

UNIVERSITY OF CALGARY

Biodegradation of Starch by Microbial Communities in Soil Collected from Olduvai Gorge in

Northern Tanzania

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN BIOLOGICAL SCIENCES

CALGARY, ALBERTA

JULY, 2020

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Abstract

Starch is the main component of most dietary intake by humans and it is synthesized as starch granules in different plant parts. Once exposed to the soil, starch is susceptible to biodegradation by enzymes produced by communities of soil bacteria and fungi. However, the preservation of starch granules in the archaeological record (thousands of years) has been documented. This preservation is poorly understood and there is a paucity of information in the scientific literature on the effects of oxygen and moisture on the enzymatic degradation of starches from different botanical sources in the soil. This study attempts to fill these knowledge gaps by examining soil–starch microcosms subjected to different moisture and oxygen conditions. These microcosms were sampled at regular intervals to observe the metabolic activity of the soil microbes using gas chromatography and also to identify the dominant microbial clades degrading the starch granules using 16S rRNA and 18S rRNA gene analyses.

Commercial wheat, corn and potato starches were used in addition to a starch of ethnobotanical interest named *Ipomoea longituba* collected from Oldupai Gorge. Results indicated that wheat starch and *Ipomoea longituba* were the most susceptible to microbial degradation while potato starch was more recalcitrant. Starch degradation rates were significantly affected by starch type, soil moisture content, oxygen and calcium phosphate. 16S and 18S rRNA gene analyses showed that starch degradation involves both bacteria and fungi and important taxa that were involved in starch breakdown include *Ascomycota*, *Alkalibacterium*, *Streptomyces* and others, mostly Gram-positive bacteria. In addition, from the preliminary results on the microbial communities living in the rhizosphere of *Ipomoea longituba* and bulk soil, we can conclude that the microbiome of the rhizosphere of *Ipomoea longituba* is very different from the microbiome of the surrounding bulk soil that was collected from around 1 m² of the plant.

Acknowledgements

I wish to thank the Almighty God for bringing me to the end of this master's program. My sincere gratitude goes to my supervisor Professor Peter Dunfield and to my co-supervisor Professor Julio Mercader. I wish to say a big thank you to the two of them for the opportunity to carry out this research project under their supervision and for reviewing my research proposal and the manuscript of this thesis. It has been a great experience working under their supervision and I learnt a lot on how to approach problems from a scientific perspective. In addition, I want to thank Karen Barron and Anna Urbanska for all the administrative support they gave me throughout my program, particularly when I wanted to renew my study permit. Special thanks go to NSERC and SSHRC for funding this research project.

I would like to thank members of the Dunfield lab where I learnt techniques in Molecular Biology. Particularly, I wish to thank Felix Nwosu for showing me how to process my soil samples for 16S rRNA gene sequencing and for teaching me how to use the QIIME software to analyze sequenced data. My profound appreciation goes to Andriy Sheremet for helping out in the lab whenever I have problems with any laboratory procedure and also for helping me with QIIME, especially on the command line. Thank you Andriy for all the fun discussions we had about European soccer and international soccer in general; it's been a pleasure to work with you all through these years. I also want to thank Chantel Biegler and Dr Ilona Ruhl for helping around in the Dunfield lab and I extend my sincere appreciation to Dr Angela Smirnova for helping me with the sequencing and the analysis of 18S rRNA gene sequence data using QIIME.

I say a big thank you to members of Professor Julio Mercader's research group called the stone tools, diet and sociality (SDS) group. I especially want to thank Dr Jamie Inwood for collecting the *Ipomoea longituba* starch and soil samples that I used during this thesis during SDS's

annual summer visit to Oldupai Gorge in Tanzania. Also, I wish to thank Dr Maria Soto Quesada for being a good friend and for precipitating the starch I used in the first experiment with calcium phosphate. Many thanks to Dr Makarius Itambu Peter for being a friend and for all the jokes we have shared over the years and I wish him the best in his post-PhD endeavours. Also, I want to thank Julien Favreau for providing the map of Oldupai Gorge that I used in this thesis. I cannot but mention Siobhan Clarke for all the support she provided during the early days of my master's program when I was starting the preliminary experiment. To Aloyce Mwambwiga, I say thank you for being a friend and I wish you the best as you continue your PhD journey.

Finally, I wish to thank my parents back in Nigeria for their continuous prayers and moral support throughout the two and half years that I have spent in Canada in pursuit of this degree. I also want to thank my siblings for being there when it mattered most. I thank my sister, her husband and their three children for being so accommodative since I have been living with them here in Canada. Life here in Canada has been an interesting adventure and I would not have been able to live it to the fullest without you guys!

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
$C_6nH_{10n}O_{5n}$	Starch
CP	Calcium Phosphate
DNA	Deoxyribonucleic Acid
FID	Flame Ionization Detector
GC	Gas Chromatography
GC-MS	Gas Chromatography Mass Spectrometry
HSD	Honestly Significant Difference
LPSN	List of Prokaryotic names with Standing in Nomenclature
LSD	Least Significant Difference
NMDS	Non-metric Multidimensional Scaling
NSERC	Natural Sciences and Engineering Research Council of Canada
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
QIIME	Quantitative Insights Into Microbial Ecology
RNA	Ribonucleic Acid
RODI	Reverse Osmosis Deionized
rRNA	Ribosomal Ribonucleic Acid
SCFA	Short Chain Fatty Acids
SDS	Stone tools, Diet and Sociality
SIP	Stable Isotope Probing
SSHRC	Social Sciences and Humanities Research Council of Canada
UNESCO	United Nations Educational, Scientific and Cultural Organization

CHAPTER ONE: INTRODUCTION

1.1 Starch

Starch is the most important source of carbohydrate in the human diet and it accounts for more than 50% of our carbohydrate intake (Macdonald 2003). It occurs in plants in the form of semi-crystalline granules and these starch granules can be found in seeds (especially the cereal grains) and tubers where it serves as a storage form of carbohydrate (A. M. Smith 2001). Apart from being a major constituent of the human diet, starch serves as a renewable raw material for industry. When heated in water, starch gelatinizes to form pastes and this paste is used extensively as a thickener and texturizer in processed foods (Pfister and Zeeman 2016). Starch has many uses in the industrial sector as it is used in the production of biodegradable plastics, packaging materials and paper (Pfister and Zeeman 2016).

Starches from different botanical sources possess different functional properties e.g. gelatinization onset temperature, paste final viscosity and paste stickiness (Pfister and Zeeman 2016). These variations arise from differences in starch structure, such as the granule size, granule composition and molecular architecture of the starch's constituent glucose polymers named amylose and amylopectin (Copeland et al. 2009). Starch can be utilized in its native or modified form. Native starch refers to starch in its natural state as extracted from its plant source (A. M. Smith 2010). However, native starches are unsuitable for many industrial processes as a result of their poor solubility and inability to withstand many industrial conditions, hence the need for modification through chemical, physical and enzymatic treatments to enhance their functional properties (A. M. Smith 2010; Pfister and Zeeman 2016).

Starch is a stable polymer and can be preserved in a dry environment (Barton 2009). However, once exposed to soil, starch is very susceptible to biodegradation by enzymes (e.g. α -

amylases) produced by soil bacteria and fungi (Haslam 2004). Most soils contain microbial communities capable of producing these starch degrading enzymes (Haslam 2004). Despite the fact that starch can be easily biodegraded in most types of soils, archaeological literature has reported the preservation of starch granules on stone tools and dental calculus but there is a lack of plausible explanation on the mechanisms of starch preservation in archaeological contexts (Mercader et al. 2018; Pearsall 2016). In addition, there is also a paucity of information in the scientific literature on the tempo and mode of microbial decay of starch granules (Mercader et al. 2018). Several scientific efforts have investigated the enzymatic degradation of cereal starches under anoxic conditions *in vivo* where it was discovered that most cereal seeds (e.g. wheat and barley) are unable to produce α -amylases under anoxic conditions (Guglielminetti et al. 1995). Even though rice is an exception as it is able to produce amylase required for the degradation of starch under anoxic conditions, amyolytic activities in rice seeds under aerobic or oxic conditions were found to be greater than that in anoxic or anaerobic conditions (Perata et al. 1992; Guglielminetti et al. 1995).

However, no attempt has been made to understand the effect of anoxia on the enzymatic degradation of starch by bacteria in the soil *in vitro*. This thesis attempts to fill these knowledge gaps by investigating the effects of soil oxygen conditions and moisture levels on the biodegradation of starch granules from different botanical sources by microbial communities in Tanzanian soils. Four incubatory conditions were created by combining oxic and anoxic conditions together with low and high soil moisture levels. Three of the most commonly and commercially available starches, namely corn, wheat and potato were used in the first experiment while potato starch and starch from an underground storage organ from a plant species called *Ipomoea longituba* were used in the second experiment.

1.2 Hypothesis

The main hypothesis of this thesis is that the botanical source of the starch and the conditions of incubation will affect the microbial community structure i.e. the bacterial or fungal species growing on the starches as well as the biodegradation or preservation of the starches over time. It is also hypothesized that the biodegradation of the tested starches will be greatly enhanced under oxic and high moisture level conditions.

1.3 Study area

All the soil samples used for experimental purposes during this thesis came from Oldupai Gorge in Tanzania. Oldupai Gorge is one of the most important paleoanthropological sites in the world preserving the best records of human history spanning almost 2 million years (Njau 2014). Located in the Great Rift Valley, Oldupai Gorge is placed between the Ngorongoro crater and the Serengeti National Park. The location of Oldupai Gorge in northern Tanzania is shown in Figure 1.1. Oldupai Gorge in northern Tanzania is famous for the discovery of early humans and magnificent antiquities by Louis and Mary Leakey that documents the evolutionary history of our ancestor's usage of stone tools (Leakey 1978). Paleoanthropologists have discovered hundreds of fossilized bones and stone tools at Oldupai Gorge that date back millions of years and have concluded that humans evolved in Africa. Soil samples and tuber plants (*Ipomoea longituba*) for the second experiment were collected from two sites in Kesile which is located in Oldupai Gorge. The first site had an elevation of 1606 m and a GPS coordinate of -2.81234/35.32621 while the second site had an elevation of 1617 m and a GPS coordinate of -2.81245/35.32695. Annual rainfall in Oldupai Gorge ranges from between 331 mm – 531 mm but the surrounding Serengeti Plains has a mean annual rainfall of 800 mm (Mercader et al. 2019; Mercader et al. 2017). The dry

season in Oldupai Gorge is from June to October while the wet season extends from November to May with peaks in December and April (Mercader et al. 2017). The soil at Oldupai Gorge has a low moisture content and a mean temperature of 22 °C (Mercader et al. 2017). Also, the soil has a high sodium content and possesses a high pH (making it alkaline) that sometimes exceed 10.8. Soils are yellowish-brown with high humic content (< 1% organic carbon) (Mercader et al. 2019; Mercader et al. 2017). Soil samples were also collected from the roots (rhizosphere) of the tuber plant (*Ipomoea longituba*) and from within a 1 m² area around the plant. The soil samples and tuber plants were collected by Jamie Inwood, a postdoctoral fellow in the Stone tools, Diet and Sociality (SDS) research group in 2018 and 2019.

1.4 Experimental Design

1.4.1 Detection of CO₂ production

The biodegradation of starches from different botanical sources by soil microbiota was studied by setting up soil–starch incubations in gas-tight sealed serum bottles (microcosms). Because CO₂ production is a reliable proxy of metabolic activity by microorganisms, these microcosms were sampled at regular intervals to measure the amount of CO₂ produced in the headspace of the serum bottles using gas chromatography.

1.4.2 Molecular-based community Analysis

The microbial community structure in the microcosms was examined by sampling the starch–soil incubations every one or two weeks and extracting DNA from these soil samples. The extracted DNA was then amplified with primers targeting the 16S rRNA genes of bacteria and 18S rRNA genes of fungi and other eukaryotes. 16S rRNA and 18S rRNA gene amplicons were then

prepared for sequencing in accordance to the Illumina 16S Metagenomic Sequencing Library Preparation protocol and the 18S Illumina Amplicon Protocol. Thereafter, they were run on a MiSeq (Illumina) at the Centre for Health Genomics and Informatics, University of Calgary.

1.5 Research Objectives

1. *To begin the experimental exploration of how microbial communities degrade starch over time under different conditions.* It would be helpful to know the different microbes growing on the starch and also the dominant bacteria or fungal species actually undertaking the decomposition. This objective can be achieved by extracting the DNA of the microbes in the soil-starch incubations and performing 16S and 18S ribosomal RNA (rRNA) gene analysis.
2. *To establish and compare conditions for the enzymatic degradation of the starch granules by microorganisms.* Because oxygen is the strongest oxidant in soils and because most microbes need moisture to carry out their metabolic activities, it is expected that degradation rates are faster under oxic and wet conditions than under anaerobic conditions.
3. *To determine the relative susceptibilities of the tested starches to biodegradation.* The susceptibility of the starch granules to microbial enzymatic degradation is expected to vary depending on the botanical origin of the starch (Srichuwong et al. 2005). This variation could be due to the crystallinity and size of the starch granule type. For example, cereal starches like corn are known to be more easily degraded than tuber starches like potato (Sarian et al. 2012).

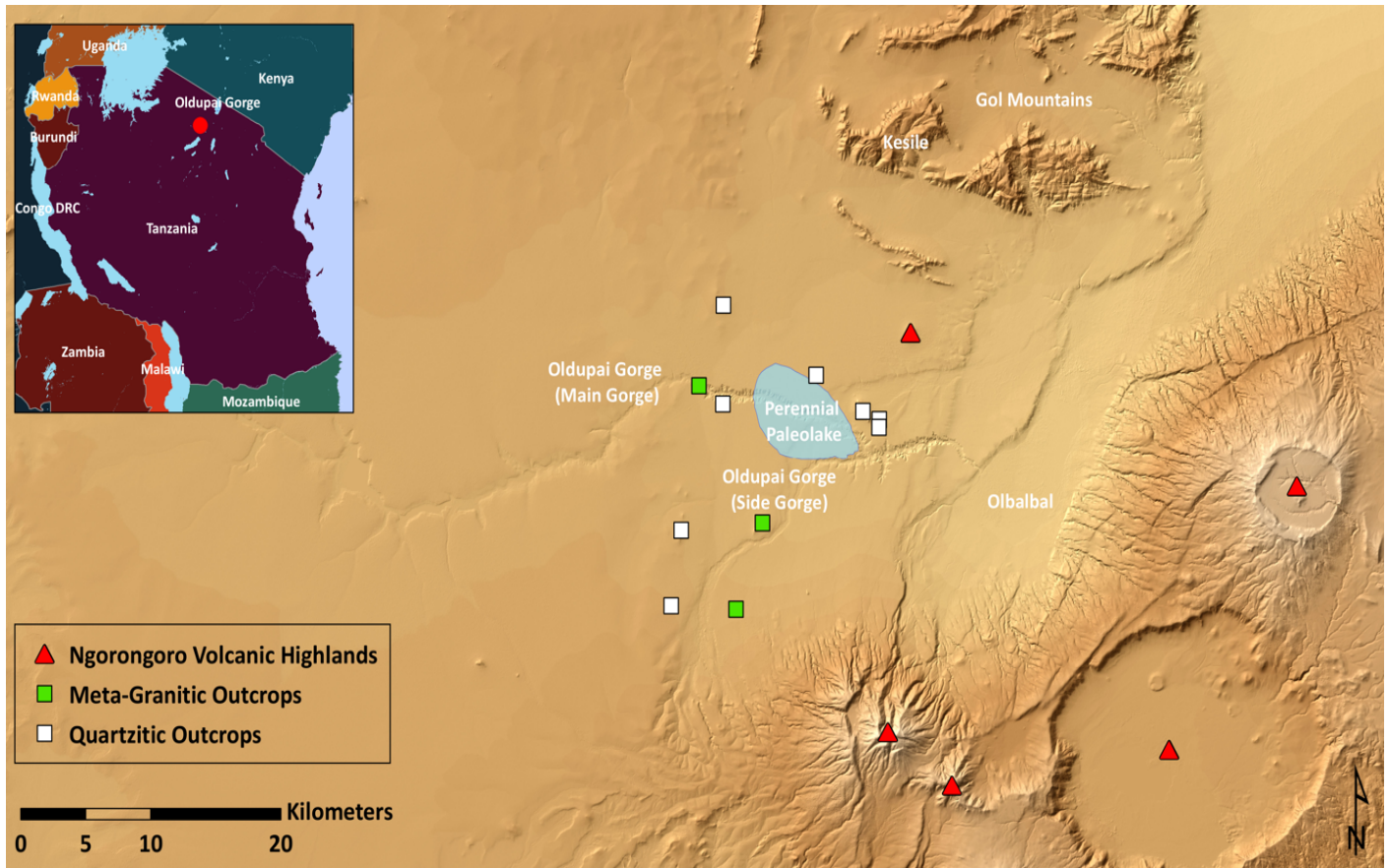


Figure 1.1 Location of the Kesile site in the Oldupai Gorge area. Inset is the location of Oldupai Gorge in Northern Tanzania, East Africa (Image source: Stone Tools, Diet and Sociality Research Group). Photo Credit: Julien Favreau

CHAPTER TWO: LITERATURE REVIEW

2.1 Starch Structure

Next to cellulose, starch is the second most abundant natural polysaccharide on earth and an important source of energy for animals, including humans (Naguleswaran et al. 2014). Starch is the main component of most dietary intake by humans. It is synthesized as starch granules by photosynthetic plants and stored in plant parts such as roots, leaves, tubers, stems, fruits and seeds (Gallant et al. 1992). Two main forms of starch exist – transitory starch is synthesized in leaves and acts as a temporary source of energy while storage starch is formed in amyloplasts as long-term energy source. The biosynthesis of starch granules begins at the hilum and these granules occur in all shapes and sizes with diameters ranging from 0.1 μm to 200 μm depending on the plant source (Pérez and Bertoft 2010; Haslam 2004). Generally, potato starch granules are larger (around 100 μm) in diameter when compared to granules from cereals like corn or wheat (Vamadevan and Bertoft 2015).

Starch is semi-crystalline (i.e. consists of both crystalline and amorphous regions) in nature and is basically composed of two glucose polymers – amylose and amylopectin. From Figure 2.1, it can be seen that amylose is essentially a linear polymer whereby the glucose units are bound together by α -(1,4)-linkages but there exists a very small amount of branching with α -(1,6)-bonds (Stevnebø et al. 2006; Vamadevan and Bertoft 2015). Amylopectin is the main component of starch granules and is much larger than amylose. In fact, amylopectin is one of the most abundant naturally occurring polymers. It is a highly branched macromolecule of α -(1,6)-bonds in addition to the α -(1,4)-linkages in linear chains (see Figure 2.1). The crystallinity of starch granules is mainly associated with amylopectin while amylose is linked to the amorphous parts (Singh et al. 2003; Gallant et al. 1992). Native starch granules usually contain about 20–30% amylose and 70–

80% amylopectin but amylose content can be less than 1% in waxy starches and greater than 70% in certain high amylose starches (Martens et al. 2018).

Using X-ray diffractometry, the crystalline structure of starch granules has been found to exist in three different forms or patterns: A, B and C. Generally, starch granules from cereals (and also small granules from tropical tubers) are known to exhibit the A pattern while potato and other tropical tuber starches (as well as some amylose-rich starch granules like wrinkled pea, amylo maize and barley) typically show the B pattern (Gallant et al. 1992). Starch granules from other tropical tubers like *Manihot utilissima* and *Ipomoea batatas*, together with legume starches exhibit the C pattern (Gallant, Bouchet, and Baldwin 1997; Singh et al. 2003; Gallant et al. 1992). The C pattern is known by some to be a distinct crystallographic pattern, but others consider it to be a mixture of populations in which the starch granules have the two A and B structural patterns (Gallant, Bouchet, and Baldwin 1997). Finally, a V pattern can appear after gelatinization and it is due to the amylose component forming complexes with fatty acids, phospholipids or other polar molecules (Gallant et al. 1992).

Therefore, it is clear that starch granules have complicated structures as a result of the variations in their compositions and variations in their amorphous and crystalline structures. Compositionally, amylose and amylopectin make up 98-99% of the dry weight of native starch granules but starches (e.g. cereals) also contain small amounts of lipids (in the form of phospholipids), proteins and small quantities of minerals like calcium, magnesium, potassium, sodium and phosphorus all of which may affect their functional properties (Alcázar-Alay and Meireles 2015; Copeland et al. 2009)

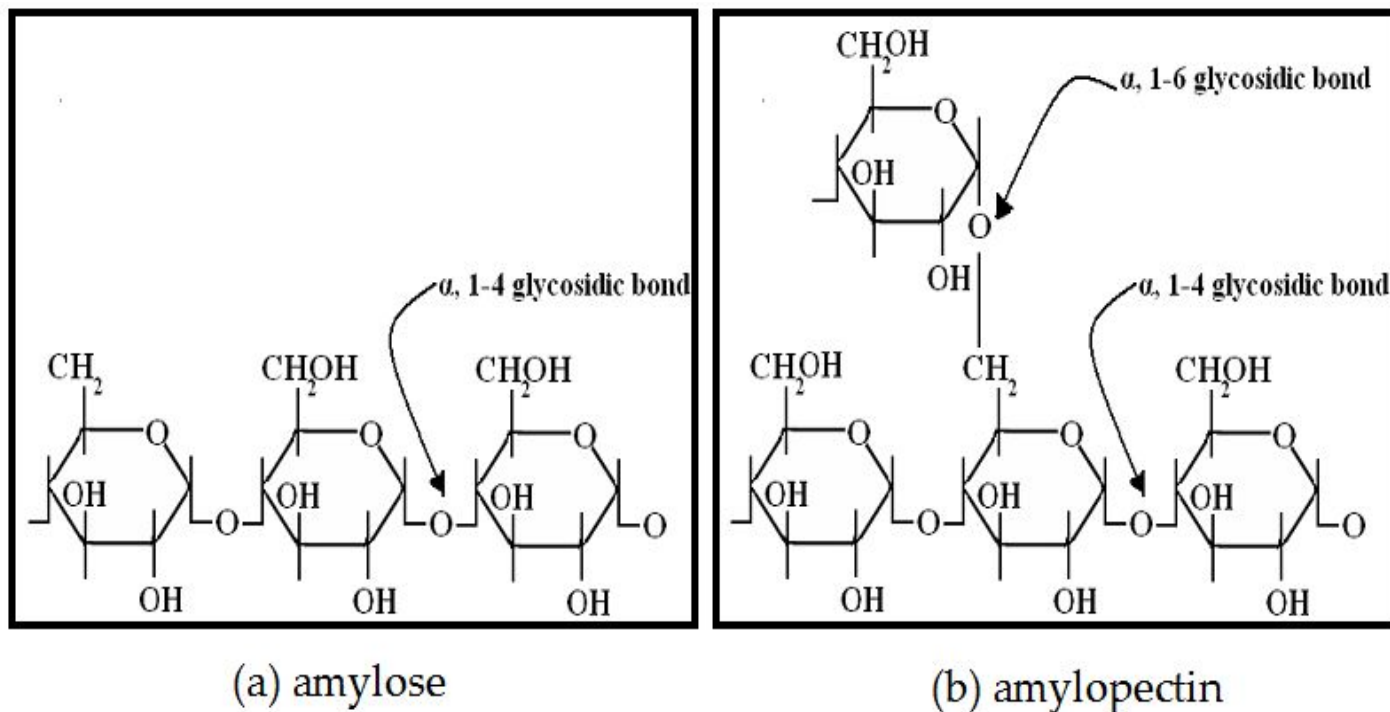


Figure 2.1: The structure of amylose and amylopectin (El-Fallal et al. 2012) (Open Access)

Although a minor component by weight, lipids play a very important role in determining the functionality and properties of starch. The content and composition of lipids in different starch granules varies with respect to the botanical source or plant species. Also, the lipid content of native starch granules correlates with amylose content: the higher the amylose content, the greater the amount of lipid present (Copeland et al. 2009).

2.2 Gelatinization and Retrogradation

Starch granules are insoluble in cold water. However, when starch is heated in water, the granules absorb water, swell and burst. Water enters the amorphous regions of the granule and then transmits disruptive forces into the crystalline regions leading to a complete collapse of the

granule architecture to form a paste. The absorption of water by the amorphous regions within the starch granules alters the crystalline structure and results in the loss of birefringence leading to a process called gelatinization (Ratnayake and Jackson 2006). Gelatinization eventually results in the complete separation of amylose and amylopectin and the temperature whereby the starch granules lose their birefringence is known as the gelatinization temperature. This temperature partly depends on the botanical source of the starch. Starch granules show birefringence in the form of a ‘maltese cross’ when viewed in polarized light under a microscope, indicating that there is a heavy degree of molecular orientation within the starch granules. This birefringence is destroyed when gelatinization occurs and granules become less crystalline, and also when microbes damage the granules (Cai et al. 2014; Schirmer, Jekle, and Becker 2015; Bertoft 2017). A ‘maltese cross’ occurs when four translucent sections of a starch granule are separated from each other by a dark cross when viewed in polarized light under a microscope (Lisinska and Leszczynski 1989).

When starch is cooled after gelatinization, the disrupted amylose and amylopectin chains gradually re-arrange themselves to form a different semi-ordered crystalline structure in a process called retrogradation. Retrogradation takes time to occur – in minutes to hours for amylose and hours to days for amylopectin and is an ongoing process that initially involves the rapid recrystallization of amylose chains followed by a slow recrystallization of amylopectin molecules (Wang et al. 2015; Copeland and Hardy 2018). Retrogradation of amylose molecules facilitates the initial hardness of a starch gel and the long-term development of starch gels and crystallinity of processed starch is attributed to the retrogradation of amylopectin and is responsible for the staling of bread and cakes. Because of its industrial significance, starch retrogradation and ways to retard it have been the subject of many scientific investigations (Ottenhof and Farhat 2004;

Wang et al. 2015). Starch retrogradation is also of nutritional significance because of the slower enzymatic digestion of retrograded starch and the controlled release of glucose into the blood stream. The digestibility of retrograded starch is mainly related to the extent of gelatinization, the rate of cooling and temperature of storage. Therefore, the fast and slow retrogradation of amylose and amylopectin molecules consecutively causes a rapid and gradual decrease in the digestibility of retrograded starch respectively (Annison and Topping 1994; Wang et al. 2015). Research efforts have also analyzed the effect of storage temperature on retrogradation. For amylose, the rate of retrogradation has been shown to decrease with an increase in the temperature of storage. In fact, the degree of retrogradation decreased within 24 hours from 58.8% to 7.1% as storage temperature increased from 5°C to 45°C. After incubating starch at 25°C, 4°C and -18°C, the rate of starch retrogradation was known to be highest at 4°C (Lu, Jane, and Keeling 1997; Aguirre et al. 2011).

2.3 Enzymatic degradation of starch by microorganisms in the soil

Although starch is a stable polymer and can be preserved over long periods of time in a dry environment, it is susceptible to enzymatic attack when exposed to conditions that facilitate its biodegradation. Starch degrading enzymes are produced by microbial communities in all types of soil and the activity of these enzymes has been found to be greater at the topsoil than at the subsoil (at greater depth). Besides enzymatic attack, other factors impacting the degradation of starch in soils to a lower degree include differences in soil pH, moisture and temperature which could alter the accessibility of starch granules to soil microbes (Haslam 2004).

Once exposed to the soil, starch is very susceptible to biodegradation by α -amylases produced by soil bacteria such as those in the genus *Bacillus* (Hutschenreuther et al. 2017).

Amylases are exoenzymes, hydrolytic enzymes released from the cell to degrade high molecular weight substrates (e.g. polysaccharides such as starch and cellulose) while endoenzymes are mainly used for further breakdown of carbohydrates and synthesis by the cell (Singh and Kumari 2016). The degradation of starch granules with amylases occurs in three steps which include diffusion of the enzyme to the granule surface, adsorption and catalytic reaction (Naguleswaran et al. 2014). Smaller monosaccharides and oligosaccharides are then imported into the cell. The rate of hydrolysis is initially fast but after some time, it proceeds at a slower and more persistent rate (Naguleswaran et al. 2014). Amylases are a class of enzymes capable of hydrolyzing starch to give various products such as dextrin and other oligosaccharides and are of two types: endoamylases and exoamylases (Hii et al. 2012). Endoamylases are known to cleave α -(1,4) linkages present in the internal part of amylose or amylopectin chains in a random fashion while exoamylases cleave both α -(1,4) and α -(1,6) linkages near the ends of amylose or amylopectin chains from the nonreducing terminal bond (Windish and Mhatre 1965; Hii et al. 2012). α -amylase is an endoamylase, β -amylase is an exoamylase producing a maltose disaccharide, and glucoamylase is an exoamylase capable of producing individual glucose molecules from the non-reducing ends of starch. The nonreducing end of starch is a glucose molecule with free OH group on its C4 element. Native starch granules are very resistant to amylolytic degradation and so the action of α - and β -amylase on raw starch granules is not very effective (Shariffa et al. 2009). Consequently, degrading starch with amylases requires a long period of hydrolysis between 24 – 72 hours. α -amylase has been shown to degrade starch granules more efficiently than β -amylase (Sujka and Jamroz 2007; Shariffa et al. 2009; Sarikaya et al. 2000), and is capable of directly attacking native starch granules to produce oligosaccharides which can further be degraded into carbon dioxide and water (Planchot et al. 1995; Haslam 2004).

Generally, the susceptibility of starch granules to amylolysis has been shown to vary with the botanical source. Starch granules that exhibit the 'A' crystallographic pattern (e.g. cereals) are known to be more susceptible to amylolysis than starch granules exhibiting the 'B' and 'C' patterns (e.g. potato) (Planchot et al. 1995; Adejumo et al. 2013; Gallant et al. 1992). Potato starches especially have a high level of crystallinity and are commonly known to resist bacterial and fungal breakdown (Valk et al. 2015). However, a few bacteria like the *Mycobacterium aurum* strain B8.A have been reported to degrade potato starch albeit rather slowly compared to wheat and tapioca starch (Valk et al. 2015; Sarian et al. 2012). Apart from the crystalline structure, other factors like the granule size also affect the enzymatic degradation of starch granules (Qi and Tester 2016); enzymes are more easily attached to smaller starch granules due to a relatively large surface area to volume ratio than when compared to larger starch granules with a small surface area. Smaller starch granules are therefore more susceptible to degradation (Franco et al. 1992; Sarian et al. 2012). Therefore, scientific investigations have showed that the rate and extent of α -amylase degradation of wheat > maize > pea > potato, reflecting increasing granule sizes for these starches from different botanical sources (Tester, Qi, and Karkalas 2006).

The porosity of starch granules is an important feature that affects their susceptibility to enzymatic degradation. Granules of certain starches (e.g. corn, sorghum, barley, millet, wheat) are characterized with surface pores as a natural feature and enzymatic hydrolysis may influence the number and/or size of these pores (Sujka and Jamroz 2010). It has been hypothesized that pores are openings to channels that lead to the interior of the starch granule. Due to these openings, enzymes are able to digest starch granules from the inside-out, leading to a more rapid enzymatic hydrolysis (Martens et al. 2018; Sujka and Jamroz 2010). Also, it has been suggested that enzymatic degradation of starch granules may begin at the surface pores, openings that offer

enzyme molecules direct entry to the granule interior (hilum). In wheat starch granules, it was observed that enzymatic attack starts at cracked regions or at surface pores and then proceeds towards the center of the granule with very minute change in the lateral dimensions of the pore that was formed (Sujka and Jamroz 2007).

