactions in the floodwater may have been accelerated.

Chapter 8: CONCLUSIONS

8.1 KEY FINDINGS

This thesis set out to test the hypotheses, aims and objectives outlined in the introduction chapter, section 1.2.2. These hypotheses related to factors of crop response, soil and water geochemistry, and soil microbiology and their response to biosolid applications and an extreme weather event leading to short-term flooding. The strength of the project lies in its study of a closely controlled holistic system which resembles field conditions, rather than individual elements in isolation. The experimental system which was designed, built and implemented for this project performed well, and a broad range of high-quality data was collected. The key findings mainly related to the geochemistry and the redox potential of the flooded soil system, and the effects of flooding and biosolid application on the soil bacterial populations. Though some trends were identified in the crop response data, there was no conclusive evidence supporting any of the crop effects. The key findings of this project were:

- O_2 and NO_3^- control the redox potential of a flooded soil system, which in turn governs the mobility of P in the soil porewater and surface water. O_2 is depleted quickly in all soils, causing Eh to drop and then is maintained at NO_3 reduction, around +200mV, until its depletion. Systems with higher NO_3 contents mean the system is poised at +200mV for longer, preventing more highly reducing conditions from occurring. This prevents the reduction of Fe^{3+} to Fe^{2+}, therefore preventing the mobilisation of Fe sorbed P to floodwater.

- The bacterial populations of soils and biosolids were very dissimilar. Biosolids contained far more bacterial DNA on a sample weight/weight basis than soils. However, there was little carryover of these biosolid populations to the soil on application, with the soil microbiome appearing very resilient to new bacterial species. The nutrient addition provided by biosolids did affect the relative abundances of native soils populations, but treated soils still closely resembled control soils.

- A 10-day flood altered the relative abundances of soil bacteria, with more adaptable phyla able to capitalise on the changing conditions, some of which also favoured biosolid-applied soils. However, after a 20-day recovery period flooded soils had recovered to a pre-flood state and no lasting effects on the bacterial populations were observed.

8.2 RECOMMENDATIONS

Current wastewater management and controls means risk of PTE contamination to soils from biosolids is low. Modern wastewater treatment and digestion methods mean transfer of pathogens is also very low, as supported by the bacterial characterisation of biosolid-applied soils in this project. Some anaerobes from the digestion process may survive in very small quantities in the soil, and could increase under flooding, but these effects did not persist in the soil after flooding, and the species identified were not pathogenic. Biosolids are therefore a safe soil input, with minimal risk to the health of the soil microbiome or to humans, with no increased risk induced by flooding.

Soil N release to floodwater after 10 days of flooding supports current maximum organic N application limits for protecting watercourses from NO_3^- pollution. Soils applied with the maximum application rate of biosolids saw floodwater soluble N begin to increase. Rates above the maximum application then saw very high soluble N in floodwaters, indicating these rates led to a high risk of N loss from soils. However, the NO_3^- present in the floodwater was likely responsible for maintaining the soil porewater ORP and preventing SRP release, with more NO_3^- leading to a longer time before elevated SRP loss occurs. The current guidance relating to organic N additions to land with a high N leaching risk therefore appears to be supported by the results gathered.

The P contained in biosolids appeared to be very stable, with no increased SRP loss to floodwater from biosolid-applied soils than control soils over the 10-day flood, even when biosolid P was applied at extreme rates. This was likely due to much of the biosolid contained P being sorbed to Fe, and conditions not becoming reducing enough to see this P released due to porewater NO_3^- maintaining the ORP above the reduction threshold. On the other hand, an application of mineral fertiliser P to soils prior to biosolid application showed a rise in SRP release to floodwater from all soils. Biosolids therefore appear to be a more suitable source of crop fertiliser P than conventional mineral P fertiliser for application to flood-risk land with high watercourse P pollution potential.

Despite the low mobility of P from biosolids, applications still saw increased soil plant available Olsen P, as well as increases in SOM and TKN. Biosolid application also mitigated poor crop establishment when soil quality was low, and high application rates saw increases in plant dry-weight biomass. These results suggest that biosolids are an effective method for improving soil nutrient status and crop performance. However, low applications of biosolids showed some indication of negatively affecting crop flood survival rates, possibly due to weaker plants establishing, with flood stress then leading to their death. Given the crop effect observed, low applications of biosolids could be detrimental to crop flood survival, therefore it would be more beneficial to apply maximal or near-maximal rates of biosolids to flood-risk land, governed by existing N application guidance.

8.3 FURTHER WORK

8.3.1 Further exploration of geochemical factors

Further exploration of the mechanisms controlling P solubility and N transformations could be incorporated into the experimental setup for further understanding of the system. This could be achieved by carrying out more frequent and extensive measurements of key factors that were identified as important geochemical influences in this project. Although the ORP, pH and DO_2 were measured regularly throughout the flood, improvements to the methods could still be made. Introducing an autologger system to monitor pH, DO_2 and Eh would increase the accuracy and reliability of those results. This method was considered, but the resources were not available to monitor all the boxes in this way at the same time. Having probes which are permanently fixed in position would remove any issues of disturbing the system by inserting probes each time. Fixed probes and electrodes would also allow them to be maintained at equilibrium in the system rather than requiring time to equilibrate when measurements were undertaken.

Improvements could also be made to the soluble N and P measurements which were only taken once at the end of the flood. Measuring floodwater NO_3^-, SRP and P adsorbing minerals in solution (Mn, Fe, Ca) regularly throughout flooding would provide more detailed support to many of the assumptions made in this project. The conditions leading to the reduction of Mn^{4+} and Fe^{3+}, and the release of their associated P, could be further investigated by measuring the Mn^{2+} and Fe^{2+} dissolved in solution. Knowing the NO_3^- content of floodwaters at each stage of the flood could also show its link to maintaining Eh. The mechanisms leading to the depletion of NO_3^- could be monitored by measuring gas fluxes from flooded soils, such as N_2O loss as a consequence of denitrification. Furthermore, the sampling methods for floodwaters could be improved to more accurately distinguish between surface water and porewater. Rather than using surface water and whole floodwater measurements, use of capillary samplers could allow for more accurate geochemical characterisation and monitoring of soil porewater.

Further to the improved monitoring and measurement of geochemical factors, independent variables in the experiment such as flood length, soil type and biosolid type could be manipulated. This could allow for a wider range of environmental conditions to be observed and understood. A longer flood period could allow more highly reducing conditions to be achieved, enabling longer-term changes in Eh caused by NO_3^- depletion to be observed at a range of biosolid application rates. Soil types with different native P, N and SOM content could be used to observe any differences between native and biosolid-applied nutrients. Soils with lower pH could lead to the dissolution of Ca minerals and the release of any P they contained to floodwaters. Finally, different biosolid types,

or even a range of different organic inputs, could be studied within the experimental system to further understand differences in pollution risk between these inputs under flooding.

8.3.2 Transfer of anaerobic bacterial species from biosolids to anaerobic soils

Further exploration of the bacterial responses observed in this project could be carried out with a focus on more extreme flooding conditions. Some anaerobic bacterial populations were transferred from biosolids to soils and inducing anaerobic flood conditions caused several of these bacteria to increase in abundance in the soil. These included bacteria in the phyla *Firmicutes, Synergistetes* and *Atribacteria,* but all populations returned to pre-flood relative abundances 20 days after flooding. This project focused on a short-term flood of 10 days, but extension of the flood period may lead to a greater increase in some bacteria and an increased threat that they could persist in the wider environment. A longer flood with potentially more extreme changes to microbial populations could require a longer recovery period which could periodically be monitored to assess recovery progress. More frequent sampling in the recovery period of the experiments in this project was discussed but was outside the scope of resources, and a focus was made on more replicates at fewer sample timings. Accurately assessing the rate of microbial recovery from different flood lengths could offer more valuable insights into the extent of flood and biosolid environmental impacts.

Another topic which could be important to explore is the potential presence and survival of pathogenic anaerobic bacteria from any secondary wastewater treatment process. If these species enter soils through biosolid application they could increase in abundance due to anaerobic flood conditions. Anaerobic human pathogenic bacteria are known to belong in the genera *Clostridium* and *Bacillus,* both of which belong in the *Firmicutes* phylum. In fact, the most abundant OTU of the biosolid samples of this project belonged to a species in the order 'Clostridales' which can contain the *Clostridium* genus, and this OTU was present in biosolid-applied soils. Hence, there is precedent to show that species in the same order as known human pathogens can survive in soils and may be increased in abundance with flooding. Despite this potential concern, no pathogenic bacterial species were detected in the biosolid or soil samples of this project. However, the soil bacterial population characterisation in this project was carried out using primers to target the hypervariable 16S rRNA gene, which allowed for a general characterisation of bacterial species. This method may not have been sensitive enough to detect small, novel populations of potential pathogens. Methods to target known pathogenic bacteria could be used to detect the presence of any of these species are present in biosolids and soils after biosolid application, and if they increase in abundance with flooding. Targeted analysis such as this could help ensure that biosolids are safe for application to land under even extreme environmental conditions when properly managed. This could further assure of the safety of biosolids as an agricultural soil organic input.

APPENDIX A: EXPERIMENTAL SETUP

A.1 FIELD CROPPING HISTORY

Table A.1 – Details of the cropping history for Big Substation field, Spen Farm, 1970-2019.

Year	Crop	Year	Crop	Year	Crop	Year	Crop	Year	Crop
1970	Oats	1980	Spring barley	1990	Potatoes	2000	Winter wheat	2010	Winter wheat
1971	Winter wheat	1981	Set aside	1991	Winter barley	2001	Winter wheat	2011	Oilssed rape
1972	Potatoes	1982	Winter wheat	1992	Grass ley	2002	Potatoes	2012	Winter wheat
1973	Winter wheat	1983	Potatoes	1993	Permanent pasture	2003	Winter wheat	2013	Vining peas
1974	Beet	1984	Winter wheat	1994	Permanent pasture	2004	Oilssed rape	2014	Winter wheat
1975	Winter wheat	1985	Winter wheat	1995	Winter wheat	2005	Winter wheat	2015	Winter wheat
1976	Set aside	1986	Spring barley	1996	Potatoes	2006	Beet	2016	Spring barley
1977	Set aside	1987	Winter barley	1997	Unknown	2007	Winter wheat	2017	Winter barley
1978	potatoes	1988	Unknown	1998	Winter wheat	2008	Winter wheat	2018	Oilssed rape
1979	Beet	1989	Oats	1999	Winter wheat	2009	Potatoes	2019	Winter wheat

A.2 GROWTH BOX PHOTOSYNTHETICALLY ACTIVE RADIATION

	BOX 1						BOX 2						BOX 3				
	Lights			Row total	Average		Lights			Row total	Average		Lights			Row total	Average
Back	5.5	8.58	7.2	21.28	7.09		6.77	6.23	7.4	20.4	6.80		6.97	7.25	6.15	20.37	6.79
Middle	8.23	12.34	12.92	33.49	11.16		9.92	9	9.78	28.7	9.57		11.66	10.65	8.29	30.6	10.20
Front	4.52	6.38	6.7	17.6	5.87		4.53	4.93	5.43	14.89	4.96		5.28	5.3	4.9	15.48	5.16
			Box Total	72.37	8.04				Box Total	63.99	7.11				Box Total	66.45	7.38
	Range =	4.52-12.92					Range =	4.53-9.78					Range =	4.90-11.66			
	BOX 4						**BOX 5**						**BOX 6**				
	Lights			Row total	Average		Lights			Row total			Lights			Row total	Average
Back	6.94	8.3	8.35	23.59	7.86		5.55	5.62	6.73	17.9	5.97		6.9	6.71	5.61	19.22	6.41
Middle	8.62	10.22	10.4	29.24	9.75		8.2	8.24	10.45	26.89	8.96		10.54	9.04	7.03	26.61	8.87
Front	4.2	5.6	5.97	15.77	5.26		4.44	4.39	5.17	14	4.67		6.56	4.97	4.64	16.17	5.39
			Box Total	68.6	7.62				Box Total	58.79	6.53				Box Total	62	6.89
	Range =	4.20-10.22					Range =	4.39-10.45					Range =	4.64-10.54			

Figure A.0.1 - Photosynthetically active radiation measured at the soil surface in growth boxes using a Skye SKP 200 light meter, containing a SKP 215 sensor. All units displayed are x10 µmol m^{-2} s^{-1}.

Table A.2 – Photosynthetically active radiation summary of measurements across all boxes. All units displayed are x10 µmol m^{-2} s^{-1}.

	Low	High	Range	Mean	STDEV.P
Back	17.90	23.59	5.69	20.46	1.76
Middle	26.61	33.49	6.88	29.26	2.33
Front	14.00	17.60	3.60	15.65	1.11
Total	58.79	72.37	13.58	65.37	4.42

A.3 GROWTH CHAMBER AMBIENT TEMPERATURE

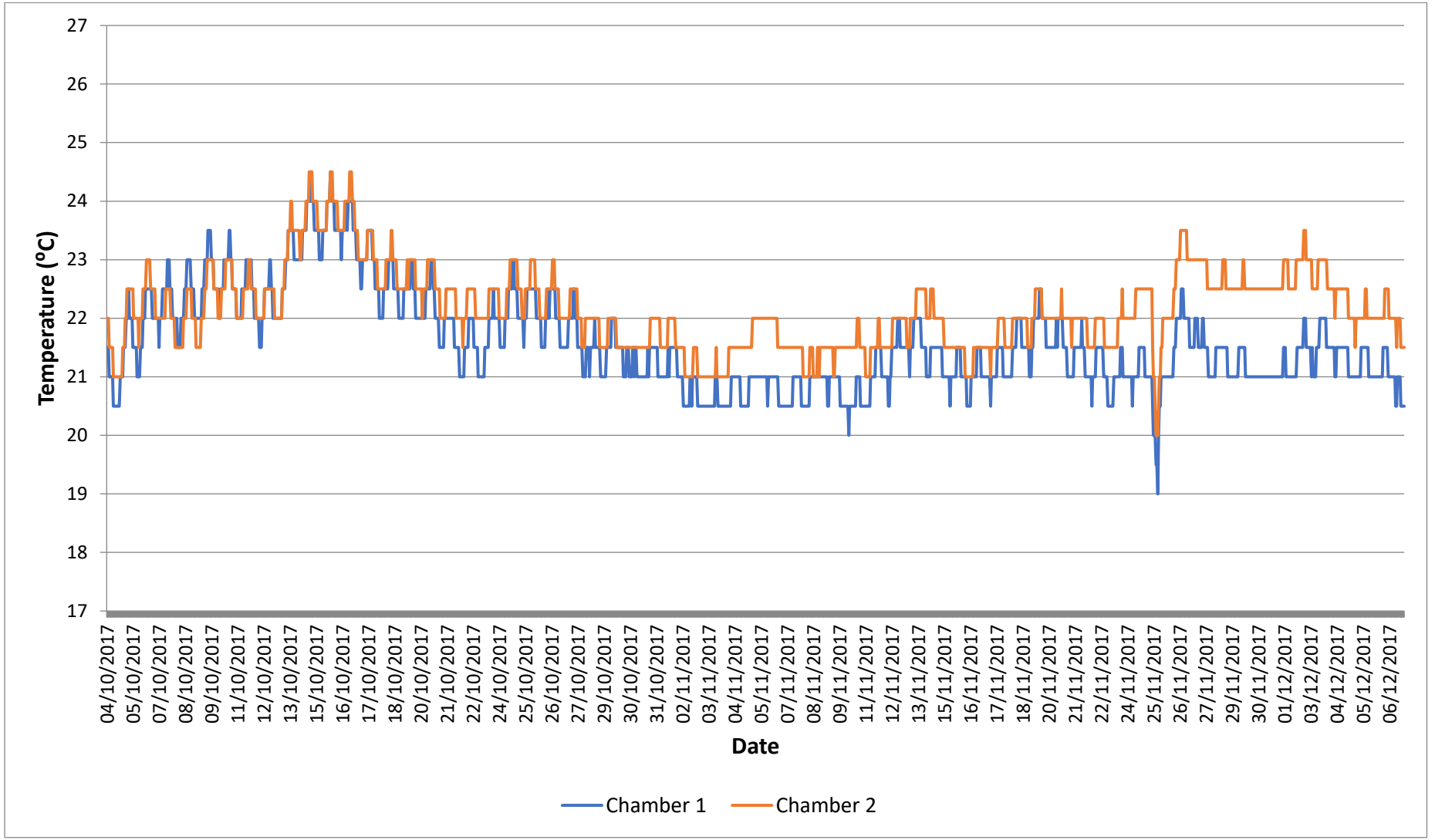

Figure A.2 - Experiment 1 ambient temperature recorded in growth chambers during experiment using autologger.

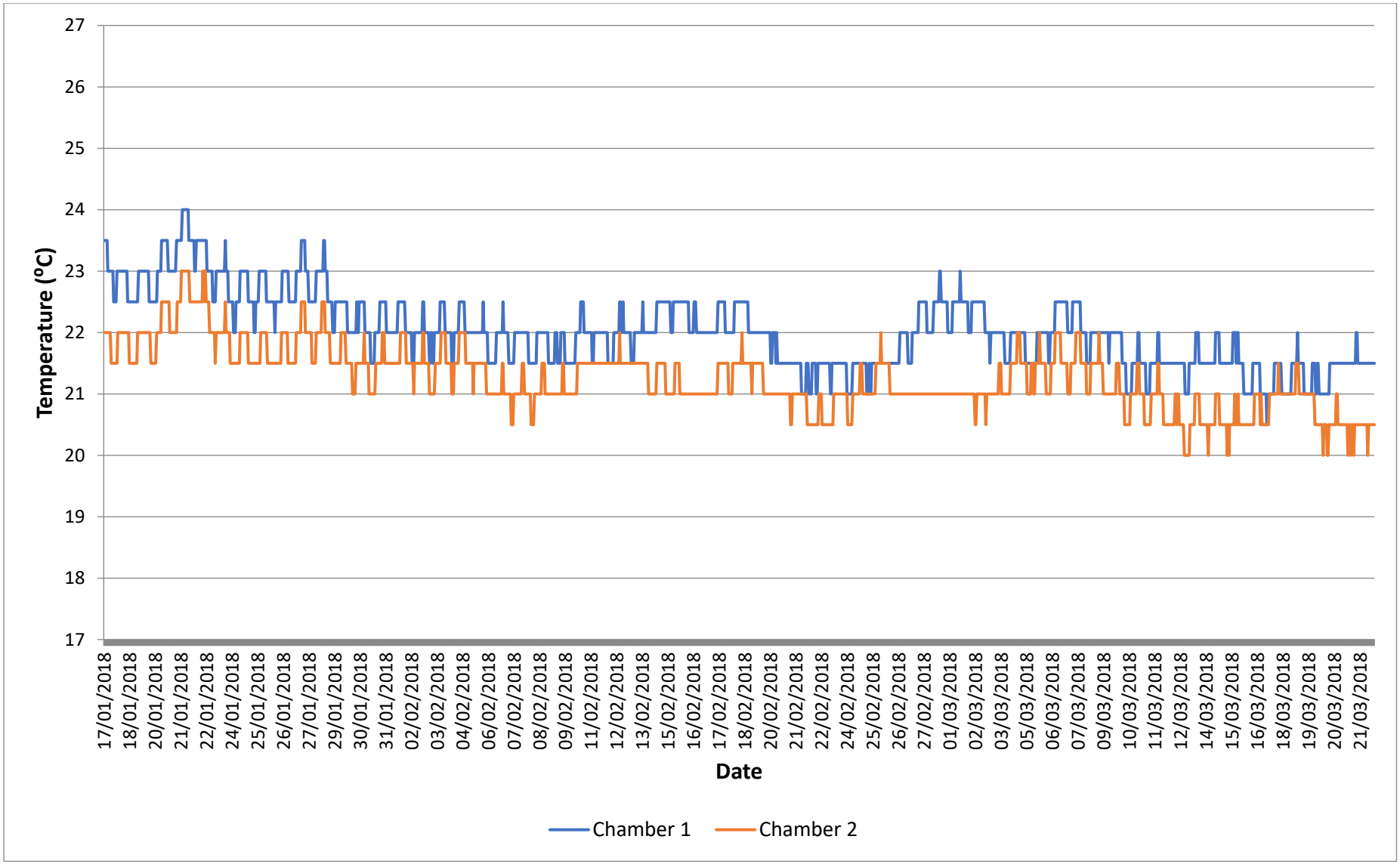

Figure A.3 - Experiment 2 ambient temperature recorded in growth chambers during experiment using autologger.