Two main forms of enzymatic attack by microbial and pancreatic amylases have been described in starch granules: exo-corrosion and endo-corrosion. Exo-corrosion occurs when enzymes cause surface alterations, eroding the entire granule surface or sections of it while endo-corrosion happens when enzymes penetrate the granule, eroding the internal sections through small pores observed on the surface of the starch granule (Bird et al. 2009; Sujka and Jamroz 2007). Potato starch granules which are resistant to amylolytic degradation are slowly but gradually eroded by exo-corrosion without the formation of pores on the granular surface. However, the enzymatic degradation of cereal starches is characterized by the formation of holes whereby the core of the granule is readily disrupted (Gallant et al. 1992; Sujka and Jamroz 2007). For instance, starch granules from wheat, rye, barley and also tropical tubers (e.g. cassava, sweet potato) have specific susceptible zones on the granule surface which become pitted because of endo-corrosion. The pits become enlarged and numerous channels of endo-corrosion are formed inside the granule. Similarly, high-amylose starches show little exo-corrosion and extensive endo-corrosion (Gallant et al. 1992; Gallant, Bouchet, and Baldwin 1997; Bird et al. 2009).

2.4 Starch Degradation – Archaeological Implications

Several factors influence the preservation of starches in the archaeological record. Starch has been shown to retain its physical structure for up to 100 years when stored as dried tuber or processed flour (Barton 2007), an indicator that moisture or water is an important factor in starch

degradation because enzyme activity is reduced in the absence of water. Apart from desiccation, starch is also better preserved in water-logged and basic (high pH) contexts than in acidic ones. Research has shown that there are two categories of factors affecting the degradation of starch in soils: soil properties (e.g. soil moisture, pH, temperature, texture) and soil constituents (e.g. enzymes, bacteria, fungi, earthworms) (Pearsall 2016). In addition, the enzymatic breakdown of starch has been shown to follow an asymptotic curve whereby most of the organic material is decomposed within the first few days or so following soil burial. If starch must persist for thousands of years on stone tools or artefacts in the archaeological record, there must be some mechanism protecting it from the early onslaught of microbial enzymes (Barton 2007).

Most decomposition of starch in soils is carried out by microorganisms (Haslam 2004) and scientific research has established that microbial decomposition of starch in the soil modifies native starch granules. Microbes inflict a series of diagenetic alterations to starch granules during burial in the soil. The most common diagenetic process is the disruption of the crystallinity of starch granules (Mercader et al. 2017). The second most common indicator of diagenesis is centric cavitation of the starch granule, a feature that points to the culinary modification of starches. However, other forms of damage to the starch granule through bacterial degradation include centric implosion, pitting, clefting (splitting inside granule), fissuring, creasing and cracking (Mercader et al. 2017).

When preserved, starch granules can provide a direct indicator of ancient tool use, diet and agricultural activities. Ever since the discovery of starch granules on stone tools about 40 years ago, starch granules have been very important in studies of ancient diet and culture and have been recovered from several archaeological artefacts, sediments and dental calculus (Copeland and Hardy 2018; Hardy et al. 2009). For example, archaeological researchers have been able to

determine past diet from starch grain analysis of excavated charred residues at Zhejiang province in China and from the surfaces of stone tools recovered from the humid neotropical regions of Panama (Piperno and Holst 1998; Yang and Jiang 2010).

However, the mechanisms by which some starch granules in the soil survive biodegradation through the archaeological record are poorly understood (Copeland and Hardy 2018; Hutschenreuther et al. 2017). Several reasons have been given in archaeological literature to explain the preservation of starch grains in soil. Individual starch granules may survive by pure chance due to their presence in large numbers or the formation of starch clusters that ensure long-term survival. In addition, a couple of protective mechanisms like the formation of soil aggregates and the presence of clays and heavy metals have been identified to reduce the availability of native starch granules to enzymatic attack by bacterial and fungal amylases in the soil (Haslam 2004).

Starch granules have very small sizes and so it is easy for them to be trapped in places (such as microscopic artefact crevices or cracks in rocks) that could protect them from biodegradation (Barton and Matthews 2006). However, recent archaeological literature cast doubt on the idea that starch could be preserved inside rock crevices. For one thing, the different groups of microbes which colonize rock surfaces produce α -amylases which are exoenzymes, implying that these enzymes work outside the cell (Mercader et al. 2018). Since these enzymes (4 – 8 nm) are much smaller than the smallest microbial cells (200 nm), they can easily gain access to rock crevices to colonize it (Mehta and Satyanarayana 2016; Mercader et al. 2018). Furthermore, organic material and water which are known to be present in stone crevices create attractive living conditions for fungal and microbial communities. Therefore, the conditions in rock crevices are not expected to preserve starch granules and could facilitate degradation instead (Mercader et al. 2018).

Dental calculus has been shown to provide a protective environment that increases the chances for the preservation of starch granules and the extraction of starch granules from archaeological dental calculus offers a direct link to the ancient consumption of starchy foods by humans (Weyrich, Dobney, and Cooper 2015; Marston, D'Alpoim, and Warinner 2014). Dental calculus forms rapidly in the mouth through the dental plaque biofilms around the teeth of most people and becomes steadily mineralized over time which contributes to its protective effect. The rate of dental calculus formation varies and is associated with several factors such as diet, salivary flow, local pH and genetic factors (Leonard et al. 2015; Hardy et al. 2009). Although saliva is a good source of amylase and salivary amylase begins the digestion of uncooked or partially cooked starch granules in the mouth, some starch granules become trapped in dental plaque before degradation (Hendy et al. 2018; Hardy et al. 2009). Once trapped in the plaque, the starch granules are protected from salivary amylase. Dental calculus builds up around the surface of the teeth, calcifying into supragingival (above the gum line) or subgingival area (below the gum line) calculus in the gingival crevice (Leonard et al. 2015; Hardy et al. 2009). Subgingival calculus can slowly build-up for long periods if it is not eliminated and the gingival crevice offers an environment that is suitable for starch preservation because it is protected from salivary amylase and microbial communities that live there are primarily proteolytic (using proteins as substrates) rather than saccharolytic (using sugars as substrates) (Hardy et al. 2009; Radini et al. 2017). Proteolytic metabolism leads to release of by-products such as ammonia which results in pH increase. The increased pH facilitates the precipitation of calcium phosphate (plaque mineralization). Calculus is as hard as bone once calcified and is preserved on archaeological teeth (Barton and Torrence 2015; Henry and Piperno 2008; Hardy et al. 2009).

2.5 Diversity of microbial communities in soils with starch degrading enzymes

The soil is the richest habitat in terms of microbial diversity, and it is estimated that there are about 10 billion microorganisms per gram of soil. This includes thousands of bacterial, archaeal and fungal species (Souza et al. 2018). Ninety percent of the soil microbial biomass is composed of bacteria and fungi which are the main agents of decomposition of soil organic matter. However, bacteria have been found to be the main starch degrading microbes and this finding is backed by several studies (Rinnan and Bååth 2009; Žifčáková et al. 2017). In one study that considered soil microbial community compositions and functional genes, it was discovered that *amyA* – the gene that encodes α -amylase, was ubiquitous in all three types of forest soils studied. The *amyA* genes were mainly derived from bacteria, although some were derived from fungal phyla named *Ascomycota* and *Basidiomycota* while others were derived from the archaeal phylum Euryarchaeota (Cong et al. 2015).

In another research effort that studied the microbial community structure and functional potential of North American prairie soils (cultivated and never cultivated) using 16S rRNA gene sequencing and metagenomic analyses, scientists revealed that starch degradation genes were prevalent among all soils (Mackelprang et al. 2018). By comparing raw reads to the glycoside hydrolase (GH) sequences from the carbohydrate-active enzyme (CAZy) database, it was discovered that the most dominant GH gene family was the GH13 (to which α -amylase belongs), making up 36% of all GH sequences and it was followed by GH15 at 8% (Plaza-Vinuesa et al. 2019; Mackelprang et al. 2018). Other metagenomic soil studies have also shown the GH13 to be very abundant among biomes and one study in particular found GH13 to be more abundant in desert and semiarid biomes than in forest and grassland soils (Manoharan et al. 2015; Noronha et al. 2017). While GH13 is the main α -amylase family in the CAZy database, glucoamylases (which

are enzymes also involved in starch breakdown) constituted the majority of the GH15 enzymes and in combination with the GH13 enzymes, the data from this study suggest a high amylolytic capacity for the prairie soils (Plaza-Vinuesa et al. 2019; Mackelprang et al. 2018; Cantarel et al. 2009).

Microbial α -amylase is one of the most important industrial amylases and it accounts for more than 30% of the global enzyme market (Kumari et al. 2019). Different microbial species produce α -amylase but for commercial purposes, α -amylase is mainly derived from the genus *Bacillus*. For example, α -amylases produced from *Bacillus licheniformis*, *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens* are thermostable and find potential applications in the food, fermentation, textile and paper industries. Fungal sources of thermostable α -amylase include strains from the *Aspergillus* and *Penicillium* genera. Thermostable enzymes have a high applicability in commercial settings because of their stability under high temperatures (Asgher et al. 2007; de Souza and de Oliveira Magalhães 2010). As starch hydrolysis is performed at high temperatures (100 – 110°C) during industrial processes, thermostable amylolytic enzymes are presently under intense investigations to improve starch degradation in industrial settings for the commercial production of valuable products such as sugar, maltose, crystalline dextrose and maltodextrins. Thermostable α -amylases can be obtained from *Bacillus licheniformis*, *Bacillus* sp. ANT-6, *Bacillus subtilis* JS-200 and various other strains from the *Bacillus* genus (Asgher et al. 2007; de Souza and de Oliveira Magalhães 2010).

2.6 Bacterial communities in the rhizosphere of starchy tuber plants

Plant roots generally exert a strong influence on the microbial communities that are found in the rhizosphere of tuber plants which mainly come from the secretion of organic compounds in

root exudates. The nature and extent of this influence are most likely related to the composition of the root exudates. Through their root systems, plants secrete a range of chemical compounds including sugars, amino acids, organic acids and several other diverse signalling molecules (Weinert et al. 2010; Inceoğlu, Salles, and van Elsas 2012). This means that through the active release of these organic compounds, plants can selectively attract certain microorganisms to the rhizosphere, and this includes the main consumers of the root exudates. The microorganisms that plant root secretions attract may be beneficial (defense against harmful organisms) or they may be harmful/pathogenic (Inceoğlu et al. 2011; Inceoğlu, Salles, and van Elsas 2012). As a result of these plant effects on soils, microbial communities are expected to differ in the rhizospheres of different plants especially if those plants produce different root exudates. In fact, bacterial communities associated with plant roots can be different among cultivars of the same plant species (Inceoğlu et al. 2011).

In a study that analyzed the cultivable diversity and functional activities of the rhizospheric and endophytic bacteria of cilembu sweet potato during its growth stages, it was discovered that the diversity and genera richness in the early growth stages for the rhizospheric bacteria was higher than in the late stages and this finding might be due to changes in secreted root exudates (Tangapo, Astuti, and Aditiawati 2018). The diversity of the rhizosphere bacteria was high, featuring species from the phyla *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes*. In addition, the genus *Bacillus* was found to be dominant in the rhizosphere and endophytes of cilembu sweet potato (Tangapo, Astuti, and Aditiawati 2018). This study corroborates the findings of a previous and similar study that analyzed the influence of plant age and genotype on the bacterial community composition of the tuber rhizosphere in sweet potato plants grown in the field (Marques et al. 2014). In this previous study, scientists discovered that the bacterial communities present in the

bulk soil were significantly different from the bacterial communities found in the tuber rhizosphere of all the three sweet potato genotypes which is an indication of a typical ‘rhizosphere effect’ (Marques et al. 2014). Additionally, the genus *Bacillus* was highly dominant in the tuber rhizosphere samples of all sweet potato genotypes studied while bacteria from other genera showed an abundance dependent on the genotype of the sweet potato plant. The enrichment of *Bacillus* in the tuber rhizosphere of sweet potato is thought to be due to the ability of *Bacillus* species to produce beneficial antimicrobial substances which can help the tuber plant avoid the destructive effect of phytopathogen agents and most *Bacillus* species produce α -amylase, the enzyme responsible for starch hydrolysis (Marques et al. 2014).

Using metagenomics, another study analyzed the composition of the microbial communities inhabiting the rhizospheres of three Andean tuber crops named Oca (*Oxalis tuberosa*), Ullucu (*Ullucus tuberosus*) and Mashua (*Tropaeolum tuberosum*). Results showed that the bacterial communities colonizing the rhizosphere of the three Andean tuber crops and bulk soil were different (Chica et al. 2019). It was noted that the composition of the rhizospheric microbiome was greatly influenced by the surrounding bulk soil. Diversity of the operational taxonomic units (OTUs) revealed that while *Proteobacteria* and *Bacteroidetes* were the most abundant phyla in the Andean tuber crop soils, *Chloroflexi* and *Acidobacteria* were dominant in the bulk soil (Chica et al. 2019). A similar study that was aimed at understanding the effects of soil and climatic conditions on the community structure of sweet potato endophytes using metagenomics also found that *Proteobacteria* (85%), *Bacteroidetes* (6.6%) and *Actinobacteria* (6.3%) were the three most dominant phyla, making up 97.9% of the reads (Puri et al. 2019). At the genus level, *Pseudomonas* was found to be most dominant. Overall, the endophytic

microbiome was similar under different soil and climatic conditions suggesting that soil and climate were not major factors affecting the totality of the endophytic community (Puri et al. 2019).

For potato tuber plants, there was a recent study that analyzed the bacterial communities associated with tubers of different potato cultivars that were cultivated in different soils over several generations, particularly addressing the community changes and inheritance of the tuber microbiome (Buchholz et al. 2019). At the phylum level, the potato tuber bacteria were dominated by *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes*. Although alpha diversity analysis showed that tubers of different potato varieties differed only in bacterial richness and evenness, beta diversity analysis showed that the bacterial community composition was influenced by the genotype of the potato variety and also by planting in the same soil type (Buchholz et al. 2019). Results also indicated that the bacteria that colonized the potato tubers were recruited from the soil and that the community was independent of the plant tuber genotype. Generally, this study discovered that the soil is the main reservoir for bacteria communities that colonize potato tubers and that the rhizosphere microbiome is transmitted from one tuber generation to the next (Buchholz et al. 2019).

2.7 Conclusion

Starch is the major component of most dietary intake by humans and it is synthesized as starch granules in different plant parts such as roots, leaves, fruits, seeds, etc. Starch is basically composed of amylose and amylopectin. It is semi-crystalline in nature, consisting of both amorphous and crystalline regions. Once exposed to the soil, starch is very susceptible to biodegradation by enzymes (alpha amylases) produced by communities of soil bacteria. These starch degrading enzymes are known to be present in all types of soil. Amylases are extracellular

enzymes secreted by microorganisms that are capable of hydrolyzing starch to produce various products such as dextrin and other oligosaccharides. Most decomposition of starch in soil is carried out by microorganisms and microbes modify starch granules by inflicting a series of diagenetic changes in starch during soil burial. Starch is a stable polymer and starch granules can provide a reliable indication of ancient diet and tool use when preserved. However, the preservation of starch granules through the archaeological record (thousands of years) is poorly understood and several reasons have been proposed by archaeologists to explain the preservation of starch grains in soil. The dental calculus complex formed by the calcification of dental plaque provides a protective environment to starch granules on archaeological teeth.

CHAPTER THREE: MATERIALS AND METHODS

3.1 First Experimental Setup

There were two experiments with the starch-soil incubations. The first one was conducted with commercial starches derived from three different plants namely wheat, corn and potato. Artificial soil was composed of nanospheres and clay mixed with natural soil from Oldupai Gorge in Tanzania that served as inoculum. The second experiment was conducted with potato starch and starch from *Ipomoea longituba*, a plant of ethnobotanical interest. Artificial soil was discarded in the second experiment. Only soil from Oldupai Gorge in Tanzania was used in the second experiment.

3.1.1 Starches, Natural and Artificial Soil

Commercial starches from three different botanical sources (corn, wheat and potato) were subjected to four different incubatory conditions by altering soil water content and oxygen conditions. The commercial starches came from the College of Agriculture and Bioresources, University of Saskatchewan. This first experiment was carried out using artificial soil as the substrate and natural soil as the inoculum. The natural soil was collected from different quadrant sections and plant covers in Oldupai Gorge, a world-renowned paleoanthropological site in Tanzania that has proven invaluable to our understanding of early human evolution. The natural soil was not exposed to light and was stored at room temperature with a moisture content of 4%. The artificial soil was composed of clay and nanospheres: the big-sized nanospheres (150 – 250 μm) represent sand while the small-sized nanospheres (10 – 50 μm) represent silt. The clay was composed of the montmorillonite mineral and the nanospheres are made up of silicon dioxide. The artificial soil was sterilized in the autoclave before use in this experiment. From the 20.0 g of total

soil matrix that was placed in each 100-ml serum test bottle, the percentage composition of the constituents was 2% clay (0.4 g), 20% silt (4.0 g) and 78% sand (15.6 g). The proportions of the clay and nanospheres that were used here came from known textural parameters for the grain size of local soils (Mercader et al. 2019). Finally, 1.6 g of natural soil from Tanzania and 0.1 g of starch (0.0037 moles of carbon) was added to the soil substrate. The test bottles were covered with butyl rubber stoppers and crimp sealed.

3.1.2 Incubatory Conditions: Soil Moisture and Oxygen Levels

The baseline moisture content of the natural soil used as the inoculum was measured by weighing a sample of the soil from Oldupai Gorge and placing it in the oven to dry at 105°C for 24 hours. The dried soil sample was reweighed, and the percentage moisture content was calculated using the equation below:

$$\text{Percentage (\%)} \text{ moisture content} = \frac{\text{weight of moist soil} - \text{weight of dry soil}}{\text{weight of dry soil}}$$

The 4% baseline soil moisture content was measured this way. Four different treatments or incubatory groups were created by altering soil moisture and O₂ levels – the two variables used in this experiment (see Figure 3.1). For the baseline and high-level water content, 0.8 g (4% of 20 g of artificial soil) and 4.0 g (20% of 20 g of artificial soil) of reverse osmosis de-ionized (RODI) water was added to the soil matrix respectively. To establish anoxic conditions, the air in the headspace of the test bottles was evacuated using a vacuum pump and replaced with nitrogen gas (Praxair, Calgary, Purity grade 4.6). The evacuation and refilling with nitrogen were done in three cycles: for each cycle the air was evacuated for 10 minutes followed immediately by refilling with nitrogen gas for 30 seconds. This procedure was undertaken to ensure that the microcosms were devoid of oxygen in order to mimic anaerobic degradation which is much slower compared to

aerobic degradation. From Figure 3.1, it can be seen that the four different incubatory groups obtained were as follows: 4% water–oxic; 4% water – anoxic; 20% water – oxic and 20% water – anoxic. Each incubatory group contains 11 test bottles – two replicates of potato, corn and wheat starch (added to the soil matrices), 2 controls (here starch was not added to the soil matrix) and 1 replicate each of calcium phosphate treated wheat, corn and potato starch – making a total of 44 test bottles (see Figure 3.1). All test bottles were incubated at room temperature (20-22°C). The calcium phosphate treatments were added to determine the effect of calcium phosphate on the susceptibility of the starches to enzymatic degradation by microorganisms. The treatment bottles were sampled for gas chromatography every week to measure or quantify the CO₂ produced in order to have an idea of how fast the starch degradation is taking place. In addition, aliquots of the soil in the microcosms were collected for 16S rRNA gene sequencing in order to identify the dominant bacterial species undertaking the biodegradation of the starch granules. After collecting soil samples for DNA analysis, anoxic conditions were re-established in the anoxic microcosms by evacuating the air in the bottles and refilling with nitrogen gas in three cycles as stated above. This first experiment was made to last for 70 days.

3.1.3 Artificial Dental Calculus (Precipitating Starch with Calcium Phosphate)

Starch was precipitated with calcium phosphate following the procedure of Soto et al. (2019). First, 0.5 g of unmodified potato, wheat and corn starch were collected with the tip of a Pasteur pipette (3 cm³ volume) and added to 5% calcium chloride [CaCl₂] (Fisher C79-500) solution that had been prepared by dissolving 0.5 g of calcium chloride in 9 ml of water (15 ml tube). The preparation was vortexed for 30 s and the volume was brought to 10 ml by adding reverse osmosis deionized (RODI) water. Then the pH was adjusted to 7 – 8 by the addition of

sodium hydroxide [NaOH] (Home Hardware Canada #3226-431). The tube was vortexed for 2 minutes. 0.5g of ammonium phosphate [$\text{NH}_4\text{H}_2\text{PO}_4$] (Fisher A684-500) was added with 0.5 g of glycine (Sigma-Aldrich, G7126) and NaOH to help with increasing the pH and binding properties of the eventual precipitate with starch (Soto et al. 2019).

Second, calcium carbonate blocks (Acros-Organics, CAS: 471.34-1) were used as a substrate to facilitate calcium phosphate precipitation. The blocks were autoclaved, rinsed twice with RODI water, air-dried and placed in 50 ml centrifuge tubes. The CaCl_2 + starch + glycine mixture prepared during the first step were poured into the carbonate blocks in tubes and vortexed for 5 minutes, stirring vigorously with a glass stirring rod. Tubes were capped and placed in an upright stand for 24 hours. The decantation of the supernatant left a precipitate at the bottom of the tube and this precipitate (along with the carbonate block) were transferred to a petri-dish and air-dried for 72 hours. The mass of the precipitated material was recorded in grams, along with its appearance and cohesiveness. Microscopic analysis of the artificial calculus was completed at 10–40 \times magnification (light microscopy, System Olympus BX-51) (Soto et al. 2019). After the formation of artificial dental calculus, 0.1 g of the precipitated material was added to the soil to form the calcium phosphate precipitated wheat, corn and potato microcosms.

<p>4% H₂O – oxic (11 bottles) 2 W; 2 C; 2 P 2 controls 1 Pcp; 1Ccp; 1Wcp</p>	<p>4% H₂O – anoxic (11 bottles) 2 W; 2 C; 2 P 2 controls 1 Pcp; 1Ccp; 1Wcp</p>
<p>20% H₂O – oxic (11 bottles) 2 W; 2 C; 2 P 2 controls 1 Pcp; 1Ccp; 1Wcp</p>	<p>4% H₂O – anoxic (11 bottles) 2 W; 2 C; 2 P 2 controls 1 Pcp; 1Ccp; 1Wcp</p>

W = Wheat;
C = Corn
P = Potato
cp = Calcium phosphate

Figure 3.1: The experimental set-up showing the distribution and the number of replicates of the treatment bottle across the four incubation conditions

<p>7% H₂O – oxic (9 bottles) 3 <i>Ipomoea</i> starch; 3 potato starch; 3 controls</p>	<p>7% H₂O – anoxic (9 bottles) 3 <i>Ipomoea</i> starch; 3 potato starch 3 controls</p>
<p>12% H₂O – oxic (9 bottles) 3 <i>Ipomoea</i> starch; 3 potato starch 3 controls</p>	<p>12% H₂O – anoxic (9 bottles) 3 <i>Ipomoea</i> starch; 3 potato starch 3 controls</p>

Figure 3.2: The experimental set-up showing the distribution and the number of replicates of the treatment bottle across the four incubation conditions



Figure 3.3: *Ipomoea longituba* tubers in the soil being collected at the Kesile site of Oldupai Gorge, located in Northern Tanzania. Source: Stone tools, Diet and Sociality (SDS) Research Group. Photo Credit: Jamie Inwood

3.2 Second Experimental Setup

3.2.1 Starches and Soil Collection

Based on the results of the first experiment, a second experiment was set-up to better mimic real-life starch degradation scenarios that will normally be encountered in the Oldupai Gorge region and also to reduce the variables encountered in the first experiment as a result of the use of artificial soil. Oldupai Gorge is located within the Ngorongoro conservation area and it is a

UNESCO world heritage site. This time around, artificial soil was discarded and only natural soil from Oldupai Gorge was used. A plant of ethnobotanical interest named *Ipomoea longituba* (see Figure 3.3) was collected from Oldupai Gorge and the tuber from the plant was cut into small pieces and dried in the sun to preserve it. The other starch used in this second experiment was potato starch that was obtained as a commercial preparation. The crystallographic pattern of *Ipomoea longituba* was unknown at the time of this writing but because it is a tuber, potato starch was chosen as a homologue. Since most tubers have the B or C diffraction pattern, we considered using potato starch because of its B diffraction pattern and also its commercial availability.

The natural soil was collected from a designated sampling area in Kesile located in Oldupai Gorge. Two sites were selected within the sampling area and bulk soil was collected from around 10 – 30 cm deep into two 1 L bottles during the dry season. The first site had an elevation of 1606 m and a GPS coordinate of -2.81234/35.32621 while the second site had an elevation of 1617 m and a GPS coordinate of -2.81245/35.32695. We avoided collecting insects and the soils were stored at ambient temperature. Bottles were uncapped briefly every week to replenish air. The baseline soil moisture content was determined to be 7% using the procedure and the equation stated in the first experiment above. A second level of soil moisture was chosen at 12%.

In addition to the bulk soil collected above, soil was also collected from a 1 m² area around the plant. Within each 1 m² area, three subsamples were taken and pooled into sterile 50 mL polypropylene tubes (Falcon, Fischer Scientific). The soil was collected from around 0 – 20 cm deep. Finally, soil from the roots or the rhizosphere of the *Ipomoea* plant was also collected whereby sections of plastic wrap were spread over the plants. Either the root or the entire plant was pulled out and the root/plant was gently shaken to dislodge any loosely attached soil. The detached soil was then collected in a sterile 50 mL tube.

3.2.2 Incubatory Conditions: Soil Moisture and Oxygen Levels

Just like in the first experiment, four different treatments or incubatory groups were created by altering soil moisture and O₂ levels – the two variables in this second experiment (see Figure 3.2). For the second level of moisture content (12%), 0.61 g of RODI water (the amount of water added was derived from the moisture content equation above by setting the moisture content to 12%) was added to 15.0 g of soil in serum bottles. Finally, 0.6 g of starch (2.2 millimoles of carbon) was added to 15.0 g of soil in serum bottles. The starch compound – (C₆H₁₀O₅)_n – has a molecular weight of 162 g mol⁻¹ per glucose unit. The bottles were capped and sealed and stored at room temperature. Anoxic conditions were established as stated above for the first experiment.

From Figure 3.2, it can be seen that the four different incubatory groups obtained were as follows: 7% water–oxic; 7% water – anoxic; 12% water – oxic and 12% water – anoxic. Each incubatory group contains 9 test bottles – three replicates of potato starch, three replicates of *Ipomoea longituba* starch and controls (here starch was not added to the soil). All 36 test bottles were incubated at room temperature. Almost every week, serum bottles were sampled for gas chromatography (to measure the carbon dioxide produced), DNA analysis and light microscopy.

3.3 Measurement of Carbon dioxide (CO₂)

The CO₂ produced by the microbial communities in the headspace of the test bottles was measured by sampling through the rubber stopper of each bottle with a syringe and transferring 1 ml of the gas sample to a 0.10 ml sample loop injector of a SRI 8610C Gas Chromatograph (SRI Instruments, Torrance, CA, USA) fitted with a HayeSep-D column (column temperature: 100°C) coupled to a methanizer (temperature: 300°C) and flame ionization detector (FID) (temperature: 300°C), with N₂ as the carrier gas. The detected peaks of CO₂ were analyzed using the PeakSimple

software package and compared against peaks from a standard gas mixture of known concentration (1.0% CO₂; 5014ppm CO; 5087 ppm CH₄ and balance nitrogen) (Praxair, Calgary). The areas of the detected peaks of CO₂ were converted to percentage (mixing ratio) of headspace, and then converted into number of moles of CO₂ using the ideal gas equation $PV = nRT$, where P, V and T are the pressure, volume and absolute temperature; n is the number of moles of gas; and R is the ideal gas constant. The cumulative percentage of CO₂ was calculated for each day that the test bottles were sampled because the CO₂ gas produced was lost each time the test bottles were uncapped for microscopic and DNA sampling.

Initial rates of CO₂ production were calculated by linear regression of millimoles of CO₂ produced versus time (days) in the first 40 days of incubations. In this phase (40 days), CO₂ increases were linear although they later decreased in some incubations. In order to test the significance of treatments (type of starch, incubatory conditions and calcium phosphate) on CO₂ production rates, the analysis of variance (ANOVA) was performed using the SPSS package (IBM Corporation). Post-hoc tests to determine the effects of different treatments used the Tukey HSD (Honestly Significant Difference) and Fisher's LSD (Least Significant Difference) methods.

3.4 Microbial Community Analysis or DNA Analysis

3.4.1 DNA Extraction

DNA was extracted from 0.5 g of each soil sample (stored at – 80 °C immediately after sampling) using the FastDNA Extraction Kit for Soil (MP Biomedicals, Santa Ana, CA, USA). To optimize DNA yield and remove humic acids, an additional purification procedure was utilized using 5.5 M guanidine thiocyanate (Knief et al. 2003). DNA concentration was measured using a Qubit Fluorometer with a Quant-iT dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA)

3.4.2 16S and 18S ribosomal RNA (rRNA) gene PCR and Illumina Sequencing

The extracted DNA was PCR amplified to obtain 16S rRNA and 18S rRNA gene amplicons for community analysis. Different primers and PCR reaction programs were used for the 16S and 18S rRNA gene PCR. The PCR procedures followed previous protocols in our laboratory (Ruhl et al. 2018). For 16S rRNA genes, the universal 341 forward and 785 reverse primers were used to amplify the V3-V4 region of the 16S rRNA genes (Klindworth et al. 2013). A PCR reaction mixture containing 5 μ L of 1 μ M forward primer (341), 5 μ L of 1 μ M reverse primer (785) and 12.5 μ L of the enzyme called SparQ HiFi mix 2X (Quantabio, Beverly, MA, USA) and 2.5 μ L of template DNA for a total volume of 25 μ L per reaction was loaded into a PCR tube. The first PCR reaction program for 16S rRNA genes was carried out with initial denaturation for 3 min at 95°C, 30 cycles of 30 s at 95°C, 55°C, 72°C and a prolonged step for 5 min at 72°C.

The primers Euk_Ill_F and Euk_Ill_R were used to amplify the eukaryotic 18S rRNA genes (Richardson et al. 2020). A PCR reaction mixture containing 0.5 μ L of 10 μ M forward primer (Euk_Ill_F), 0.5 μ L of 10 μ M reverse primer (Euk_Ill_R) and 15 μ L of enzyme (SparQ HiFi mix 2X, Quantabio) and 2 μ L of template DNA for a total volume of 30 μ L per reaction was loaded into a PCR tube. The first PCR reaction program was carried out as follows: initial denaturation for 3 minutes at 98°C, 10 cycles of touchdown PCR: 98°C for 20s, 60°C for 30s (decreasing at 0.5C/cycle), and 72°C for 30s, followed by 30 cycles: 98°C for 30s, 55°C for 30s, and 72°C for 30s, and 72°C for 5 minutes.