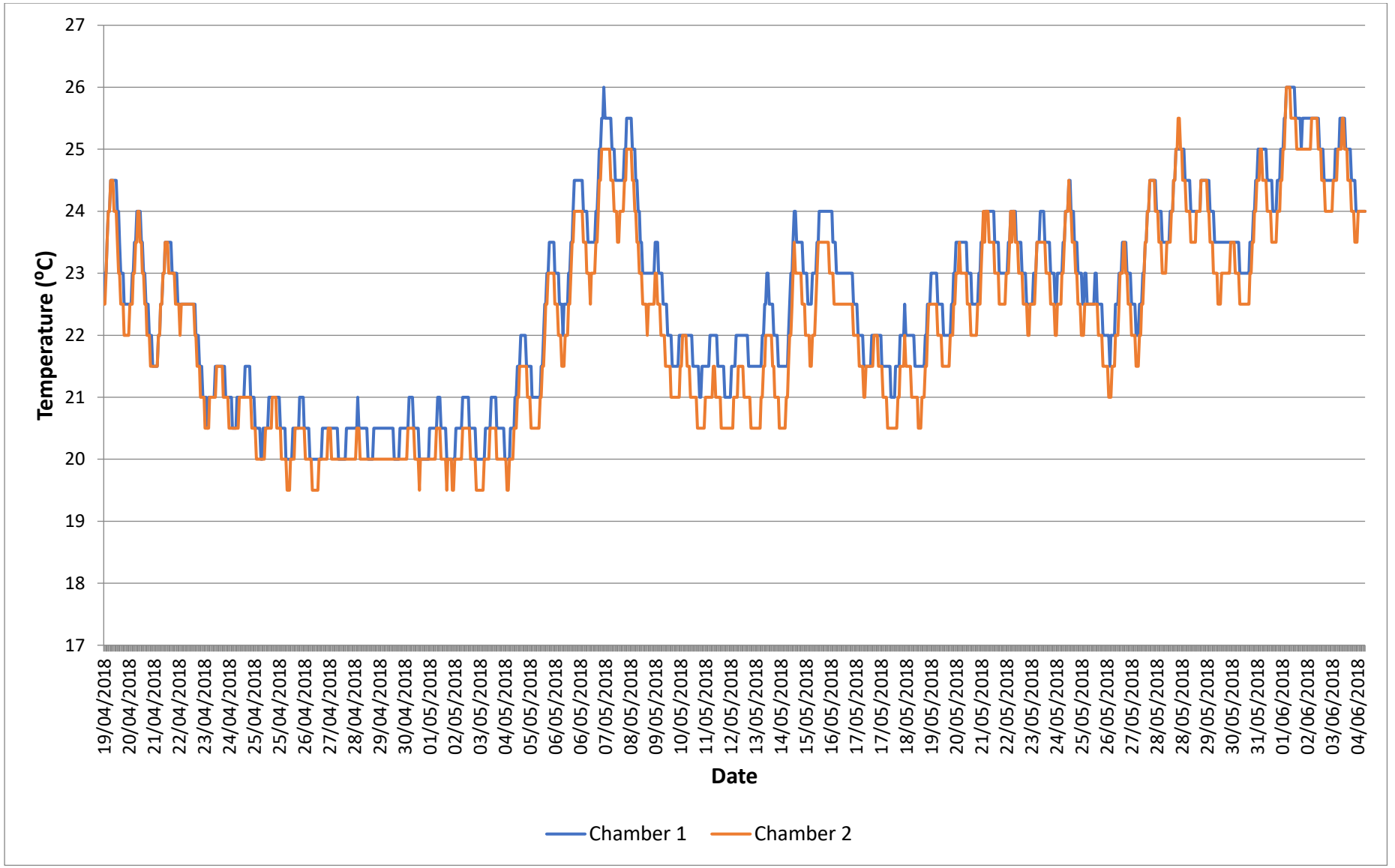

Figure A.4 - Experiment 3 ambient temperature recorded in growth chambers during experiment using autologger.

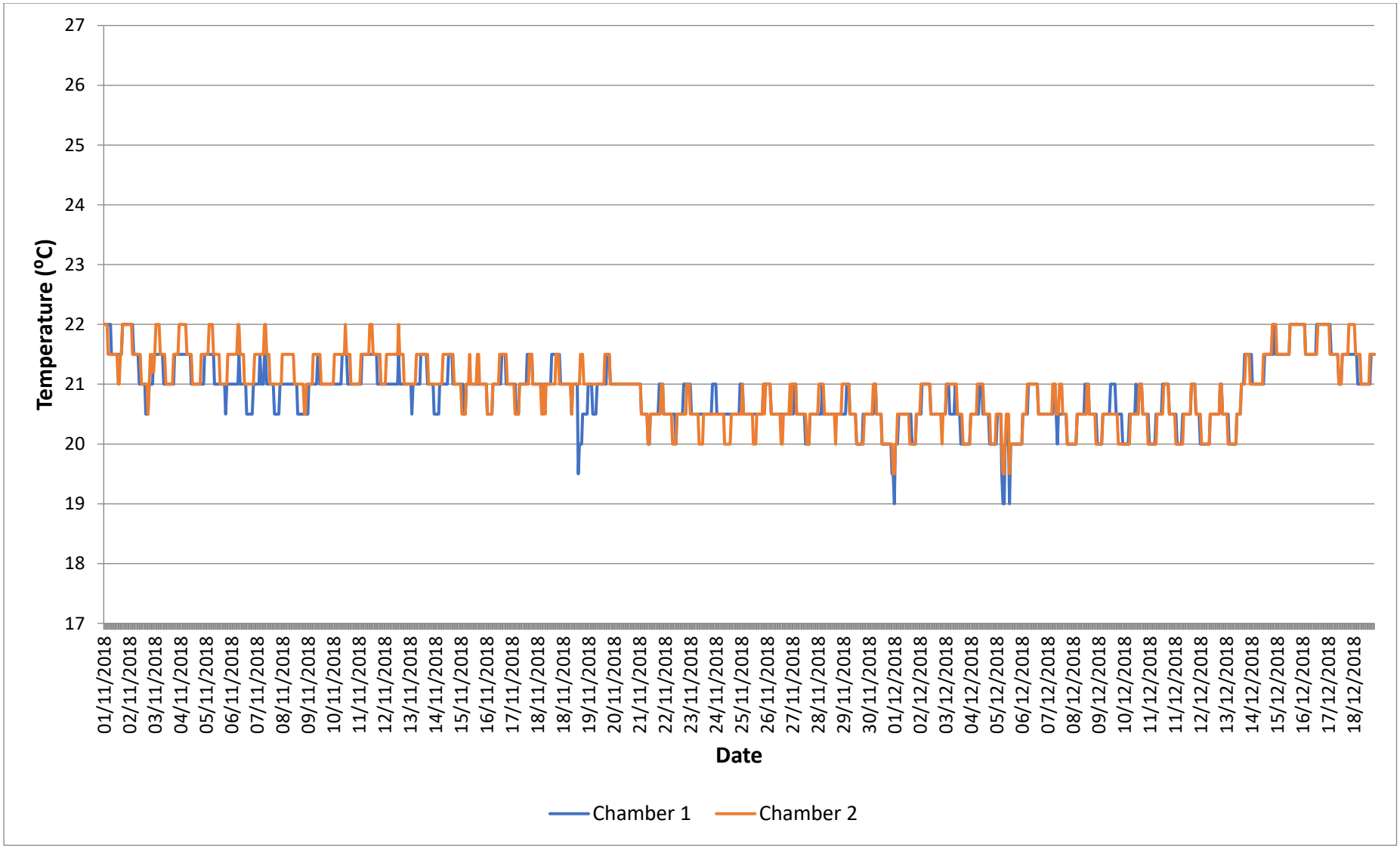

Figure A.5 - Experiment 4 ambient temperature recorded in growth chambers during experiment using autologger.

APPENDIX B: SOIL ANALYSIS RESULTS

B.2 SOIL ORGANIC MATTER

Table B.1 - Experiment 1 SOM analysis results in full.

Timing	Box biosolid application (g)	Box	Sample	SOM (%)	SOC (%)	Mean SOM (%)	Mean SOC (%)	SOM Standard deviation
Initial			1	2.93	1.64			
			2.1	2.78	1.56			
			2.2	2.64	1.48			
			3	2.78	1.56	2.78	1.56	0.10
Pre-Flood			1	2.63	1.47			
			2	2.59	1.45			
	0	6	3	2.66	1.49	2.63	1.47	0.03
			1	2.68	1.50			
			2	2.85	1.60			
	100	3	3	2.66	1.49	2.73	1.53	0.08
			1	2.86	1.60			
			2	2.88	1.61			
	200	4	3	2.90	1.63	2.88	1.61	0.02
			1	2.96	1.66			
			2	2.86	1.60			
	400	5	3	2.84	1.59	2.88	1.61	0.05
			1	3.09	1.73			
			2	3.04	1.70			
	800	2	3	2.99	1.68	3.04	1.70	0.04
			1	3.84	2.15			
			2	3.88	2.17			
	1600	1	3	3.19	1.79	3.64	2.04	0.32
Post-Flood			1	2.78	1.56			
			2	2.81	1.58			
	0	6	3	2.72	1.52	2.77	1.55	0.04
			1	2.84	1.59			
			2	2.85	1.60			
	100	3	3	2.82	1.58	2.84	1.59	0.01
			1	2.88	1.61			
			2	2.81	1.57			
	200	4	3	2.87	1.61	2.85	1.60	0.03
			1	2.93	1.64			
			2	2.97	1.66			
	400	5	3	3.11	1.74	3.01	1.68	0.08
			1	3.14	1.76			
			2	3.09	1.73			
	800	2	3	3.26	1.83	3.17	1.77	0.07
			1	4.00	2.24			
			2	3.63	2.03			
	1600	1	3	3.23	1.81	3.62	2.03	0.31
Final			1	2.80	1.57			
			2	2.95	1.65			
	0	6	3	2.93	1.64	2.89	1.62	0.06
			1	2.73	1.53			
			2	2.78	1.56			
	100	3	3	2.82	1.58	2.78	1.56	0.04
			1	2.86	1.60			
			2	3.22	1.80			
	200	4	3	3.09	1.73	3.06	1.71	0.15
			1	3.15	1.76			
			2	3.17	1.77			
	400	5	3	3.03	1.70	3.11	1.74	0.06
			1	3.34	1.87			
			2	3.37	1.89			
	800	2	3	3.21	1.80	3.31	1.85	0.07
			1	3.35	1.88			
			2	3.57	2.00			
	1600	1	3	3.84	2.15	3.59	2.01	0.20

Table B.2 - Experiment 2 SOM analysis results in full.

Timing	Box biosolid application (g)	Box	Sample	SOM (%)	SOC (%)	Mean SOM (%)	Mean SOC (%)	SOM Standard deviation
Initial			1	2.81	1.58			
			2	2.70	1.51			
			3	2.88	1.61	2.80	1.57	0.07
Pre-Flood			1	2.69	1.50			
			2	2.69	1.51			
	0	4	3	2.82	1.58	2.73	1.53	0.06
			1	2.81	1.57			
			2	2.91	1.63			
	100	1	3	2.93	1.64	2.88	1.61	0.05
			1	2.91	1.63			
			2	2.94	1.65			
	200	3	3	2.93	1.64	2.93	1.64	0.01
			1	2.89	1.62			
			2	3.10	1.73			
	400	2	3	3.18	1.78	3.05	1.71	0.12
			1	2.95	1.65			
			2	3.33	1.86			
	800	6	3	3.28	1.84	3.19	1.78	0.17
			1	3.62	2.03			
			2	3.62	2.02			
	1600	5	3	3.71	2.07	3.65	2.04	0.04
Post-Flood			1	2.91	1.63			
			2	2.69	1.50			
	0	4	3	2.83	1.59	2.81	1.57	0.09
			1	2.87	1.61			
			2	2.81	1.57			
	100	1	3	2.94	1.64	2.87	1.61	0.05
			1	2.98	1.67			
			2	3.17	1.78			
	200	3	3	3.22	1.80	3.13	1.75	0.10
			1	3.01	1.69			
			2	3.12	1.75			
	400	2	3	3.02	1.69	3.05	1.71	0.05
			1	3.38	1.89			
			2	3.24	1.81			
	800	6	3	3.33	1.86	3.32	1.86	0.06
			1	3.67	2.06			
			2	3.53	1.97			
	1600	5	3	3.70	2.07	3.63	2.04	0.08
Final			1	2.69	1.50			
			2	2.80	1.57			
	0	4	3	2.72	1.52	2.74	1.53	0.05
			1	2.88	1.61			
			2	2.57	1.44			
	100	1	3	2.88	1.61	2.77	1.55	0.15
			1	2.92	1.63			
			2	3.05	1.71			
	200	3	3	2.88	1.61	2.95	1.65	0.07
			1	3.06	1.72			
			2	3.02	1.69			
	400	2	3	2.96	1.66	3.02	1.69	0.04
			1	3.43	1.92			
			2	3.13	1.75			
	800	6	3	3.20	1.79	3.25	1.82	0.13
			1	3.65	2.04			
			2	3.34	1.87			
	1600	5	3	3.29	1.84	3.43	1.92	0.16

Table B.3 - Experiment 3 SOM analysis results in full.

Timing	Box biosolid application (g)	Box	Sample	SOM (%)	SOC (%)	Mean SOM (%)	Mean SOC (%)	SOM Standard deviation
Initial			1	2.96	1.66			
			2	2.80	1.57			
			3	2.64	1.48	2.80	1.57	0.13
Pre-Flood			1	2.68	1.50			
			2	2.72	1.52			
	0	6	3	2.69	1.51	2.69	1.51	0.02
			1	2.82	1.58			
			2	2.96	1.66			
	100	4	3	2.78	1.56	2.85	1.60	0.08
			1	2.97	1.66			
			2	3.06	1.71			
	200	2	3	3.12	1.75	3.05	1.71	0.06
			1	3.05	1.71			
			2	3.24	1.81			
	400	1	3	3.02	1.69	3.10	1.74	0.10
			1	3.16	1.77			
			2	2.99	1.67			
	800	3	3	3.37	1.89	3.17	1.78	0.16
			1	3.62	2.03			
			2	3.75	2.10			
	1600	5	3	3.41	1.91	3.59	2.01	0.14
Post-Flood			1	2.69	1.51			
			2	2.70	1.51			
	0	6	3	2.55	1.43	2.64	1.48	0.07
			1	2.82	1.58			
			2	2.80	1.57			
	100	4	3	2.75	1.54	2.79	1.56	0.03
			1	2.95	1.65			
			2	2.80	1.57			
	200	2	3	3.02	1.69	2.92	1.64	0.09
			1	2.93	1.64			
			2	2.86	1.60			
	400	1	3	3.10	1.74	2.96	1.66	0.10
			1	3.48	1.95			
			2	3.15	1.76			
	800	3	3	3.60	2.02	3.41	1.91	0.19
			1	3.76	2.10			
			2	3.48	1.95			
	1600	5	3	3.07	1.72	3.44	1.93	0.28

Table B.4 - Experiment 4 SOM analysis results in full.

Timing	Box biosolid application (g)	Box	Sample	SOM (%)	SOC (%)	Mean SOM (%)	Mean SOC (%)	SOM Standard deviation
Initial			1	2.75	1.54			
			2	2.95	1.65			
			3	2.76	1.55	2.82	1.58	0.09
Pre-Flood			1	2.93	1.64			
			2	2.84	1.59			
	0 (1)	3	3	2.86	1.60	2.88	1.61	0.04
			1	2.73	1.53			
			2	2.86	1.60			
	0 (2)	5	3	2.73	1.53	2.77	1.55	0.06
			1	3.27	1.83			
			2	3.03	1.70			
	400 (1)	2	3	2.90	1.62	3.07	1.72	0.15
			1	2.95	1.65			
			2	3.13	1.75			
	400 (2)	6	3	2.98	1.67	3.02	1.69	0.08
			1	3.69	2.06			
			2	3.24	1.81			
	800 (1)	1	3	3.39	1.90	3.44	1.93	0.19
			1	3.41	1.91			
			2	3.47	1.94			
	800 (2)	4	3	3.38	1.89	3.42	1.92	0.04
Post-Flood			1	2.86	1.60			
			2	2.84	1.59			
	0 (1)	3	3	2.78	1.56	2.83	1.58	0.03
			1	2.87	1.61			
			2	2.89	1.62			
	0 (2)	5	3	2.80	1.57	2.85	1.60	0.04
			1	3.45	1.93			
			2	3.03	1.70			
	400 (1)	2	3	3.06	1.71	3.18	1.78	0.19
			1	3.07	1.72			
			2	3.03	1.70			
	400 (2)	6	3	2.97	1.66	3.02	1.69	0.04
			1	3.60	2.01			
			2	3.23	1.81			
	800 (1)	1	3	2.84	1.59	3.22	1.80	0.31
			1	3.40	1.91			
			2	3.17	1.78			
	800 (2)	4	3	3.21	1.80	3.26	1.83	0.10
Final			1	2.86	1.60			
			2	2.82	1.58			
	0 (1)	3	3	2.71	1.52	2.80	1.57	0.06
			1	2.72	1.52			
			2	2.71	1.52			
	0 (2)	5	3	2.70	1.51	2.71	1.52	0.01
			1	2.98	1.67			
			2	3.02	1.69			
	400 (1)	2	3	2.97	1.66	2.99	1.67	0.02
			1	2.97	1.66			
			2	2.87	1.61			
	400 (2)	6	3	3.00	1.68	2.95	1.65	0.05
			1	3.38	1.89			
			2	3.54	1.98			
	800 (1)	1	3	3.33	1.86	3.41	1.91	0.09
			1	3.24	1.81			
			2	3.25	1.82			
	800 (2)	4	3	3.04	1.70	3.18	1.78	0.10

B.3 SOIL TOTAL KJELDAHL NITROGEN

Table B.5 - Experiment 1 soil TKN analysis results in full.