For both 16S and 18S rRNA gene amplicons, 5 μ L of the unpurified PCR product was run on a 2% agarose gel and then purified with Omega Mag-Bind RXNPure Plus beads (Omega Bio-tek, Norcross, GA, USA). Next, a second PCR reaction program was set up to attach individual

barcodes to each sample using index primers for both 16S and 18S rRNA gene amplicons. The reaction mixture contained 5 μ L of bead-purified PCR product, 5 μ L of forward index primer (1 μ M), 5 μ L of reverse index primer (1 μ M), 25 μ L of Premix F, 0.2 μ L of Taq Polymerase enzyme and 9.8 μ L of water. The second PCR reaction involved an initial denaturation for 3 min at 95°C followed by 8 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s; and a final elongation step of 72 °C for 5 min. Amplified PCR products were re-run on agarose gels and purified using Omega Mag-Bind RXNPure Plus beads (Omega Bio-tek, Norcross, GA, USA). DNA samples were measured using Qubit BR kit (Thermo Fisher Scientific) and then normalized to 4 nM. Normalized samples were then pooled and run for quality control with a BioAnalyzer (Agilent). Samples were prepared for high throughput sequencing in accordance to the Illumina 16S Metagenomic Sequencing Library Preparation protocol and run on a MiSeq (Illumina) at the Centre for Health Genomics and Informatics, University of Calgary.

3.5 QIIME Analysis

The Illumina raw sequence reads were analyzed with the Quantitative Insights Into Microbial Ecology (QIIME) pipeline version 1.9. QIIME is a bioinformatics pipeline used to analyze raw sequenced 16S and 18S rRNA gene data (Caporaso et al. 2010). Forward and reverse reads were paired using a minimum overlap of 20 base pairs. The QIIME command ‘split_libraries_fastq.py’ with the qualifiers: -q 19, --barcode_type ‘not barcoded’ was used to perform the quality control whereby sequences with a phred score lower than 20 were removed.

Next, sequences were clustered at a sequence identity of 97%. The most abundant member of each cluster was selected as a representative sequence for that cluster. For this step, the ‘pick_open_reference_otus.py’ command was used and sequences with a 97% similarity were clustered into operational taxonomic units (OTUs) and were subjected to BLAST searches against

the SILVA119 database for bacterial and archaeal 16S rRNA gene amplicons. Those sequences that could not be matched with a reference sequence in the Silva database were assigned a de novo OTU. Taxonomy was assigned based on the closest representative detected on the Silva reference database (McDonald et al. 2012). The resulting .biom output files were converted to tab delimited text files through the use of the ‘biom convert’ function and the ‘-to-tsv -header-key taxonomy’ qualifier.

Core diversity analysis and relative abundance computations were run on the OTU table (rarefied to the minimum number of reads depending on the Illumina lane that was being analyzed) with ‘core_diversity_analysis.py’ script. Rarefying each dataset meant that some samples with insufficient reads were excluded from the core diversity analysis. The alpha diversity metrics calculated using QIIME include Shannon, Observed Species, Faith’s Phylogenetic Diversity, Simpson, and Chao1. Finally, the ‘nmds.py’ script was used to output the Nonmetric Multidimensional Scaling (NMDS) file from the input distance matrix file called the weighted unifrac distance matrix that was obtained from the beta diversity computations. The genus L6 table from QIIME (where OTUs of the same genus are combined) was used to determine the relative abundance of bacteria that changed over time.

CHAPTER FOUR: RESULTS

4.1 Production of carbon dioxide in the wheat, corn and potato microcosms

Starches from three different botanical sources (wheat, corn and potato) were added to the soil substrate (artificial soil + natural soil) in serum bottles and subjected to different soil moisture levels and oxygen conditions to investigate their susceptibility to enzymatic degradation by soil microbes. Gas chromatography (GC) was used to measure the production of carbon dioxide (CO₂) in the headspace of the treatment bottles. All treatment bottles (44 in total) were sampled for GC at days 2, 6, 14, 27, 41, 49, 55 and 68 and the mixing ratio (percentage) of carbon dioxide (CO₂) in the gas headspace of each treatment bottle was recorded. Thereafter, the cumulative percentage CO₂ in each of the four incubatory conditions (i.e. 4% water – oxic, 4% water – anoxic, 20% water – oxic and 20% water – anoxic) was converted to millimoles of CO₂ to keep track of the amount of starch oxidised to CO₂ by the microbes. For this first experiment, it is important to recall from the methods section that the 0.1 g of starch added is equivalent to 3.7 millimoles of carbon. It is expected that some of the carbon will be converted into microbial biomass while the rest will be oxidized to CO₂. Therefore, the amount of CO₂ released should be less than the amount of starch added to each microcosm.

Results obtained showed that across the four starch treatments (wheat, corn, potato and control), the initial rate of CO₂ production in the headspace of the wheat starch treatment bottles was the highest. The rate of CO₂ production from corn starch was the second highest while potato had the slowest rate (see Figure 4.1). After 68 days for instance, the cumulative amount of CO₂ produced by wheat starch was 3.33 ± 0.33 mmol (4% water – oxic), 0.86 ± 0.36 mmol (4% water – anoxic), 2.61 ± 0.20 mmol (20% water – oxic) and 1.17 ± 0.20 mmol (20% water – anoxic). These values are much higher than what was obtained for corn starch and potato starch. The

analysis of variance (ANOVA) output in Table 4.1 shows that there was a statistically significant effect of starch on the initial rate of CO₂ production. The initial rate of CO₂ production in wheat starch was significantly higher than CO₂ production in all controls or in corn and potato starch, based on post-hoc tests. However, the rates in corn and potato starch treatments were not initially higher than the controls across all incubatory conditions (although they may have been in particular moisture/O₂ conditions, particularly the oxic incubations).

Similar patterns of CO₂ production were shown with potato, wheat and corn starches precipitated with calcium phosphate. Across the four incubatory groups, wheat starch treated with calcium phosphate (Wheat-cp) showed the highest production of CO₂. Production of CO₂ in corn starch treated with calcium phosphate (Corn-cp) was second highest while potato starch treated with calcium phosphate (Potato-cp) showed the lowest production of CO₂ (see Figure 4.2).

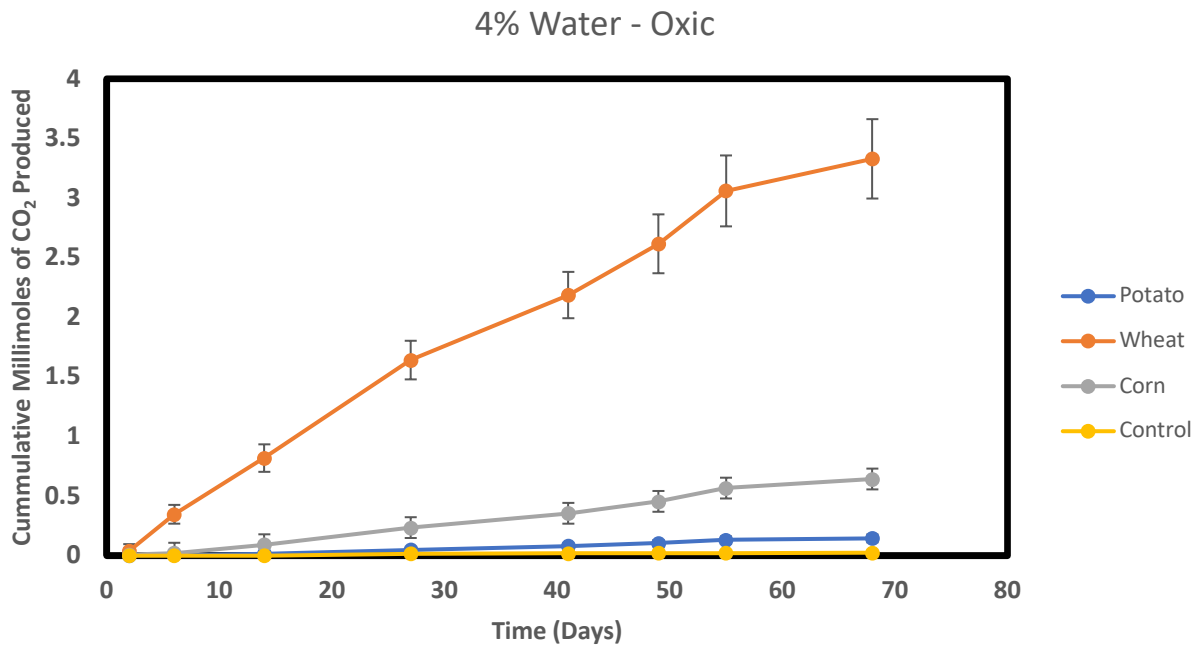
Thereafter, in order to know which incubatory condition was most suitable for microbial degradation of the starches tested, the rate of production of CO₂ in potato, wheat and corn starches for 41 days was calculated and comparisons were made among the four incubatory groups. Results from Figure 4.3 indicate that the 4% water – oxic incubatory condition was most suitable for microbial degradation for all the three starches tested, followed by the 20% water – oxic incubatory group, the 20% water – anoxic condition, and finally the 4% water – anoxic condition, which was the least suitable incubatory condition for the microbial degradation of these starches. The ANOVA output in Table 4.1 shows that the type of incubatory condition had a statistically significant effect on the rate of CO₂ production. Post hoc tests shown in Table 4.2 specifically show that the rate of CO₂ production in the 4% water – oxic condition was significantly higher than the rate of CO₂ production in the two anoxic conditions. Table 4.2 also shows that the rate of

CO₂ production after 41 days in the 4% water – oxic condition was not significantly higher than CO₂ production rate in the 20% water – oxic condition (significance value > 0.05).

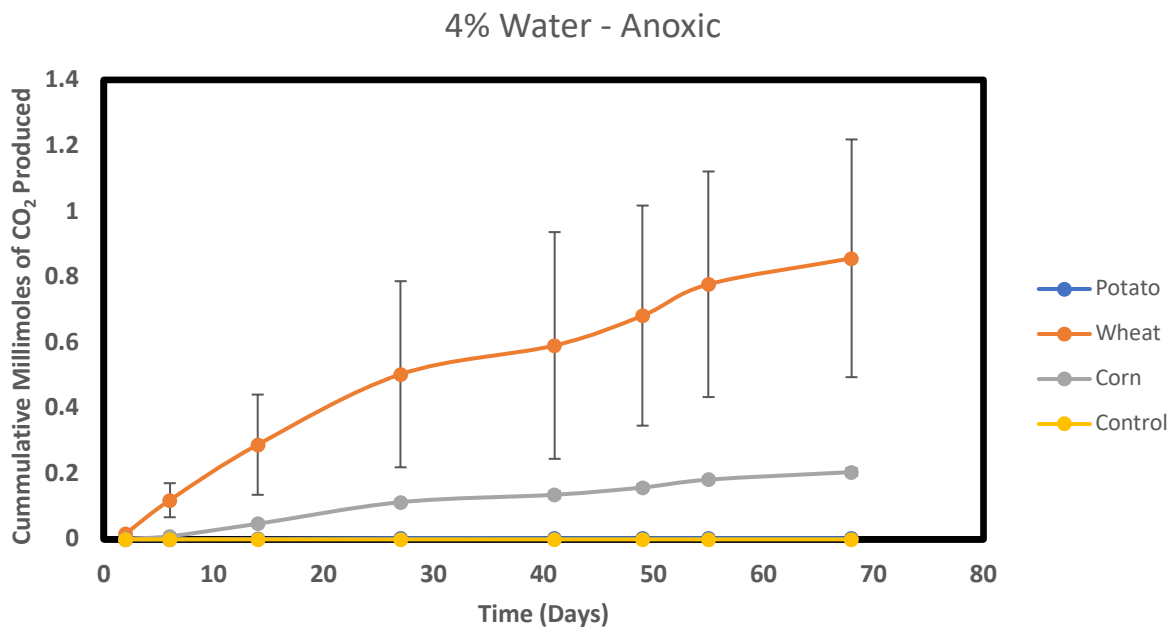
For the starches precipitated with calcium phosphate, the 4% water – oxic group gave the highest rate of production of CO₂ (after 41 days) for Wheat-cp and Potato-cp at 0.0309 mmol/day and 0.0028 mmol/day respectively (see Figure 4.4). For Corn-cp, the rate of CO₂ production was highest in the 20% water – oxic group at 0.0114 mmol/day. Just as with the normal starches, the least suitable incubatory group for the microbial degradation of calcium phosphate treated starches was the 4% water – anoxic group as it yielded the lowest production levels of CO₂ for Potato-cp, Wheat-cp and Corn-cp. It is important to note here that oxic groups still yielded the most CO₂ and were therefore more conducive to microbial degradation. Except for Corn-cp, the 4% water – oxic group showed higher CO₂ production compared to the 20% water – oxic group. The anoxic groups yielded the least amounts of CO₂ and the 4% water – anoxic group showed less CO₂ production when compared to the 20% water – anoxic group.

From Figure 4.5, it can be seen that generally the native starches are more susceptible to microbial degradation than their calcium phosphate treated counterparts. Potato starch seems to be an exception here as Figure 4.5 shows that the cumulative CO₂ produced for over 68 days for calcium phosphate treated potato starch was found to be similar to that for native or normal potato starch. Table 4.1 shows that there is a statistically significant effect of calcium phosphate on CO₂ production and this confirms the information in Figure 4.5 that indicates that calcium phosphate does reduce the rate of CO₂ production significantly. Although individual calcium phosphate Ca₃(PO₄)₃ vials were not replicated, the overall effect of calcium phosphate addition was proven to be statistically significant because it operated consistently over all starch/incubation treatment combinations (Figure 4.5).

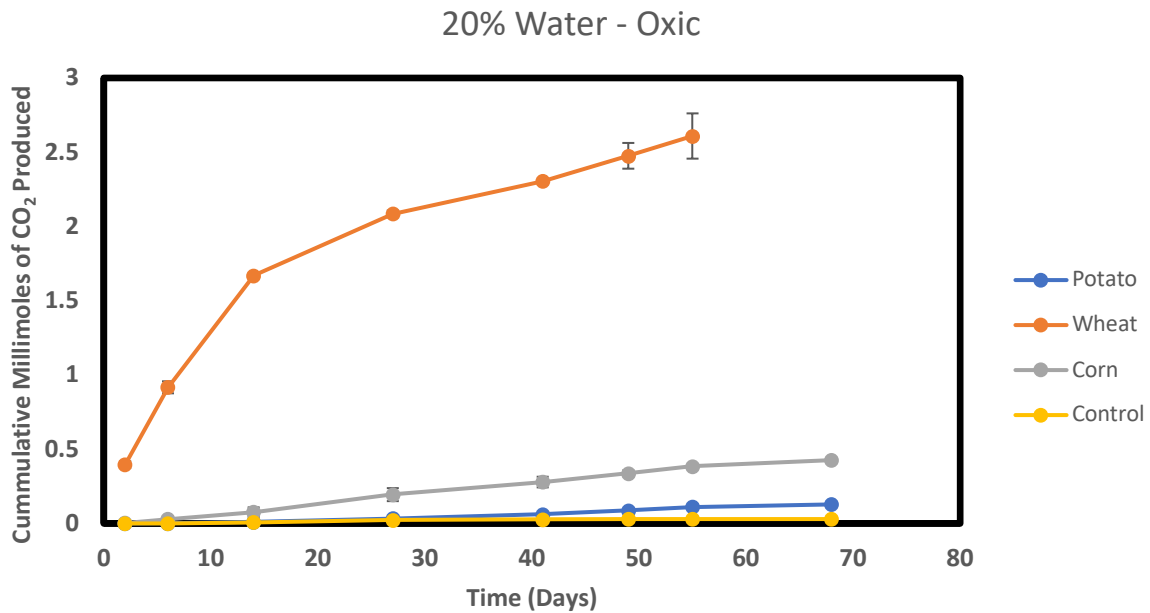
(A)



(B)



(C)



(D)

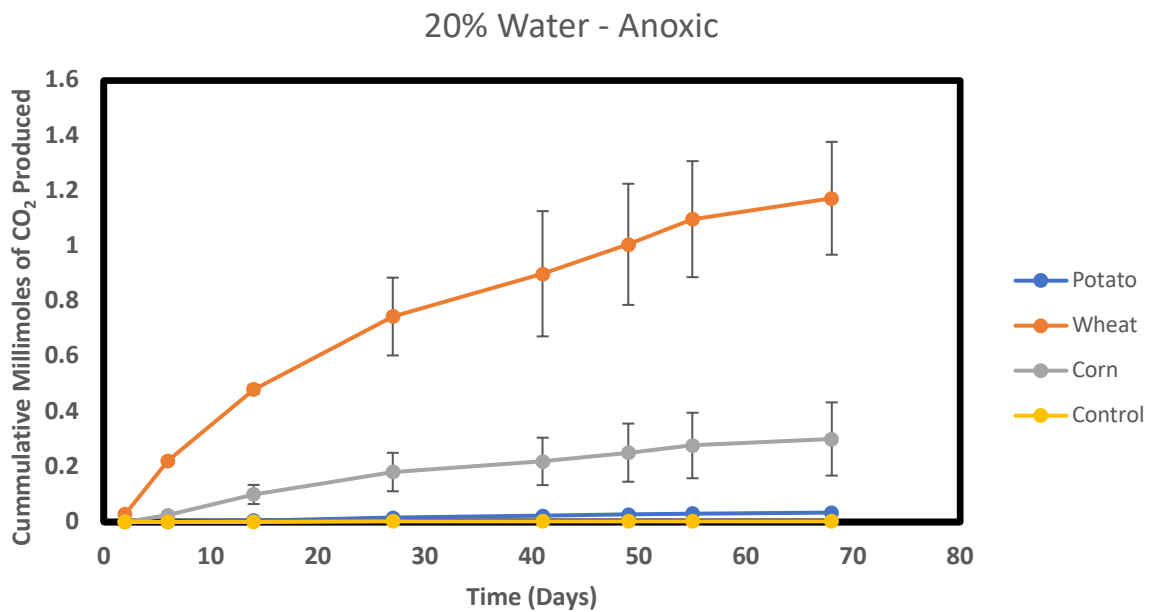
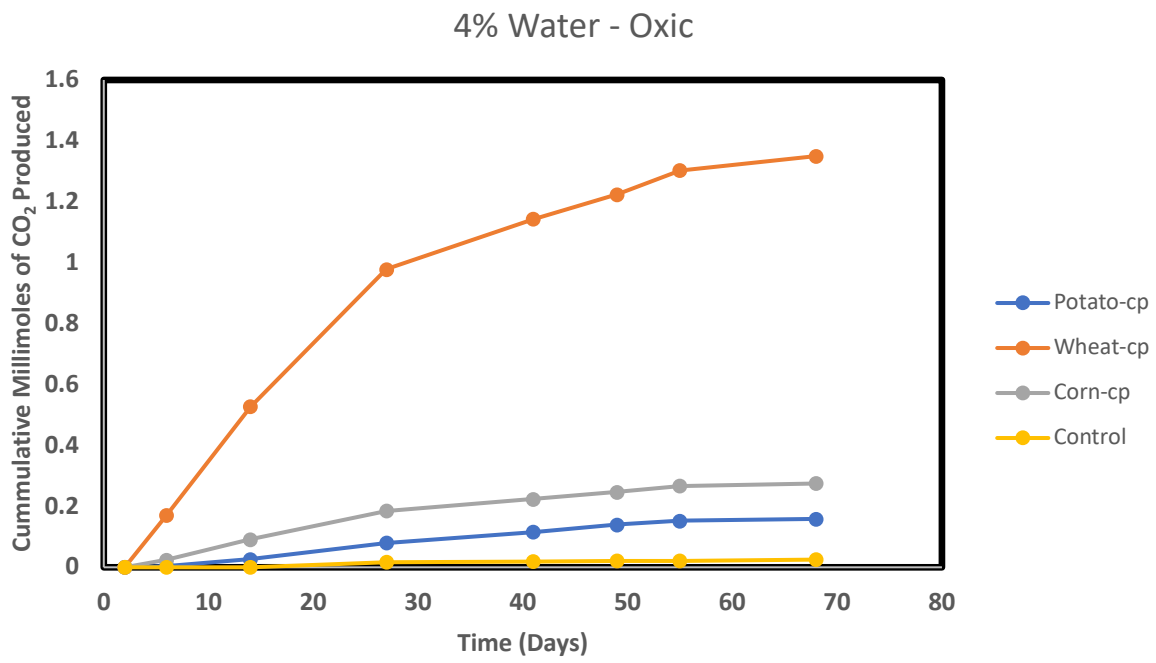
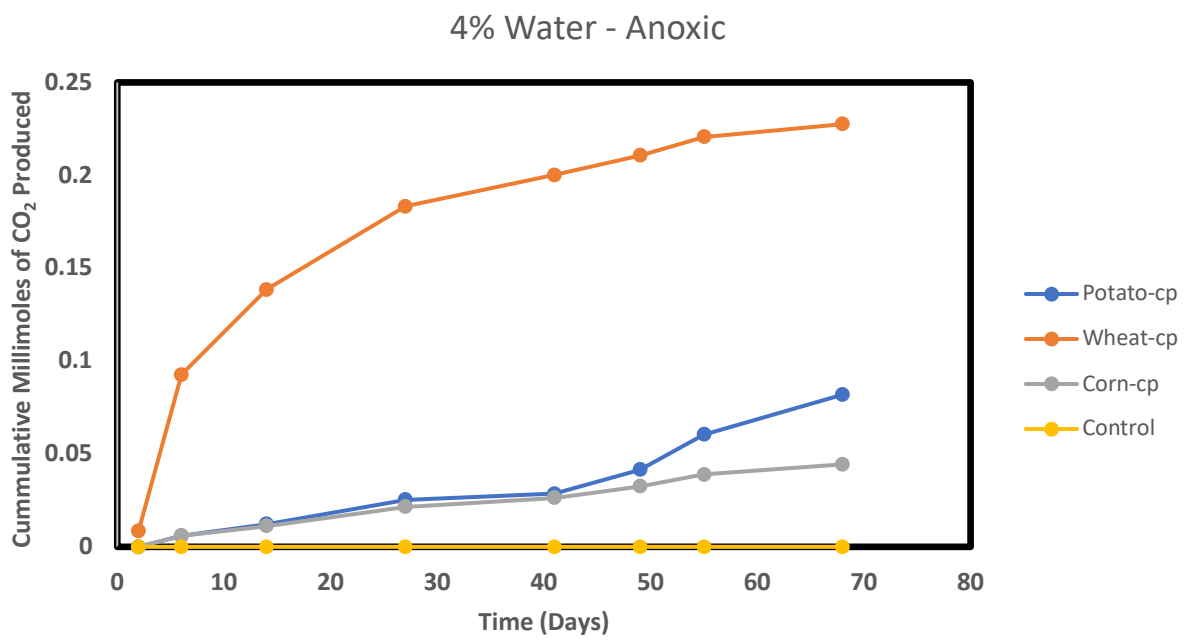


Figure 4.1: Cumulative millimoles of CO₂ produced in the headspace of the potato, wheat, corn and control treatment bottles or microcosms across the four incubatory conditions (A – D) in Experiment 1. Error bars represent ± 1 standard error of the mean of two replicates. Where error bars are not seen they are smaller than the diameter of the symbol.

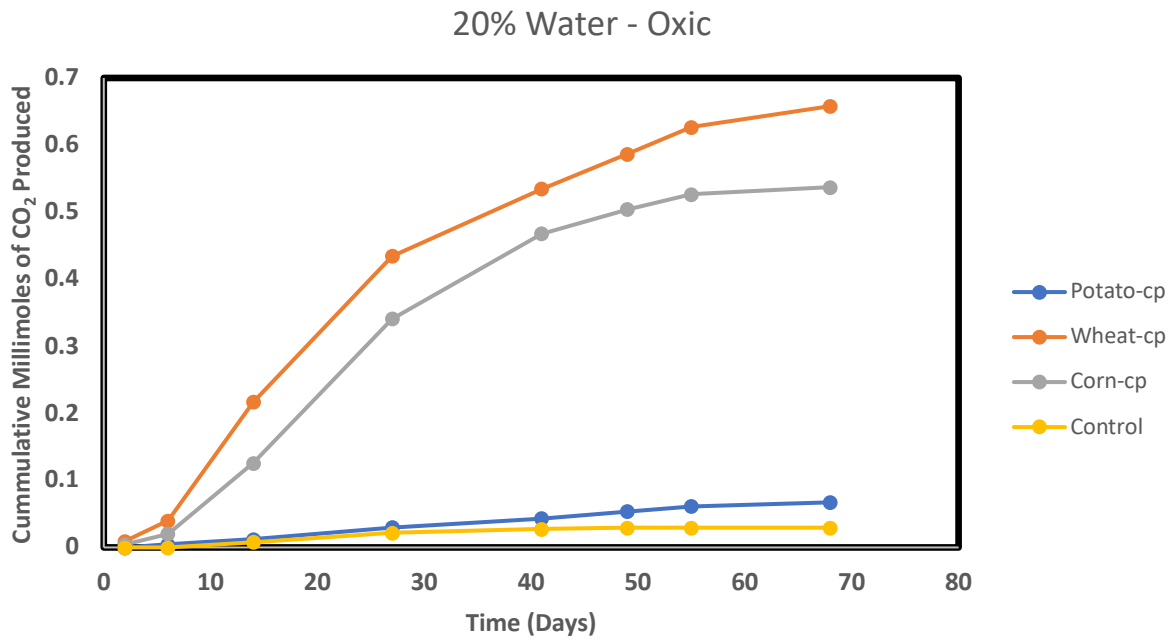
(A)



(B)



(C)



(D)

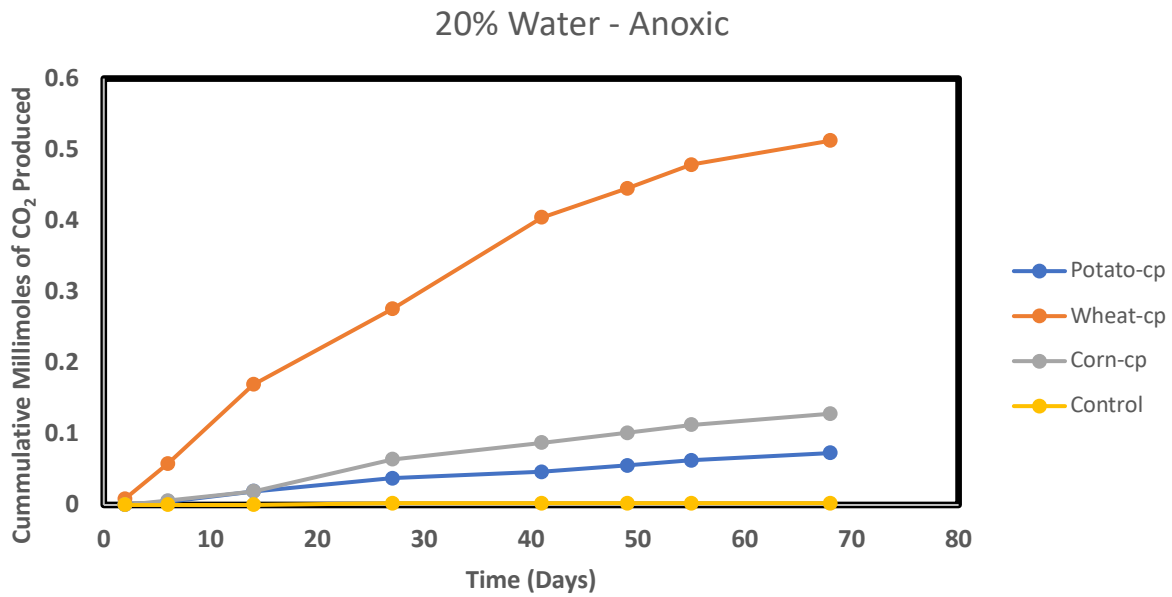


Figure 4.2: Cumulative millimoles of CO₂ produced in the headspace of potato, wheat and corn starches treatment bottles (microcosms) treated with calcium phosphate across the four incubatory conditions (A – D) of Experiment 1. Each point on the line graph represents a single replicate.

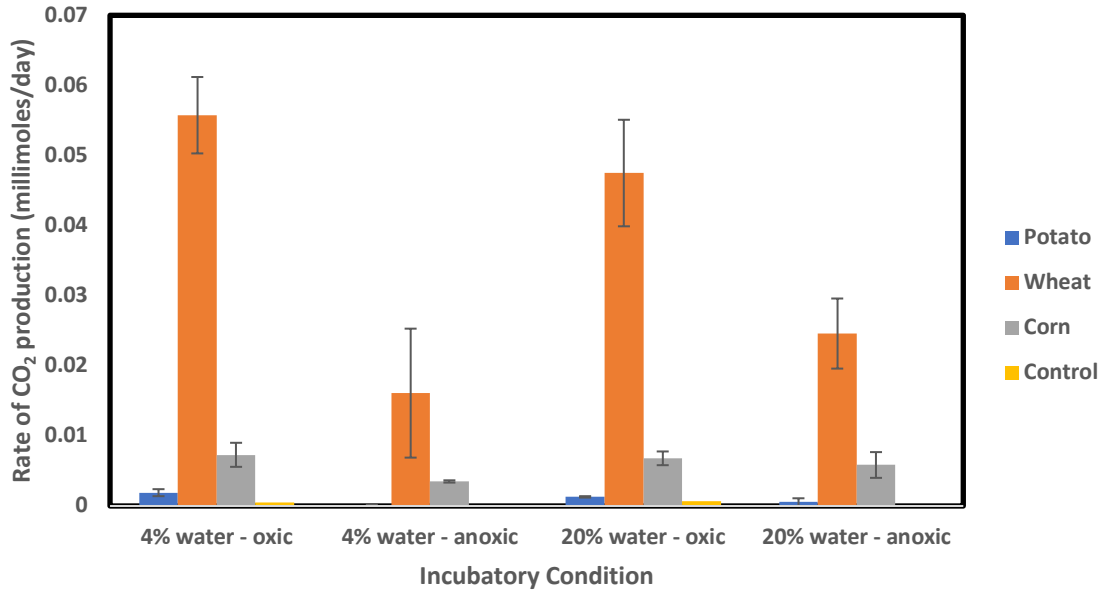


Figure 4.3: Bar graph comparing the initial rate (0-41 days) of CO₂ production in the headspace of potato, wheat and corn treatment bottles or microcosms in Experiment 1. Error bars represent ± 1 standard error of the mean of two replicates. Wheat produced significantly more CO₂ than all other treatments.

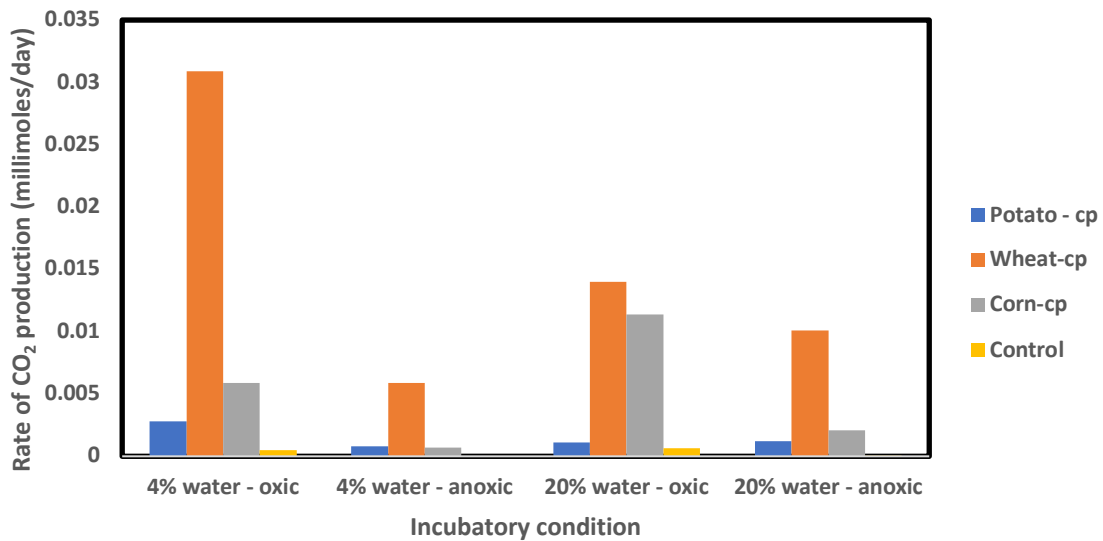


Figure 4.4: Bar graph comparing initial rate (0-41 d) of CO₂ production in the headspace of calcium phosphate – treated potato, wheat and corn treatment bottles or microcosms after 41 days in Experiment 1.

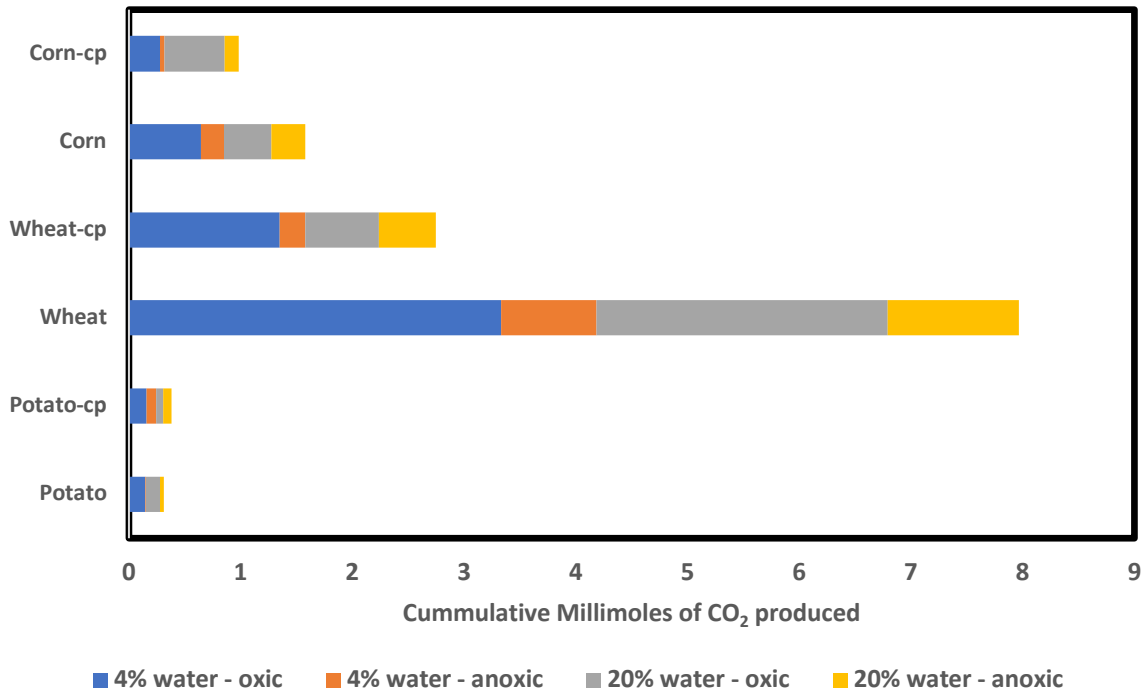


Figure 4.5: Bar chart comparing the cumulative millimoles of CO₂ produced by the native potato, wheat and corn starches, and their calcium phosphate treated counterparts across the four incubatory conditions after 68 days in Experiment 1.

Table 4.1: Three-way univariate analysis of variance (ANOVA) table (test of between-subject effect) showing the effect of starch, incubation condition and calcium phosphate on the dependent variable (rate of CO₂ production) in Experiment 1.

Source	Type III Sum of Squares	df	Mean Square	F	Significance
Corrected Model	.009 ^a	16	.001	10.661	.000
Intercept	.002	1	.002	32.043	.000
Starch	.007	3	.002	41.611	.000
Incubation condition	.001	3	.000	4.578	.010
Calcium Phosphate	.000	1	.000	7.502	.011
Starch * Incubation condition	.001	9	.000	2.999	.013
Error	.001	27	5.269E-5		
Total	.015	44			
Corrected Total	.010	43			

^a. R Squared = .863 (Adjusted R Squared = .782)

Table 4.2: Post-hoc analyses using the Tukey HSD and Fisher LSD post hoc tests, of the four different incubatory conditions (moisture/O₂) from the ANOVA comparing the rate of CO₂ production in Experiment 1 (See Table 4.1).

	(I) Incubation condition	(J) Incubation condition	Mean Difference (I-J)	Std. Error	Significance	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	20% anoxic	20% oxic	-0.006	0.003	0.266	-0.014	0.003
		4% anoxic	0.003	0.003	0.834	-0.006	0.011
		4% oxic	-0.009*	0.003	0.044	-0.017	0.000
	20% oxic	20% anoxic	0.006	0.003	0.266	-0.003	0.014
		4% anoxic	0.008	0.003	0.053	0.000	0.017
		4% oxic	-0.003	0.003	0.792	-0.011	0.006
	4% anoxic	20% anoxic	-0.003	0.003	0.834	-0.011	0.006
		20% oxic	-0.008	0.003	0.053	-0.017	0.000
		4% oxic	-.0112*	0.003	0.006	-0.020	-0.003
	4% oxic	20% anoxic	0.009*	0.003	0.044	0.000	0.017
		20% oxic	0.003	0.003	0.792	-0.006	0.011
		4% anoxic	0.011*	0.003	0.006	0.003	0.020
LSD	20% anoxic	20% oxic	-0.006	0.003	0.073	-0.012	0.001
		4% anoxic	0.003	0.003	0.407	-0.004	0.009
		4% oxic	-0.009*	0.003	0.01	-0.015	-0.002
	20% oxic	20% anoxic	0.006	0.003	0.073	-0.001	0.012
		4% anoxic	0.008*	0.003	0.012	0.002	0.015
		4% oxic	-0.003	0.003	0.363	-0.009	0.003
	4% anoxic	20% anoxic	-0.003	0.003	0.407	-0.009	0.004
		20% oxic	-0.008*	0.003	0.012	-0.015	-0.002
		4% oxic	-0.011*	0.003	0.001	-0.018	-0.005
	4% oxic	20% anoxic	0.009*	0.003	0.01	0.002	0.015
		20% oxic	0.003	0.003	0.363	-0.003	0.009
		4% anoxic	0.011*	0.003	0.001	0.005	0.018

Based on observed means.
The error term is Mean Square (Error) = 5.269E-5.
*. The mean difference is significant at the .05 level.

4.2 Production of carbon dioxide in the *Ipomoea longituba* and potato microcosms

Starch of ethnobotanical interest (*Ipomoea longituba*) and potato starch were added to natural soil collected from Oldupai Gorge in Tanzania in serum bottles and subjected to four different incubatory conditions by altering the soil moisture content and oxygen level. Similar to the first experiment, gas chromatography (GC) was used to measure the production of CO₂ in the headspace of the treatment bottles. All treatment bottles (36 in total) were sampled for GC at days 2, 5, 9, 13, 19, 26, 33, 40, 48, 55, 61, 69, 76, 82, 89, 96, 104, 118, 125. Thereafter, the cumulative percentage CO₂ in each of the four incubatory conditions (i.e. 7% water – oxic, 7% water – anoxic, 12% water – oxic and 12% water – anoxic) was calculated and converted to millimoles of CO₂ to keep track of the amount of starch consumed by the microbes. For this second experiment, it is important to note that the 0.6 g of starch added to the natural soil is equivalent to 2.2 millimoles of carbon.

Across the four incubatory conditions, the amount of CO₂ produced in the *Ipomoea longituba* treatment bottles was the highest for each day the bottles were sampled for GC. From Figure 4.6A and Figure 4.6C, it can be seen that the curves for *Ipomoea longituba* in the 7% water – oxic and 12% water – oxic conditions were hyperbolic, meaning that the rate of CO₂ release in the first few days was very rapid and declined over time. This trend continued until day 61 when the CO₂ release curve seemed to level off, probably because the degradable starch was exhausted. Unlike what was obtained for the oxic groups, CO₂ was more slowly released initially from the 7% water – anoxic and 12% water – anoxic conditions. The patterns over time were less predictable, and the curves did not seem to level off in the 120-day incubations (Figure 4.6 B and Figure 4.6 D). The ANOVA output in Table 4.3 shows that there was a statistically significant effect of starch on the rate of

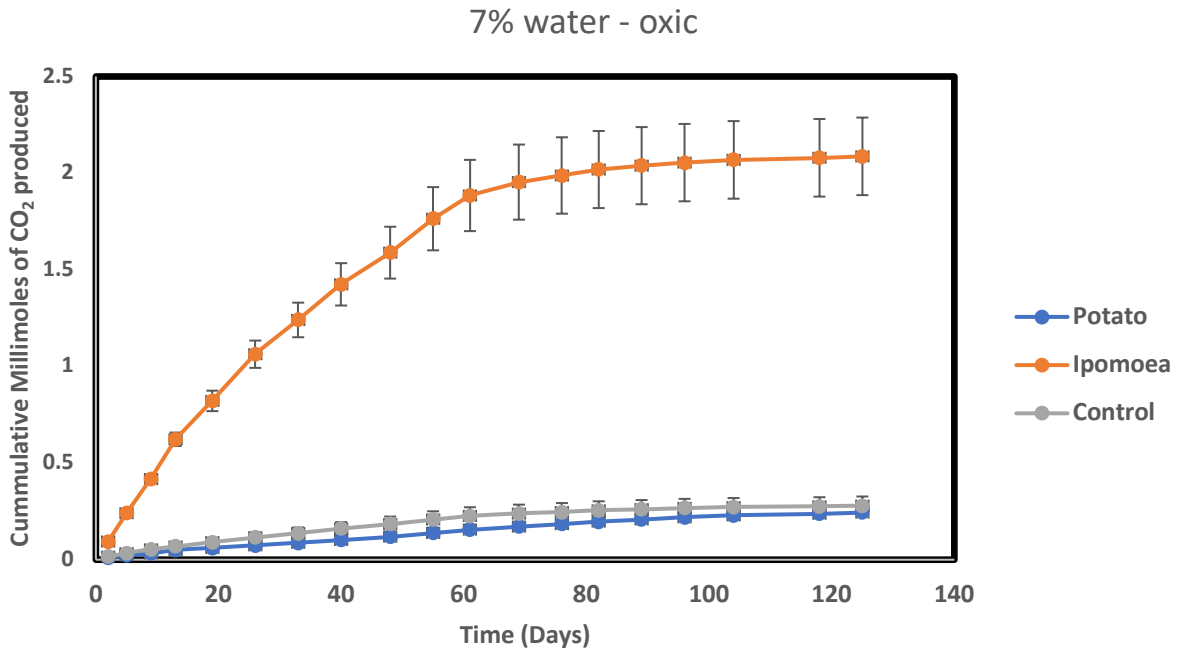
CO₂ production. The rate of CO₂ production in *Ipomoea longituba* starch was significantly higher than CO₂ production in the potato starch and the control treatments.

Potato starch produced little CO₂ and from Figure 4.6, it can be seen that the curves for potato starch could almost not be distinguished from those of the control in three of the incubatory conditions (i.e. 7% water – oxic, 7% water – anoxic and 12% water – anoxic). It is interesting to note that for the 12% water – oxic incubatory condition, potato starch initially released very little amounts of CO₂ and was indistinguishable from the control until day 33 when the release of CO₂ began to increase. This rapid release of CO₂ continued from day 33 to day 60 when it slowed down and the curve seemed to level off (see Figure 4.6C). As expected, the controls produced little to no CO₂ primarily because no starch was added to the control treatment bottles.

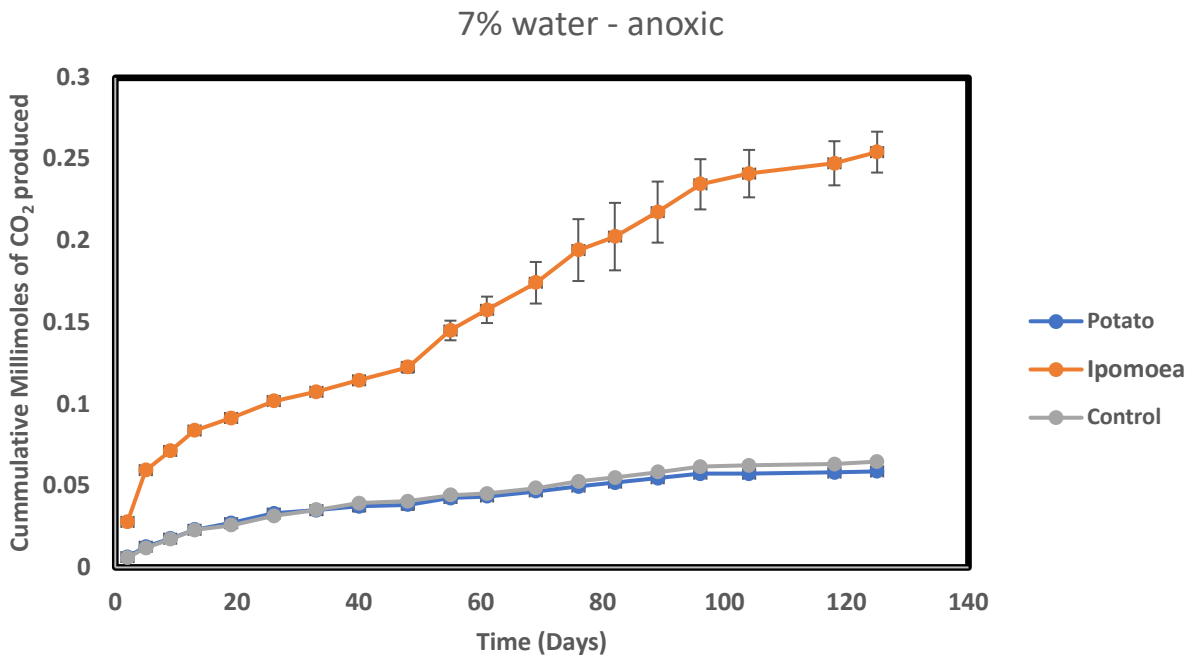
There were also significant differences in the CO₂ produced among the four incubatory groups (Table 4.3). It can be seen from Figure 4.7 and Table 4.4 that the most suitable incubatory group for the degradation of the starches tested was the 12% water – oxic group as it showed the highest rate of production of CO₂ for the potato starch, the *Ipomoea longituba* starch, and the controls. The second most suitable treatment was the 7% water – oxic group. The least suitable incubatory conditions were the two anoxic treatments, which were not significantly different from each other (Table 4.4).

In addition, Figure 4.7 shows that after 40 days, the rate of production of CO₂ was highest for *Ipomoea longituba* across all four incubatory groups. In the 12% water – oxic incubatory condition, the rate of production of CO₂ was higher in the potato starch microcosms compared to the controls. In the three other incubatory conditions (i.e. the 7% water – oxic, 7% water – anoxic and 12% water – anoxic), the rate of production of CO₂ in potato starch was almost indistinguishable from that of the controls.

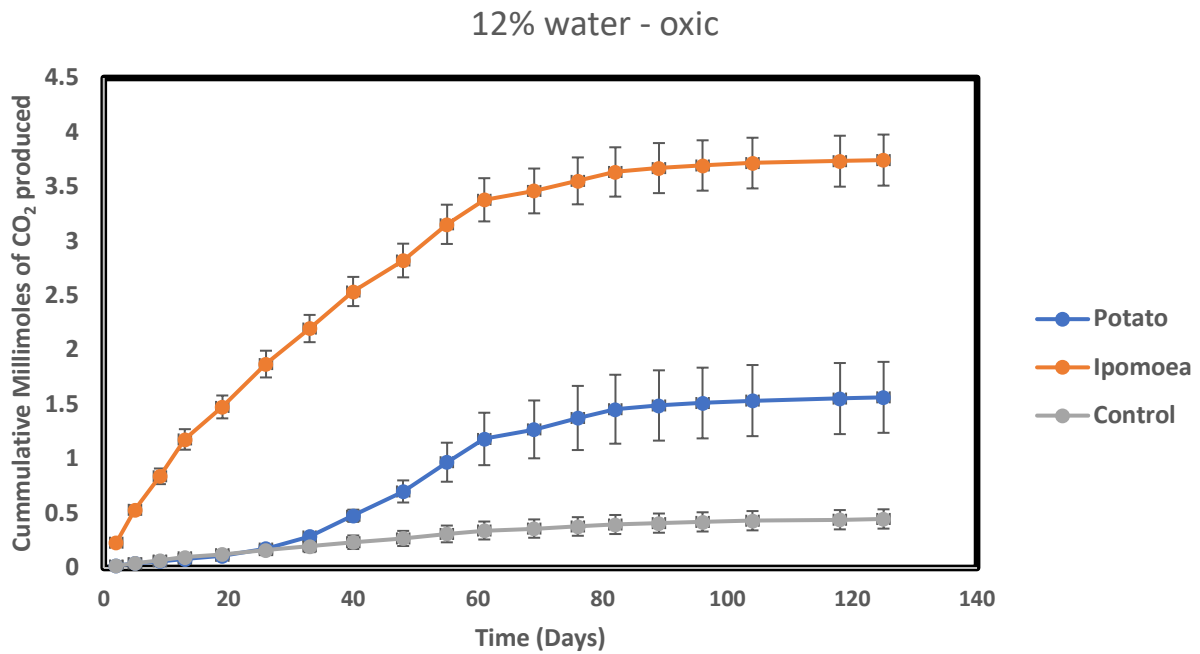
(A)



(B)



(C)



(D)

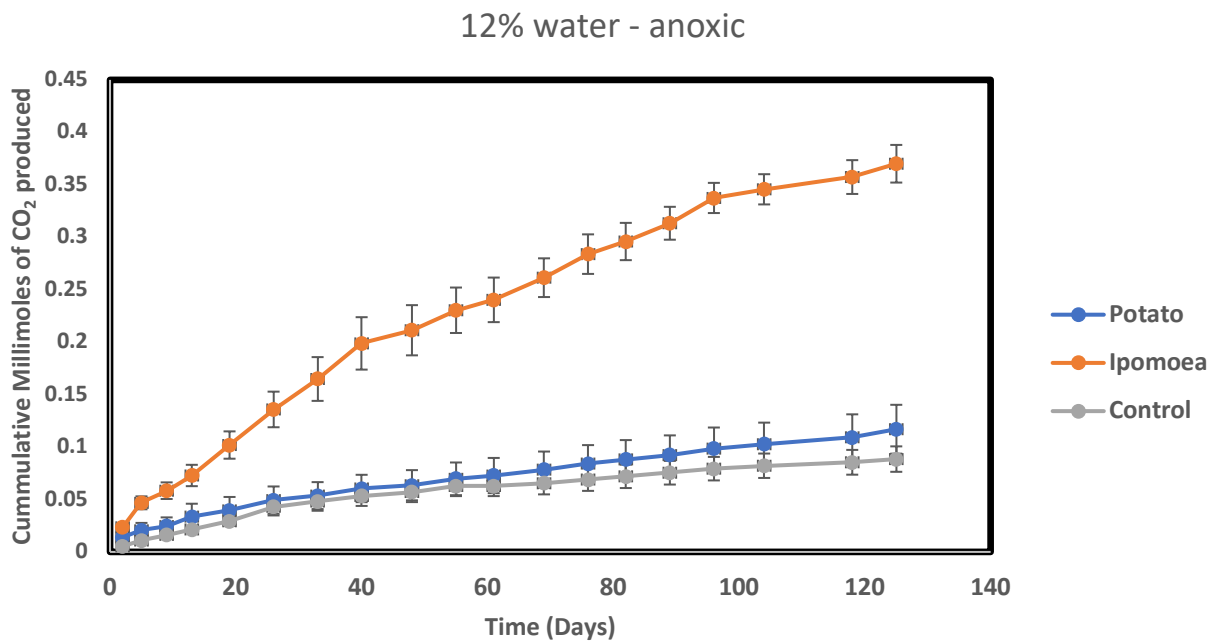


Figure 4.6: Cumulative millimoles of CO₂ produced in the headspace of potato, *Ipomoea longituba* and control treatment bottles or microcosms across the four incubatory conditions (A – D). Error bars represent ± 1 standard error of the mean of three replicates.

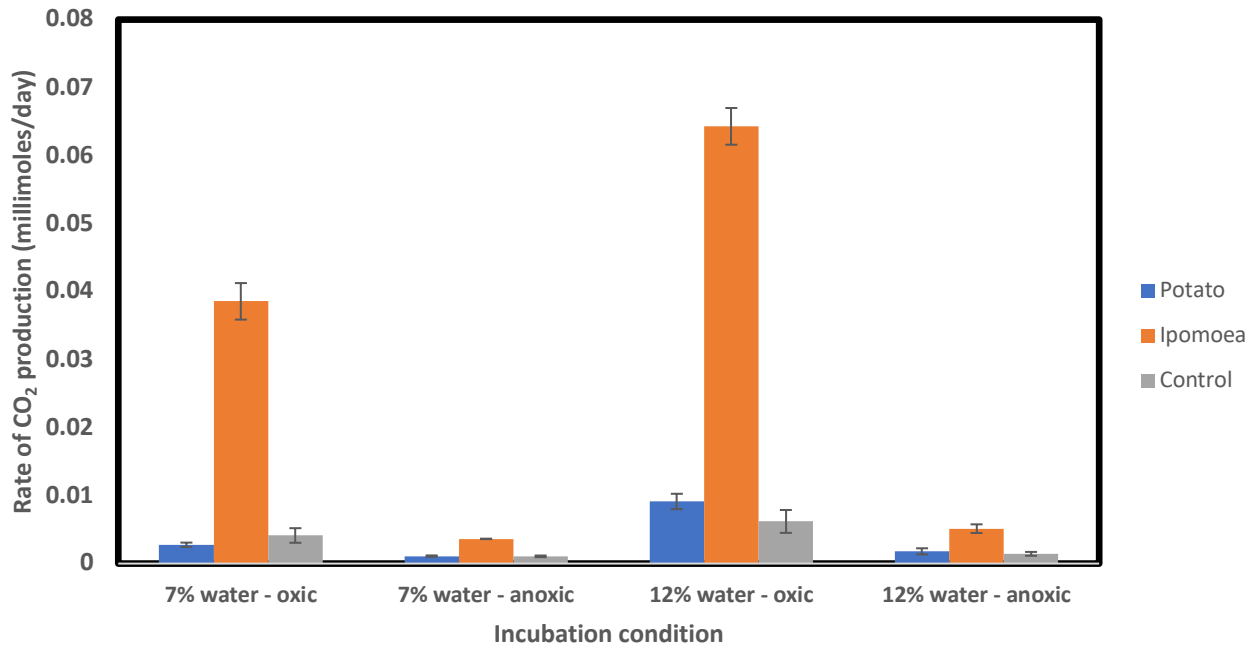


Figure 4.7: Bar graph comparing the rate of CO₂ produced in the headspace of potato, *Ipomoea longituba* and control treatment bottles or microcosms among the four incubatory conditions in the first 40 days. Error bars represent the standard error of the mean of three replicates. CO₂ production was significantly higher in the *Ipomoea* treatments compared to the control and potato treatments.

Table 4.3: Two-way univariate analysis of variance (ANOVA) table (test of between-subject effects) showing the effect of starch and incubation condition on the initial rate of CO₂ production in Experiment 2.

Source	Type III Sum of Squares	df	Mean Square	F	Significance
Corrected Model	.013 ^a	11	.001	222.543	.000
Intercept	.005	1	.005	942.175	.000
Starch	.005	2	.002	464.938	.000
Incubation condition	.004	3	.001	237.288	.000
Starch * Incubation condition	.004	6	.001	134.372	.000
Error	.000	24	5.146E-6		
Total	.018	36			
Corrected Total	.013	35			

^a. R Squared = .990 (Adjusted R Squared = .986)

Table 4.4: Post-hoc analyses using the Tukey HSD and Fisher LSD post hoc tests, of the four different incubatory conditions (moisture/O₂) from the ANOVA comparing the rate of CO₂ production in Experiment 2 (See Table 4.3).

	(I) Incubation condition	(J) Incubation condition	Mean Difference (I-J)	Std. Error	Significance	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	12% anoxic	12% oxic	-0.024*	0.001	0.00	-0.027	-0.021
		7% anoxic	0.001	0.001	0.854	-0.002	0.004
		7% oxic	-0.012*	0.001	0.00	-0.015	-0.009
	12% oxic	12% anoxic	0.024*	0.001	0.00	0.021	0.027
		7% anoxic	0.025*	0.001	0.00	0.022	0.028
		7% oxic	0.011*	0.001	0.00	0.008	0.014
	7% anoxic	12% anoxic	-0.001	0.001	0.854	-0.004	0.002
		12% oxic	-0.025*	0.001	0.00	-0.028	-0.022
		7% oxic	-0.013*	0.001	0.00	-0.016	-0.010
	7% oxic	12% anoxic	0.012	0.001	0.00	0.009	0.015
		12% oxic	-0.011*	0.001	0.00	-0.014	-0.008
		7% anoxic	0.013*	0.001	0.00	0.010	0.016
LSD	12% anoxic	12% oxic	-0.024*	0.001	0.00	-0.026	-0.022
		7% anoxic	0.001	0.001	0.432	-0.001	0.003
		7% oxic	-0.012*	0.001	0.00	-0.015	-0.010
	12% oxic	12% anoxic	0.024*	0.001	0.00	0.022	0.026
		7% anoxic	0.025*	0.001	0.00	0.022	0.027
		7% oxic	0.011*	0.001	0.00	0.009	0.014
	7% anoxic	12% anoxic	-0.001	0.001	0.432	-0.003	0.001
		12% oxic	-0.025*	0.001	0.00	-0.027	-0.022
		7% oxic	-0.013*	0.001	0.00	-0.015	-0.011
	7% oxic	12% anoxic	0.012*	0.001	0.00	0.010	0.015
		12% oxic	-0.011*	0.001	0.00	-0.014	-0.009
		7% anoxic	0.013*	0.001	0.00	0.011	0.015

Based on observed means.
 The error term is Mean Square (Error) = 5.146E-6.
 *. The mean difference is significant at the .05 level.

4.3 Bacterial communities present in the wheat, corn and potato microcosms

For the first experiment, soil samples collected from the soil–starch microcosms at days 15 and day 70 were processed for 16S rRNA gene sequencing. The major phyla that occurred across all treatment groups and starch type are Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes while the major genera are *Promicromonospora*, *Bacillus*, *Streptomyces*, *Alkalibacterium* and *Pseudomonas*. *Bacillus* was often dominant, but these bacteria were highest in the original soil at day 0 and did not seem to increase their relative abundance over time. Therefore, *Bacillus* was not likely involved in starch degradation. They were likely present in high numbers as spores that do not germinate.

Table 4.5 profiles the taxonomy of each genus that will be discussed in this section. Also, Figure 4.8 to Figure 4.13 summarize the changes in relative abundances over time across the different treatments. The genera that showed at least 2-fold increases in relative abundance over time are also summarized in Table 4.6. More detailed tables of the genera that increased in response to starch treatments are given in the Appendix (Supplementary Tables 1 – 3), ordered in terms of their fold increases.

For potato starch, the genera that most increased over time in the 4% water – anoxic treatment group were: *Promicromonospora* and *Nitriliruptor* with their average relative abundance increasing between day 15-70 (see Figure 4.10A). In the 4% water – oxic condition, the relative abundance of *Streptomyces* increased over time from day 15 to day 70 while the average relative abundance of *Promicromonospora* increased from day 0 to day 70. Also, the relative abundance of *Nocardiopsis* increased from day 15 to day 70 (see Figure 4.10B).

For corn starch, the genus that most increased over time in the 4% water – anoxic treatment group was *Promicromonospora* with its average relative abundance increasing from day 15 to day

70 (see Figure 4.9A). In the 4% water – oxic condition, relative abundance of *Streptomyces* increased over time from day 15 to day 70 while in the 20% water – anoxic treatment, *Promicromonospora* increased from 2% on day 0 to 10% on day 70 while *Rhizobium* increased from day 15 to day 70 (see Figure 4.9C).

For wheat starch, the genus that mostly increased over time in the 4% water – anoxic treatment group was *Alkalibacterium* with its average relative abundance increasing from < 2% on day 0 to 36% on day 15 before decreasing to 14% on day 70 (see Figure 4.8A). In the 20% water – oxic condition, *Pseudomonas* increased over time from an average relative abundance of 9% on day 15 to 20% on day 70 while in *Alkalibacterium* increased from < 2% on day 0 to 18% on day 15 before decreasing to 7% on day 70 (see Figure 4.8D).

In the calcium–phosphate precipitated starches, we see a microbial community structural pattern similar to that for the normal starches. For corn-cp, the relative abundance of *Sinomonas* (31%) and *Planococcus* increased in the 20% water – oxic condition at day 15 (see Figure 4.12D). In the wheat-cp microcosms, *Sinomonas* increased to dominate the microbial community with relative abundances of 34% and 31% in the 4% water – anoxic (see Figure 4.11A) and 4% water – oxic conditions respectively (see Figure 4.11B). In the 20% water – oxic condition on day 15, *Pseudomonas* and an uncultured genus of *Corynebacteriales* became the most dominant clades with relative abundances of 20% and 23% respectively (see Figure 4.11D). In the potato-cp microcosms, *Planococcus*, *Citricoccus* and *Sinomonas* became the most dominant genera in the 4% water – oxic treatment group as their average abundances increased from < 5% on day 0 to 5%, 12% and 15% respectively on day 15 (see Figure 4.13B).