Timing	Box biosolid application (g)	Box	Sample	Sample Weight (g)	10mM H_2SO_4 titrated - blank (ml)	NH_3-N (mg kg^{-1})	Moisture corrected NH_3-N (mg kg^{-1})	Mean NH_3-N (mg kg^{-1})	Standard deviation
Initial			1	0.50	3.70	2088	2129		
			2	0.52	3.50	1883	1919		
			3	0.50	3.60	2035	2075	2041	89
Pre-Flood			1	1.06	7.30	1926	1963		
			2	0.98	6.70	1920	1957		
	0	6	3	0.99	6.90	1960	1999	1973	18
			1	1.08	7.20	1871	1907		
			2	1.00	7.30	2037	2076		
	100	3	3	1.04	7.20	1932	1970	1984	70
			1	0.98	7.00	2004	2043		
			2	1.05	7.70	2061	2101		
	200	4	3	1.03	7.50	2030	2070	2071	24
			1	1.04	7.60	2047	2087		
			2	1.01	7.70	2126	2167		
	400	5	3	0.98	7.10	2025	2065	2106	44
			1	0.98	7.40	2118	2159		
			2	1.04	8.10	2192	2235		
	800	2	3	0.99	7.50	2130	2171	2188	33
			1	1.07	10.70	2807	2862		
			2	1.05	10.80	2880	2936		
	1600	1	3	1.05	8.30	2220	2263	2687	301
Post-Flood			1	0.98	6.80	1947	1985		
			2	0.99	7.10	2010	2049		
	0	6	3	0.99	7.00	1976	2014	2016	26
			1	1.05	7.10	1888	1925		
			2	1.00	6.90	1932	1969		
	100	3	3	0.99	6.90	1948	1986	1960	26
			1	0.99	6.90	1949	1987		
			2	0.98	7.70	2204	2247		
	200	4	3	1.02	7.50	2068	2108	2114	106
			1	0.99	7.20	2040	2079		
			2	1.02	8.00	2187	2230		
	400	5	3	0.97	8.00	2300	2345	2218	109
			1	0.99	7.60	2160	2202		
			2	1.03	8.00	2179	2222		
	800	2	3	1.00	8.40	2359	2405	2276	92
			1	0.99	10.30	2910	2967		
			2	1.05	9.60	2574	2624		
	1600	1	3	1.06	8.60	2278	2323	2638	263
Final			1	1.00	6.40	1791	1826		
			2	1.00	6.90	1928	1965		
	0	6	3	1.05	7.00	1862	1898	1897	57
			1	1.00	6.40	1795	1830		
			2	1.01	6.30	1755	1789		
	100	3	3	1.02	7.00	1915	1952	1857	70
			1	0.98	7.10	2028	2067		
			2	1.00	7.60	2125	2167		
	200	4	3	1.05	6.90	1837	1873	2036	122
			1	0.96	7.50	2182	2224		
			2	1.03	8.30	2265	2309		
	400	5	3	1.08	7.90	2053	2093	2209	89
			1	0.99	8.00	2268	2312		
			2	0.98	7.75	2214	2258		
	800	2	3	0.99	7.20	2046	2085	2218	96
			1	1.02	8.45	2330	2376		
			2	1.01	8.80	2443	2490		
	1600	1	3	0.99	9.40	2648	2700	2522	134

Table B.6 - Experiment 2 soil TKN analysis results in full.

Timing	Box biosolid application (g)	Box	Sample	Sample Weight (g)	10mM H_2SO_4 titrated - blank (ml)	NH_3-N (mg kg^{-1})	Moisture corrected NH_3-N (mg kg^{-1})	Mean NH_3-N (mg kg^{-1})	Standard deviation
Initial			1	1.05	6.2	1660	1692		
			2	1.05	6.4	1710	1743		
			3	0.98	6.4	1836	1872	1769	76
Pre-Flood			1	1.07	7	1827	1862		
			2	1.07	6.6	1735	1769		
	0	4	3	1.07	7.1	1851	1887	1839	51
			1	1.04	6.3	1694	1727		
			2	1.01	6.3	1746	1780		
	100	1	3	1.00	6.55	1843	1878	1795	63
			1	1.04	7.1	1919	1956		
			2	1.01	7.1	1960	1998		
	200	3	3	0.98	6.6	1878	1914	1956	34
			1	1.02	6.55	1802	1837		
			2	1.06	7.4	1960	1998		
	400	2	3	1.05	7.7	2055	2095	1977	106
			1	1.02	6.7	1845	1881		
			2	0.98	8.1	2308	2353		
	800	6	3	1.01	8.1	2244	2288	2174	209
			1	0.97	7.1	2048	2088		
			2	1.01	9.3	2590	2641		
	1600	5	3	1.02	8.9	2434	2482	2404	232
Post-Flood			1	1.02	6.7	1841	1877		
			2	1.02	6.2	1705	1738		
	0	4	3	1.02	5.85	1604	1635	1750	99
			1	1.04	6.6	1782	1817		
			2	1.00	6.8	1908	1945		
	100	1	3	1.06	7.1	1877	1914	1892	55
			1	1.07	5.9	1544	1574		
			2	1.06	7	1855	1891		
	200	3	3	0.99	7	1986	2024	1830	189
			1	1.08	7.1	1848	1884		
			2	1.02	6.5	1786	1821		
	400	2	3	0.96	6.9	2015	2054	1920	99
			1	1.02	7.6	2081	2121		
			2	0.99	7.6	2142	2184		
	800	6	3	0.97	8	2312	2357	2221	100
			1	0.97	9.45	2729	2783		
			2	1.06	9.3	2457	2504		
	1600	5	3	1.02	8.4	2305	2349	2545	179
Final			1	1.00	6.4	1787	1822		
			2	1.05	6.5	1739	1773		
	0	4	3	1.02	6.5	1780	1815	1803	22
			1	0.99	6.1	1723	1757		
			2	1.05	6.2	1652	1684		
	100	1	3	0.99	6.3	1778	1813	1751	53
			1	1.02	6.3	1726	1759		
			2	1.00	6.5	1819	1854		
	200	3	3	1.04	6.8	1831	1867	1827	48
			1	1.04	6.65	1798	1833		
			2	1.00	6.8	1903	1940		
	400	2	3	1.00	6.6	1857	1893	1889	44
			1	1.05	8.3	2221	2265		
			2	1.00	8.3	2319	2364		
	800	6	3	0.96	7.3	2134	2175	2268	77
			1	1.06	9.6	2549	2599		
			2	0.97	8.1	2351	2396		
	1600	5	3	1.00	7.6	2138	2179	2392	171

Table B.7 - Experiment 3 soil TKN analysis results in full.

Timing	Box biosolid application (g)	Box	Sample	Sample Weight (g)	10mM H_2SO_4 titrated - blank (ml)	NH_3-N (mg kg^{-1})	Moisture corrected NH_3-N (mg kg^{-1})	Mean NH_3-N (mg kg^{-1})	Standard deviation
Initial			1	0.97	6.2	1782	1817		
			2	0.96	6	1759	1794		
			3	1.04	6.4	1731	1765	1792	21
Pre-Flood			1	0.98	5.6	1598	1629		
			2	1.00	6	1722	1755		
	0	6	3	0.99	6.3	1712	1745	1710	57
			1	1.04	6.45	1840	1875		
			2	1.00	7.15	1912	1949		
	100	4	3	0.98	6.55	1782	1816	1880	54
			1	1.00	6.7	1798	1833		
			2	1.07	6.8	1901	1938		
	200	2	3	1.04	7.5	2154	2195	1989	152
			1	0.98	6.6	1881	1917		
			2	1.05	6.95	1939	1977		
	400	1	3	1.03	6.35	1805	1840	1911	56
			1	0.96	8.05	2245	2288		
			2	1.02	7.9	2073	2113		
	800	3	3	1.04	8.6	2321	2366	2256	105
			1	0.98	8.35	2442	2490		
			2	0.98	9.3	2552	2602		
	1600	5	3	1.03	8.3	2246	2289	2460	129
Post-Flood			1	0.97	6.5	1785	1820		
			2	0.96	6.5	1787	1822		
	0	6	3	1.04	5.9	1644	1676	1772	68
			1	1.05	6.65	1829	1864		
			2	0.99	6.75	1846	1881		
	100	4	3	1.04	6.65	1814	1850	1865	13
			1	0.98	6.9	1844	1880		
			2	0.98	6.4	1814	1849		
	200	2	3	1.00	7.2	1937	1974	1901	53
			1	1.02	6.75	1942	1980		
			2	1.02	6.05	1765	1799		
	400	1	3	1.03	7.1	1910	1947	1909	78
			1	0.99	8.5	2420	2467		
			2	1.04	7.7	2200	2242		
	800	3	3	1.02	9.2	2588	2638	2449	162
			1	1.02	9.25	2625	2676		
			2	1.02	8.9	2408	2455		
	1600	5	3	1.01	7.7	2117	2159	2430	212

Table B.8 - Experiment 4 soil TKN analysis results in full.

Timing	Box biosolid application (g)	Box	Sample	Sample Weight (g)	10mM H_2SO_4 titrated - blank (ml)	NH_3-N (mg kg^{-1})	Moisture corrected NH_3-N (mg kg^{-1})	Mean NH_3-N (mg kg^{-1})	Standard deviation
Initial			1	1.02	6.6	1813	1848		
			2	1.04	7.2	1939	1977		
			3	1.00	6.6	1849	1885	1903	54
Pre-Flood			1	1.01	6.6	1831	1866		
			2	0.99	6.6	1761	1795		
	0 (1)	3	3	1.02	6.4	1811	1846	1836	30
			1	1.00	6.3	1783	1817		
			2	1.05	6.7	1840	1876		
	0 (2)	5	3	1.00	6.6	1849	1885	1859	30
			1	1.01	7.7	2157	2199		
			2	1.05	7.15	1908	1945		
	400 (1)	2	3	0.99	6.45	1807	1842	1995	150
			1	1.00	7.3	2066	2106		
			2	1.01	8.6	2385	2432		
	400 (2)	6	3	1.05	7.3	1985	2024	2187	176
			1	0.99	9.1	2524	2573		
			2	1.02	7.6	2151	2192		
	800 (1)	1	3	1.00	8.5	2335	2380	2382	155
			1	0.99	8.8	2465	2513		
			2	1.01	9.1	2524	2573		
	800 (2)	4	3	1.03	8.5	2268	2312	2466	112
Post-Flood			1	1.02	6.6	1813	1848		
			2	1.00	6.9	1859	1895		
	0 (1)	3	3	1.05	6.5	1785	1820	1854	31
			1	1.00	6.8	1886	1923		
			2	0.99	6.6	1831	1866		
	0 (2)	5	3	0.99	6.9	1914	1951	1913	35
			1	1.02	8.1	2269	2313		
			2	1.04	7.6	2151	2192		
	400 (1)	2	3	1.02	7.4	2094	2135	2213	74
			1	1.01	7.2	2058	2098		
			2	1.01	7.7	2035	2075		
	400 (2)	6	3	1.02	7.5	2080	2121	2098	19
			1	1.01	10.1	2774	2828		
			2	1.01	8	2241	2285		
	800 (1)	1	3	1.01	7.5	2001	2040	2384	329
			1	0.98	9	2496	2545		
			2	1.06	8.1	2247	2290		
	800 (2)	4	3	1.01	8	2197	2240	2358	133
Final			1	1.05	6.5	1839	1875		
			2	1.02	6.75	1872	1909		
	0 (1)	3	3	1.03	6.65	1882	1918	1901	19
			1	0.99	6.8	1814	1850		
			2	1.04	6.3	1783	1817		
	0 (2)	5	3	1.03	6.9	1933	1971	1879	66
			1	0.99	7.1	2009	2048		
			2	1.01	7.6	2047	2087		
	400 (1)	2	3	0.99	7.3	1985	2024	2053	26
			1	1.03	8.05	2233	2276		
			2	1.01	7.7	2074	2114		
	400 (2)	6	3	1.02	7.65	2101	2142	2178	71
			1	1.05	9	2401	2448		
			2	0.99	9.3	2554	2604		
	800 (1)	1	3	1.00	8.4	2285	2329	2460	113
			1	1.01	8.8	2393	2440		
			2	1.04	8.4	2330	2375		
	800 (2)	4	3	1.02	7.7	2115	2156	2324	122

B.4 SOIL OLSEN PHOSPHORUS

Table B.9 - Experiment 1 soil Olsen P analysis results in full.

Timing	Box biosolid application (g)	Box	Sample	Soil P (mg kg^{-1})	Moisture Corrected Soil P (mg kg^{-1})	Mean Soil P (mg kg^{-1})	Standard deviation
Initial			1	21.84	22.26		
			2	24.19	24.66		
			3	15.46	15.76	20.89	3.76
Pre-Flood			1	29.85	30.43		
			2	30.24	30.82		
	0	6	3	33.08	33.72	31.66	1.47
			1	38.25	38.99		
			2	39.80	40.57		
	100	3	3	37.73	38.46	39.34	0.90
			1	35.02	35.70		
			2	37.08	37.81		
	200	4	3	40.19	40.97	38.16	2.17
			1	41.61	42.42		
			2	42.64	43.47		
	400	5	3	38.63	39.39	41.76	1.73
			1	45.48	46.37		
			2	40.96	41.76		
	800	2	3	42.12	42.94	43.69	1.95
			1	45.10	45.97		
			2	53.49	54.53		
	1600	1	3	42.64	43.47	47.99	4.74
Post-Flood			1	31.08	31.69		
			2	34.09	34.75		
	0	6	3	29.83	30.41	32.28	1.82
			1	35.09	35.77		
			2	37.85	38.58		
	100	3	3	37.97	38.71	37.69	1.36
			1	32.46	33.09		
			2	33.46	34.11		
	200	4	3	36.47	37.18	34.79	1.74
			1	35.22	35.90		
			2	43.24	44.08		
	400	5	3	43.24	44.08	41.35	3.85
			1	43.24	44.08		
			2	43.36	44.21		
	800	2	3	48.50	49.44	45.91	2.50
			1	53.14	54.17		
			2	52.26	53.28		
	1600	1	3	40.10	40.88	49.44	6.06
Final			1	26.47	26.98		
			2	25.85	26.35		
	0	6	3	27.46	27.99	27.11	0.68
			1	36.36	37.07		
			2	35.87	36.57		
	100	3	3	37.11	37.83	37.16	0.52
			1	35.13	35.81		
			2	34.26	34.93		
	200	4	3	36.12	36.82	35.85	0.77
			1	41.06	41.86		
			2	48.36	49.30		
	400	5	3	39.83	40.60	43.92	3.84
			1	50.22	51.19		
			2	54.18	55.23		
	800	2	3	45.39	46.28	50.90	3.66
			1	36.86	37.58		
			2	48.24	49.18		
	1600	1	3	55.41	56.49	47.75	7.79

Table B.10 - Experiment 2 soil Olsen P analysis results in full.

Timing	Box biosolid application (g)	Box	Sample	Soil P (mg kg^{-1})	Moisture Corrected Soil P (mg kg^{-1})	Mean Soil P (mg kg^{-1})	Standard deviation
Initial			1.1	30.58	31.17		
			1.2	46.17	47.07		
			2.1	25.75	26.25		
			2.2	30.21	30.80		
			3.1	34.54	35.21		
			3.2	42.54	43.37	35.64	7.33
Pre-Flood			1	47.84	48.77		
			2	44.38	45.24		
	0	4	3	44.76	45.63	46.55	1.58
			1	56.95	58.05		
			2	55.54	56.62		
	100	1	3	55.41	56.49	57.05	0.71
			1	45.53	46.42		
			2	50.41	51.39		
	200	3	3	52.71	53.74	50.51	3.05
			1	57.97	59.10		
			2	45.40	46.29		
	400	2	3	52.33	53.35	52.91	5.24
			1	47.07	47.99		
			2	48.61	49.56		
	800	6	3	51.69	52.69	50.08	1.96
			1	64.26	65.51		
			2	64.51	65.77		
	1600	5	3	55.15	56.22	62.50	4.44
Post-Flood			1	40.69	41.48		
			2	44.51	45.38		
	0	4	3	40.20	40.98	42.61	1.97
			1	51.49	52.49		
			2	46.55	47.45		
	100	1	3	53.39	54.43	51.46	2.94
			1	45.53	46.42		
			2	53.14	54.17		
	200	3	3	51.11	52.11	50.90	3.28
			1	59.10	60.25		
			2	52.76	53.79		
	400	2	3	52.38	53.40	55.81	3.14
			1	53.63	54.68		
			2	50.31	51.28		
	800	6	3	57.83	58.95	54.97	3.14
			1	55.73	56.81		
			2	60.05	61.21		
	1600	5	3	58.57	59.71	59.25	1.83
Final			1	42.29	43.11		
			2	40.32	41.10		
	0	4	3	41.67	42.49	42.23	0.84
			1	44.32	45.18		
			2	47.66	48.58		
	100	1	3	40.35	41.14	44.97	3.04
			1	44.07	44.93		
			2	48.28	49.22		
	200	3	3	42.95	43.79	45.98	2.34
			1	51.12	52.12		
			2	50.75	51.74		
	400	2	3	50.26	51.23	51.70	0.36
			1	61.03	62.22		
			2	51.91	52.92		
	800	6	3	59.55	60.71	58.62	4.08
			1	66.70	68.00		
			2	58.94	60.08		
	1600	5	3	55.48	56.56	61.55	4.78

Table B.11 - Experiment 3 soil Olsen P analysis results in full.

Timing	Box biosolid application (g)	Box	Sample	Soil P (mg kg^{-1})	Moisture Corrected Soil P (mg kg^{-1})	Mean Soil P (mg kg^{-1})	Standard deviation
Initial			1	27.35	27.88		
			2	30.15	30.74		
			3	35.20	35.88	31.50	3.31
Pre-Flood			1	40.96	41.75		
			2	43.68	44.53		
	0	6	3	42.46	43.29	43.19	1.14
			1	41.54	42.35		
			2	42.34	43.16		
	100	4	3	38.94	39.70	41.74	1.48
			1	38.27	39.02		
			2	41.85	42.67		
	200	2	3	39.22	39.98	40.56	1.54
			1	39.84	40.62		
			2	46.69	47.60		
	400	1	3	39.49	40.26	42.83	3.38
			1	45.50	46.38		
			2	40.76	41.55		
	800	3	3	50.48	51.46	46.46	4.05
			1	50.21	51.19		
			2	55.67	56.75		
	1600	5	3	47.61	48.53	52.16	3.43
Post-Flood			1	42.73	43.56		
			2	44.10	44.96		
	0	6	3	38.57	39.32	42.61	2.40
			1	37.45	38.18		
			2	35.37	36.06		
	100	4	3	34.70	35.38	36.54	1.19
			1	35.72	36.42		
			2	36.07	36.77		
	200	2	3	38.38	39.13	37.44	1.20
			1	37.14	37.86		
			2	34.98	35.66		
	400	1	3	37.11	37.83	37.12	1.03
			1	50.08	51.06		
			2	42.35	43.17		
	800	3	3	47.37	48.29	47.51	3.27
			1	57.57	58.69		
			2	54.40	55.46		
	1600	5	3	42.86	43.70	52.61	6.44

Table B.12 - Experiment 4 soil Olsen P analysis results in full.