The major starch-responsive genera within the oxic conditions across all the starch treatment combinations are schematically summarized in Figure 4.14

Table 4.5: Taxonomic profiles of all genera in Experiment 1 whose average relative abundance increase over time as described in section 4.3

Genus	Phylum	Class	Order	Family
<i>Bacillus</i>	<i>Firmicutes</i>	<i>Bacilli</i>	<i>Bacillales</i>	<i>Bacillaceae</i>
<i>Alkalibacterium</i>	<i>Firmicutes</i>	<i>Bacilli</i>	<i>Lactobacillales</i>	<i>Carnobacteriaceae</i>
<i>Promicromonospora</i>	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Micrococcales</i>	<i>Promicromonosporaceae</i>
<i>Pseudomonas</i>	<i>Proteobacteria</i>	<i>Gammaproteobacteria</i>	<i>Pseudomonadales</i>	<i>Pseudomonadaceae</i>
<i>Sinomonas</i>	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Micrococcales</i>	<i>Micrococcaceae</i>
<i>Streptomyces</i>	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Streptomycetales</i>	<i>Streptomycetaceae</i>
<i>Nocardiosis</i>	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Streptosporangiales</i>	<i>Nocardiosaceae</i>
<i>Planococcus</i>	<i>Firmicutes</i>	<i>Bacilli</i>	<i>Bacillales</i>	<i>Planococcaceae</i>
<i>Ornithinimicrobium</i>	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Micrococcales</i>	<i>Intrasporangiaceae</i>
<i>Citricoccus</i>	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Micrococcales</i>	<i>Micrococcaceae</i>
<i>Rhizobium</i>	<i>Proteobacteria</i>	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	<i>Rhizobiaceae</i>
<i>Pelagibacterium</i>	<i>Proteobacteria</i>	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	<i>Hyphomicrobiaceae</i>
<i>Paracoccus</i>	<i>Proteobacteria</i>	<i>Alphaproteobacteria</i>	<i>Rhodobacterales</i>	<i>Rhodobacteraceae</i>
<i>Mongoliicoccus</i>	<i>Bacteroidetes</i>	<i>Cytophagia</i>	<i>Cytophagales</i>	<i>Cyclobacteriaceae</i>
<i>Nitriliruptor</i>	<i>Actinobacteria</i>	<i>Nitriliruptoria</i>	<i>Nitriliruptorales</i>	<i>Nitriliruptoraceae</i>

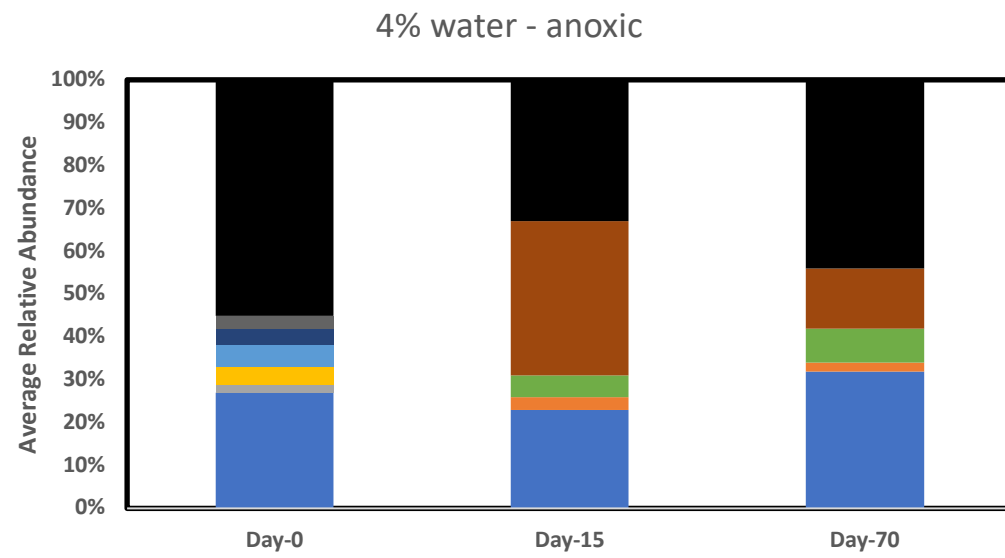
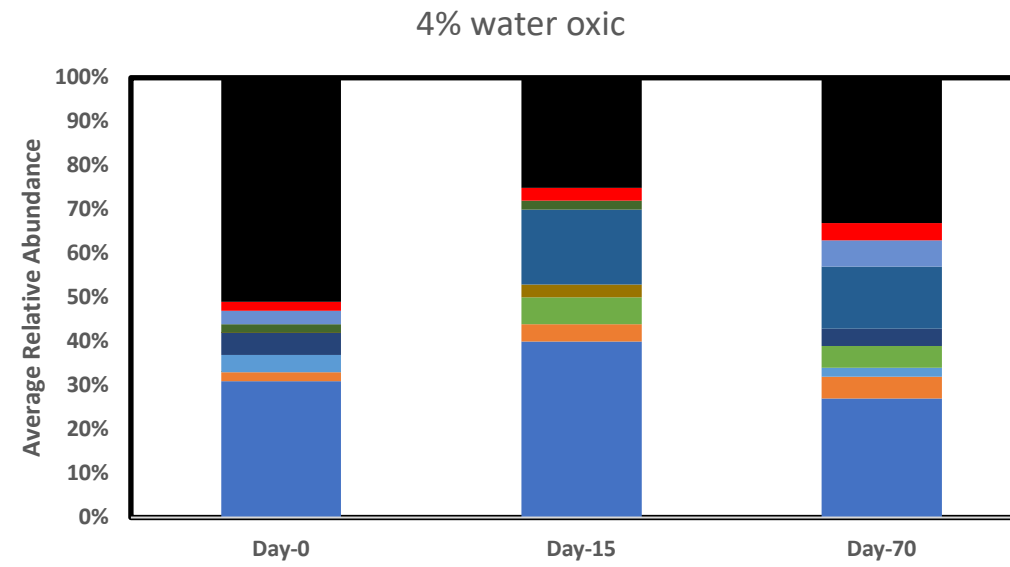
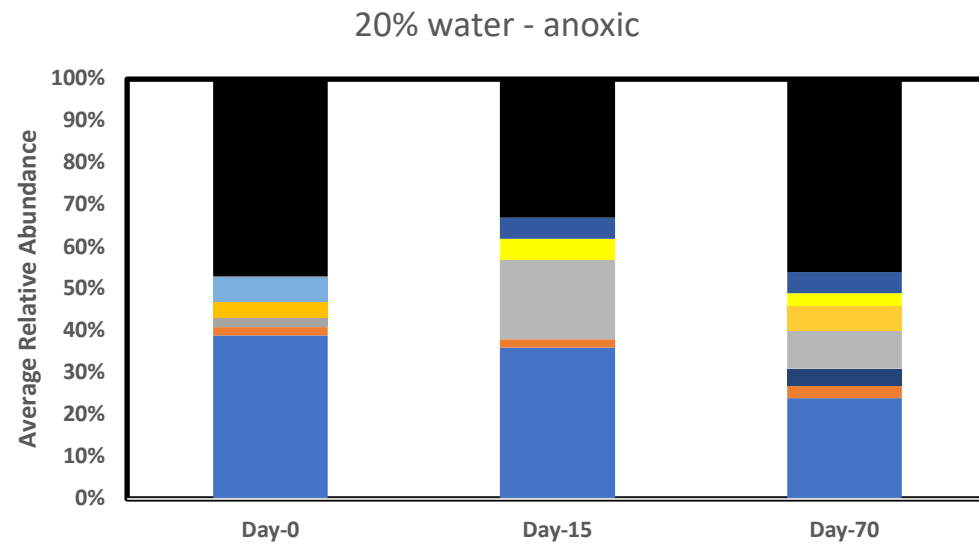
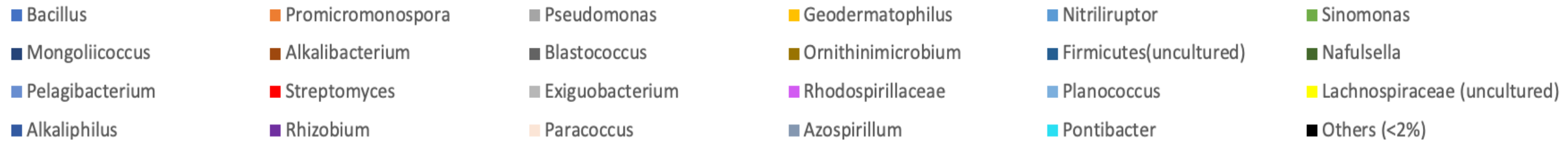
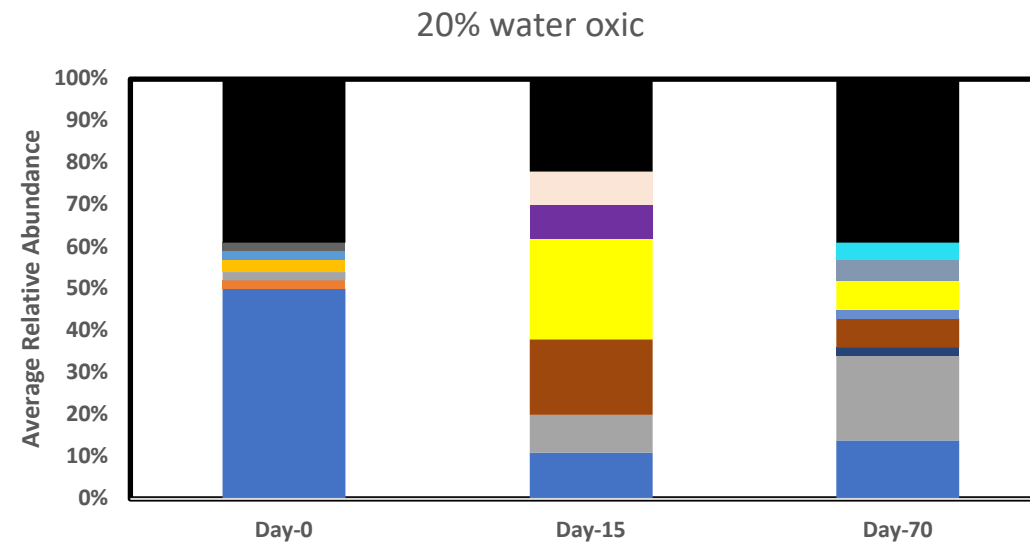
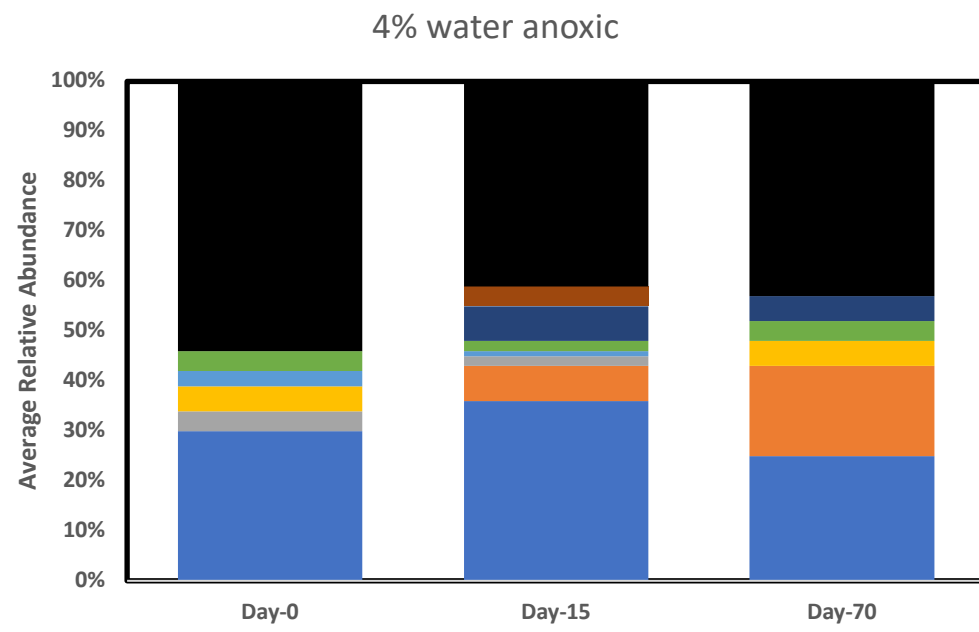
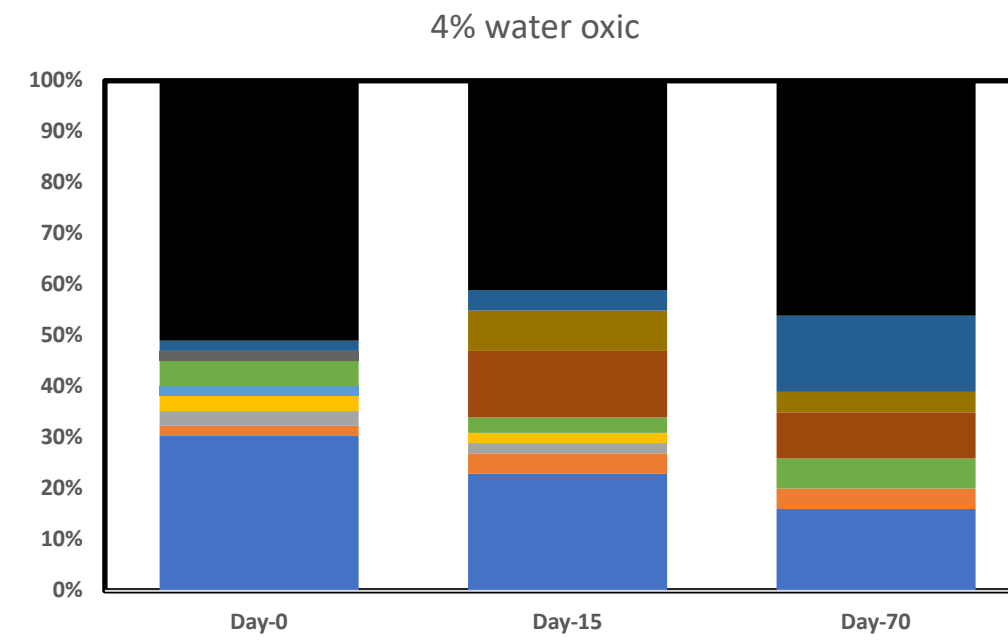
(A)**(B)****(C)****(D)**

Figure 4.8: Genus level representation of bacterial community found in the wheat starch microcosms on days 0, 15 and 70 under the four incubatory conditions (A – D). Each treatment is the average of two replicates. Community analyses are based on the sequencing of 16S rRNA gene amplicons. Samples were rarified to 1,100 reads. Only genera that represented >1% of the total relative abundance are shown.

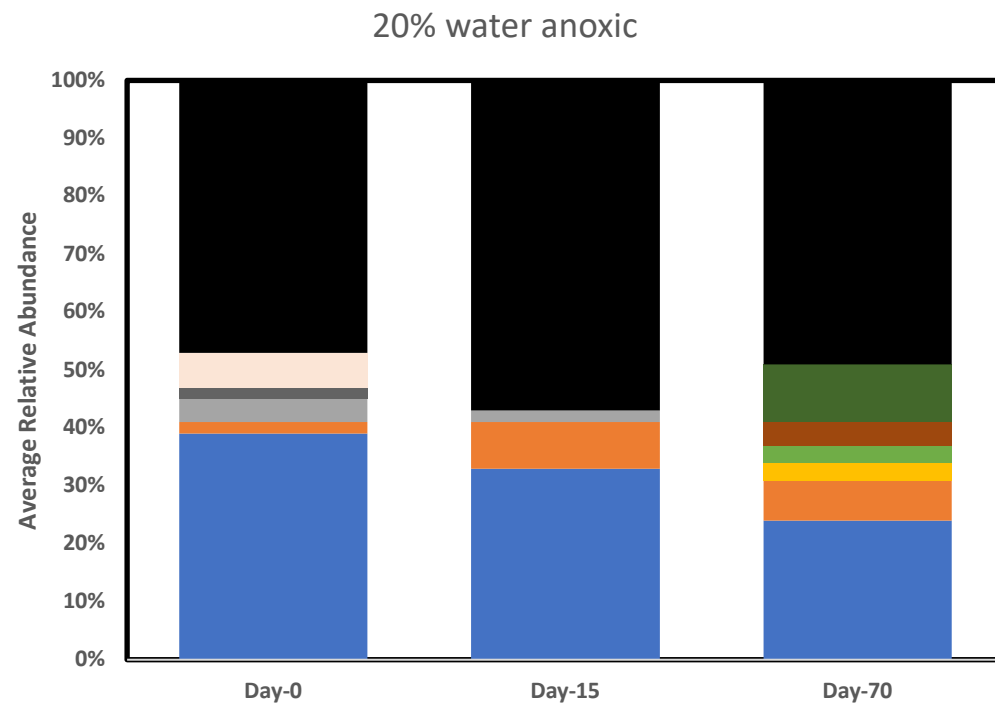
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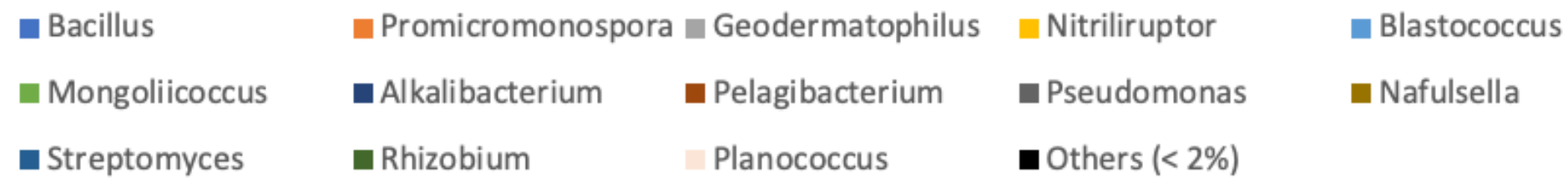
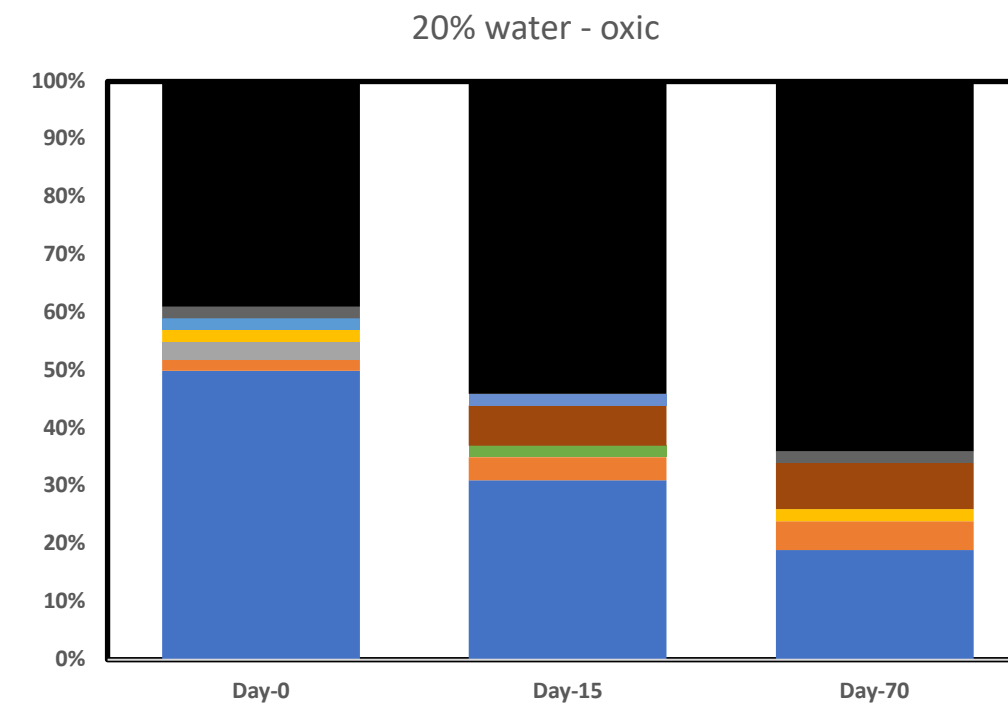
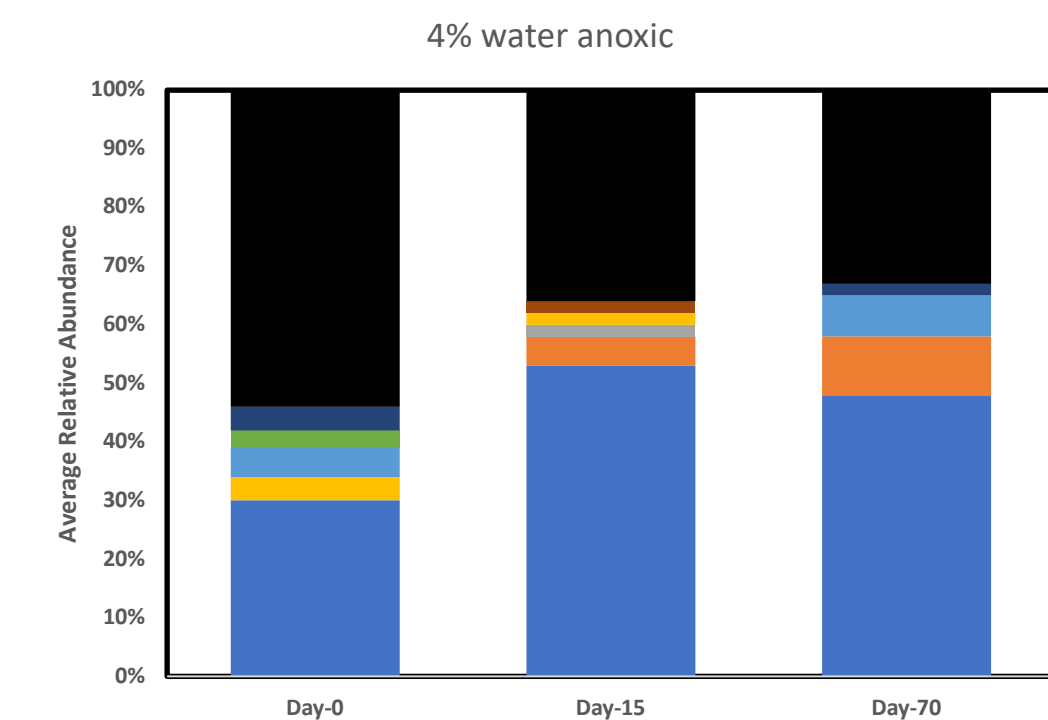


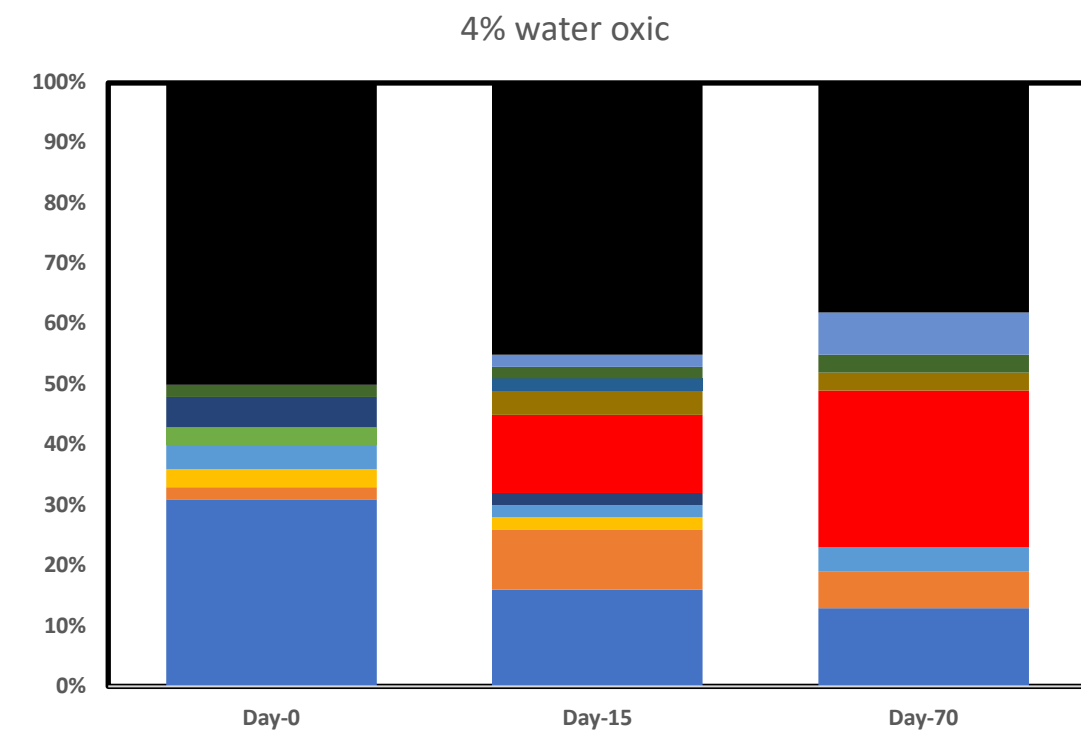
Figure 4.9: Genus level representation of the bacterial community found in the corn starch microcosms on days 0, 15 and 70 under the four incubatory conditions (A – D). Each treatment is the average of two replicates. Community analyses are based on the sequencing of 16S rRNA gene amplicons. Samples were rarified to 1,100 reads. Only genera that represented >1% of the total relative abundance are shown

(A)

(B)



(C)



(D)

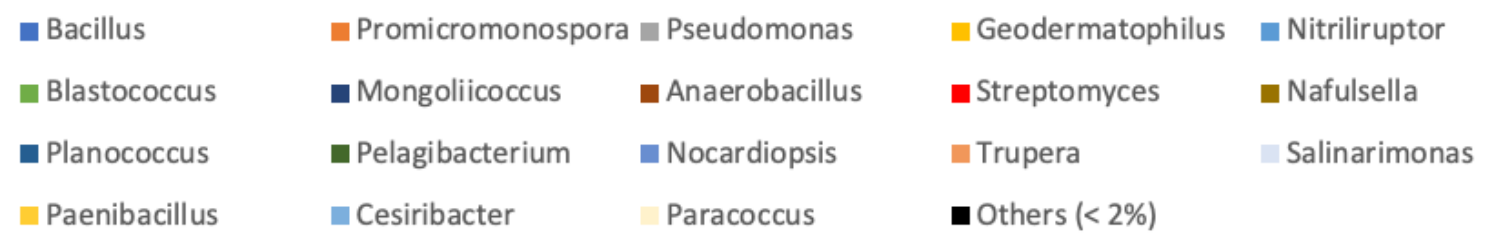
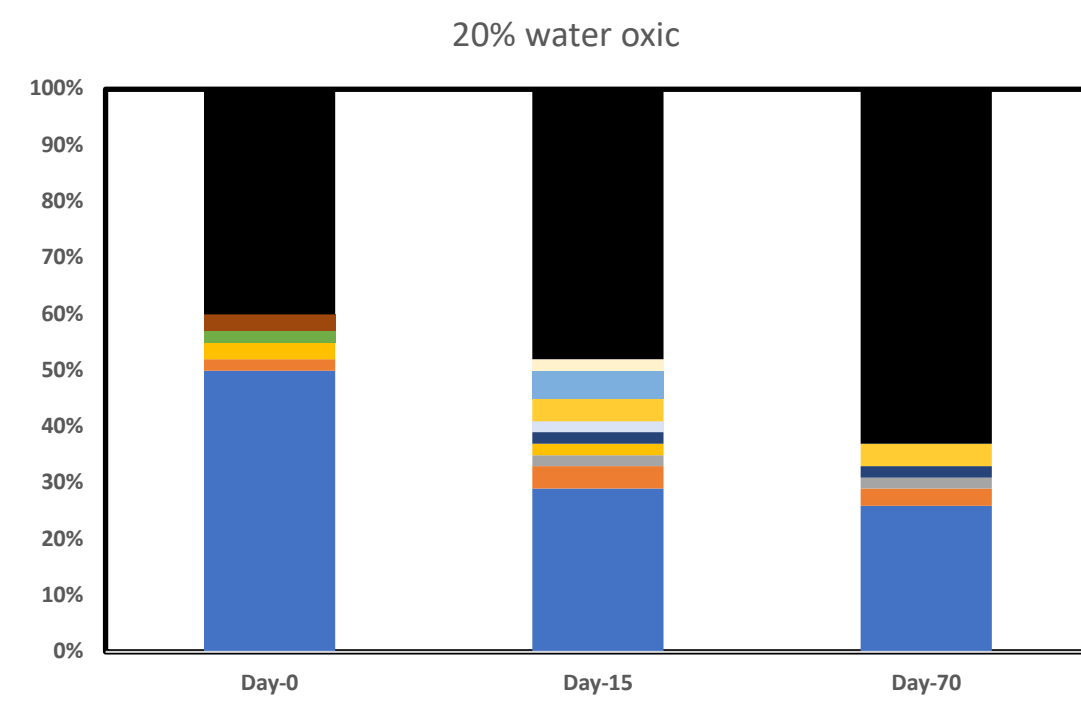
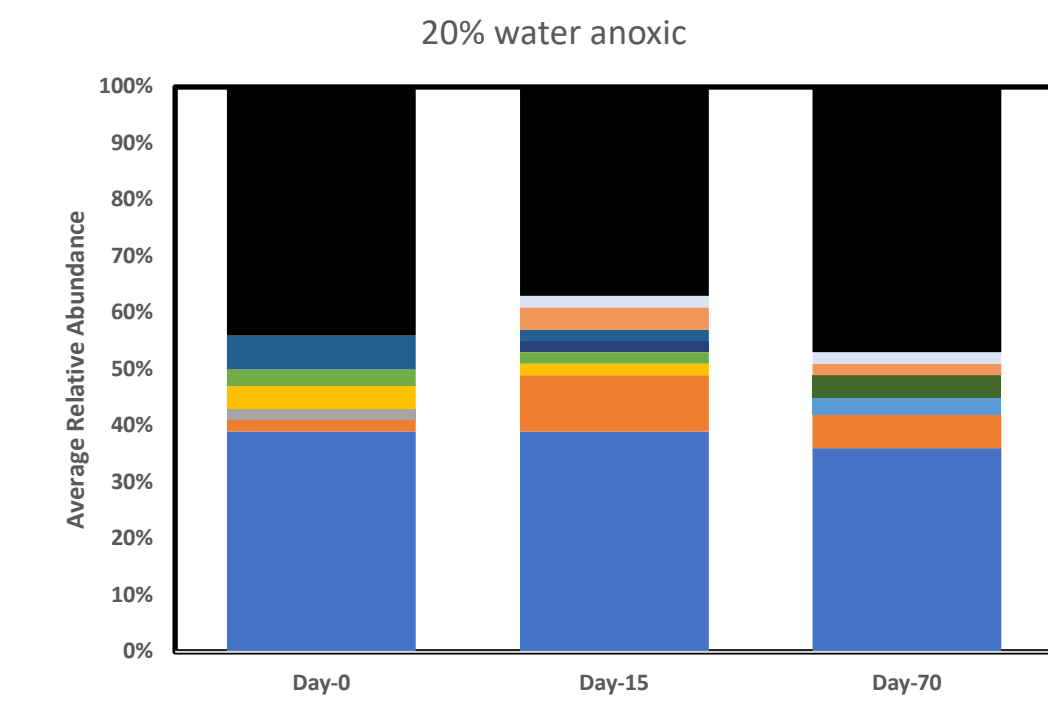
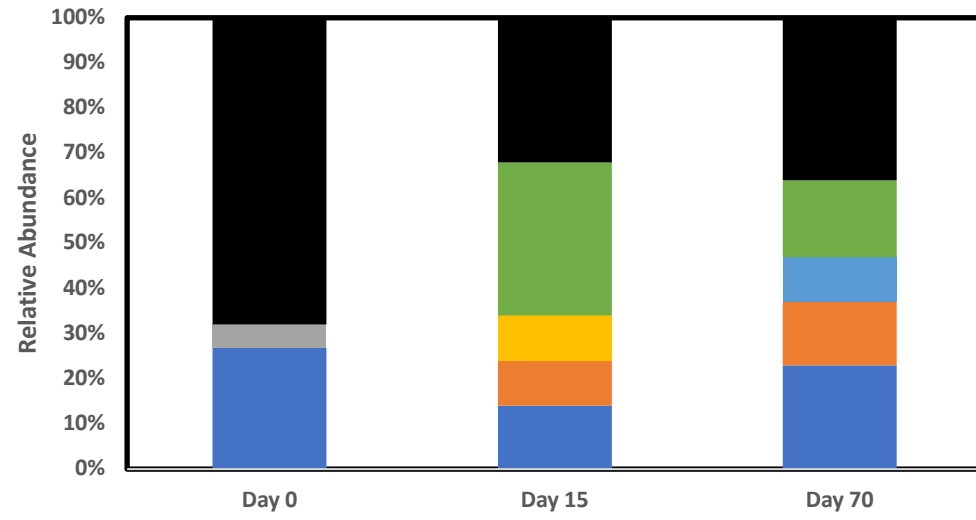


Figure 4.10: Genus level representation of the bacterial community found in the potato starch microcosms on days 0, 15 and 70 under the four incubatory conditions (A – D). Each treatment is the average of two replicates. Community analyses are based on the sequencing of 16S rRNA gene amplicons. Samples were rarified to 1,100 reads. Only genera that represented >1% of the total relative abundance are shown

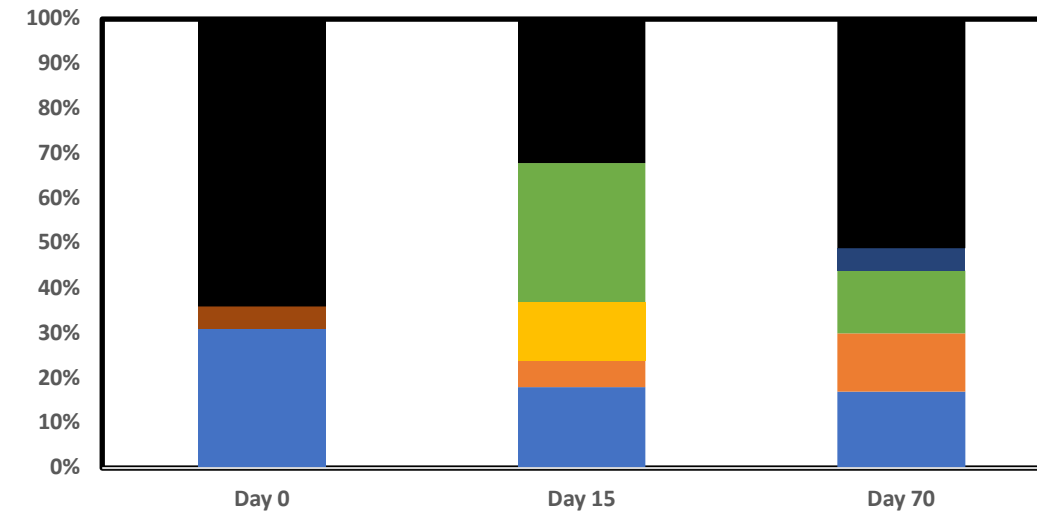
(A)

4% water anoxic



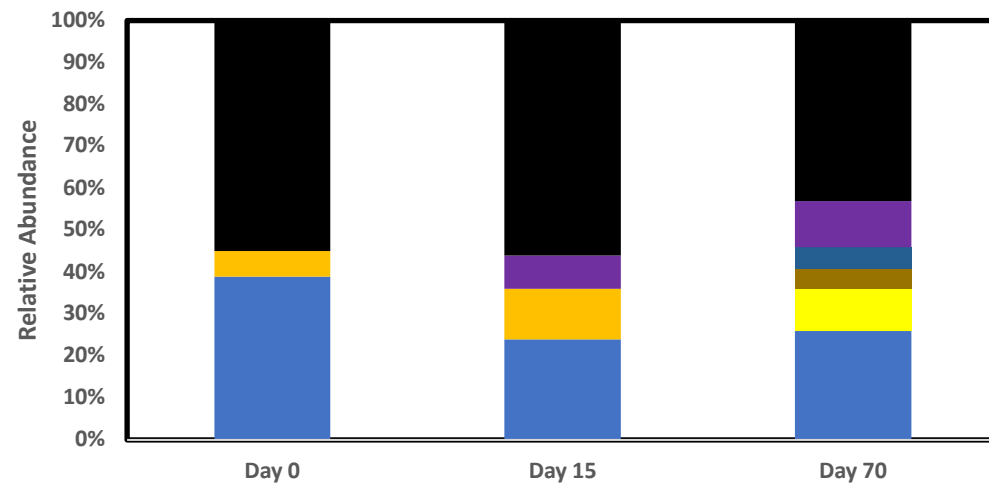
(B)

4% water oxic



(C)

20% water anoxic



(D)

20% water oxic

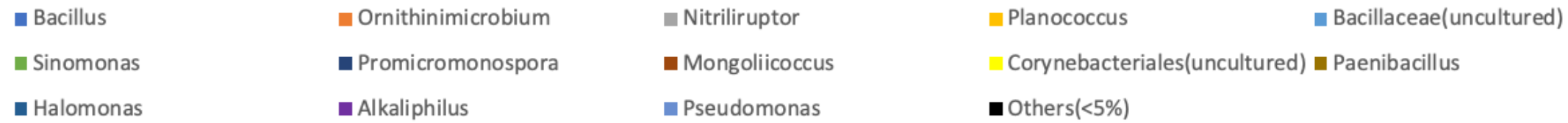
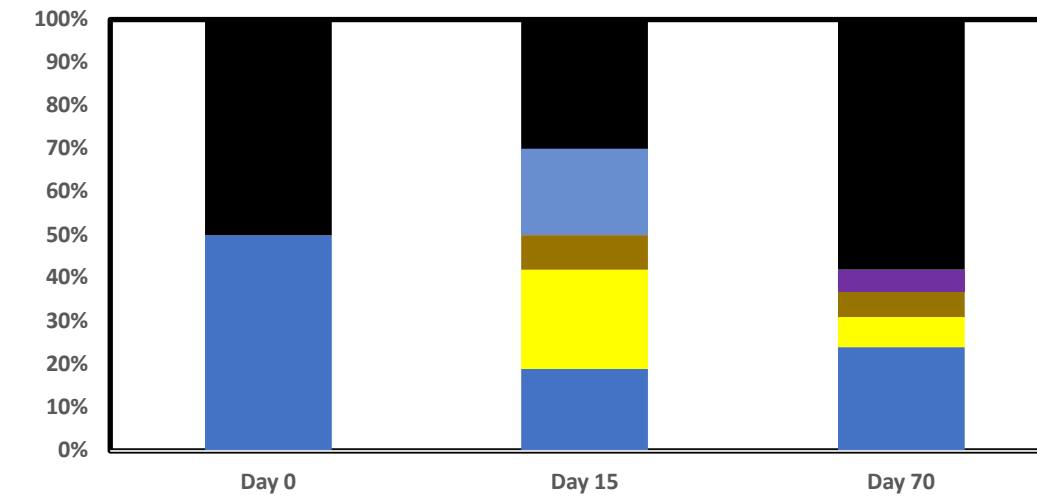


Figure 4.11: Composition of the dominant genera present in the bacterial community found in the calcium phosphate-treated wheat starch microcosms on days 0, 15 and 70 under the four incubatory conditions. Each treatment is made up of just one replicate. Community analyses are based on the sequencing of 16S rRNA gene amplicons. Only genera that represented >1% of the total relative abundance are shown

(A)

(B)

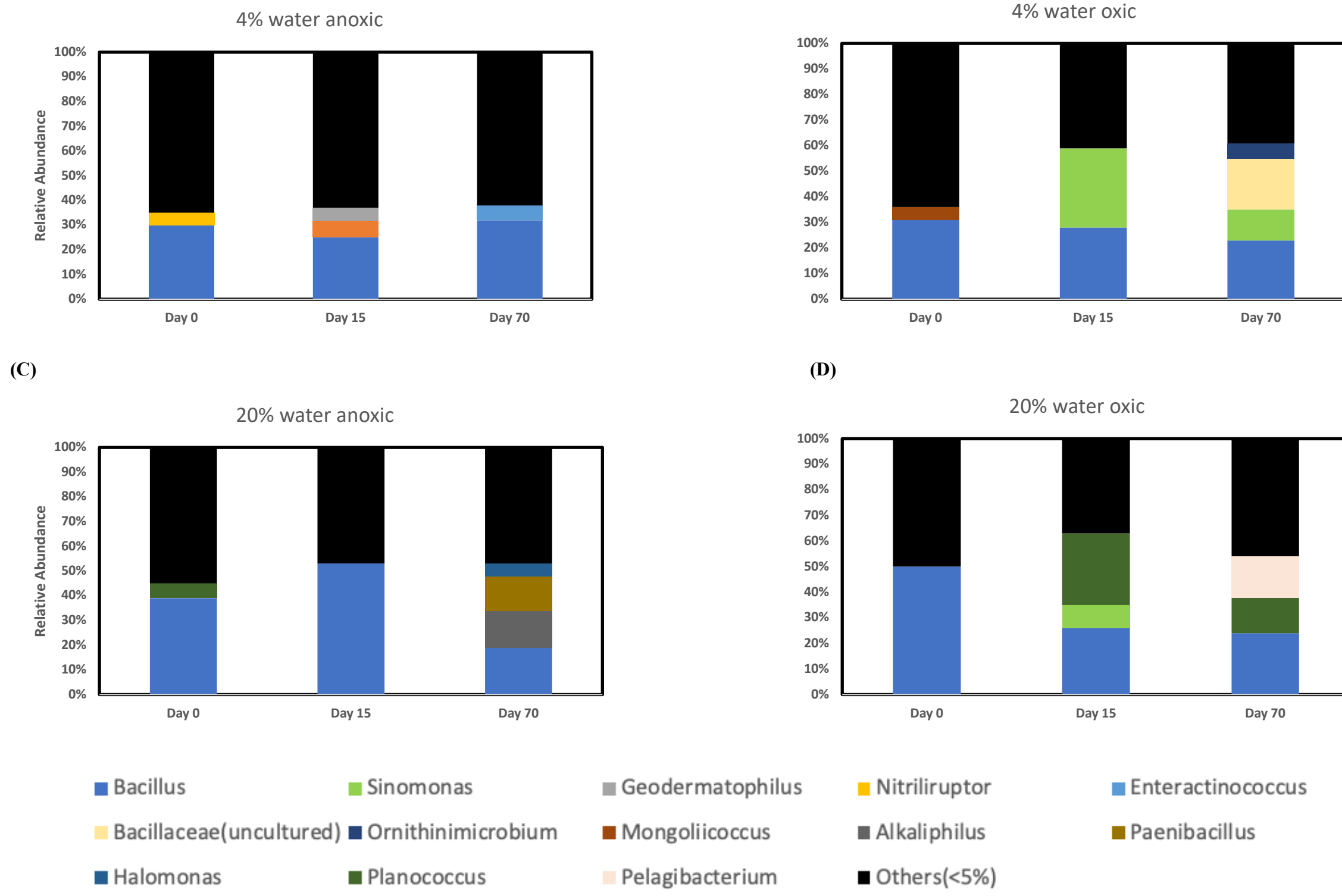


Figure 4.12: Composition of the dominant genera present in the bacterial community found in the calcium phosphate-treated corn starch microcosms on days 0, 15 and 70 under the four incubatory conditions. Each treatment is made up of just one replicate. Community analyses are based on the sequencing of 16S rRNA gene amplicons. Only genera that represented >1% of the total relative abundance are shown

(A) (B)

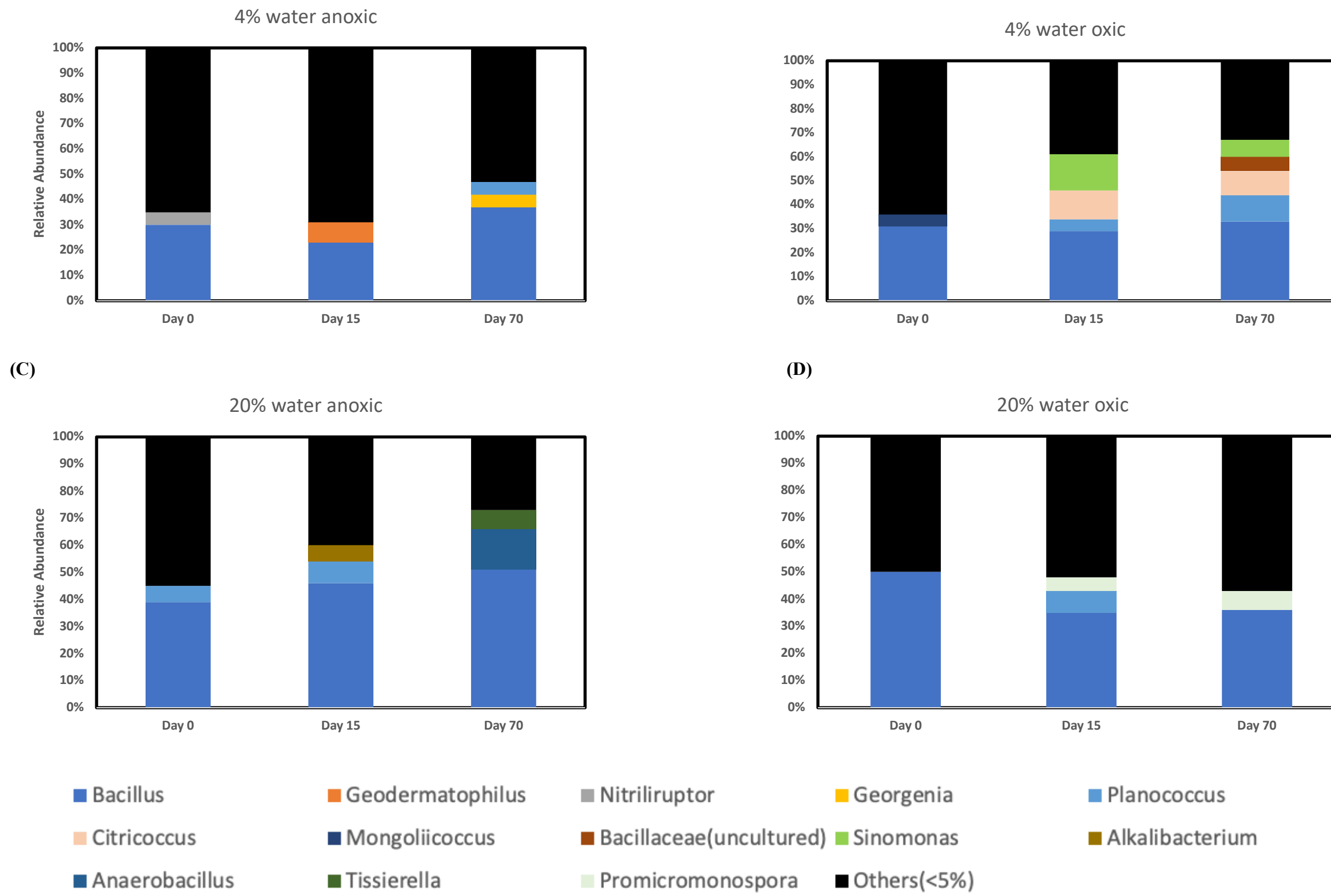


Figure 4.13: Composition of the dominant genera present in the bacterial community found in the calcium phosphate-treated potato starch microcosms on days 0, 15 and 70 under the four incubatory conditions. Each treatment is made up of just one replicate. Community analyses are based on the sequencing of 16S rRNA gene amplicons. Only genera that represented >1% of the total relative abundance are shown

A non-metric multidimensional scaling (NMDS) plot in Figure 4.15 shows that the microbial communities present in the wheat starch microcosms were different from the microbial communities in the controls on both days 15 and 70. This confirms that microbial communities differentiate based on the addition of starch to the microcosms. Furthermore, the NMDS plot in Figure 4.16 shows the clustering of microbial communities based on water content and day of sampling. It can be seen that samples with 4% water content are clustered differently from samples with 20% water content. This shows that soil moisture content exerts a strong influence on microbial communities degrading starches from different botanical sources in the soil.

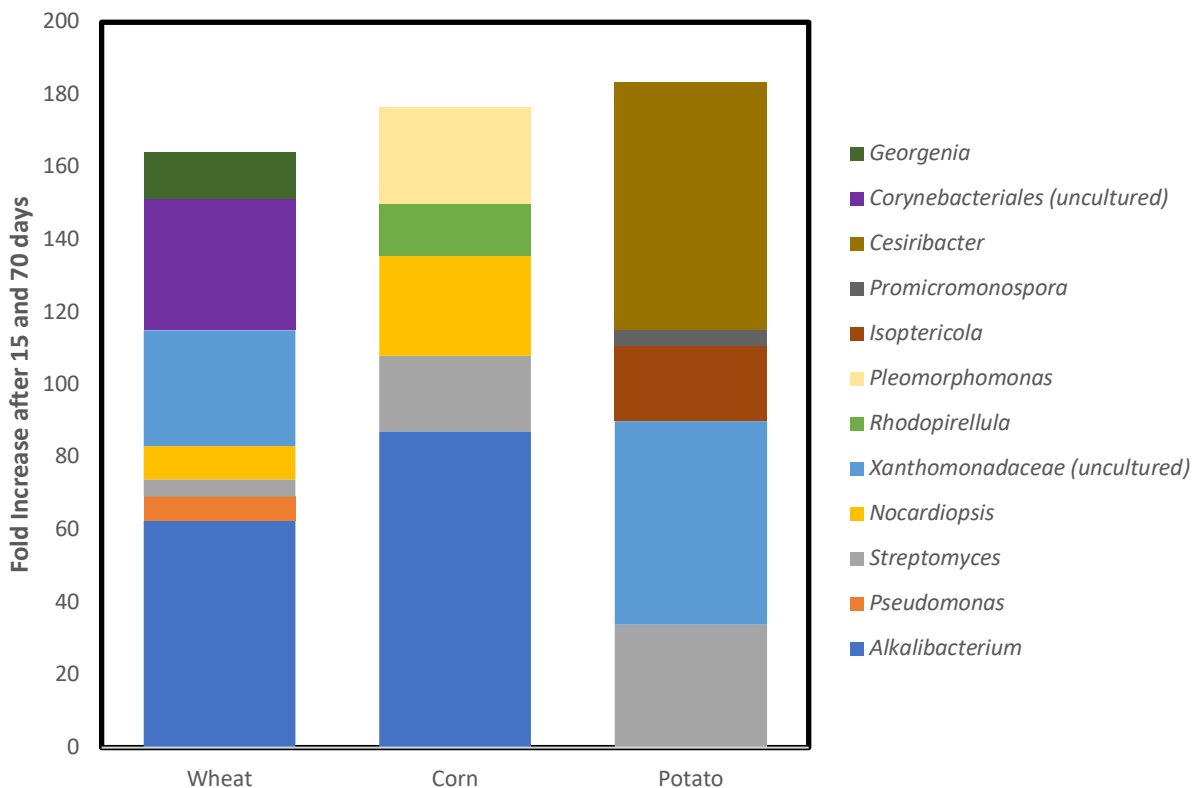


Figure 4.14: Schematic graph of the major starch-responsive genera with their fold increases (in the oxic conditions) across the three starch treatments in Experiment 1. ‘Uncultured’ indicates that the genus is undescribed and can only be identified to a higher taxonomic level

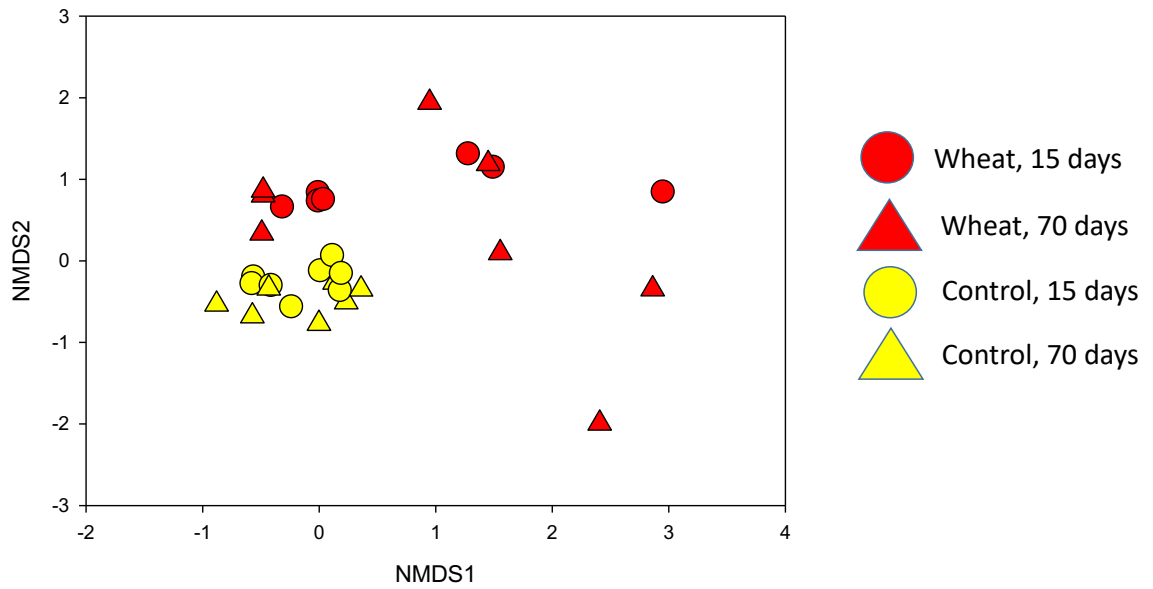


Figure 4.15: Non-metric multidimensional scaling plot (NMDS) of microbial communities present in the wheat starch and control microcosms. Plot is based on a Weighted Unifrac comparison and shows the separation of microbial communities in the wheat starch microcosms from that of the control microcosms.

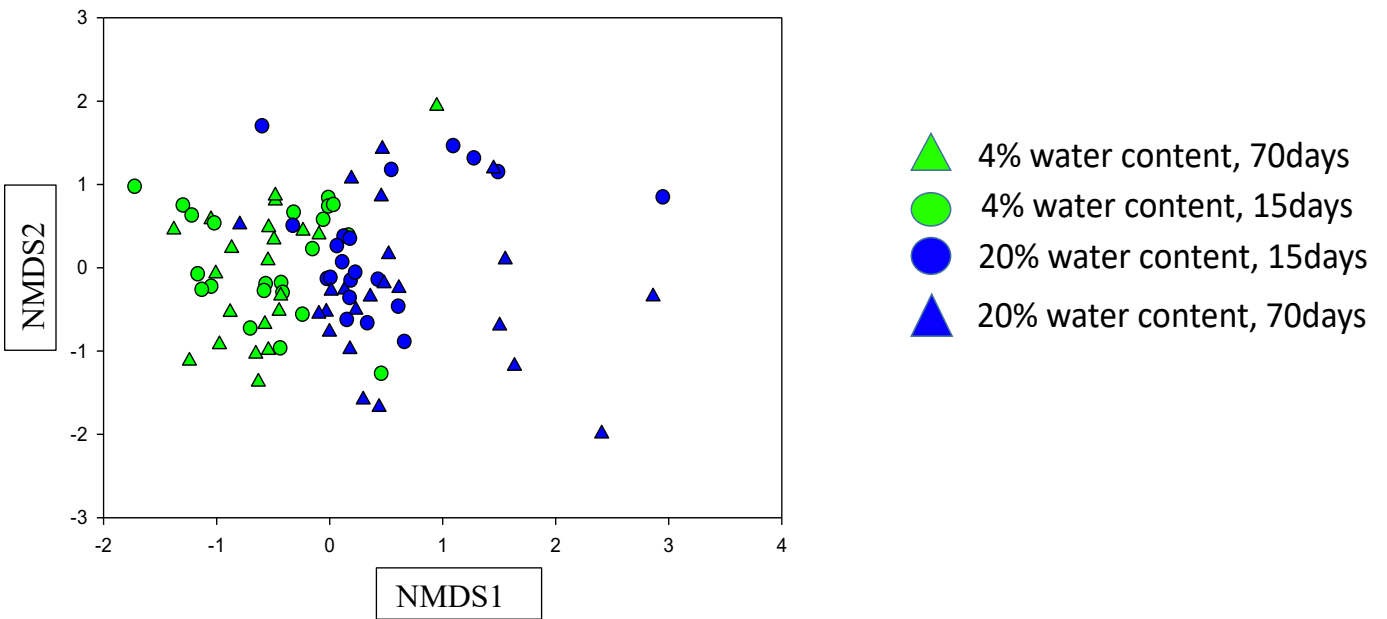


Figure 4.16: Non-metric multidimensional scaling (NMDS) plot of microbial communities present in the wheat, potato and corn microcosms at the 4% and 20% water content on day 15 and 70. Plot is based on a Weighted Unifrac comparison and shows the differentiation of microbial communities based on water content as communities of the 4% water content are clustered separately from the communities of the 20% water content.

Table 4.6: Genera with fold increases ≥ 2 in all four treatment groups for wheat, corn and potato starches in Experiment 1, when comparing day 0 to day 15. More detailed tables ordered by the fold increases are given in the appendix (Supplementary Tables 1-3). "Uncultured" indicates that the genus is undescribed and can only be identified to a higher taxonomic level.

Starch	Incubation condition	Most responding Genera or OTUs (Enclosed in parentheses are the fold increases)
Wheat	4% water – anoxic	<i>Alkaliphilus</i> (327.0), <i>Alkalibacterium</i> (64.9), <i>Georgenia</i> (24.8), <i>Exiguobacterium</i> (13.6), <i>Actinotalea</i> (4.8), <i>Sinomonas</i> (3.0), <i>Kocuria</i> (2.9)
Wheat	4% water – oxic	<i>Alkalibacterium</i> (29.4), <i>Georgenia</i> (4.3), <i>Sinomonas</i> (4.2), <i>Kocuria</i> (3.5), <i>Nocardiopsis</i> (9.3), <i>Streptomyces</i> (5.6), <i>Nesterenkonia</i> (26.4)
Wheat	20% water – anoxic	<i>Lachnospiraceae</i> (346.1), <i>Anaerovirgula</i> (283.8), <i>Exiguobacterium</i> (64.1), <i>Anaerobranca</i> (58.9), <i>Rhizobium</i> (52.2), <i>Alkaliphilus</i> (22.5), <i>Peptostreptococcaceae (uncultured)</i> (11.3)
Wheat	20% water – oxic	<i>Xanthomonadaceae (uncultured)</i> (31.8), <i>Pseudomonas</i> (10.7), <i>Devosia</i> (9.6), <i>Mongoliicoccus</i> (4.9), <i>Pelagibacterium</i> (3.7), <i>Geobacillus</i> (2.7), <i>Pseudaminobacter</i> (3.2)
Corn	4% water – anoxic	<i>Alkalibacterium</i> (7.2), <i>Promicromonospora</i> (4.8), <i>Actinotalea</i> (3.1)
Corn	4% water – oxic	<i>Pleomorphomonas</i> (26.6), <i>Pelagibacterium</i> (7.4), <i>Nocardiopsis</i> (6.7), <i>Rhizobiales (uncultured)</i> (9.1), <i>Lysobacter</i> (4.4), <i>Actinotalea</i> (2.4), <i>Pseudaminobacter</i> (5), <i>Streptomyces</i> (4.2)
Corn	20% water – anoxic	<i>Alkalibacterium</i> (15.5), <i>Promicromonospora</i> (5.2), <i>Actinotalea</i> (5.2), <i>Lachnospiraceae (uncultured)</i> (232.3), <i>Clostridiales (uncultured)</i> (38.3), <i>Paenibacillus</i> (7.2), <i>Gemmatimonadetes (uncultured)</i> (5.4), <i>Salinibacterium</i> (23.3)

Corn	20% water – oxic	<i>Alkalibacterium</i> (87.2), <i>Pontibacter</i> (33.1), <i>Rhodopirellula</i> (14.1), <i>Pelagibacterium</i> (11.6), <i>Salinibacterium</i> (12.2), <i>Paenibacillus</i> (4.9)
Potato	4% water – anoxic	<i>Anaerobacillus</i> (3.8), <i>Bacillus</i> (2), <i>Promicromonospora</i> (2.0)
Potato	4% water – oxic	<i>Isoptericola</i> (20.8), <i>Streptomyces</i> (16.4), <i>Nocardiopsis</i> (9.7), <i>Promicromonospora</i> (4.4), <i>Actinotalea</i> (3.5), <i>Gemmatimonadetes</i> (uncultured) (2.6)
Potato	20% water – anoxic	<i>Anaerobranca</i> (35.4), <i>Aquamicrobium</i> (11.7), <i>Promicromonospora</i> (7.1), <i>Alkaliphilus</i> (5.9), <i>Devosia</i> (5.8), <i>Actinotalea</i> (5.3), <i>Pelagibacterium</i> (2.9), <i>Salinibacterium</i> (2.8)
Potato	20% water – oxic	<i>Cesiribacter</i> (754.7), <i>Alkaliphilus</i> (111.1), <i>Xanthomonadaceae</i> (uncultured) (56.2), <i>Lachnospiraceae</i> (uncultured) (45.1), <i>Rhizobium</i> (39.2), <i>Rhodopirellula</i> (71.5), <i>Rhizobiales</i> (uncultured) (42.1), <i>Mongoliicoccus</i> (16.2)

4.4 Bacterial communities in the *Ipomoea longituba* and potato starch microcosms

In the second experiment, soil samples collected from the soil–starch microcosms at days 0, 6, 14, 41, 77 and 133 were processed for 16S rRNA gene sequencing. Figure 4.17 to Figure 4.19 summarize relative abundance data from the community analysis using QIIME and shows the dominant microorganisms (at the genus taxonomic level) growing on the soil–starch incubations based on the starch type and incubatory conditions in the second experiment. The genera that showed at least 2-fold increases in relative abundance over time are also summarized in Table 4.7. More detailed tables of the genera that increased in response to starch treatments are given in the Appendix (Supplementary Tables 4 – 5), ordered in terms of their fold increases. Table 4.8 shows the taxonomy of each genus that will be discussed in this section.