Timing	Box biosolid application (g)	Box	Sample	Soil P (mg kg^{-1})	Moisture Corrected Soil P (mg kg^{-1})	Mean Soil P (mg kg^{-1})	Standard deviation
Initial			1.1	15.35	15.65		
			2.1	22.94	23.38		
			3.1	23.04	23.49		
			1.2	14.83	15.12		
			2.2	20.33	20.73		
			3.2	20.21	20.60	19.83	3.34
Pre-Flood			1	30.01	30.60		
			2	32.21	32.83		
	0 (1)	3	3	31.61	32.22	31.88	0.94
			1	22.69	23.13		
			2	25.70	26.20		
	0 (2)	5	3	25.31	25.80	25.04	1.36
			1	38.71	39.47		
			2	32.39	33.02		
	400 (1)	2	3	33.96	34.62	35.70	2.74
			1	34.18	34.85		
			2	42.25	43.07		
	400 (2)	6	3	35.63	36.33	38.08	3.58
			1	46.41	47.31		
			2	36.92	37.64		
	800 (1)	1	3	39.08	39.84	41.60	4.14
			1	37.56	38.29		
			2	44.82	45.69		
	800 (2)	4	3	39.32	40.09	41.35	3.15
Post-Flood			1	32.96	33.60		
			2	30.24	30.83		
	0 (1)	3	3	29.05	29.62	31.35	1.67
			1	27.75	28.29		
			2	27.98	28.52		
	0 (2)	5	3	24.24	24.71	27.18	1.75
			1	40.40	41.19		
			2	42.19	43.02		
	400 (1)	2	3	38.64	39.39	41.20	1.48
			1	41.58	42.38		
			2	39.81	40.59		
	400 (2)	6	3	38.01	38.75	40.57	1.48
			1	46.61	47.52		
			2	41.00	41.80		
	800 (1)	1	3	30.75	31.35	40.22	6.70
			1	40.61	41.40		
			2	36.68	37.40		
	800 (2)	4	3	35.65	36.34	38.38	2.18
Final			1	27.23	27.76		
			2	27.28	27.81		
	0 (1)	3	3	29.40	29.98	28.52	1.03
			1	23.73	24.19		
			2	19.26	19.64		
	0 (2)	5	3	22.34	22.77	22.20	1.90
			1	31.93	32.55		
			2	38.47	39.22		
	400 (1)	2	3	35.42	36.11	35.96	2.72
			1	43.30	44.14		
			2	39.97	40.74		
	400 (2)	6	3	37.72	38.45	41.11	2.34
			1	43.48	44.33		
			2	44.34	45.20		
	800 (1)	1	3	37.78	38.51	42.68	2.97
			1	34.47	35.14		
			2	41.00	41.80		
	800 (2)	4	3	28.71	29.27	35.40	5.12

B.5 SOIL PH

Table B.13 - Experiment 1 soil pH analysis results in full.

Timing	Box biosolid application (g)	Box	Sample	pH	Mean pH	Standard Deviation
Initial			1	7.53		
			2	7.7		
			3	7.69	7.64	0.08
Pre-Flood			1	7.81		
			2	7.82		
	0	6	3	7.82	7.82	0.00
			1	7.65		
			2	7.75		
	100	3	3	7.84	7.75	0.08
			1	7.69		
			2	7.63		
	200	4	3	7.71	7.68	0.03
			1	7.6		
			2	7.65		
	400	5	3	7.54	7.60	0.04
			1	7.35		
			2	7.31		
	800	2	3	7.42	7.36	0.05
			1	7.19		
			2	7.21		
	1600	1	3	7.23	7.21	0.02
Post-Flood			1	7.77		
			2	7.69		
	0	6	3	7.65	7.70	0.05
			1	7.75		
			2	7.68		
	100	3	3	7.85	7.76	0.07
			1	7.72		
			2	7.74		
	200	4	3	7.72	7.73	0.01
			1	7.66		
			2	7.72		
	400	5	3	7.55	7.64	0.07
			1	7.6		
			2	7.63		
	800	2	3	7.54	7.59	0.04
			1	7.48		
			2	7.52		
	1600	1	3	7.57	7.52	0.04
Final			1	7.77		
			2	7.8		
	0	6	3	7.86	7.81	0.04
			1	7.8		
			2	7.74		
	100	3	3	7.77	7.77	0.02
			1	7.79		
			2	7.71		
	200	4	3	7.79	7.76	0.04
			1	7.89		
			2	7.66		
	400	5	3	7.75	7.77	0.09
			1	7.62		
			2	7.62		
	800	2	3	7.66	7.63	0.02
			1	7.57		
			2	7.43		
	1600	1	3	7.41	7.47	0.07

Table B.14 - Experiment 2 soil pH analysis results in full.

Timing	Box biosolid application (g)	Box	Sample	pH	Mean pH	Standard Deviation
Initial			1	7.49		
			2	7.55		
			3	7.54	7.53	0.03
Pre-Flood			1	7.68		
			2	7.76		
	0	4	3	7.68	7.71	0.04
			1	7.57		
			2	7.65		
	100	1	3	7.6	7.61	0.03
			1	7.65		
			2	7.54		
	200	3	3	7.58	7.59	0.05
			1	7.51		
			2	7.48		
	400	2	3	7.48	7.49	0.01
			1	7.46		
			2	7.43		
	800	6	3	7.37	7.42	0.04
			1	7.3		
			2	7.21		
	1600	5	3	7.36	7.29	0.06
Post-Flood			1	7.6		
			2	7.7		
	0	4	3	7.7	7.67	0.05
			1	7.63		
			2	7.66		
	100	1	3	7.58	7.62	0.03
			1	7.59		
			2	7.61		
	200	3	3	7.59	7.60	0.01
			1	7.63		
			2	7.53		
	400	2	3	7.6	7.59	0.04
			1	7.54		
			2	7.55		
	800	6	3	7.5	7.53	0.02
			1	7.46		
			2	7.37		
	1600	5	3	7.39	7.41	0.04
Final			1	7.71		
			2	7.67		
	0	4	3	7.7	7.69	0.02
			1	7.51		
			2	7.56		
	100	1	3	7.59	7.55	0.03
			1	7.76		
			2	7.75		
	200	3	3	7.68	7.73	0.04
			1	7.62		
			2	7.59		
	400	2	3	7.65	7.62	0.02
			1	7.63		
			2	7.72		
	800	6	3	7.65	7.67	0.04
			1	7.47		
			2	7.48		
	1600	5	3	7.54	7.50	0.03

Table B.15 - Experiment 3 soil pH analysis results in full.

Timing	Box biosolid application (g)	Box	Sample	pH	Mean pH	Standard Deviation
Initial			1	7.56		
			2	7.66		
			3	7.71	7.64	0.06
Pre-Flood			1	7.79		
			2	7.75		
	0	6	3	7.74	7.76	0.02
			1	7.66		
			2	7.61		
	100	4	3	7.71	7.66	0.04
			1	7.52		
			2	7.57		
	200	2	3	7.56	7.55	0.02
			1	7.5		
			2	7.54		
	400	1	3	7.49	7.51	0.02
			1	7.42		
			2	7.42		
	800	3	3	7.5	7.45	0.04
			1	7.41		
			2	7.3		
	1600	5	3	7.39	7.37	0.05
Post-Flood			1	7.69		
			2	7.72		
	0	6	3	7.87	7.76	0.08
			1	7.76		
			2	7.69		
	100	4	3	7.74	7.73	0.03
			1	7.6		
			2	7.6		
	200	2	3	7.65	7.62	0.02
			1	7.75		
			2	7.69		
	400	1	3	7.56	7.67	0.08
			1	7.61		
			2	7.67		
	800	3	3	7.64	7.64	0.02
			1	7.58		
			2	7.61		
	1600	5	3	7.63	7.61	0.02

Table B.16 - Experiment 4 soil pH analysis results in full.

Timing	Box biosolid application (g)	Box	Sample	pH	Mean pH	Standard Deviation
Initial			1	7.44		
			2	7.41		
			3	7.35	7.40	0.04
Pre-Flood			1	7.64		
			2	7.64		
	0 (1)	3	3	7.64	7.64	0.00
			1	7.71		
			2	7.61		
	0 (2)	5	3	7.58	7.63	0.06
			1	7.39		
			2	7.52		
	400 (1)	2	3	7.52	7.48	0.06
			1	7.4		
			2	7.43		
	400 (2)	6	3	7.52	7.45	0.05
			1	7.27		
			2	7.32		
	800 (1)	1	3	7.35	7.31	0.03
			1	7.23		
			2	7.28		
	800 (2)	4	3	7.28	7.26	0.02
Post-Flood			1	7.64		
			2	7.55		
	0 (1)	3	3	7.5	7.56	0.06
			1	7.44		
			2	7.5		
	0 (2)	5	3	7.55	7.50	0.04
			1	7.25		
			2	7.34		
	400 (1)	2	3	7.4	7.33	0.06
			1	7.47		
			2	7.47		
	400 (2)	6	3	7.48	7.47	0.00
			1	7.23		
			2	7.25		
	800 (1)	1	3	7.42	7.30	0.09
			1	7.31		
			2	7.32		
	800 (2)	4	3	7.32	7.32	0.00
Final			1	7.68		
			2	7.72		
	0 (1)	3	3	7.77	7.72	0.04
			1	7.79		
			2	7.68		
	0 (2)	5	3	7.68	7.72	0.05
			1	7.46		
			2	7.52		
	400 (1)	2	3	7.6	7.53	0.06
			1	7.57		
			2	7.57		
	400 (2)	6	3	7.56	7.57	0.00
			1	7.25		
			2	7.32		
	800 (1)	1	3	7.31	7.29	0.03
			1	7.45		
			2	7.42		
	800 (2)	4	3	7.44	7.44	0.01

APPENDIX C: FLOODWATER MEASUREMENTS

C.1 UNFILTERED FLOODWATER ANALYSIS

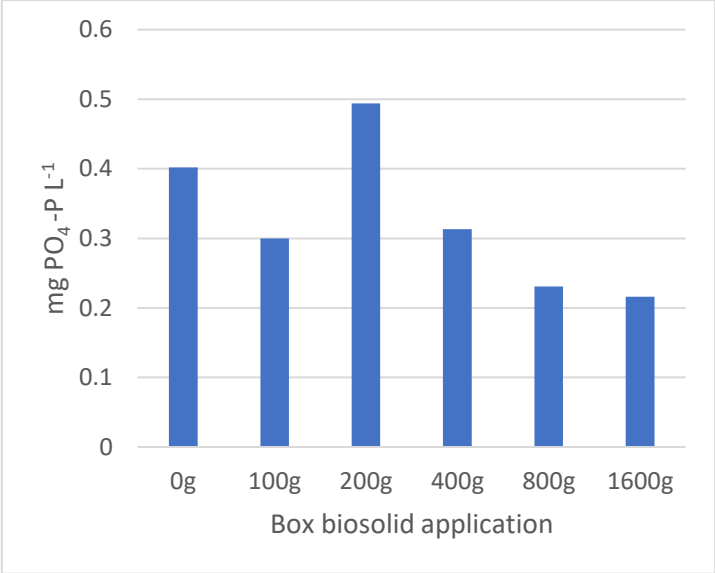

Figure C.1 - Experiment 1 floodwater total P analysis results. Water was not filtered prior to analysis and so was not presented alongside floodwater measurements from other experiments due to the possible contamination by P associated with soil particles in solution

C.2 EXPERIMENT 1

C.2.1 Surface water

Table C.1 - Experiment 1 surface water pH measurements in full.

	Time	1100	2000	0800	2000	1100	1100	1300	1500	2000	1700	1500	1200	1000
Box biosolid application (g)	**Hours**	**3**	**12**	**24**	**36**	**51**	**75**	**101**	**127**	**156**	**177**	**199**	**220**	**242**
	Reading	**pH**												
	1	7.13	7.43	7.64	7.73	7.86	7.73	8.11	8.13	8.05	8.36	8.16	8.16	7.84
	2	7.29	7.44	7.76	7.84	8.02	8.30	8.38	8.60	8.21	8.33	8.56	8.20	8.26
	3	7.15	7.21	7.67	8.08	7.91	7.90	8.55	8.33	8.11	8.66	8.33	8.50	7.95
0	**Mean**	7.19	7.36	7.69	7.88	7.93	7.98	8.35	8.35	8.12	8.45	8.35	8.29	8.02
	1	7.00	7.55	7.64	7.63	7.82	7.79	8.27	8.40	8.23	8.65	8.45	8.56	8.41
	2	7.06	7.35	7.61	7.70	7.48	7.96	8.25	8.53	8.24	8.70	8.66	8.58	8.40
	3	6.93	7.24	7.50	7.46	7.76	7.86	8.16	8.31	8.09	8.60	8.35	8.56	8.27
100	**Mean**	7.00	7.38	7.58	7.60	7.69	7.87	8.23	8.41	8.19	8.65	8.49	8.57	8.36
	1	7.28	6.98	7.36	7.39	7.48	7.40	7.52	8.37	7.93	8.19	8.52	8.41	8.36
	2	7.00	6.84	7.39	7.35	7.51	7.48	8.08	8.20	8.12	8.51	8.54	8.55	8.38
	3	7.02	6.81	7.59	7.52	7.62	7.76	7.99	8.48	8.03	8.52	8.70	8.53	8.60
200	**Mean**	7.10	6.88	7.45	7.42	7.54	7.55	7.86	8.35	8.03	8.41	8.59	8.50	8.45
	1	6.89	6.88	7.28	7.07	7.26	7.57	7.76	7.90	7.87	8.17	8.29	8.13	8.16
	2	6.78	7.14	7.22	7.15	7.39	7.48	7.81	7.98	7.81	8.21	8.46	8.30	8.33
	3	6.72	7.19	7.34	7.16	7.37	7.42	7.73	8.11	7.82	8.33	8.52	8.33	8.36
400	**Mean**	6.80	7.07	7.28	7.13	7.34	7.49	7.77	8.00	7.83	8.24	8.42	8.25	8.28
	1	6.64	6.89	7.23	7.20	7.32	7.42	7.60	8.02	7.79	8.13	8.20	8.08	7.94
	2	6.78	7.28	7.42	7.42	7.38	7.61	8.02	7.93	7.85	8.34	8.14	8.27	7.97
	3	7.01	6.78	7.48	7.37	7.50	7.67	7.69	8.35	8.05	8.00	8.44	8.00	8.20
800	**Mean**	6.81	6.98	7.38	7.33	7.40	7.57	7.77	8.10	7.90	8.16	8.26	8.12	8.04
	1	6.91	6.98	7.02	7.26	7.14	7.40	7.69	8.02	7.50	7.88	8.01	7.75	8.14
	2	7.25	7.15	7.19	7.48	7.40	7.15	7.93	7.82	7.71	8.11	7.92	8.06	7.89
	3	7.12	6.70	7.38	7.12	7.15	7.16	7.61	7.74	7.76	7.85	8.16	7.78	7.84
1600	**Mean**	7.09	6.94	7.20	7.29	7.23	7.24	7.74	7.86	7.66	7.95	8.03	7.86	7.96

Table C.2 - Experiment 1 surface water dissolved oxygen measurements in full.

	Time	1100	2000	0800	2000	1100	1100	1300	1500	2000	1700	1500	1200	1000
Box biosolid application (g)	**Hours**	**3**	**12**	**24**	**36**	**51**	**75**	**101**	**127**	**156**	**177**	**199**	**220**	**242**
	Reading	**Dissolved oxygen (mg O_2 L^{-1})**												
	1	8.03	8.22	8.16	8.44	8.51	9.03	9.43	9.51	8.02	9.90	9.50	9.88	8.39
	2	7.98	8.37	8.16	8.32	8.62	10.36	9.88	10.96	8.80	9.91	11.81	9.70	9.96
	3	8.25	8.22	8.13	8.73	8.49	9.01	10.92	9.98	8.23	11.84	9.72	11.31	8.45
0	**Mean**	8.09	8.27	8.15	8.50	8.54	9.47	10.08	10.15	8.35	10.55	10.34	10.30	8.93
	1	8.20	8.00	8.33	8.18	8.51	8.74	10.93	9.99	8.91	13.16	12.77	12.12	10.89
	2	8.14	8.04	7.97	8.23	8.47	9.57	10.49	11.41	9.11	12.89	12.95	11.83	10.23
	3	8.18	8.28	8.24	8.18	8.37	8.91	10.11	10.80	8.74	12.28	11.86	11.78	10.56
100	**Mean**	8.17	8.11	8.18	8.20	8.45	9.07	10.51	10.73	8.92	12.78	12.53	11.91	10.56
	1	8.04	8.08	8.28	7.59	8.39	8.62	8.97	10.95	8.06	10.21	12.41	11.48	11.61
	2	8.50	7.91	8.27	7.88	8.47	8.60	9.90	9.92	8.58	11.32	11.15	11.62	9.99
	3	8.27	8.24	8.27	7.79	8.43	9.11	9.58	10.77	8.05	10.90	13.12	11.66	12.39
200	**Mean**	8.27	8.08	8.27	7.75	8.43	8.78	9.48	10.55	8.23	10.81	12.23	11.59	11.33
	1	8.68	7.89	7.93	7.76	7.97	8.19	8.98	9.49	8.24	10.15	11.36	10.30	9.67
	2	8.34	8.08	8.12	7.66	8.08	8.29	9.34	9.42	8.06	10.71	11.52	10.83	9.71
	3	8.30	8.26	7.97	7.70	7.95	8.04	8.91	9.83	8.03	10.46	11.58	10.63	10.23
400	**Mean**	8.44	8.08	8.01	7.71	8.00	8.17	9.08	9.58	8.11	10.44	11.49	10.59	9.87
	1	8.26	7.75	8.24	7.39	8.95	9.41	10.08	9.39	8.56	11.61	11.60	11.26	10.03
	2	8.63	7.65	8.90	7.77	9.07	8.27	10.38	10.27	8.64	12.44	10.76	11.87	8.53
	3	8.63	7.53	8.43	7.74	8.61	9.20	8.77	11.39	8.97	10.23	13.20	9.35	10.90
800	**Mean**	8.51	7.64	8.52	7.63	8.88	8.96	9.74	10.35	8.72	11.43	11.85	10.83	9.82
	1	8.42	7.96	8.14	7.87	9.43	9.24	9.03	11.49	8.74	10.29	12.78	9.38	12.34
	2	8.51	8.12	8.77	7.78	9.08	8.33	9.70	9.61	9.03	11.08	10.66	10.48	9.24
	3	8.24	8.03	8.34	7.97	8.43	8.45	8.39	9.17	9.20	9.89	11.80	8.09	9.97
1600	**Mean**	8.39	8.04	8.42	7.87	8.98	8.67	9.04	10.09	8.99	10.42	11.75	9.32	10.52

Table C.3 - Experiment 1 surface water ORP measurements in full. Eh corrected by +200mV for Pt electrode adjustment.

Time	1100	2000	0800	2000	1100	1100	1300	1500	2000	1700	1500	1200	1000
Hours	**3**	**12**	**24**	**36**	**51**	**75**	**101**	**127**	**156**	**177**	**199**	**220**	**242**
Box biosolid application (g)	**Eh (mV)**												
0	392	374	344	270	233	203	193	180	162	143	152	162	170
100	297	231	210	203	203	201	196	199	199	195	194	188	184
200	427	407	396	373	350	305	258	213	210	210	206	206	205
400	328	314	301	321	276	229	206	200	198	198	198	193	191
800	286	282	266	239	215	202	196	192	187	188	186	186	182
1600	401	377	345	303	264	228	208	204	203	203	202	203	201

C.2.2 5cm and 10cm depth porewater

Table C.4 - Experiment 1 5cm depth porewater ORP measurements in full. Eh corrected by +200mV for Pt electrode adjustment.

Time	1100	2000	0800	2000	1100	1100	1300	1500	2000	1700	1500	1200	1000
Hours	**3**	**12**	**24**	**36**	**51**	**75**	**101**	**127**	**156**	**177**	**199**	**220**	**242**
Box biosolid application (g)	**Eh (mV)**												
0	388	370	356	319	283	212	190	170	80	75	107	101	87
100	331	338	332	326	323	324	272	203	195	194	197	190	188
200	306	297	292	274	259	223	205	197	177	175	187	180	183
400	337	330	376	355	339	318	263	212	205	203	196	194	196
800	319	228	212	206	207	201	199	198	199	197	196	197	198
1600	373	237	207	202	196	196	196	194	192	194	193	193	190

Table C.5 - Experiment 1 10cm depth porewater ORP measurements in full. Eh corrected by +200mV for Pt electrode adjustment.