For *Ipomoea longituba*, *Bacillus* had a high initial abundance in the 7% water – anoxic treatment with its highest average relative abundance of 70% at day 6. This is probably because the *Ipomoea longituba* starch was not sterilized before addition to soil and may have contained *Bacillus* spores (the day 0 sample was taken before the starch addition). Therefore, we will compare later days to day 6 and not to day 0. From day 6 in the 7% water – oxic condition, the high initial *Bacillus* population gradually decreased to 41% on day 133 (see Figure 4.18B). Microbial taxa that increased to a relative abundance greater or equal to 4% included *Nitriliruptor*, *Euzebya* and *Propionibacteriaceae* (uncultured genus). *Nitriliruptor* and *Euzebya* had relative abundances that slightly increased from day 14 to day 133 (see Figure 4.18B). In the 12% water – anoxic condition, *Bacillus* had an average relative abundance of 47% at day 6 which gradually decreased as the days passed by to 15% at day 133. From Figure 4.18C, microbial taxa that increased in the 12% water – anoxic condition includes *Anaerobacillus*, *Streptomyces* and *Propionibacteriaceae* (uncultured species). Microbial clades that showed up at a relative

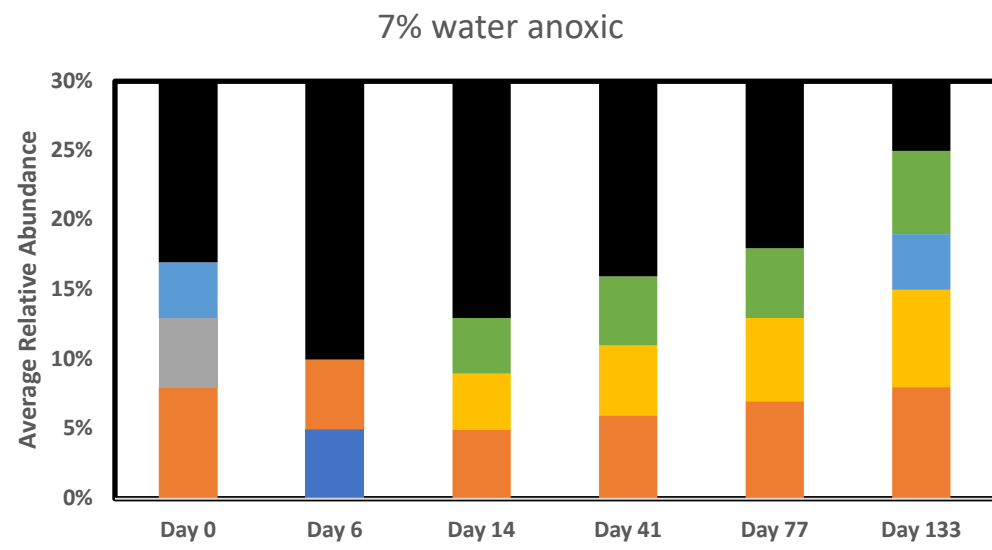
abundance level greater or equal to 4% in the 12% water – oxic condition include *Nocardioides*, *Streptomyces*, *Isoptericola* and *Bacilli* (uncultured genus).

For potato starch, *Bacillus* did not dominate the initial microbial community as it did with the *Ipomoea longituba* starch (see Figure 4.17) probably because the *Bacillus* was introduced into the microcosms when the *Ipomoea longituba* starch was introduced at the beginning of the experiment. Instead *Streptomyces* dominated the microbial community in the 7% water – oxic condition with the average relative abundance gradually increasing from day 6 to day 133. Also, in this 7% water oxic condition, the average relative abundance of *Glycomyces* increased over time from day 6 to day 133 (see Figure 4.17B). In the 12% water – anoxic condition, the average relative abundance of *Streptomyces* and *Nitriliruptor* increased over time from day 6 to day 133 (see Figure 4.16C). In the 12% water – oxic condition, the relative abundance of *Streptomyces* increased from day 6 to day 41 and it dominated the microbial community on day 41 (see Figure 4.17D). Two other clades that had an increased abundance over time in the 12% water – oxic condition were *Nocardioides* and *Isoptericola* (see Figure 4.17D).

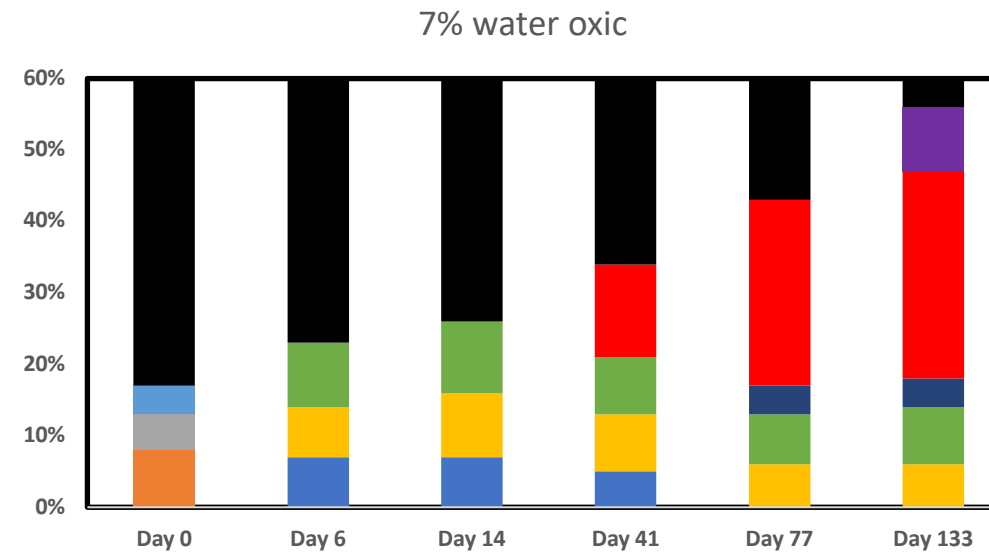
From Figure 4.19A to Figure 4.19D, we can see that most of the dominant taxa in the control microcosms had stable populations meaning that their relative abundances did not seem to increase over time except for *Nitriliruptor*. In the 12% water – anoxic condition, the average relative abundance of *Nitriliruptor* increased over time from day 6 to day 133 (see Figure 4.19C) while in the 7% water – anoxic condition, the relative abundance of *Nitriliruptor* also increased from day 41 to day 133 (see Figure 4.19A). The highest relative abundance of any clade in the control microcosms was by *Nitriliruptor* (9% at day 77) in the 12% water – anoxic condition. In the 12% water – oxic condition, *Nitriliruptor* and *Euzebya* occurred in all the days from day 6 to day 133 at an average relative abundance level that did not exceed 5% (see Figure 4.19D). Overall,

control communities were quite consistent over time, suggesting that community changes in the other treatments were due to starch metabolism rather than to incubation in the lab. The genera that showed the most increases over time in experiment 2 are shown in Table 4.7

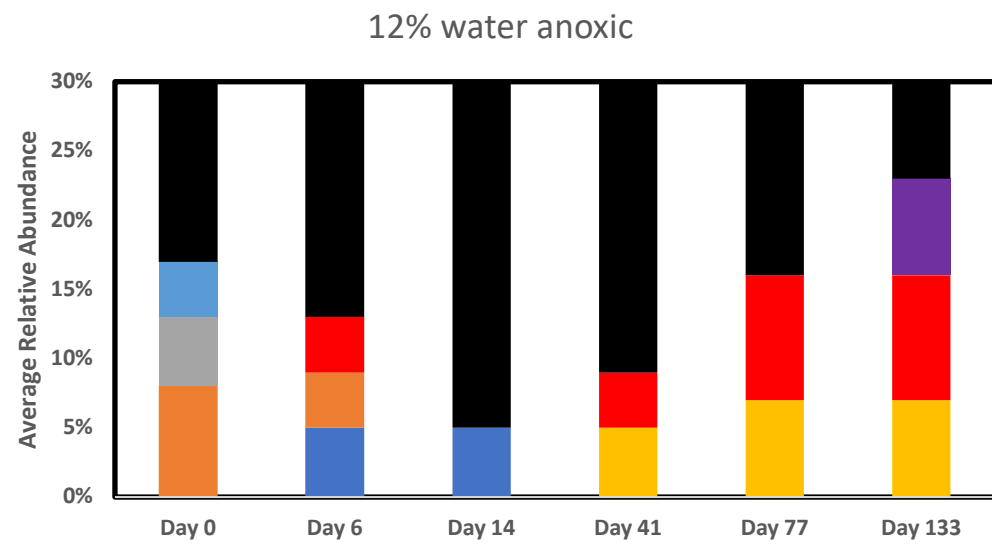
(A)



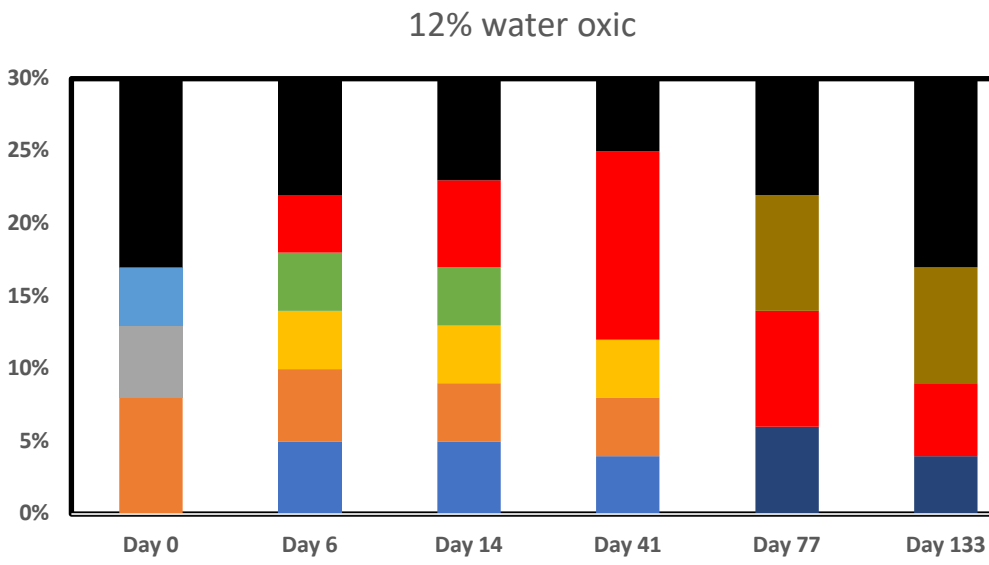
(B)



(C)



(D)

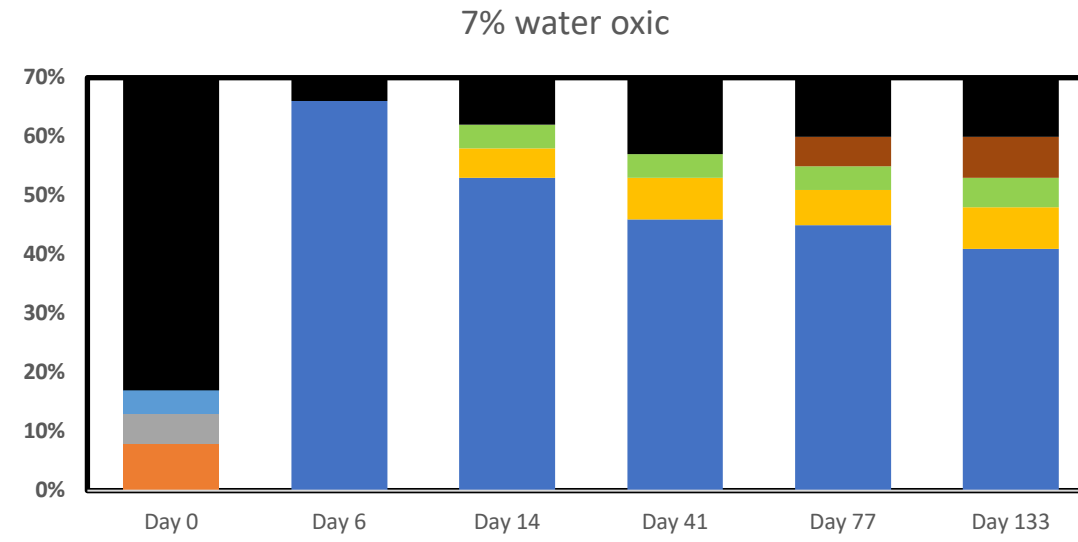
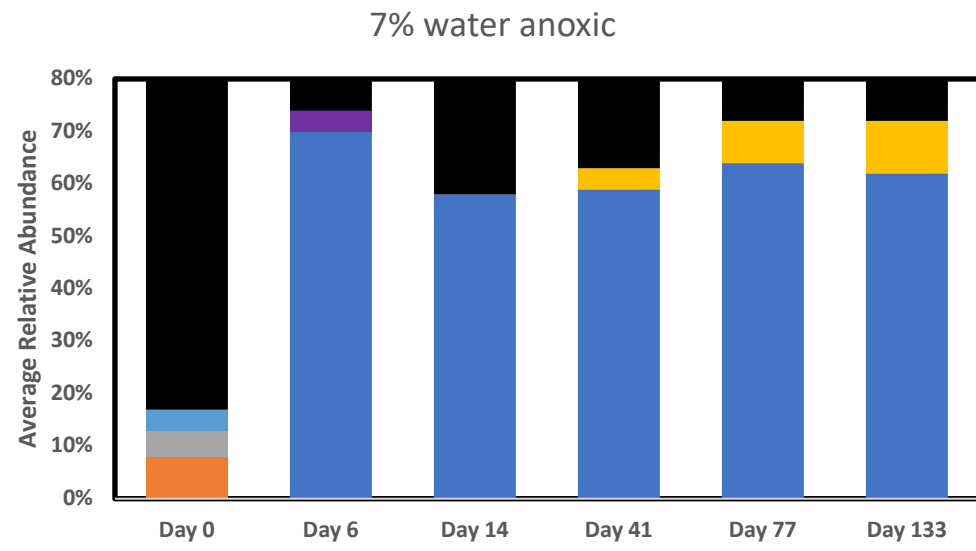


- Bacillus
- Gaiellales (uncultured)
- Gemmatimonadaceae (uncultured)
- Nitriluptor
- Solirubrobacterales (uncultured)
- Euzebya
- Nocardiosis
- Streptomyces
- Glycomyces
- Isoptericola
- Others (<4%)

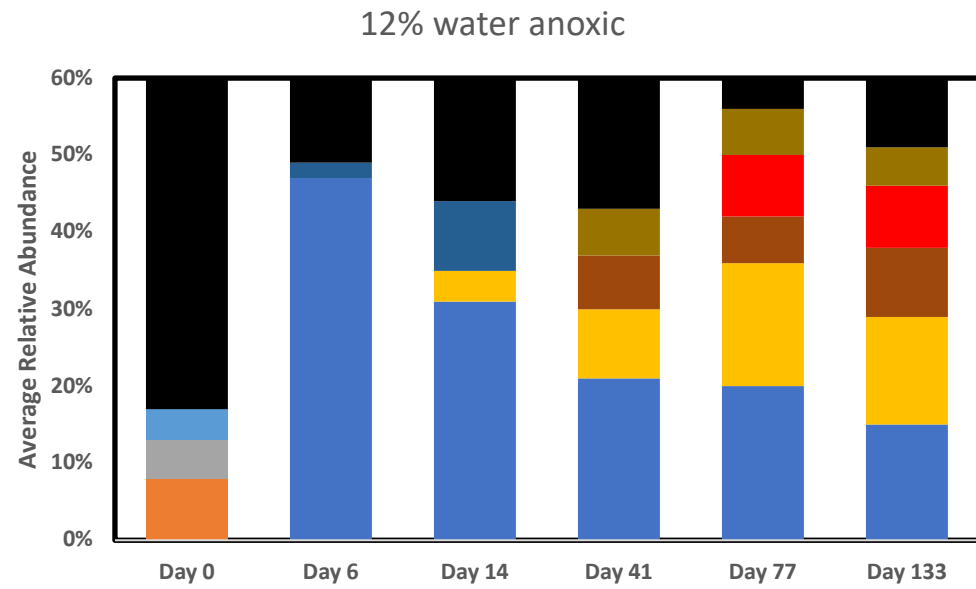
Figure 4.17: Genus-level representation of microbial communities found in the potato starch microcosms from day 0 to day 133 under the four incubatory conditions (A – D) in the second experiment. Each treatment is the average of three replicates. Community analyses are based on the sequencing of 16S rRNA genes. Samples were rarified to 1,400 reads. Only genera that represented >1% of the total relative abundance are shown.

(A)

(B)



(C)



(D)

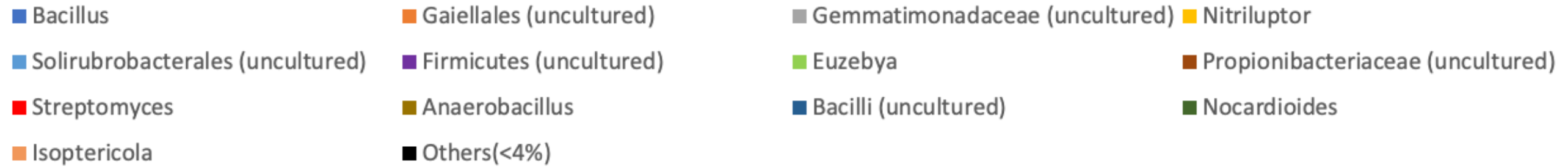
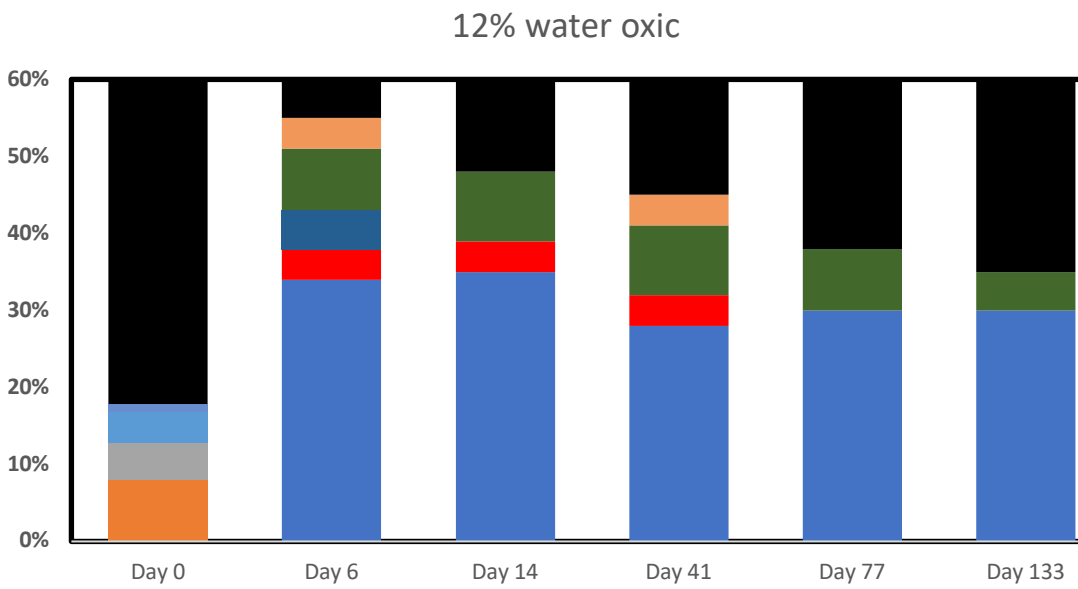
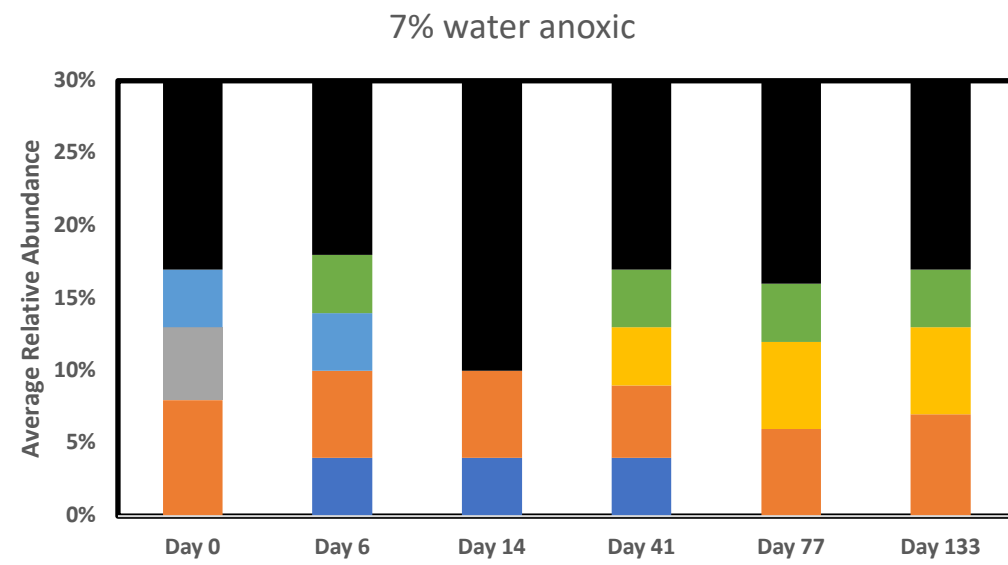
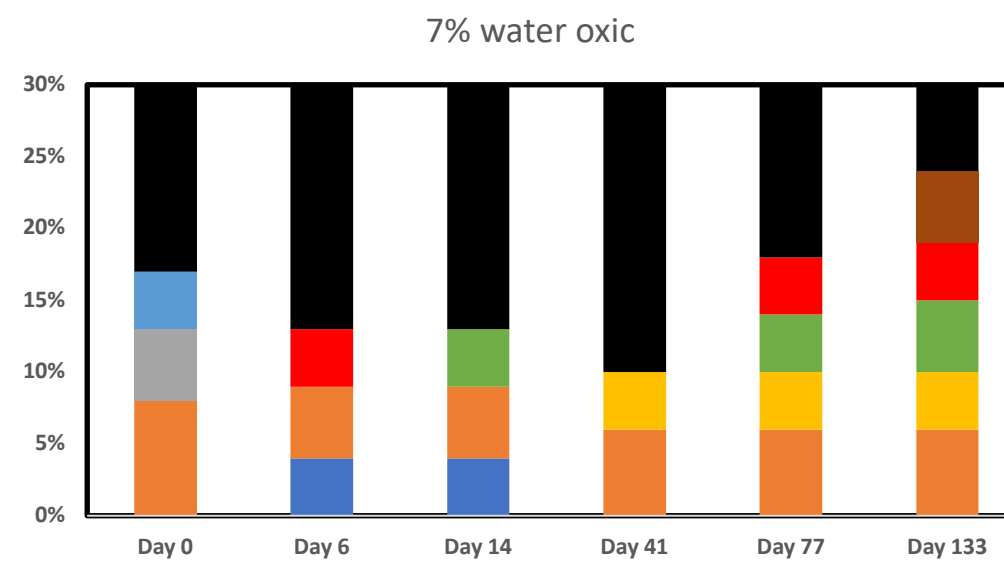
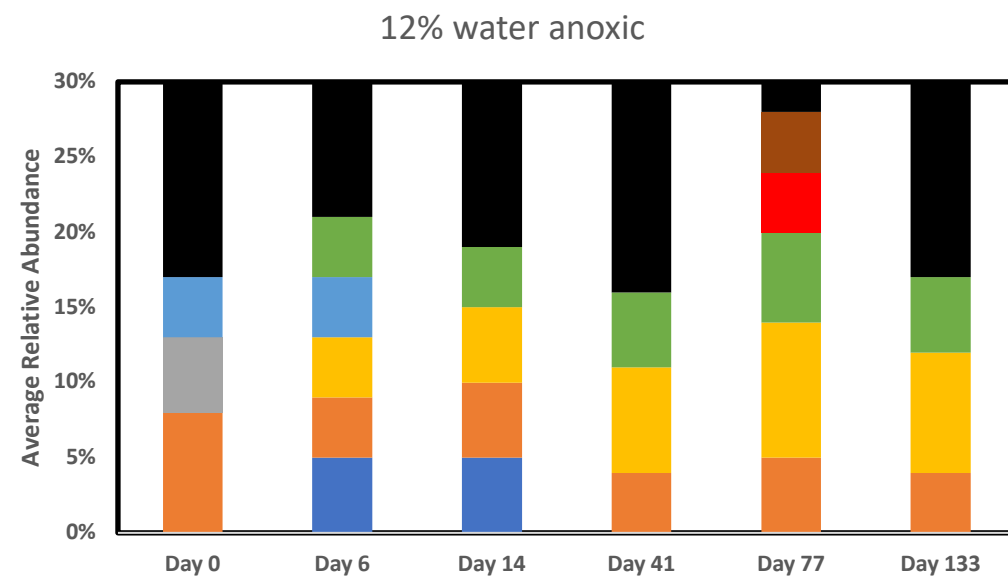
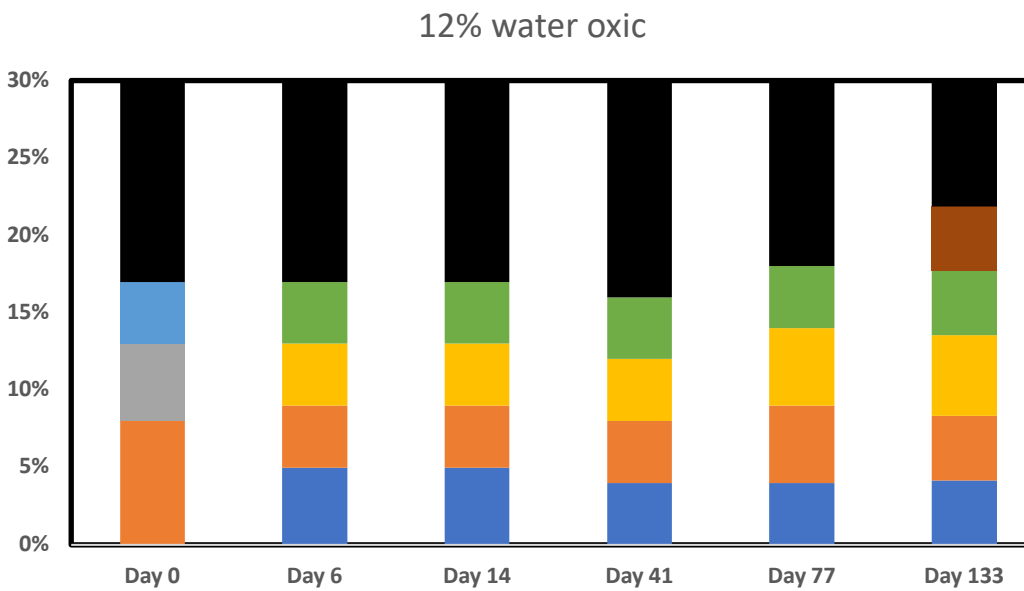


Figure 4.18: Genus-level representation of microbial communities found in the *Ipomoea longituba* starch microcosms from day 0 to day 133 under the four incubatory conditions (A – D) in the second experiment. Each treatment is the average of three replicates. Community analyses are based on the sequencing of 16S rRNA genes. Samples were rarified to 1,400 reads. Only genera that represented >1% of the total relative abundance are shown.

(A)**(B)****(C)****(D)**

■ Bacillus

■ Gaiellales (uncultured)

■ Gemmatimonadaceae (uncultured)

■ Nitriluptor

■ Solirubrobacterales (uncultured)

■ Euzebya

■ Streptomyces

■ Actinobacteria (uncultured)

■ Others (<4%)

Figure 4.19: Genus-level representation of microbial communities found in the control microcosms from day 0 to day 133 under the four incubatory conditions (A – D) in the second experiment. Each treatment is the average of three replicates. Community analyses are based on the sequencing of 16S rRNA genes. Samples were rarified to 1,400 reads. Only genera that represented >1% of the total relative abundance are shown.

The non-metric multidimensional scaling (NMDS) plot shown in Figure 4.20 shows that the microbial communities present in the *Ipomoea longituba* microcosms clustered apart from the microbial communities present in the potato, control and control day 0 microcosms. This shows that the microbial communities growing on *Ipomoea longituba* on days 6, 14, 41, 77 and 133 were different from the microbial communities growing on all other microcosms at those same days. This difference is partly due to the high initial abundance of *Bacillus* in the *Ipomoea longituba* microcosms. However, it is also interesting to note that changes in the microbial community structure over time in the 7% water – oxic condition were revealed by another NMDS plot shown in Figure 4.21 for both *Ipomoea longituba* and potato starches. Microbial communities were clustered by the day of incubation in a gradient-like manner so that the microbial communities present in the *Ipomoea* and potato microcosms on a particular day of DNA sampling were different from those present on other days.

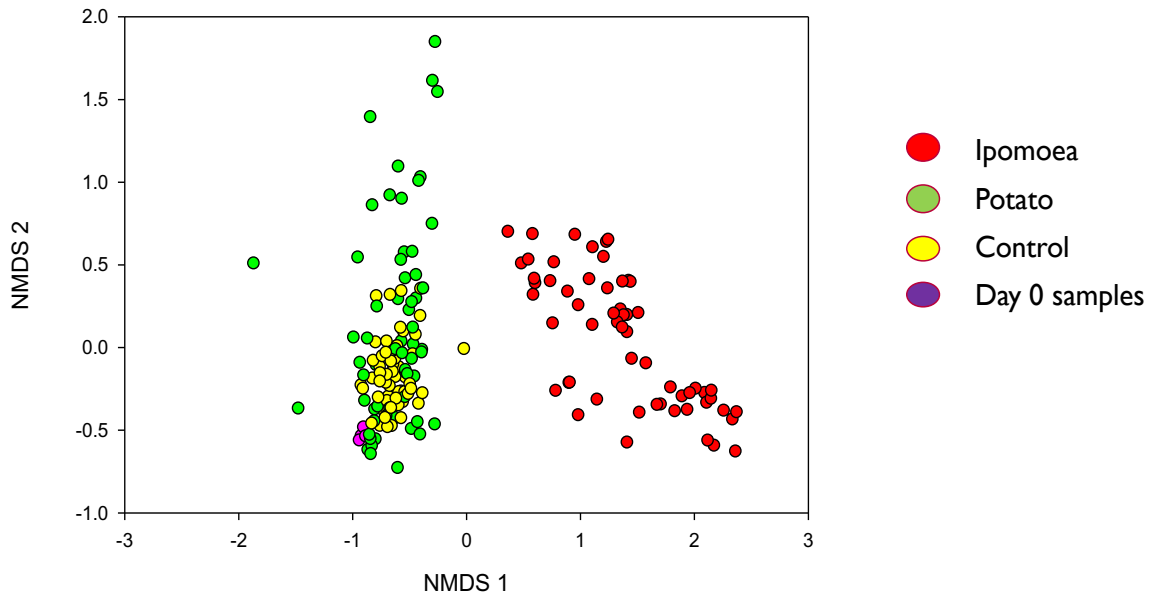


Figure 4.20: Non-metric multidimensional scaling plot (NMDS) of microbial communities present in the *Ipomoea longituba*, potato and control microcosms. Plot is based on a Weighted Unifrac comparison and shows the separation of microbial communities in the *Ipomoea longituba* microcosms from that of the potato, control and day 0 samples.