Time	1100	2000	0800	2000	1100	1100	1300	1500	2000	1700	1500	1200	1000
Hours	**3**	**12**	**24**	**36**	**51**	**75**	**101**	**127**	**156**	**177**	**199**	**220**	**242**
Box biosolid application (g)	**Eh (mV)**												
0	392	374	344	270	233	203	193	180	162	143	152	162	170
100	297	231	210	203	203	201	196	199	199	195	194	188	184
200	427	407	396	373	350	305	258	213	210	210	206	206	205
400	328	314	301	321	276	229	206	200	198	198	198	193	191
800	286	282	266	239	215	202	196	192	187	188	186	186	182
1600	401	377	345	303	264	228	208	204	203	203	202	203	201

C.3 EXPERIMENT 2

C.3.1 Surface water

Table C.6 - Experiment 2 surface water pH measurements in full.

	Time	1100	1400	1700	2000	0200	0800	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Box biosolid application (g)	**Hours**	**3**	**6**	**9**	**12**	**18**	**24**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
	Reading	**pH**															
	1	7.04	7.91	7.94	7.85	7.34	7.55	7.81	7.97	8.20	8.11	8.33	7.88	7.80	8.49	8.43	8.12
	2	7.39	8.15	8.15	7.88	7.53	7.68	7.81	8.28	8.23	8.00	8.58	8.42	8.34	8.53	8.57	8.24
	3	7.63	8.09	8.08	8.09	7.57	7.68	8.08	8.41	8.46	8.60	8.71	8.52	8.53	8.63	8.59	8.37
0	**Mean**	7.35	8.05	8.06	7.94	7.48	7.64	7.90	8.22	8.30	8.24	8.54	8.27	8.22	8.55	8.53	8.24
	1	6.74	7.47	8.05	7.19	7.27	7.46	8.11	7.92	7.93	8.43	8.12	8.36	8.13	7.85	8.26	8.06
	2	6.64	7.69	8.30	7.71	7.46	7.49	8.18	8.39	8.39	8.66	8.75	8.74	8.60	8.53	8.78	8.35
	3	6.83	7.65	8.08	7.94	7.53	7.54	8.30	8.44	8.58	8.74	8.84	8.76	8.72	8.67	8.76	8.36
100	**Mean**	6.74	7.60	8.14	7.61	7.42	7.50	8.20	8.25	8.30	8.61	8.57	8.62	8.48	8.35	8.60	8.26
	1	7.10	7.06	7.26	7.27	7.38	7.32	7.53	7.65	7.91	8.05	7.73	7.78	8.04	7.90	8.13	7.88
	2	7.27	7.37	7.43	7.54	7.52	7.52	7.67	7.78	7.97	8.03	8.10	8.00	8.21	8.10	8.27	8.01
	3	7.45	7.35	7.51	7.68	7.70	7.59	7.76	7.81	8.00	8.04	8.17	8.02	8.21	8.07	8.16	8.04
200	**Mean**	7.27	7.26	7.40	7.50	7.53	7.48	7.65	7.75	7.96	8.04	8.00	7.93	8.15	8.02	8.19	7.98
	1	7.43	7.34	7.52	7.30	7.26	7.47	7.80	7.76	7.70	7.96	8.39	7.80	8.31	8.01	8.16	7.92
	2	7.42	7.32	7.55	7.46	7.41	7.52	7.93	8.13	8.20	8.38	8.49	8.39	8.52	8.30	8.44	8.16
	3	7.42	7.33	7.56	7.53	7.46	7.57	8.01	8.19	8.25	8.40	8.52	8.40	8.53	8.46	8.50	8.25
400	**Mean**	7.42	7.33	7.54	7.43	7.38	7.52	7.91	8.03	8.05	8.25	8.47	8.20	8.45	8.26	8.37	8.11
	1	6.65	7.05	7.04	7.02	7.14	7.07	7.19	7.42	7.91	8.00	7.81	7.66	7.76	7.87	8.12	8.34
	2	7.17	7.40	7.46	7.45	7.32	7.30	7.76	8.09	8.21	8.28	8.04	7.92	7.86	8.14	7.84	8.32
	3	7.33	7.38	7.54	7.70	7.40	7.38	7.86	8.12	8.22	8.31	8.18	8.07	8.10	8.18	8.34	8.25
800	**Mean**	7.05	7.28	7.35	7.39	7.29	7.25	7.60	7.88	8.11	8.20	8.01	7.88	7.91	8.06	8.10	8.30
	1	6.96	6.88	7.22	6.99	7.07	6.92	7.17	7.59	7.57	7.61	7.97	7.84	7.99	7.92	8.02	8.02
	2	7.05	7.12	7.35	7.29	7.25	7.19	7.54	7.76	7.84	7.97	8.12	8.09	8.18	8.25	8.31	8.18
	3	7.01	7.11	7.20	7.33	7.28	7.18	7.61	7.77	7.83	7.95	8.10	8.13	8.20	8.17	8.17	8.11
1600	**Mean**	7.01	7.04	7.26	7.20	7.20	7.10	7.44	7.71	7.75	7.84	8.06	8.02	8.12	8.11	8.17	8.10

Table C.7 - Experiment 2 surface water dissolved oxygen measurements in full.

	Time	1100	1400	1700	2000	0200	0800	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Box biosolid application (g)	**Hours**	**3**	**6**	**9**	**12**	**18**	**24**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
	Reading	**Dissolved oxygen (mg O_2 L^{-1})**															
	1	8.80	8.81	8.77	8.57	6.85	3.17	7.78	8.43	10.21	8.35	9.61	7.47	9.17	10.02	10.37	9.22
	2	8.75	8.77	8.81	8.72	6.57	6.48	8.34	10.20	10.46	9.52	10.46	8.81	10.15	10.14	10.61	7.98
	3	8.81	9.06	8.83	8.80	7.20	6.62	8.81	9.40	10.99	9.89	10.46	9.77	9.72	9.91	10.09	9.36
0	**Mean**	8.79	8.88	8.80	8.70	6.87	5.42	8.31	9.34	10.55	9.25	10.18	8.68	9.68	10.02	10.36	8.85
	1	8.89	8.91	9.15	8.88	7.30	6.28	9.18	9.81	11.78	10.70	9.35	10.29	9.70	9.88	10.24	8.67
	2	8.86	8.86	9.05	9.04	6.82	6.15	8.86	10.68	12.37	10.62	9.42	9.75	10.72	10.17	11.14	8.44
	3	8.83	8.94	8.90	9.04	6.92	6.31	9.17	9.74	12.36	10.24	10.53	10.73	10.99	10.79	12.31	8.54
100	**Mean**	8.86	8.90	9.03	8.99	7.01	6.25	9.07	10.08	12.17	10.52	9.77	10.26	10.47	10.28	11.23	8.55
	1	8.67	8.54	7.98	7.02	6.70	6.09	5.48	7.15	9.25	8.01	8.36	8.42	7.74	8.34	8.20	6.26
	2	8.56	8.32	8.25	8.15	6.12	4.96	6.54	5.64	7.40	8.42	7.50	7.90	9.20	8.71	8.95	7.13
	3	8.15	8.46	7.82	7.87	6.71	4.00	5.97	7.63	9.10	7.18	8.84	7.66	9.46	9.67	8.99	6.28
200	**Mean**	8.46	8.44	8.02	7.68	6.51	5.02	6.00	6.81	8.58	7.87	8.23	7.99	8.80	8.91	8.71	6.56
	1	8.78	8.69	8.57	8.71	7.14	5.74	8.95	9.46	11.37	9.98	9.91	10.30	11.43	10.32	9.20	6.81
	2	8.69	8.59	8.36	8.33	6.98	6.37	8.82	9.62	11.57	10.08	10.02	11.46	10.35	10.42	12.03	8.97
	3	8.71	8.63	8.42	8.53	6.55	6.07	8.94	9.44	11.35	9.24	10.57	12.42	11.90	11.63	11.15	8.50
400	**Mean**	8.73	8.64	8.45	8.52	6.89	6.06	8.90	9.51	11.43	9.77	10.17	11.39	11.23	10.79	10.79	8.09
	1	8.34	8.45	8.50	8.19	6.27	5.92	8.85	9.46	12.20	9.35	9.11	7.75	8.39	8.71	11.64	9.33
	2	8.25	8.46	8.60	8.32	6.32	5.97	9.01	10.15	11.05	10.43	9.31	8.15	9.77	9.73	10.72	10.80
	3	8.40	8.16	8.10	7.56	6.61	6.14	8.28	8.82	11.27	10.25	10.74	9.93	10.30	10.41	9.35	8.67
800	**Mean**	8.33	8.36	8.40	8.02	6.40	6.01	8.71	9.48	11.51	10.01	9.72	8.61	9.49	9.62	10.57	9.60
	1	8.48	5.53	8.36	5.22	6.19	6.72	8.55	7.59	10.41	9.11	10.32	9.82	10.94	9.14	10.56	8.27
	2	7.50	4.17	8.05	6.90	6.96	7.22	8.55	7.76	10.39	9.85	10.06	9.66	11.04	9.61	10.60	9.46
	3	8.57	5.72	8.23	5.37	6.11	6.42	8.32	7.77	10.05	10.32	11.11	10.58	11.54	10.22	11.87	8.41
1600	**Mean**	8.18	5.14	8.21	5.83	6.42	6.79	8.47	7.71	10.28	9.76	10.50	10.02	11.17	9.66	11.01	8.71

Table C.8 - Experiment 2 surface water ORP measurements. Eh corrected by +200mV for Pt electrode adjustment.

Time	1100	1400	1700	2000	0200	0800	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Hours	**3**	**6**	**9**	**12**	**18**	**24**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
Box biosolid application (g)	**Eh (mV)**															
0	451	486	480	479	535	522	481	594	546	447	475	460	469	515	496	470
100	466	480	496	532	504	490	490	530	502	472	476	462	533	451	432	476
200	435	421	463	467	448	453	472	448	456	487	484	490	471	460	489	471
400	450	450	463	483	548	488	541	497	461	450	466	517	473	480	468	473
800	460	476	434	445	453	455	481	447	476	511	452	483	458	467	472	464
1600	445	418	445	472	453	464	497	481	517	544	517	490	495	538	468	473

Table C.9 - Experiment 2 surface water temperature measurements.

Time	1100	1400	1700	2000	0200	0800	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Hours	**3**	**6**	**9**	**12**	**18**	**24**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
Box biosolid application (g)	**Water temperature (ºC)**															
0	20	20	20	20	19	20	20	21	21	20	21	21	20	20	19	20
100	20	20	20	19	19	20	19	20	20	19	20	21	19	20	20	20
200	20	20	20	19	19	20	19	20	20	19	19	20	19	20	19	19
400	20	20	20	19	19	20	19	20	20	19	19	20	19	20	20	19
800	21	21	21	20	19	20	20	21	21	20	21	21	20	20	20	20
1600	20	20	20	20	19	20	20	21	21	20	21	21	20	20	19	20

C.3.2 5cm and 10cm depth porewater

Table C.10 - Experiment 2 5cm depth porewater ORP measurements. Eh corrected by +200mV for Pt electrode adjustment. The Pt electrodes in the 200g and 800g biosolid-applied boxes failed and so no measurements were recorded.

Time	1100	1400	1700	2000	0200	0800	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Hours	**3**	**6**	**9**	**12**	**18**	**24**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
Box biosolid application (g)	**Eh (mV)**															
0	514	505	512	500	475	447	441	390	318	191	132	72	87	115	116	124
100	445	444	440	416	401	382	358	239	229	200	198	180	170	161	155	130
200	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
400	499	491	478	486	469	460	433	302	224	199	200	201	200	194	200	196
800	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1600	452	448	453	435	422	410	386	291	240	215	215	207	210	211	204	212

Table C.11 - Experiment 2 5cm depth porewater temperature measurements.

Time	1100	1400	1700	2000	0200	0800	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Hours	**3**	**6**	**9**	**12**	**18**	**24**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
Box biosolid application (g)	**Water temperature (ºC)**															
0	20	20	21	20	20	21	20	21	21	20	21	21	20	20	20	20
100	20	20	20	19	19	20	19	20	20	19	20	21	19	20	20	20
200	20	20	20	19	19	20	19	20	20	19	19	20	19	20	19	19
400	20	20	20	19	19	20	19	20	20	19	19	20	19	20	20	20
800	21	21	21	20	20	21	20	21	21	20	21	21	20	20	20	20
1600	20	20	20	20	20	20	20	21	21	20	21	21	20	20	20	20

Table C.12 - Experiment 2 10cm depth porewater ORP measurements. Eh corrected by +200mV for Pt electrode adjustment.

Time	1100	1400	1700	2000	0200	0800	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Hours	**3**	**6**	**9**	**12**	**18**	**24**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
Box biosolid application (g)	**Eh (mV)**															
0	404	394	406	394	391	378	365	297	237	205	199	193	183	179	166	169
100	434	441	439	434	425	421	427	394	368	228	212	207	187	181	184	190
200	439	434	413	409	340	443	406	335	261	205	203	205	212	222	200	191
400	414	412	411	414	395	386	369	243	197	195	193	186	187	140	160	169
800	397	390	382	394	374	370	364	346	324	302	264	250	218	219	213	206
1600	490	478	479	471	457	454	445	400	347	281	256	251	265	240	262	250

Table C.13 - Experiment 2 10cm depth porewater temperature measurements.

Time	1100	1400	1700	2000	0200	0800	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Hours	**3**	**6**	**9**	**12**	**18**	**24**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
Box biosolid application (g)	**Water temperature (ºC)**															
0	20	20	21	20	20	21	20	21	21	20	21	21	20	20	20	20
100	20	20	20	19	19	20	19	20	20	19	20	21	19	20	20	20
200	20	20	20	19	19	20	19	20	20	19	19	20	19	20	20	19
400	20	20	20	19	19	20	19	20	20	19	19	20	19	20	20	20
800	21	21	21	20	20	21	20	21	21	20	21	21	20	20	20	20
1600	20	20	21	20	20	21	20	21	21	20	21	21	20	20	20	20

C.4 EXPERIMENT 3

C.4.1 Surface water

Table C.14 - Experiment 3 surface water pH measurements in full.

	Time	1100	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Box biosolid application (g)	**Hours**	**3**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
	Reading	**pH**										
	1	7.44	7.97	8.21	8.39	8.48	8.46	8.46	8.36	8.25	8.34	8.30
	2	7.49	7.97	8.18	8.35	8.44	8.53	8.36	8.35	8.26	8.36	8.25
	3	7.46	7.97	8.33	8.44	8.65	8.61	8.56	8.41	8.33	8.29	8.51
0	**Mean**	7.46	7.97	8.24	8.39	8.52	8.53	8.46	8.37	8.28	8.33	8.35
	1	7.09	7.48	8.18	8.35	8.49	8.50	8.49	8.44	8.37	8.34	8.48
	2	7.21	7.76	8.11	8.33	8.50	8.57	8.47	8.36	8.40	8.30	8.30
	3	7.09	7.62	8.23	8.34	8.45	8.51	8.49	8.44	8.37	8.27	8.27
100	**Mean**	7.13	7.62	8.17	8.34	8.48	8.53	8.48	8.41	8.38	8.30	8.35
	1	6.81	7.22	7.96	8.20	7.82	8.36	8.35	8.31	8.20	8.21	8.25
	2	7.12	7.23	8.01	8.21	7.83	8.34	8.32	8.32	8.32	8.27	8.21
	3	7.11	7.26	7.98	8.24	7.98	8.44	8.46	8.25	8.26	8.22	7.94
200	**Mean**	7.01	7.24	7.98	8.22	7.88	8.38	8.38	8.29	8.26	8.23	8.13
	1	6.84	6.75	7.89	8.10	7.83	8.21	8.32	8.22	8.18	8.14	8.11
	2	7.20	6.99	7.83	8.00	7.70	8.21	8.16	8.22	8.14	8.08	8.10
	3	6.98	7.02	7.90	8.14	7.79	8.34	8.48	8.23	8.25	8.14	8.17
400	**Mean**	7.01	6.92	7.87	8.08	7.77	8.25	8.32	8.22	8.19	8.12	8.13
	1	6.71	7.26	7.91	7.96	7.94	8.20	8.14	8.12	8.17	8.24	8.07
	2	6.69	7.29	7.91	8.12	8.00	8.14	8.29	8.20	8.13	7.98	8.14
	3	7.06	7.25	7.85	7.91	7.94	8.13	8.19	8.17	8.11	8.05	8.13
800	**Mean**	6.82	7.27	7.89	8.00	7.96	8.16	8.21	8.16	8.14	8.09	8.11
	1	6.80	7.46	7.97	8.04	8.05	8.17	8.08	8.09	8.01	7.82	8.05
	2	6.97	7.43	7.81	7.92	8.01	8.17	8.15	8.09	8.01	7.99	7.87
	3	7.19	7.52	7.75	7.93	8.01	7.97	8.08	8.09	7.94	7.94	7.98
1600	**Mean**	6.99	7.47	7.84	7.96	8.02	8.10	8.10	8.09	7.99	7.92	7.97

Table C.15 - Experiment 3 surface water dissolved oxygen measurements in full.

	Time	1100	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Box biosolid application (g)	**Hours**	**3**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
	Reading	**Dissolved oxygen (mg O_2 L^{-1})**										
	1	8.57	8.50	9.67	9.80	11.07	9.81	9.10	9.04	9.07	8.72	8.66
	2	8.45	8.50	8.96	9.33	9.22	10.65	8.73	8.74	8.40	8.48	8.24
	3	8.42	8.28	9.33	9.58	10.90	10.28	9.18	8.99	8.84	8.73	9.22
0	**Mean**	8.48	8.43	9.32	9.57	10.40	10.25	9.00	8.92	8.77	8.64	8.71
	1	8.75	8.80	9.69	10.63	11.61	11.29	10.62	10.42	10.09	10.37	10.82
	2	8.41	8.99	9.79	10.20	10.59	10.73	10.75	10.30	9.76	9.39	8.90
	3	8.24	8.67	9.94	10.52	10.57	10.62	10.52	9.87	9.33	9.12	8.85
100	**Mean**	8.47	8.82	9.81	10.45	10.92	10.88	10.63	10.20	9.73	9.63	9.52
	1	8.59	8.56	9.25	10.50	10.42	9.92	9.70	9.21	8.66	9.80	8.66
	2	8.50	8.59	9.57	10.00	10.24	10.47	9.84	9.45	9.20	9.09	8.60
	3	8.47	8.52	9.53	9.84	10.31	10.69	9.64	9.75	9.55	8.67	8.73
200	**Mean**	8.52	8.56	9.45	10.11	10.32	10.36	9.73	9.47	9.14	9.19	8.66
	1	8.50	8.79	9.56	10.38	11.12	10.54	10.60	9.91	9.56	9.52	8.48
	2	8.28	8.62	8.95	9.33	9.82	9.77	9.56	8.40	9.30	8.22	7.74
	3	8.56	8.42	9.48	10.02	11.58	10.76	10.53	11.46	10.56	9.99	8.74
400	**Mean**	8.45	8.61	9.33	9.91	10.84	10.36	10.23	9.92	9.81	9.24	8.32
	1	7.82	8.29	9.31	10.14	10.78	10.12	9.51	9.70	9.55	8.75	9.01
	2	7.71	8.46	9.54	9.87	10.30	10.27	10.25	10.20	9.90	9.11	9.16
	3	7.48	8.32	9.04	9.61	9.68	10.03	9.89	9.35	8.93	8.81	8.23
800	**Mean**	7.67	8.36	9.30	9.87	10.25	10.14	9.88	9.75	9.46	8.89	8.80
	1	8.38	8.33	9.18	9.83	10.48	11.20	10.58	9.24	8.63	7.68	7.12
	2	8.13	7.47	9.37	9.28	9.77	10.18	9.45	8.88	8.33	7.92	6.55
	3	8.01	7.88	8.50	9.06	9.10	8.66	8.48	8.08	7.68	7.37	6.18
1600	**Mean**	8.17	7.89	9.02	9.39	9.78	10.01	9.50	8.73	8.21	7.66	6.62

Table C. 16 - Experiment 3 surface water ORP measurements. Eh corrected by +200mV for Pt electrode adjustment.

Time	1100	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Hours	**3**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
Box biosolid application (g)	**Eh (mV)**										
0	500	545	495	440	453	465	453	471	481	454	477
100	513	513	460	462	480	473	442	455	480	472	479
200	593	525	498	462	486	467	483	472	497	457	493
400	539	511	541	493	502	484	466	461	517	470	493
800	461	532	515	509	470	467	468	457	487	475	473
1600	520	472	493	455	464	470	455	469	470	454	465

Table C.17 - Experiment 3 surface water temperature measurements.

Time	1100	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Hours	**3**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
Box biosolid application (g)	**Water temperature (ºC)**										
0	24	22	22	21	21	22	23	21	23	22	22
100	23	23	22	20	21	21	23	21	23	22	22
200	23	22	22	20	21	21	22	22	23	21	22
400	23	22	23	21	21	21	22	22	24	22	22
800	23	22	22	20	21	21	22	22	23	21	22
1600	23	23	23	21	21	22	22	21	23	22	22

C.4.2 5cm depth porewater

Table C.18 - Experiment 3 5cm depth porewater pH measurements.

Time	1100	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Hours	**3**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
Box biosolid application (g)	**pH**										
0	7.03	7.45	7.48	7.62	7.36	7.62	7.61	7.83	8.12	8.14	8.05
100	7.30	6.93	7.27	7.41	7.68	7.52	7.67	7.68	7.96	7.81	7.83
200	6.69	6.76	7.31	7.28	7.49	7.59	7.47	7.54	7.62	7.70	7.76
400	6.89	6.99	7.35	7.45	7.59	7.63	7.55	7.60	7.71	7.68	7.71
800	7.09	6.96	7.15	7.55	7.31	7.44	7.50	7.59	7.63	7.68	7.68
1600	7.09	6.89	6.95	7.20	7.10	7.18	7.27	7.20	7.34	7.41	7.41

Table C.19 - Experiment 3 5cm depth porewater dissolved oxygen measurements. Measured using the standpipe method with dipping probe.

Time	1100	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Hours	**3**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
Box biosolid application (g)	**Dissolved oxygen (mg O_2 L^{-1})**										
0	6.79	1.53	5.24	4.77	4.91	5.20	5.55	6.20	6.36	7.37	7.15
100	4.27	3.01	6.55	6.64	6.46	6.62	6.47	6.83	7.09	7.01	7.03
200	4.80	3.62	6.79	5.45	6.17	5.07	4.45	4.86	4.98	5.10	5.59
400	5.75	4.23	5.66	5.56	6.02	6.15	5.67	6.76	8.30	7.29	7.03
800	3.77	0.41	5.31	5.77	5.72	5.47	5.76	5.67	5.43	5.84	5.49
1600	1.33	1.06	5.10	0.62	0.47	0.30	0.36	0.21	0.10	0.14	0.21

Table C.20 - Experiment 3 5cm depth porewater ORP measurements. Eh corrected by +200mV for Pt electrode adjustment.

Time	1100	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Hours	**3**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
Box biosolid application (g)	**Eh (mV)**										
0	355	358	341	229	191	181	166	145	127	115	105
100	483	429	361	264	203	199	196	194	192	189	187
200	512	283	224	215	210	209	205	205	203	203	203
400	428	328	270	223	207	202	200	197	195	195	196
800	444	323	233	218	212	208	204	203	201	200	200
1600	365	225	208	205	204	201	202	202	201	201	201

Table C.21 - Experiment 3 5cm depth porewater temperature measurements.

Time	1100	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Hours	**3**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
Box biosolid application (g)	**Water temperature (ºC)**										
0	24	23	23	21	21	21	22	21	23	22	22
100	23	23	22	20	21	21	22	21	23	22	22
200	22	22	22	20	21	21	22	22	23	21	22
400	22	22	23	21	21	21	22	22	23	22	22
800	22	22	22	20	21	21	21	22	23	21	22
1600	23	22	23	21	21	21	22	21	23	22	22

C.4.3 10cm depth porewater

Table C.22 - Experiment 3 10cm depth porewater pH measurements.

Time	1100	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Hours	**3**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
Box biosolid application (g)	**pH**										
0	6.79	7.53	7.57	7.61	7.47	7.59	7.64	7.68	7.70	7.74	7.76
100	7.43	7.22	7.37	7.45	7.50	7.49	7.59	7.58	7.69	7.74	7.72
200	7.15	7.20	7.44	7.39	7.33	7.54	7.50	7.55	7.61	7.64	7.65
400	7.11	7.32	7.41	7.35	7.42	7.42	7.41	7.43	7.48	7.43	7.51
800	7.21	7.23	7.32	7.43	7.41	7.46	7.52	7.61	7.70	7.65	7.59
1600	7.16	7.06	7.14	7.23	7.21	7.24	7.27	7.26	7.28	7.33	7.35

Table C.23 - Experiment 3 10cm depth porewater dissolved oxygen measurments. Measured using the standpipe method with dipping probe.

Time	1100	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Hours	**3**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
Box biosolid application (g)	**Dissolved oxygen (mg O_2 L^{-1})**										
0	4.84	3.80	1.10	0.62	0.83	0.88	0.92	0.85	0.74	0.77	0.87
100	3.69	3.96	3.14	1.60	1.98	0.85	0.94	0.90	1.22	1.38	1.39
200	2.44	0.91	1.09	1.08	0.96	0.86	0.91	0.83	0.63	0.88	0.93
400	0.67	0.64	0.57	0.85	0.68	0.84	0.71	0.61	0.40	0.48	0.36
800	5.97	4.54	3.84	3.77	4.23	5.01	4.74	3.46	3.48	4.75	1.14
1600	4.95	5.79	0.55	0.49	0.56	0.55	0.48	0.63	0.41	0.20	0.08

Table C.24 - Experiment 3 10cm depth porewater ORP measurements. Eh corrected by +200mV for Pt electrode adjustment.

Time	1100	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Hours	**3**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
Box biosolid application (g)	**Eh (mV)**										
0	459	349	198	181	195	200	176	163	169	172	172
100	418	388	364	327	267	221	206	201	200	200	200
200	411	351	280	243	219	200	193	189	184	184	184
400	505	440	394	343	268	223	210	205	202	202	202
800	300	288	313	249	235	228	223	217	220	222	229
1600	390	305	264	209	200	198	195	193	190	188	186

Table C.25 - Experiment 3 10cm depth porewater temperature measurements.

Time	1100	1400	1400	1400	1400	1400	1400	1400	1400	1400	1100
Hours	**3**	**30**	**54**	**78**	**102**	**126**	**150**	**174**	**198**	**222**	**243**
Box biosolid application (g)	**Water temperature (ºC)**										
0	24	23	23	21	21	21	22	21	23	22	22
100	23	22	23	20	21	21	22	21	23	22	22
200	22	22	22	20	21	21	21	22	23	21	22
400	22	22	23	20	21	21	22	22	23	21	22
800	22	22	22	20	21	21	22	22	23	21	22
1600	23	23	23	21	21	21	22	21	23	22	22

C.5 EXPERIMENT 4

C.5.1 Surface water

Table C.26 - Experiment 4 surface water pH measurements in full.

	Time	1500	1800	2100	0000	0600	1200	1400	1400	1400	1400	1400	1400	1400	1400	1200
Box biosolid application (g)	**Hours**	**3**	**6**	**9**	**12**	**18**	**24**	**50**	**74**	**98**	**122**	**146**	**170**	**194**	**218**	**240**
	Reading	**pH**														
	1	7.81	7.45	7.73	8.15	7.69	7.76	8.22	7.89	8.17	8.18	7.86	8.16	8.20	8.21	7.89
	2	7.84	7.35	7.70	7.66	7.76	8.17	7.99	8.07	8.37	8.38	8.22	8.02	7.92	7.52	8.08
	3	7.60	7.73	7.95	7.61	7.44	8.22	8.07	8.34	8.38	8.25	8.30	8.19	7.95	7.55	7.95
0	**Mean**	7.75	7.51	7.79	7.81	7.63	8.05	8.09	8.10	8.31	8.27	8.13	8.12	8.02	7.76	7.97
	1	7.60	7.76	7.81	7.68	7.93	8.26	8.07	8.05	8.00	7.89	8.05	8.08	8.15	7.86	7.53
	2	7.61	7.88	8.05	8.04	7.88	8.20	8.12	8.39	8.20	8.33	7.78	8.30	8.19	8.01	7.61
	3	7.66	7.80	7.88	7.86	7.99	8.35	7.99	8.33	7.14	7.92	8.34	8.19	7.97	7.86	7.88
100	**Mean**	7.62	7.81	7.91	7.86	7.93	8.27	8.06	8.26	7.78	8.05	8.06	8.19	8.10	7.91	7.67
	1	6.87	7.48	7.39	7.53	7.60	7.59	7.81	7.52	7.56	7.67	8.06	7.55	7.96	7.91	7.53
	2	6.85	7.45	7.31	7.60	7.61	7.70	7.53	7.42	7.47	8.08	7.24	7.65	7.53	7.98	7.82
	3	6.88	7.56	7.50	7.67	7.78	7.61	7.72	7.70	7.82	7.99	7.90	7.54	7.64	7.54	7.89
200	**Mean**	6.87	7.50	7.40	7.60	7.66	7.63	7.69	7.55	7.62	7.91	7.73	7.58	7.71	7.81	7.75
	1	7.19	7.37	7.57	7.59	7.39	7.57	7.45	7.81	8.11	7.69	7.51	7.78	7.64	7.86	7.48
	2	7.27	7.55	7.80	7.55	7.54	7.71	7.72	7.93	8.06	7.94	8.01	7.60	7.74	7.82	7.62
	3	7.39	7.62	7.76	7.40	7.53	7.76	7.70	7.82	8.01	7.69	8.01	7.60	7.92	7.63	7.67
400	**Mean**	7.28	7.51	7.71	7.51	7.49	7.68	7.62	7.85	8.06	7.77	7.84	7.66	7.77	7.77	7.59
	1	6.86	7.22	7.23	7.24	7.47	7.29	7.55	7.48	7.30	7.46	7.45	7.31	7.38	7.47	7.49
	2	6.88	7.37	7.34	7.38	7.65	7.42	7.41	7.37	7.42	7.44	7.49	7.16	7.34	7.40	7.40
	3	6.92	7.37	7.35	7.40	7.46	7.54	7.39	7.38	7.39	7.43	7.40	7.22	7.21	7.36	7.38
800	**Mean**	6.89	7.32	7.31	7.34	7.53	7.42	7.45	7.41	7.37	7.44	7.45	7.23	7.31	7.41	7.42
	1	7.02	7.13	7.14	7.15	7.24	7.37	7.52	7.39	7.33	7.53	7.47	7.22	7.40	7.49	7.26
	2	7.06	7.17	7.21	7.27	7.31	7.45	7.42	7.45	7.30	7.30	7.29	7.37	7.37	7.38	7.21
	3	7.04	7.24	7.16	7.24	7.36	7.52	7.50	7.35	7.32	7.39	7.55	7.33	7.39	7.42	7.28
1600	**Mean**	7.04	7.18	7.17	7.22	7.30	7.45	7.48	7.40	7.32	7.41	7.44	7.31	7.39	7.43	7.25

Table C.27 - Experiment 4 surface water dissolved oxygen measurements in full.

	Time	1500	1800	2100	0000	0600	1200	1400	1400	1400	1400	1400	1400	1400	1400	1200
Box biosolid	**Hours**	**3**	**6**	**9**	**12**	**18**	**24**	**50**	**74**	**98**	**122**	**146**	**170**	**194**	**218**	**240**
application (g)	**Reading**	**Dissolved oxygen (mg O_2 L^{-1})**														
	1	8.76	8.71	8.27	7.58	7.08	8.41	9.20	9.78	10.00	9.66	10.00	9.30	9.79	9.19	8.22
	2	8.77	8.57	7.67	7.31	6.87	8.55	9.24	9.73	10.21	10.14	10.53	9.47	9.81	9.32	8.41
	3	8.73	8.61	7.75	7.29	6.74	8.48	9.21	9.80	10.18	10.19	10.42	9.18	9.47	8.96	8.27
0 (1)	**Mean**	8.75	8.63	7.90	7.39	6.90	8.48	9.22	9.77	10.13	10.00	10.32	9.32	9.69	9.16	8.30
	1	9.22	8.96	8.32	7.88	7.56	8.74	9.34	9.91	10.15	10.02	9.91	8.76	8.96	9.04	7.87
	2	9.13	8.94	8.30	7.65	7.33	8.45	9.18	9.91	9.96	9.90	9.95	9.02	9.09	8.95	7.92
	3	9.08	8.35	7.80	7.39	7.21	8.32	9.33	9.90	9.97	9.98	9.95	8.69	8.85	8.90	7.25
0 (2)	**Mean**	9.14	8.75	8.14	7.64	7.37	8.50	9.28	9.91	10.03	9.97	9.94	8.82	8.97	8.96	7.68
	1	8.01	8.66	7.62	7.24	6.61	7.49	8.64	9.04	8.85	8.90	9.19	8.31	9.81	9.37	8.34
	2	8.44	8.55	7.96	7.09	7.02	7.74	8.62	9.15	9.06	9.41	9.49	9.56	10.37	9.69	8.95
	3	8.65	8.55	7.81	7.20	6.74	8.00	8.55	9.15	8.87	9.31	9.44	8.57	9.38	8.75	7.63
400 (1)	**Mean**	8.37	8.59	7.80	7.18	6.79	7.74	8.60	9.11	8.93	9.21	9.37	8.81	9.85	9.27	8.31
	1	8.85	8.41	7.62	7.68	7.22	8.25	8.58	8.79	9.22	8.95	8.99	8.29	8.78	8.50	7.36
	2	8.30	8.37	7.62	7.32	6.74	7.63	8.32	8.73	9.00	9.37	9.10	8.56	9.00	8.64	7.84
	3	8.17	7.96	6.75	6.65	7.21	7.26	8.23	8.75	8.95	8.97	8.92	8.69	9.07	8.89	7.82
400 (2)	**Mean**	8.44	8.25	7.33	7.22	7.06	7.71	8.38	8.76	9.06	9.10	9.00	8.51	8.95	8.68	7.67
	1	8.79	7.95	7.47	7.12	6.75	7.67	8.20	8.78	8.14	8.16	8.33	7.80	7.85	7.78	6.87
	2	8.49	8.33	6.95	6.54	5.98	7.66	8.00	8.26	7.97	8.00	8.50	7.53	7.58	7.89	6.26
	3	8.67	8.55	7.91	6.34	6.76	7.30	7.93	8.13	9.81	8.30	8.33	7.31	7.27	7.42	7.00
800 (1)	**Mean**	8.65	8.28	7.44	6.67	6.50	7.54	8.04	8.39	8.64	8.15	8.39	7.55	7.57	7.70	6.71
	1	8.64	7.58	7.56	7.15	7.18	8.25	8.36	9.25	9.43	9.82	9.90	9.16	9.36	9.54	7.84
	2	8.79	8.13	7.71	7.59	7.50	7.98	8.51	8.63	9.30	9.71	10.21	9.04	9.67	9.46	8.18
	3	8.40	8.10	6.87	6.74	6.71	7.53	7.96	8.14	8.41	8.41	8.73	8.14	8.95	8.23	7.47
800 (2)	**Mean**	8.61	7.94	7.38	7.16	7.13	7.92	8.28	8.67	9.05	9.31	9.61	8.78	9.33	9.08	7.83

Table C.28 - Experiment 4 surface water ORP measurements. Eh corrected by +200mV for Pt electrode adjustment.

Time	1500	1800	2100	0000	0600	1200	1400	1400	1400	1400	1400	1400	1400	1400	1200
Hours	**3**	**6**	**9**	**12**	**18**	**24**	**50**	**74**	**98**	**122**	**146**	**170**	**194**	**218**	**240**
Box biosolid application (g)	**Eh (mV)**														
0 (1)	569	550	546	532	530	540	535	536	548	518	507	508	510	484	495
0(2)	557	561	556	557	570	558	509	537	522	507	507	522	510	486	505
400 (1)	562	556	561	534	528	548	547	549	532	510	493	502	502	480	495
400 (2)	597	548	533	539	535	537	535	542	524	513	493	513	487	496	512
800 (1)	536	566	561	536	544	566	547	542	542	520	501	513	506	494	503
800 (2)	554	566	540	550	520	553	549	546	535	520	513	519	517	502	499

Table C.29 - Experiment 4 surface water temperature measurements.

Time	1500	1800	2100	0000	0600	1200	1400	1400	1400	1400	1400	1400	1400	1400	1200
Hours	**3**	**6**	**9**	**12**	**18**	**24**	**50**	**74**	**98**	**122**	**146**	**170**	**194**	**218**	**240**
Box biosolid application (g)	**Water temperature (ºC)**														
0 (1)	20.4	20.4	20.2	20.1	20.2	20.5	20.3	19.7	19.9	19.8	19.9	20.0	19.9	20.2	20.2
0(2)	20.4	20.5	20.4	20.2	20.3	20.8	20.6	20.2	20.3	20.1	20.2	20.2	20.3	20.2	20.4
400 (1)	20.7	20.4	20.3	20.3	20.0	20.5	20.4	19.9	20.0	19.9	20.1	20.2	20.2	20.2	20.3
400 (2)	20.6	20.5	20.3	20.4	20.5	20.9	20.7	20.4	20.4	20.4	20.5	20.3	20.0	20.3	20.4
800 (1)	20.8	20.6	20.4	20.4	20.2	20.6	20.5	20.2	20.3	20.0	20.2	20.5	20.4	20.5	20.2
800 (2)	20.5	20.4	20.3	20.3	20.2	20.2	20.5	20.2	20.2	20.2	20.2	20.2	20.2	20.2	20.3

C.5.2 5cm depth porewater

Table C.30 - Experiment 4 5cm depth porewater measurements.

Time	1500	1800	2100	0000	0600	1200	1400	1400	1400	1400	1400	1400	1400	1400	1200
Hours	**3**	**6**	**9**	**12**	**18**	**24**	**50**	**74**	**98**	**122**	**146**	**170**	**194**	**218**	**240**
Box biosolid application (g)	**pH**														
0 (1)	6.93	6.83	6.82	6.95	6.98	7.44	7.53	7.58	7.68	7.68	8.11	8.09	8.10	8.27	8.04
0 (2)	6.30	6.75	6.71	7.07	6.89	7.77	7.58	7.58	7.59	7.62	7.84	7.57	7.72	7.88	7.54
400 (1)	6.59	6.70	6.83	7.06	7.09	7.35	7.42	7.42	7.45	7.50	7.75	7.56	7.53	7.70	7.63
400 (2)	6.52	6.81	6.81	6.87	7.44	7.42	7.55	7.63	7.78	7.88	8.34	8.04	8.18	8.35	7.81
800 (1)	6.72	6.84	6.94	7.00	7.43	7.15	7.46	7.68	7.67	7.73	7.71	7.90	7.65	7.73	7.67
800 (2)	6.48	6.49	6.56	6.69	6.68	7.22	7.47	7.39	7.28	7.25	7.32	7.46	7.32	7.49	7.40

Table C.31 - Experiment 4 5cm depth porewater dissolved oxygen measurements. Measured using the oxygen sensor spot method.

Time	1500	1800	2100	0000	0600	1200	1400	1400	1400	1400	1400	1400	1400	1400	1200
Hours	**3**	**6**	**9**	**12**	**18**	**24**	**50**	**74**	**98**	**122**	**146**	**170**	**194**	**218**	**240**
Box biosolid application (g)	**Dissolved oxygen (mg O_2 L^{-1})**														
0 (1)	3.14	0.81	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0 (2)	4.27	1.50	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
400 (1)	0.53	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
400 (2)	3.38	1.19	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
800 (1)	0.45	0.53	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
800 (2)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table C.32 - Experiment 4 5cm depth porewater ORP measurements. Eh corrected by +200mV for Pt electrode adjustment. The 400g (2) biosolid applied box Pt electrode failed and readings are marked with an asterisk (*) to highlight this. These failed electrode readings were recorded throughout the experiment but were not included in the results of Chapter 4.

Time	1500	1800	2100	0000	0600	1200	1400	1400	1400	1400	1400	1400	1400	1400	1200
Hours	**3**	**6**	**9**	**12**	**18**	**24**	**50**	**74**	**98**	**122**	**146**	**170**	**194**	**218**	**240**
Box biosolid application (g)	**Eh (mV)**														
0 (1)	448	445	442	440	432	439	406	321	217	190	178	196	183	176	171
0 (2)	429	417	411	399	458	391	268	219	210	203	200	197	193	187	179
400 (1)	351	348	338	334	326	343	332	270	227	212	188	189	194	196	196
400 (2)	272*	258*	250*	241*	245*	228*	209*	199*	191*	183*	161*	133*	92*	34*	-22*
800 (1)	384	380	378	373	372	381	353	257	210	204	173	109	106	121	141
800 (2)	480	464	460	451	452	437	268	265	210	203	200	200	197	196	194

Table C.33 - Experiment 4 5cm depth porewater temperature measurements.

Time	1500	1800	2100	0000	0600	1200	1400	1400	1400	1400	1400	1400	1400	1400	1200
Hours	**3**	**6**	**9**	**12**	**18**	**24**	**50**	**74**	**98**	**122**	**146**	**170**	**194**	**218**	**240**
Box biosolid application (g)	**Water temperature (ºC)**														
0 (1)	20.5	20.5	20.3	20.2	20.1	20.5	20.3	19.6	19.8	19.7	19.9	19.9	19.9	20.0	20.2
0(2)	20.4	20.5	20.3	20.3	20.3	20.7	20.6	20.1	20.2	20.2	20.2	20.2	20.2	20.1	20.2
400 (1)	20.7	20.5	20.4	20.2	19.9	20.5	20.5	19.8	19.9	19.8	20.0	20.0	20.0	20.1	20.0
400 (2)	20.7	20.7	20.5	20.5	20.5	20.9	20.7	20.3	20.4	20.4	20.4	20.3	20.3	20.2	20.4
800 (1)	20.9	20.7	20.5	20.5	20.2	20.7	20.8	20.0	20.1	19.9	20.2	20.2	20.2	20.1	20.1
800 (2)	20.6	20.6	20.5	20.4	20.3	20.7	20.5	20.1	20.2	20.2	20.1	20.2	20.2	20.0	20.2

C.5.3 10cm depth porewater

Table C.34 - Experiment 4 10cm depth porewater pH measurements.

Time	1500	1800	2100	0000	0600	1200	1400	1400	1400	1400	1400	1400	1400	1400	1200
Hours	**3**	**6**	**9**	**12**	**18**	**24**	**50**	**74**	**98**	**122**	**146**	**170**	**194**	**218**	**240**
Box biosolid application (g)	**pH**														
0 (1)	7.08	7.04	7.05	7.11	7.16	7.31	7.50	7.44	7.46	7.45	7.51	7.49	7.50	7.52	7.47
0 (2)	6.76	7.07	7.08	7.18	7.16	7.32	7.27	7.42	7.48	7.35	7.41	7.34	7.35	7.40	7.29
400 (1)	6.68	6.93	6.99	7.10	7.10	7.23	7.35	7.46	7.40	7.38	7.42	7.30	7.23	7.29	7.30
400 (2)	6.90	7.13	7.17	7.23	7.35	7.37	7.30	7.32	7.35	7.43	7.49	7.46	7.47	7.55	7.40
800 (1)	6.51	6.98	7.05	7.11	7.34	7.11	7.17	7.28	7.23	7.22	7.40	7.28	7.26	7.24	7.23
800 (2)	6.64	6.84	6.91	6.96	6.99	7.23	7.35	7.36	7.34	7.48	7.60	7.59	7.56	7.60	7.34

Table C.35 - Experiment 4 10cm depth porewater dissolved oxygen measurements. Measured using the oxygen sensor spot method.

Time	1500	1800	2100	0000	0600	1200	1400	1400	1400	1400	1400	1400	1400	1400	1200
Hours	**3**	**6**	**9**	**12**	**18**	**24**	**50**	**74**	**98**	**122**	**146**	**170**	**194**	**218**	**240**
Box biosolid application (g)	**Dissolved oxygen (mg O_2 L^{-1})**														
0 (1)	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0 (2)	5.04	1.34	0.02	0.29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
400 (1)	4.85	0.91	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
400 (2)	2.06	0.08	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
800 (1)	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
800 (2)	6.28	3.31	0.19	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table C.36 - Experiment 4 10cm depth porewater ORP measurements. Eh corrected by +200mV for Pt electrode adjustment. The 0g (1) biosolid applied box Pt electrode failed and readings are marked with an asterisk (*) to highlight this. These failed electrode readings were recorded throughout the experiment but were not included in the results of Chapter 4.

Time	1500	1800	2100	0000	0600	1200	1400	1400	1400	1400	1400	1400	1400	1400	1200
Hours	**3**	**6**	**9**	**12**	**18**	**24**	**50**	**74**	**98**	**122**	**146**	**170**	**194**	**218**	**240**
Box biosolid application (g)	**Eh (mV)**														
0 (1)	266*	244*	223*	216*	211*	210*	205*	201*	201*	200*	198*	196*	193*	153*	165*
0 (2)	457	447	443	436	462	433	370	230	195	190	188	187	183	184	181
400 (1)	477	473	469	465	450	450	343	243	222	212	200	206	204	197	188
400 (2)	496	490	482	472	476	444	300	247	229	223	218	215	213	211	209
800 (1)	438	434	432	425	421	426	369	252	215	207	204	203	202	202	200
800 (2)	494	480	471	454	413	373	258	244	230	226	228	227	220	218	213

Table C.37 - Experiment 4 10cm depth porewater temperature measurements.

Time	1500	1800	2100	0000	0600	1200	1400	1400	1400	1400	1400	1400	1400	1400	1200
Hours	**3**	**6**	**9**	**12**	**18**	**24**	**50**	**74**	**98**	**122**	**146**	**170**	**194**	**218**	**240**
Box biosolid application (g)	**Water temperature (ºC)**														
0 (1)	20.6	20.7	20.5	20.4	20.3	20.6	20.4	19.7	19.9	19.9	19.9	19.9	19.8	20.0	20.0
0(2)	20.6	20.6	20.5	20.4	20.4	20.7	20.9	20.1	20.2	20.2	20.3	20.2	20.2	20.0	20.2
400 (1)	20.6	20.7	20.5	20.4	20.3	20.5	20.6	19.8	19.9	19.8	20.0	19.9	19.9	20.0	20.0
400 (2)	20.8	20.7	20.8	20.7	20.6	20.9	21.0	20.3	20.4	20.4	20.4	20.4	20.4	20.1	20.4
800 (1)	20.8	20.7	20.5	20.5	20.3	20.6	20.7	19.9	20.0	19.9	20.1	20.1	20.1	20.1	20.0
800 (2)	20.5	20.6	20.5	20.4	20.4	20.7	20.7	20.1	20.2	20.2	20.2	20.2	20.2	20.0	20.2

APPENDIX D: CROP DRY WEIGHT BIOMASS DETERMINATION

0g biosolid application (lost)

100g biosolid application

200g biosolid application

400g biosolid application

800g biosolid application

1600g biosolid application (lost)

Figure D.1 - Photographs of plants from Experiment 2 which were used in the ImageJ analysis to determine plant dry weight biomass after loss of the samples indicated on the image.

APPENDIX E: BACTERIAL CHARACTERISATION

E.1 PHYLUM RELATIVE ABUNDANCE

Table E.1 - Bacterial phyla relative abundances in biosolid and initial soil samples. Phyla marked with and asterisk (*) were included as 'Other bacteria' in the main analysis.

	Biosolid					Initial soil				
	Biosolid1	Biosolid2	Biosolid3	Biosolid4	Biosolid5	Initial1	Initial2	Initial3	Initial4	Initial5
Phylum	Relative abundance (%)									
Acidobacteria	0.117	0.191	0.121	0.191	0.168	35.1	37.1	30.6	30.3	29.4
Proteobacteria	0.46	0.451	0.533	0.456	0.412	18.5	17.4	19.9	17.2	19.4
Chloroflexi	3.7	3.17	3.56	3.57	3.6	8.11	8.55	8.35	10.5	10.3
Planctomycetes	5.26	5.31	5.45	5.01	6.07	9.21	9.45	9.77	9.32	8.4
Actinobacteria	0.0808	0.126	0.0939	0.128	0.0796	7.33	6.68	12.4	16.7	14
Bacteroidetes	12.6	13.9	14	12.9	12.4	4.37	5.04	5.13	3.35	3.45
Verrucomicrobia	0.374	0.162	0.266	0.0851	0.107	6.57	5.76	4.86	4.17	4.47
Nitrospirae	0.0012	0.00205	0.00174	0	0.00356	3.17	2.31	1.94	2.27	2.87
Firmicutes	36.2	37.7	38.3	37.7	38.1	0.253	0.441	0.229	0.593	1.38
Gemmatimonadetes	0.0018	0.00205	0.00058	0.000956	0.00158	3.52	3.21	2.73	1.82	2.63
Latescibacteria	0.0018	0.00102	0.00058	0.000956	0.00158	1.53	0.893	0.948	0.971	1.17
Saccharibacteria	8.71	7.72	5.79	6.01	5.53	0.698	0.911	1.18	0.731	0.732
Synergistetes	12.6	12.7	11.7	14.6	13	0.00205	0.0785	0.00134	0.0958	0.118
Armatimonadetes	2.12	2.39	2.14	2.41	2.35	0.689	1.03	0.72	0.721	0.649
Atribacteria	7	6.31	6.1	6.66	6.93	0.00512	0.044	0.00134	0.0586	0.09
(Unassigned)*	3.34	3.51	3.95	3.66	3.87	0.246	0.2	0.278	0.437	0.338
Hydrogenedentes*	0.871	1.02	0.817	1.07	0.926	0.113	0.158	0.174	0.17	0.0801
Cloacimonetes*	3.05	2.24	3.16	2.19	2.85	0	0.018	0.000672	0.0243	0.06
Cyanobacteria*	0	0	0	0	0	0.134	0.167	0.293	0.142	0.174
Lentisphaerae*	1.47	1.52	1.93	1.79	1.82	0.00102	0.0142	0.00134	0.0157	0.0189
SHA-109*	0.105	0.122	0.0887	0.152	0.128	0.0933	0.116	0.0907	0.0701	0.0378
Chlorobi*	0	0	0	0	0	0.1	0.139	0.136	0.107	0.0477
JL-ETNP-Z39*	0	0	0	0	0	0.0543	0.106	0.0658	0.0615	0.06
Candidate division SR1*	0.858	0.774	0.885	0.793	0.753	0	0.00568	0	0.00858	0.00616
Fibrobacteres*	0.138	0.0492	0.069	0.022	0.0372	0.0482	0.0397	0.0551	0.03	0.06
Spirochaetae*	0.586	0.4	0.637	0.336	0.56	0	0.00426	0	0.00429	0.0111
Elusimicrobia*	0	0	0	0	0	0.0471	0.0506	0.0249	0.0315	0.0329
Aerophobetes*	0	0	0	0	0	0.00102	0.00237	0.041	0.0172	0
Omnitrophica*	0.0748	0.0779	0.0847	0.0803	0.0934	0.00922	0.00237	0.0195	0.00572	0.00986
Parcubacteria*	0.0365	0.0338	0.0452	0.0421	0.0313	0.0205	0.0118	0.00605	0.00715	0.00781
WCHB1-60*	0	0	0	0	0	0.0225	0.018	0.0208	0.0214	0.00945
Thermotogae*	0.144	0.0922	0.111	0.0449	0.0693	0	0.000946	0	0.00286	0.00123
TA06*	0.0862	0.0789	0.0847	0.0659	0.076	0	0	0	0	0.023
Gracilibacteria*	0	0	0	0	0	0.00512	0.0109	0.0134	0.00286	0.00493
Candidate division OP3*	0	0	0	0	0	0	0.00142	0.00739	0.00143	0
TM6*	0	0	0	0	0	0.00307	0.00899	0	0.00572	0.00123
Deinococcus-Thermus*	0	0	0	0	0	0.0297	0.0109	0	0.00429	0
Marinimicrobia*	0.0126	0.00205	0.0122	0.00382	0.000396	0	0.000473	0	0	0.00164
Chlamydiae*	0	0	0	0	0	0	0	0.00134	0	0.00493

Table E.2 - Pre-Flood soil sample bacterial phyla relative abundances. Phyla marked with and asterisk (*) were included as 'Other bacteria' in the main analysis.

	Pre-Flood 0g						Pre-Flood 400g						Pre-Flood 800g					
	PreB3S1	PreB3S2	PreB3S3	PreB5S1	PreB5S2	PreB5S3	PreB2S1	PreB2S2	PreB2S3	PreB6S1	PreB6S2	PreB6S3	PreB1S1	PreB1S2	PreB1S3	PreB4S1	PreB4S2	PreB4S3
Phylum	Relative abundance (%)																	
Acidobacteria	34.5	34.2	36.3	33.7	26.6	28.3	28.8	27.1	30.4	29.6	31.9	28.4	29.8	28.9	25.1	26.4	26.4	26.6
Proteobacteria	19.2	17.8	18.3	18.9	19.9	19.3	22.8	24.7	22.3	20.6	21.1	23.1	24.5	23.2	29.8	26.5	28.2	23.9
Chloroflexi	9.31	9.81	8.94	9.51	10.1	12.3	9.38	9.33	9.54	11.7	9.97	9.83	9.98	9.69	8.33	9.98	9.14	11
Planctomycetes	10.7	10.1	10.4	11.3	12.6	10.8	10.3	9.23	10.4	10.5	10.9	10.5	8.74	10.2	9.51	8.83	8.43	8.73
Actinobacteria	5.95	6.83	6.94	6.68	10.6	13	6.69	6.4	6.18	7.63	6.39	6.27	3.86	4.45	4.77	7.04	5.54	6.85
Bacteroidetes	3.37	3.05	3.03	3.45	3.88	2.38	2.57	5.16	5.19	3.79	3.3	4.11	4.43	4.38	5.4	3.97	5.07	4.91
Verrucomicrobia	4.43	4.88	3.87	4.57	6.1	4.53	3.83	4.52	4.28	4.29	4.29	3.94	3.83	4.37	4.65	4.12	4.08	4.08
Nitrospirae	4.78	5.5	4.71	4.65	4.04	3.58	4.27	3.65	3.62	3.56	4.37	4.34	4.75	5.13	3.59	3.48	2.87	3.58
Firmicutes	0.138	0.122	0.191	0.39	0.655	0.573	0.708	0.91	0.207	0.654	0.32	0.958	0.814	0.578	0.4	0.785	0.681	0.93
Gemmatimonadetes	2.77	3.08	2.95	2.23	1.69	1.94	3.76	2.44	3.15	2.69	2.85	2.77	2.54	2.8	2.69	2.54	3.14	2.99
Latescibacteria	2.08	2.18	1.92	2.34	2.02	1.44	2.42	1.54	1.64	1.95	2.14	2.5	2.24	2.47	1.41	1.64	1.32	1.95
Saccharibacteria	0.618	0.485	0.439	0.408	0.369	0.314	1.4	0.987	0.922	0.836	0.51	0.838	1.51	0.966	1.48	1.88	2.34	1.7
Synergistetes	0.00212	0.0016	0	0.0083	0.0151	0.0128	0.45	0.465	0.0299	0.182	0.0638	0.373	0.338	0.205	0.173	0.341	0.162	0.316
Armatimonadetes	0.989	0.964	0.922	0.69	0.529	0.632	0.873	0.826	0.922	0.581	0.72	0.72	0.961	1.02	0.808	0.929	0.987	0.829
Atribacteria	0	0.0016	0	0.00332	0.00339	0.000799	0.132	0.217	0.029	0.124	0.0387	0.133	0.324	0.281	0.162	0.358	0.305	0.435
(Unassigned)*	0.287	0.25	0.288	0.268	0.31	0.439	0.45	0.334	0.284	0.331	0.222	0.328	0.288	0.302	0.187	0.259	0.213	0.266
Hydrogenedentes*	0.203	0.149	0.128	0.211	0.162	0.14	0.275	0.255	0.199	0.251	0.216	0.184	0.329	0.219	0.394	0.193	0.322	0.198
Cloacimonetes*	0	0	0	0.00166	0.000616	0.00479	0.0331	0.0537	0.00561	0.0236	0.00258	0.0777	0.0581	0.0476	0.00941	0.0531	0.0287	0.0615
Cyanobacteria*	0.123	0.0971	0.133	0.297	0.19	0.115	0.136	0.152	0.144	0.134	0.101	0.163	0.115	0.161	0.614	0.255	0.133	0.145
Lentisphaerae*	0	0.000534	0	0	0.00246	0.00399	0.0731	0.0235	0.00561	0.0143	0.00709	0.0134	0.0214	0.0258	0.0141	0.0293	0.0335	0.0487
SHA-109*	0.195	0.156	0.111	0.161	0.0585	0.102	0.252	0.121	0.168	0.118	0.2	0.0911	0.129	0.179	0.152	0.0892	0.0944	0.133
Chlorobi*	0.143	0.14	0.124	0.105	0.0878	0.0639	0.18	0.0772	0.136	0.148	0.157	0.163	0.187	0.151	0.191	0.155	0.226	0.13
JL-ETNP-Z39*	0.131	0.0779	0.15	0.0871	0.0542	0.0375	0.0906	0.073	0.0607	0.048	0.0644	0.0742	0.103	0.0968	0.0788	0.0763	0.103	0.0747
Candidate division SR1*	0	0	0	0.00166	0.00154	0	0	0.021	0.00187	0.00758	0.000644	0.00848	0.0122	0.00156	0.00235	0.00545	0.00547	0.00954
Fibrobacteres*	0.0301	0.0405	0.0517	0.0299	0.0185	0.016	0.0157	1.34	0.0701	0.0817	0.0445	0.0664	0.0291	0.0273	0.047	0.0347	0.0486	0.0334
Spirochaetae*	0	0.000534	0	0.00083	0	0.000799	0.00174	0.0118	0	0	0	0.00777	0.00153	0.0164	0.00118	0.00953	0.0103	0.017
Elusimicrobia*	0.0354	0.0395	0.0353	0.0307	0.0231	0.0359	0.0586	0.0319	0.0402	0.0413	0.0296	0.0268	0.0214	0.039	0.0235	0.0204	0.0335	0.045
Aerophobetes*	0.0476	0.0155	0.0232	0.0207	0.00616	0.024	0.0244	0.0193	0.0271	0.0152	0.0103	0.0134	0.00306	0.0398	0.00353	0	0.00342	0.00371
Omnitrophica*	0.0037	0.016	0.0103	0.0149	0.004	0.00399	0	0.0109	0.0028	0.00758	0.00644	0.00636	0.00918	0.00937	0.00118	0.0034	0.00205	0.00159
Parcubacteria*	0.01	0.00534	0.00517	0.00415	0.00277	0.0016	0	0.00756	0.0159	0.00168	0.00387	0.00141	0.00153	0.0187	0.00235	0.0157	0.00958	0.0053
WCHB1-60*	0.00529	0.0107	0.00344	0.0124	0.00339	0.024	0.0104	0.00084	0.0121	0.0118	0.0103	0.00989	0.00918	0.00546	0.00353	0.000681	0.00684	0.00742
Thermotogae*	0	0	0	0.00166	0.000308	0	0	0.0084	0	0.00589	0	0.00212	0.00612	0.0125	0.00118	0.00477	0.0041	0.00212
TA06*	0	0	0	0	0	0.000799	0	0	0	0.000842	0.00129	0	0.00459	0.00702	0.0047	0.0034	0.00821	0.00636
Gracilibacteria*	0.00212	0.0048	0	0.00083	0.00185	0.00399	0.00058	0.00084	0.000935	0	0.00387	0.00424	0.00459	0	0.00118	0.000681	0.00889	0.00212
Candidate division OP3*	0.00264	0.0016	0	0	0.000616	0	0.00058	0.0042	0.00467	0.00337	0	0.000706	0.00612	0.0039	0	0	0	0
TM6*	0	0	0.000861	0	0.00185	0.000799	0	0	0.000935	0	0	0	0.0122	0.00078	0	0.00272	0	0
Deinococcus-Thermus*	0	0	0	0	0	0	0	0	0.00374	0	0	0	0	0	0	0	0.00479	0
Marinimicrobia*	0	0	0	0	0	0	0	0	0	0	0	0	0.00153	0.00156	0	0.000681	0	0
Chlamydiae*	0	0	0	0	0.000308	0	0	0	0	0	0	0	0	0	0	0	0.00342	0

Table E.3 - Post-Flood soil samples bacterial phyla relative abundances. Phyla marked with and asterisk (*) were included as 'Other bacteria' in the main analysis.

	Post-Flood 0g						Post-Flood 400g						Post-Flood 800g					
	PostB3S1	PostB3S2	PostB3S3	PostB5S1	PostB5S2	PostB5S3	PostB2S1	PostB2S2	PostB2S3	PostB6S1	PostB6S2	PostB6S3	PostB1S1	PostB1S2	PostB1S3	PostB4S1	PostB4S2	PostB4S3
Phylum	Relative abundance (%)																	
Acidobacteria	27.6	28.5	27.7	32	32.6	29.6	21.6	28.8	26.2	29.3	26.9	22.1	18.4	23.5	27.1	17.7	16.8	18.4
Proteobacteria	22.7	21.7	23.1	19.2	19.6	20.8	27.5	20.3	24	20.5	24.5	29.6	35.4	30.2	25.5	37.5	33.7	34.1
Chloroflexi	11.4	10.8	11.4	10.5	9.77	9.12	10.5	11.1	10.4	10.5	9.9	8.96	9.72	9.47	10.5	10.2	10.6	11.4
Planctomycetes	10.8	10	10.3	10.4	10	12.4	8.43	10.9	9.48	10.3	9.55	8.85	7.33	8.77	9.82	7.29	7.04	7.15
Actinobacteria	8.61	9.79	7.72	5.8	5.37	6.29	8.62	9.09	9.03	6.67	7.9	4.79	7.13	6.99	8.58	4.96	6.23	5.83
Bacteroidetes	3.29	3.27	3.1	3.73	3.51	3.38	4.51	2.91	3.62	3.34	4.42	5.63	5.67	4.86	3.24	6.25	6.85	5.91
Verrucomicrobia	4.33	4.07	4.06	4.47	4.25	5.06	3.52	4.03	3.8	3.66	3.9	3.61	2.87	3.36	3.57	3.57	3.96	3.66
Nitrospirae	4	3.99	4.2	4.88	5.31	4.53	3.22	4.49	4.56	5.11	3.56	3.25	2.29	3.57	4.06	2.35	2.23	2.35
Firmicutes	0.699	0.708	1.17	0.417	0.423	0.443	2.83	0.899	1.34	0.818	1.01	2.53	1.98	1.58	0.486	1.39	2.24	1.27
Gemmatimonadetes	2.41	2.71	2.61	3.77	3.97	3.22	2.49	2.32	2.39	3.57	3.13	3.25	2.63	2.48	3.26	2.9	2.64	2.86
Latescibacteria	1.71	2.05	2.19	2.05	2.21	2.72	1.45	2.04	2.09	2.41	1.77	1.65	0.904	1.57	1.46	0.978	1.04	0.952
Saccharibacteria	0.387	0.402	0.534	0.586	0.44	0.618	1.36	0.56	0.559	0.934	1.04	1.93	2	1.12	0.375	1.53	2.79	1.82
Synergistetes	0	0	0.00113	0	0.00476	0.000471	1.13	0.147	0.276	0.171	0.211	0.717	0.73	0.293	0.0152	0.45	0.773	0.686
Armatimonadetes	0.861	0.824	0.838	0.882	1.19	0.822	0.719	0.921	0.793	1.19	0.731	0.961	0.76	0.872	0.895	0.705	0.624	0.777
Atribacteria	0	0	0.00563	0	0.00381	0	0.582	0.179	0.279	0.146	0.177	0.582	0.84	0.194	0.0325	1.04	1.12	1.26
(Unassigned)*	0.35	0.336	0.268	0.353	0.347	0.3	0.421	0.403	0.409	0.33	0.36	0.298	0.24	0.241	0.276	0.227	0.276	0.259
Hydrogenedentes*	0.225	0.19	0.212	0.412	0.256	0.249	0.258	0.203	0.199	0.191	0.43	0.254	0.328	0.191	0.145	0.293	0.331	0.343
Cloacimonetes*	0	0	0	0.000951	0	0	0.0837	0.0104	0.0243	0.0226	0.0266	0.0774	0.105	0.0187	0.00247	0.064	0.113	0.0467
Cyanobacteria*	0.226	0.122	0.222	0.157	0.222	0.0762	0.238	0.138	0.0688	0.171	0.115	0.266	0.272	0.223	0.145	0.184	0.223	0.336
Lentisphaerae*	0.000871	0.00117	0	0	0.000951	0	0.0413	0.0114	0.0152	0.0277	0.0108	0.0516	0.0278	0.024	0.00124	0.0568	0.0366	0.0782
SHA-109*	0.109	0.139	0.0923	0.0932	0.128	0.0814	0.0967	0.137	0.14	0.141	0.0998	0.0926	0.0792	0.0695	0.148	0.0692	0.0653	0.136
Chlorobi*	0.111	0.129	0.108	0.0999	0.0951	0.14	0.128	0.0975	0.0688	0.214	0.124	0.204	0.131	0.178	0.101	0.196	0.15	0.201
JL-ETNP-Z39*	0.0958	0.0996	0.0709	0.0809	0.104	0.0809	0.0684	0.0986	0.076	0.118	0.061	0.0828	0.045	0.0861	0.0746	0.0589	0.0366	0.0544
Candidate division SR1*	0	0	0	0	0	0	0.0507	0	0.00797	0.00252	0.00443	0.0289	0.00643	0.00282	0	0.00103	0.00637	0.00763
Fibrobacteres*	0.0374	0.0246	0.018	0.0419	0.0295	0.0226	0.0401	0.0187	0.0246	0.0377	0.0433	0.0471	0.045	0.0353	0.084	0.0496	0.051	0.0305
Spirochaetae*	0	0	0	0	0	0	0.00943	0.00104	0.00579	0.00503	0.000984	0.00835	0.0107	0.000706	0	0.0031	0.00637	0.00954
Elusimicrobia*	0.0453	0.0633	0.0338	0.0381	0.0247	0.0259	0.033	0.027	0.0384	0.0226	0.0615	0.0676	0.0214	0.0272	0.0408	0.0155	0.0175	0.0277
Aerophobetes*	0.0192	0.0258	0.018	0.0333	0.0228	0.00659	0.00825	0.0457	0.0272	0.0101	0.0103	0.00152	0	0.00529	0.0556	0	0.00159	0.000954
Omnitrophica*	0.00348	0.00938	0.00675	0.0114	0.00571	0.00565	0.00825	0.0249	0.00724	0.00755	0.0128	0.0121	0	0.00529	0.0144	0.00516	0.00478	0.00286
Parcubacteria*	0.00435	0.00352	0.00338	0.00571	0.0133	0.00282	0.00354	0.0114	0.00253	0.0101	0.00443	0.00911	0.00643	0.00388	0.00742	0.0134	0.0127	0.00191
WCHB1-60*	0.0157	0.0164	0.0101	0.00476	0.00571	0.00988	0.00471	0.00934	0.0167	0.0176	0.0123	0.00228	0.0107	0.00988	0.019	0.00516	0.00478	0.00954
Thermotogae*	0	0	0	0	0	0	0.00943	0.0125	0.00543	0.00252	0	0.0205	0.00857	0.00318	0	0.0031	0.0112	0.00477
TA06*	0	0	0	0	0	0	0.00471	0.00104	0.00217	0.00252	0.00197	0.0038	0.0107	0.00494	0	0.0176	0.0159	0.02
Gracilibacteria*	0.00609	0.00469	0.0045	0	0.000951	0.000941	0.00236	0.00519	0.0119	0.0101	0.00295	0.000759	0.00428	0.00212	0.0033	0	0.00159	0.00477
Candidate division OP3*	0	0	0.00675	0	0	0.000941	0.00825	0	0.00181	0.00252	0.00148	0.000759	0	0.000353	0.00206	0	0	0.00191
TM6*	0.000871	0	0	0	0	0	0	0	0	0.0101	0.00393	0.00759	0.00214	0.000706	0	0	0	0.000954
Deinococcus-Thermus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Marinimicrobia*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chlamydiae*	0	0	0	0	0	0.000941	0	0.00311	0	0	0	0	0	0	0	0	0	0

Table E.4 - Final soil samples bacterial phyla relative abundances. Phyla marked with and asterisk (*) were included as 'Other bacteria' in the main analysis.

	Final 0g						Final 400g						Final 800g					
	FinalB3S1	FinalB3S2	FinalB3S3	FinalB5S1	FinalB5S2	FinalB5S3	FinalB2S1	FinalB2S2	FinalB2S3	FinalB6S1	FinalB6S2	FinalB6S3	FinalB1S1	FinalB1S2	FinalB1S3	FinalB4S1	FinalB4S2	FinalB4S3
Phylum	Relative abundance (%)																	
Acidobacteria	34.3	30.2	32.8	31.3	32.4	33.1	30.5	24.1	30.3	29.8	26.3	27.5	26.2	21.6	24.5	25.1	29.5	31.7
Proteobacteria	19.5	17.8	18.2	17.9	19	18	17	26.3	20	22.1	23.3	21.5	25.2	33.2	26.9	22.9	20	18.5
Chloroflexi	10.2	13.6	12.5	10.1	9.87	11.1	12.4	10.3	11.2	8.61	10.3	11.3	10.6	7.8	12.2	13.1	12.6	12.6
Planctomycetes	9.9	11.8	11.6	11.2	10.8	11.8	11.1	10	10.6	11.9	10.4	10.6	10.9	11	9.76	10.1	10.7	10.2
Actinobacteria	6.09	7.89	5.27	6.72	6.74	5.64	5.91	6.08	6.34	5.17	4.94	4.92	4.01	3.89	3.9	6.04	4.26	3.55
Bacteroidetes	2.82	2.73	3.01	2.1	3.33	2.92	3.33	4.36	3.47	3.64	4.52	3.97	4.08	5.59	4.75	4.26	3.73	2.72
Verrucomicrobia	4.15	4.01	4.06	4.18	4.4	4.08	3.94	3.92	3.77	4.5	4.19	3.84	4.03	4.69	3.27	3.58	3.8	3.6
Nitrospirae	4.53	3.51	4.11	5.72	4.7	4.65	4.62	4.17	4.5	4.98	4.23	4.45	4.66	2.85	4.2	3.73	4.33	6.1
Firmicutes	0.399	0.349	0.184	1.1	0.183	0.204	0.196	0.895	0.549	0.262	1.69	0.986	0.547	0.296	0.69	0.355	0.225	0.259
Gemmatimonadetes	2.99	3.29	3.04	3.67	3.65	3.24	3.28	2.9	3.27	3.23	3.68	3.54	3.64	2.41	3.23	4.27	4.31	3.84
Latescibacteria	2.31	1.94	2.24	3.23	2.15	2.43	2.3	1.87	2.37	2.46	2.04	2.5	2.08	1.24	1.88	1.91	1.72	3.11
Saccharibacteria	0.309	0.261	0.363	0.324	0.366	0.375	0.674	1.64	0.542	0.795	1.08	1.42	1.27	2.79	1.56	1.76	1.56	1.13
Synergistetes	0.00183	0.00085	0.000339	0	0.00487	0	0.0273	0.327	0.0423	0.0735	0.421	0.38	0.144	0.211	0.266	0.0917	0.0636	0.0639
Armatimonadetes	1.21	1.25	1.23	1.04	1.2	1.12	1.36	1.13	1.24	0.966	1.17	1.11	1.09	0.707	1.17	1.23	1.53	1.38
Atribacteria	0	0	0.000339	0	0.00244	0	0.033	0.326	0.0912	0.0997	0.248	0.213	0.172	0.266	0.303	0.334	0.334	0.175
(Unassigned)*	0.271	0.329	0.344	0.324	0.261	0.331	0.339	0.293	0.304	0.232	0.281	0.406	0.231	0.178	0.234	0.246	0.214	0.208
Hydrogenedentes*	0.445	0.436	0.339	0.497	0.339	0.241	0.321	0.56	0.619	0.456	0.55	0.487	0.368	0.551	0.433	0.388	0.386	0.273
Cloacimonetes*	0	0	0	0	0	0	0.00456	0.042	0.0029	0.0111	0.0386	0.0519	0.0229	0.0323	0.0356	0.0133	0.00757	0.00655
Cyanobacteria*	0.152	0.136	0.175	0.0544	0.154	0.138	2.16	0.176	0.132	0.192	0.092	0.194	0.111	0.144	0.0868	0.232	0.0908	0.0655
Lentisphaerae*	0	0	0.00102	0	0	0	0.00228	0.021	0.0104	0.00111	0.0117	0.0106	0.0056	0.0129	0.0145	0.0229	0.00227	0.00164
SHA-109*	0.113	0.0922	0.199	0.205	0.168	0.216	0.137	0.162	0.194	0.121	0.131	0.22	0.193	0.137	0.212	0.119	0.188	0.165
Chlorobi*	0.161	0.103	0.118	0.0654	0.134	0.0994	0.159	0.134	0.131	0.107	0.118	0.106	0.115	0.169	0.215	0.133	0.175	0.154
JL-ETNP-Z39*	0.0769	0.0778	0.0859	0.144	0.134	0.12	0.0831	0.0893	0.111	0.133	0.0742	0.15	0.103	0.0698	0.0868	0.0793	0.0734	0.123
Candidate division SR1*	0	0	0	0	0	0	0	0.00657	0	0	0.0224	0.00568	0	0.00763	0.00557	0	0	0
Fibrobacteres*	0.00732	0.0162	0.0319	0.0378	0.039	0.0506	0.0353	0.0486	0.0527	0.0407	0.0244	0.0435	0.0803	0.0675	0.0712	0.0271	0.053	0.0508
Spirochaetae*	0	0	0	0	0	0	0.00114	0.00788	0	0	0.00457	0.00795	0.000934	0.00293	0.00223	0.000459	0	0.00164
Elusimicrobia*	0.0183	0.0353	0.0512	0.0322	0.0244	0.0314	0.049	0.0591	0.0634	0.0529	0.121	0.0375	0.0322	0.0305	0.0245	0.0417	0.0439	0.0311
Aerophobetes*	0.022	0.0463	0.0529	0.0157	0.0268	0.0349	0.0387	0.0171	0.0373	0.00557	0.00915	0.014	0.0107	0.00704	0.00111	0.0119	0.0144	0.0164
Omnitrophica*	0.0146	0.0251	0.0207	0.0101	0.00244	0.0244	0.0171	0.0315	0.0141	0.00334	0.00102	0.00303	0.0201	0.00939	0.00557	0.016	0.0053	0.00491
Parcubacteria*	0.00183	0.0323	0.0017	0.00369	0.00244	0.00174	0.0194	0.00788	0.00663	0.0106	0.00661	0.00984	0.007	0.0111	0.00668	0.00642	0.0129	0.00983
WCHB1-60*	0.00732	0.0451	0.00848	0.00276	0.00731	0.00872	0.0148	0.0105	0.00705	0.00724	0.0117	0.00757	0.0229	0.0334	0.00223	0.00642	0.00454	0
Thermotogae*	0	0	0	0	0	0	0	0	0.000829	0	0	0.0193	0	0.00411	0.00111	0	0	0.00491
TA06*	0	0	0	0	0	0	0	0.00525	0.000415	0.000557	0.00508	0	0.000467	0.00176	0.00557	0	0.000757	0.00491
Gracilibacteria*	0	0	0.00984	0.0138	0	0.014	0.00797	0.00394	0.0104	0.0173	0.00813	0.0087	0.000467	0.00469	0.00557	0.0174	0.0053	0.00164
Candidate division OP3*	0	0.00213	0.00136	0	0	0	0	0.00525	0.0104	0	0.0163	0.000757	0.0014	0.00763	0.00445	0.000459	0.00303	0.00328
TM6*	0	0	0	0	0.00244	0	0	0	0	0	0	0.000378	0.0014	0.000587	0	0.00321	0.000757	0
Deinococcus-Thermus*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.00151	0
Marinimicrobia*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chlamydiae*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

E.2 MICROBIOLOGY DATA SUPPLEMENT

E.2.1 OTU tables

The supplementary attachment 'Microbiology data supplement' includes all the data from the OTU table ordered in various ways. On all tables sample types are grouped and the taxonomic classification of each OTU is included. Samples are named based on their ID number, sample timing, box and sample number, for instance '01PreB1S1' indicates sample 01, pre-flood, box 1, sample 1.

The sheet 'OTU table' includes all OTUs ranked in order of their abundance across all soil and biosolid samples.

The sheet 'OTU table soil' includes all the data from the main OTU table but OTUs are ranked in order of abundance across all soil samples, including initial soils and all control, 400g and 800g biosolid applied boxes. Biosolid sample data is still included for comparison but was not used in calculating the total abundance or ranking OTUs.

The sheet 'OTU table biosolid' includes all data from the main OTU table but OTUs are presented in order of abundance across biosolid samples only. Soil sample data is still included for comparison but was not used in calculating the total abundance.

E.2.2 Bray-Curtis dissimilarity table

A copy of the full table detailing Bray-Curtis dissimilarity scores between all individual samples is included in the 'Microbiology data supplement' on the sheet 'Bray-Curtis scores'. A score of 1 indicates that samples do not share any species and a score of 0 indicates the samples are the same. The diagonal represents the comparison of a sample with itself and so shows as 0. The table is colour coded with red showing more dissimilarity and green indicating less dissimilarity between samples. Samples are named based on their sample timing, box and sample number, for instance 'PreB1S1' indicates pre-flood, box 1, sample 1.