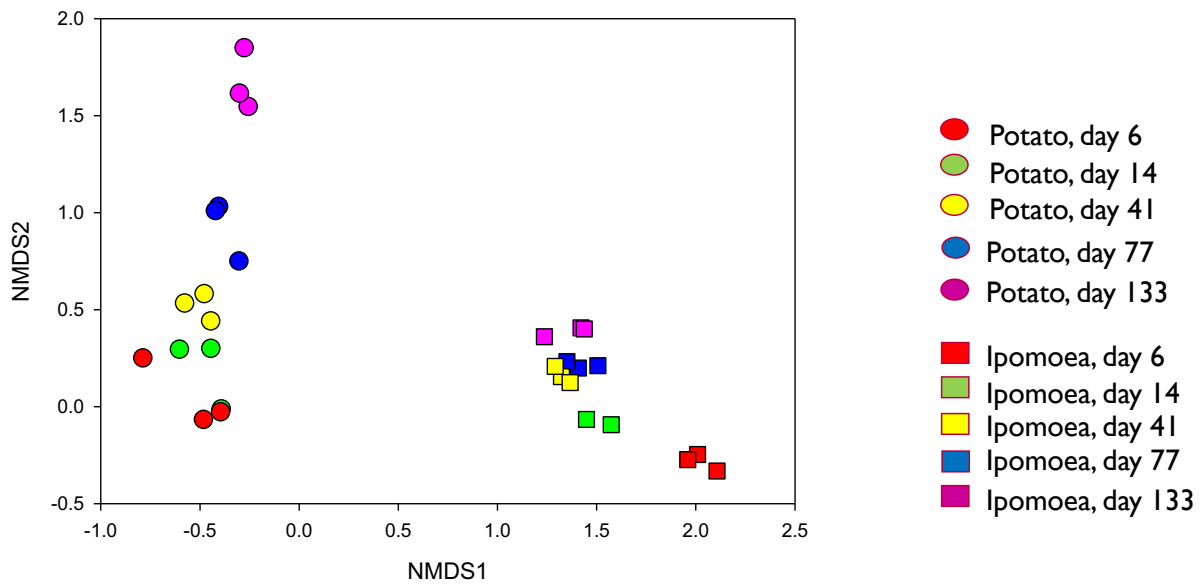


Figure 4.21: Non-metric multidimensional scaling plot (NMDS) of microbial communities present in the *Ipomoea longituba* and potato microcosms at the 7% water – oxic condition. Plot is based on a Weighted Unifrac comparison and shows the differentiation of microbial communities over time based on the day of DNA analysis for both *Ipomoea* and potato.

Table 4.7: Most responsive genera (with fold increases ≥ 2) across all four incubatory conditions for *Ipomoea longituba* and potato starches in Experiment 2, when comparing day 0 to day 41. More detailed tables ordered by the fold increases are given in the appendix (Supplementary Tables 4 – 5). Uncultured indicates that the genus is uncultured and can only be described to a higher taxonomic level.

Starch	Incubation condition	Most responding Genera or OTUs (Enclosed in parentheses are the fold increases)
<i>Ipomoea longituba</i>	7% water – anoxic	<i>Nitriliruptor</i> (3.4), <i>Geodermatophilus</i> (3), <i>Planomicrobium</i> (2.5), <i>Geobacillus</i> (8.3), <i>Lysinibacillus</i> (5.8), <i>Dermatophilaceae</i> (uncultured) (10.7), <i>Pseudonocardia</i> (4.4), <i>Propionibacteriaceae</i> (uncultured) (2.1)
<i>Ipomoea longituba</i>	7% water – oxic	<i>Nitriliruptor</i> (2.8), <i>Propionibacteriaceae</i> (uncultured) (6.3), <i>Dermatophilaceae</i> (uncultured) (6.4), <i>Geobacillus</i> (7), <i>Planomicrobium</i> (12.4), <i>Acidimicrobiales</i> (uncultured) (2.5)
<i>Ipomoea longituba</i>	12% water – anoxic	<i>Nitriliruptor</i> (4.8), <i>Planomicrobium</i> (7.2), <i>Isoptricola</i> (5.8), <i>Anaerobacillus</i> (36.9), <i>Pelagibacterium</i> (16.7), <i>Cyclobacterium</i> (34.2), <i>Dermatophilaceae</i> (uncultured) (16.2), <i>Mongoliicoccus</i> (13), <i>Halomonas</i> (21.5)
<i>Ipomoea longituba</i>	12% water – oxic	<i>Glycomyces</i> (7.3), <i>Myceligeneran</i> (4.2), <i>Cellvibrio</i> (3.8), <i>Rhodospirillaceae</i> (uncultured) (3.7), <i>Thermomicrobia</i> (uncultured) (3.5), <i>Planctomyces</i> (2.3), <i>Gemmatimonadetes</i> (uncultured) (2.6), <i>Jiangella</i> (2.7)
Potato	7% water – anoxic	<i>Nitriliruptor</i> (2), <i>Gemmatimonadetes</i> (uncultured) (2.7), <i>Anaerobacillus</i> (2), <i>Acidimicrobiaceae</i> (uncultured) (2), <i>Pseudonocardia</i> (2), <i>Propionibacteriaceae</i> (uncultured) (2)
Potato	7% water – oxic	<i>Nocardiosis</i> (4.6), <i>Haloglycomyces</i> (16.5), <i>Cytophagia</i> (uncultured) (9.8), <i>Streptomyces</i> (6.9), <i>Geobacillus</i> (5), <i>Nocardiosaceae</i> (2.6), <i>Actinomadura</i> (2.4)
Potato	12% water – anoxic	<i>Nitriliruptor</i> (2.5), <i>Mongoliicoccus</i> (3.1), <i>Gemmatimonas</i> (3.1), <i>Nocardiosis</i> (2.4), <i>Aureimonas</i> (2.4), <i>Planctomyces</i> (2), <i>Mesorhizobium</i> (2.4), <i>Jiangella</i> (2), <i>Propionibacteriaceae</i> (uncultured) (2)

Potato	12% water – oxic	<i>Isoptericola</i> (86.4), <i>Phyllobacteriaceae</i> (uncultured) (18.8), <i>Promicromonospora</i> (2.9), <i>Glycomyces</i> (5.4), <i>Nocardiopsis</i> (2.1), <i>Gemmatimonadetes</i> (uncultured) (2), <i>Pelagibacterium</i> (2.6), <i>Luteimonas</i> (7.8), <i>Rhodospirillaceae</i> (uncultured) (2)
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Table 4.8: Taxonomic profiles of all genera in Experiment 2 whose average relative abundance increase over time as described in section 4.4. Uncultured indicates that the genus is uncultured and can only be described to a higher taxonomic level

Genus	Phylum	Class	Order	Family
<i>Bacillus</i>	<i>Firmicutes</i>	<i>Bacilli</i>	<i>Bacillales</i>	<i>Bacillaceae</i>
<i>Nitriliruptor</i>	<i>Actinobacteria</i>	<i>Nitriliruptoria</i>	<i>Nitriliruptorales</i>	<i>Nitriliruptoraceae</i>
<i>Euzebya</i>	<i>Actinobacteria</i>	<i>Nitriliruptoria</i>	<i>Euzebyales</i>	<i>Euzebyaceae</i>
<i>Streptomyces</i>	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Streptomycetales</i>	<i>Streptomycetaceae</i>
<i>Nocardiodes</i>	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Propionibacteriales</i>	<i>Nocardioidaceae</i>
<i>Isoptericola</i>	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Micrococcales</i>	<i>Promicromonosporaceae</i>
<i>Uncultured</i>	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Propionibacteriales</i>	<i>Propionibacteriaceae</i>
<i>Glycomyces</i>	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Glycomycetales</i>	<i>Glycomycetaceae</i>
<i>Anaerobacillus</i>	<i>Firmicutes</i>	<i>Bacilli</i>	<i>Bacillales</i>	<i>Bacillaceae</i>
<i>Nocardiopsis</i>	<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Streptosporangiales</i>	<i>Nocardiopsaceae</i>
<i>Uncultured</i>	<i>Gemmatimonadetes</i>	<i>Gemmatimonadetes</i>	<i>Gemmatimonadales</i>	<i>Gemmatimonadaceae</i>

4.5 Eukaryotic communities in *Ipomoea longituba* and potato starch microcosms

Samples from the soil–starch microcosms that were collected at days 0, 6, 41 and 133 were processed for 18S rRNA gene sequencing to determine the dominant eukaryotic taxa, including fungi, growing on the starches. Because most fungi and other eukaryotes are aerobic organisms (there are anaerobic unicellular eukaryotes and anaerobic fungi), only samples in the oxic incubatory conditions (7% water – oxic and 12% water – oxic) were processed for 18S rRNA gene sequencing. Table 4.9 profiles the top-level classification of each eukaryotic taxon that would be discussed in this section. From Figure 4.22, it can be seen that the eukaryotic clades that dominated at day 0 (when no starch was added) include *Nematoda* and *Gregarinasina* with average relative abundances of 37% and 26% respectively. Also, *Schizoplasmodiida* had a relative abundance of 3% on day 0. All other eukaryotic taxa including fungi had relative abundances below 2% on day 0. The phylum *Nematoda* consists of nematodes while *Gregarinasina* are protozoan parasites that inhabit the intestines of many invertebrates. *Schizoplasmodiida* are slime molds belonging to the phylum *Amoebozoa* (see Table 4.9).

The most obvious and consistent pattern in the eukaryotic communities was an increase in *Ascomycota* during the degradation of both starches. *Ascomycota* increased in the later stages of the starch incubations (Figure 4.22 and Figure 4.23). Other dominant taxa declined over time or fluctuated from one sampling time to another (Figure 4.22). An increase in *Ascomycota* was not seen in the controls. The eukaryotic community in the control microcosms was different from the *Ipomoea longituba* and potato starch microcosms in the sense that the relative abundance of *Ascomycota* was lower (below 3%) in all the days in the controls compared to the soil–starch microcosms (see Figure 4.24). Instead, *Nematoda* and *Gregarinasina* were the dominant taxa in

the 7% water – oxic condition. In the 12% water – oxic condition, other taxa like *Telonema* and *Labyrinthulomycetes* also emerged over time (see Figure 4.24).

For the *Ipomoea longituba* starch microcosms, a dominant taxon at day 6 was *Streptophyta* which gradually decreased from day 41 to day 133. QIIME identified *Streptophyta* as the plant species named *Asclepias syriaca* but we believe that it was the *Ipomoea longituba* starch that was added to the soil that made up the phylum *Streptophyta* and the genus *Ipomoea* is classified under the phylum *Streptophyta*

Table 4.9: Taxonomic profiles of all eukaryotic taxa described in section 4.5 from the 18S rRNA gene sequencing for Experiment 2.

Taxon	Kingdom	Phylum	Common name
<i>Nematoda</i>	Animalia	<i>Nematoda</i>	Nematodes or round worms
<i>Streptophyta</i>	Plantae	<i>Streptophyta</i>	Green plants
<i>Annelida</i>	Animalia	<i>Annelida</i>	Segmented worms
<i>Ascomycota</i>	Fungi	<i>Ascomycota</i>	Sac fungi
<i>Gregarinasina</i>	Unknown	<i>Apicomplexa</i>	Parasitic protists
<i>Telonema</i>	Unknown	<i>Telonemia</i>	unknown
<i>Labyrinthulomycetes</i>	Unknown	<i>Heterokontophyta</i>	slime nets
<i>Syndiniales</i>	Chromista	<i>Dinoflagellata</i>	unknown
<i>Schizoplasmodiida</i>	Protista	<i>Amoebozoa</i>	slime moulds
<i>Cercomonas</i>	Chromista	<i>Cercozoa</i>	unknown
<i>Bacillariophytina</i>	Chromista	<i>Bacillariophyta</i>	diatoms

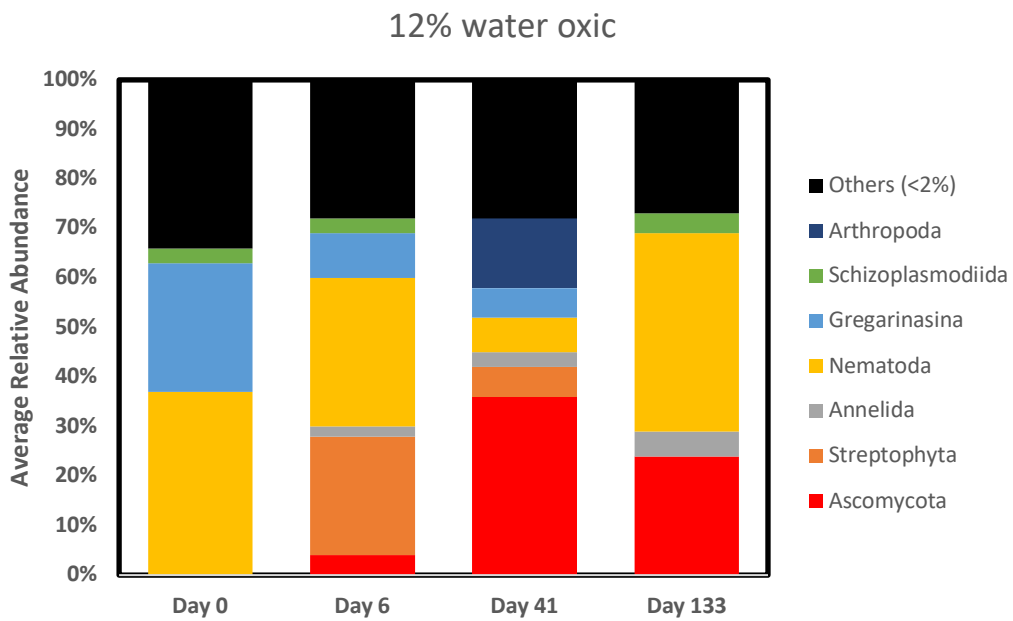
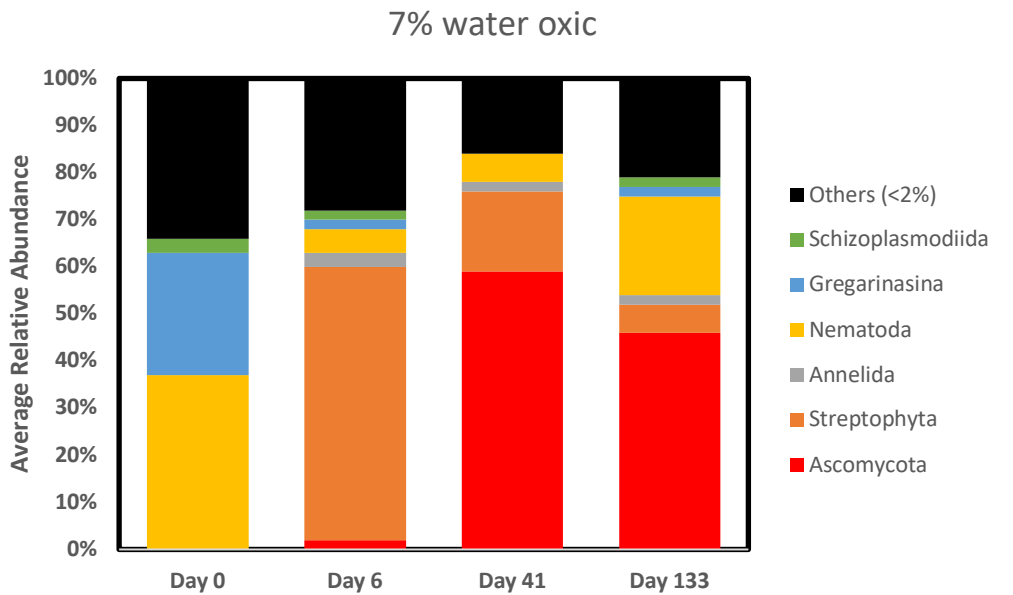


Figure 4.22: Relative abundances of the dominant phyla (>2% relative abundance) of the eukaryotic community present in the *Ipomoea longituba* microcosms under the 7% water – oxic condition and 12% water – oxic condition on day 0, 6, 41 and 133. Each treatment is the average of three replicates. Community analyses are based on the sequencing of 18S rRNA genes.

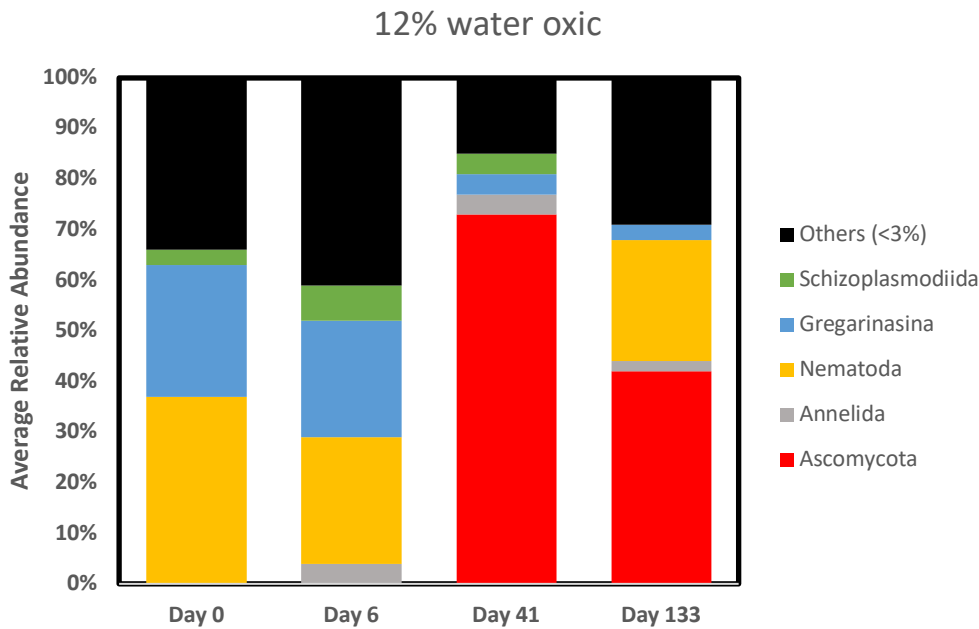
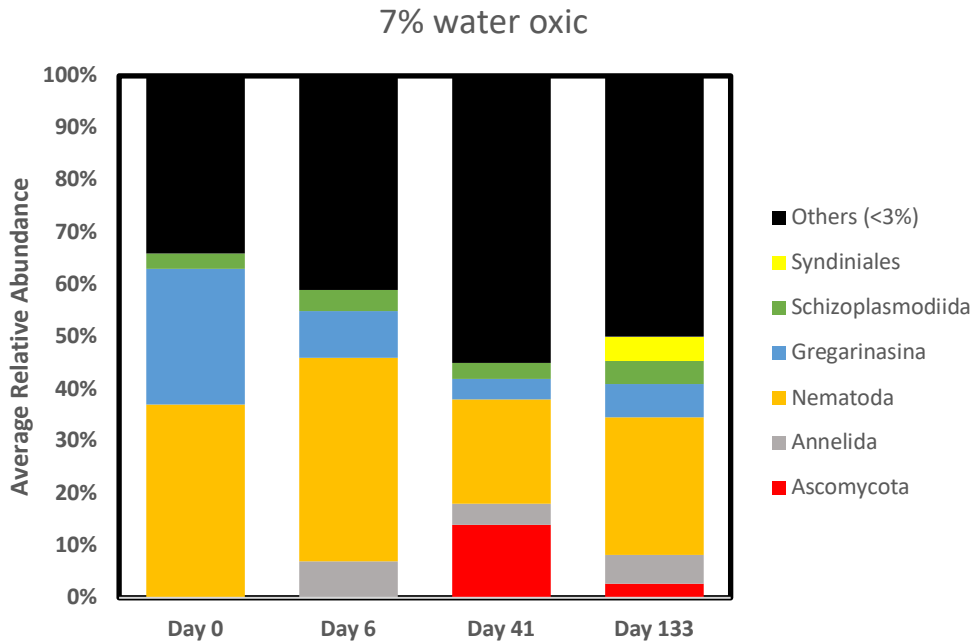


Figure 4.23: Relative abundances of the dominant phyla (>2% relative abundance) of the eukaryotic community present in the potato starch microcosms under the 7% water – oxic condition and 12% water – oxic condition on day 0, 6, 41 and 133. Each treatment is the average of three replicates. Community analyses are based on the sequencing of 18S rRNA genes.

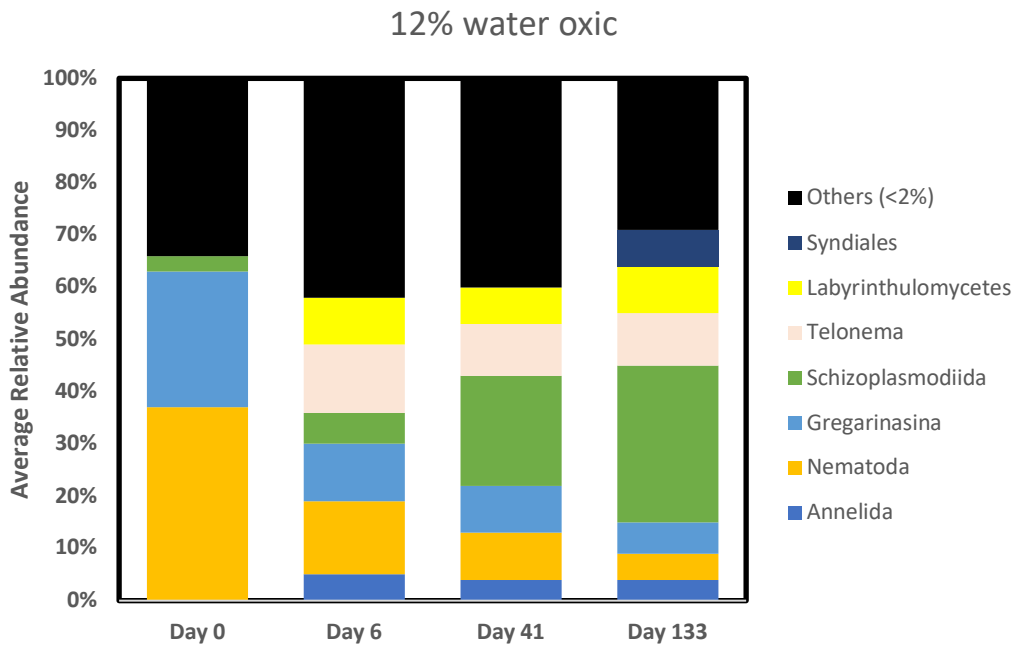
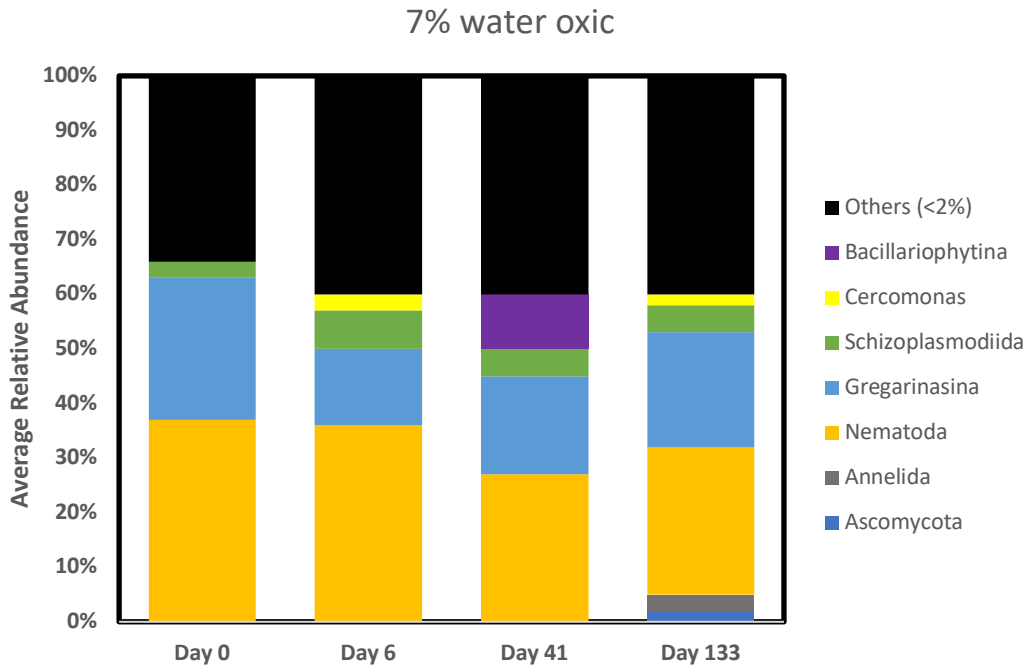


Figure 4.24: Relative abundances of the dominant phyla (>2% relative abundance) of the eukaryotic community present in the control microcosms under the 7% water – oxic condition and 12% water – oxic condition on day 0, 6, 41 and 133. Each treatment is the average of three replicates. Community analyses are based on the sequencing of 18S rRNA genes.

4.6 Bacterial communities in the rhizosphere of *Ipomoea longituba*: Preliminary results

This preliminary result section is part of a larger experiment whereby soil samples were collected from the rhizosphere and from around 1 m² of three different plants named *Cyphostemma serpens*, *Aloe volkensii*, and *Ipomoea longituba*. Soil samples from these three plants were collected from different sites in Oldupai Gorge which is located in Northern Tanzania. See section 3.2.1 in the methods chapter for details on how and when these soil samples were collected and stored. While soil samples for *C. serpens* were collected from Oleilei River, those of *A. volkensii* were collected from Ewas Oleilei River. Soil samples for *Ipomoea longituba* were collected from two different sites named Nasiu Siu and Kesile. Soil samples were processed for 16S rRNA gene analysis in the laboratory. The goal of these analysis was to discover whether the microbial communities that form the soil microbiome are affected by proximity to the roots and tuber (i.e. the rhizosphere) in different plant species (i.e. *C. serpens* or *Aloe volkensii*), and locations (i.e. Oleilei River or Nasiu Siu).

Unfortunately, although all samples were processed in the lab and submitted to the sequencing centre, sequencing of the 16S rRNA gene amplicons was delayed due to the COVID-19 pandemic. The University of Calgary was closed for several months beginning in March 2019. Prior to the shutdown we were able to obtain sequencing data for only a small subset of the soil samples for *Ipomoea longituba* that were collected at Kesile since 2018. Thus, we present the results for only this set of samples in this preliminary result section.

To determine the bacterial communities that form the microbiome of the *Ipomoea longituba* tuber, soil samples were collected from the roots (rhizosphere) of the plant and also bulk soil was collected from 1 m² around the plant. These two types of soil samples were subjected to 16 rRNA gene sequencing to determine the composition of the microbial community and the dominant taxa

that forms the microbiome. Preliminary results showed that many of the phyla that dominate in the rhizosphere also dominate in the bulk soil although their relative abundances differ (see Table 4.10). The most abundant phyla in both rhizospheric and bulk soil include *Actinobacteria*, *Proteobacteria*, *Firmicutes* and *Chloroflexi*. However, the rhizospheric soil also contain the phylum *Nitrospirae* which is in low abundance in the bulk soil bacterial community and the bulk soil has the phylum *Cyanobacteria* which is in low abundance in the rhizospheric bacterial community.

Compared to the phylum composition, there are many more differences in the bacterial communities at the OTU level in the rhizosphere versus bulk soil. For instance, the most dominant OTUs (with their relative abundance) in the rhizospheric soil include *Gaiellales* (uncultured) (10%), *Streptomyces* (5.5%), *Gemmatimonadaceae* (uncultured) (5.3%) and *Bacillus* (4.5%) while those of the bulk soil include *Bacillus* (4.7%), *Geodermatophilus* (4.1%), *Exiguobacterium* (3.3%), *Gaiellales* (uncultured) (3.0%) and *Gemmatimonadaceae* (uncultured) (2.9%). Table 4.11 shows the OTU-level representation of the bacterial community in the bulk soil and rhizosphere of *Ipomoea longituba* respectively and it can be observed from this table that certain dominant OTUs are present in both the rhizosphere and bulk soil at relative abundances greater than 1%. These include *Bacillus*, *Exiguobacterium*, *Solirubrobacter*, *Gaiellales* (uncultured), *Gemmatimonadaceae* (uncultured), *Solirubrobacterales* (uncultured), *Acidimicrobiales* (uncultured) and *Rubrobacter*. However, others differ, such as *Blastococcus*, *Microvirga*, *Bosea* among several others. An NMDS plot shown in Figure 4.25 reveals that the microbial communities in the rhizosphere of *Ipomoea longituba* are actually easily distinguished from the microbial communities in the bulk soil that was collected from around 1 m² of the tuber plant. Some of the

genera that were enriched in the rhizosphere of *Ipomoea longituba* are notable starch degraders and they include *Bacillus*, *Streptomyces*, *Exiguobacterium* and *Geodermatophilus*.

Table 4.10: Average relative abundance of the dominant phyla that constitute the microbial community of the rhizospheric soil and bulk soil of *Ipomoea longituba*. Data are based on 4 replicate samples.

Phylum	Abundance (Rhizosphere) (%)	Standard error	Abundance (Bulk 1 m ²) (%)	Standard error
<i>Actinobacteria</i>	55.57	0.01	35.45	0.01
<i>Proteobacteria</i>	12.79	0.02	21.91	0.02
<i>Firmicutes</i>	9.01	0.01	9.80	0.01
<i>Chloroflexi</i>	6.43	0.00	9.91	0.02
<i>Gemmatimonadetes</i>	5.59	0.00	3.41	0.00
<i>Acidobacteria</i>	4.63	0.01	6.81	0.00
<i>Planctomycetes</i>	3.55	0.01	6.39	0.01
<i>Nitrospirae</i>	0.81	0.00	0.50	0.00
<i>Verrucomicrobia</i>	0.56	0.00	1.25	0.00
<i>Bacteroidetes</i>	0.32	0.00	1.15	0.00
<i>Cyanobacteria</i>	0.06	0.00	2.24	0.01

Table 4.11: Average relative abundances of the dominant OTUs that make up the bacterial community of the rhizosphere and bulk soil in *Ipomoea longituba*. Some are uncultured and can only be identified to a higher taxonomic level than genus. These are indicated as taxon (uncultured). Data are based on 4 replicate samples.

Genus or OTU	Abundance (Bulk) (%)	Standard error	Abundance (Rhizosphere) (%)	Standard error
<i>Bacillus</i>	4.67	0.01	4.47	0.00
<i>Geodermatophilus</i>	4.11	0.01	1.05	0.00
<i>Exiguobacterium</i>	3.25	0.01	1.00	0.00
<i>Gaiellales (uncultured)</i>	3.02	0.01	10.10	0.01
<i>Gemmatimonadaceae (uncultured)</i>	2.87	0.00	5.30	0.00
<i>Rubrobacter</i>	2.80	0.00	2.36	0.00
<i>Phycisphaerae (uncultured)</i>	2.77	0.00	0.80	0.00
<i>Blastococcus</i>	2.54	0.00	0.51	0.00
<i>Acidobacteria (uncultured)</i>	2.15	0.00	0.51	0.00
<i>Microvirga</i>	1.90	0.00	0.51	0.00
<i>Solirubrobacterales (uncultured)</i>	1.76	0.00	3.39	0.00
<i>Solirubrobacter</i>	1.71	0.00	2.11	0.00
<i>Chloroflexia (uncultured)</i>	1.71	0.00	0.00	0.00
<i>Planctomycetaceae (uncultured)</i>	1.59	0.00	1.00	0.00
<i>Bosea</i>	1.22	0.00	0.08	0.00
<i>Acidimicrobiales (uncultured)</i>	1.21	0.00	1.61	0.00
<i>Solirubrobacterales (uncultured)</i>	1.08	0.00	0.08	0.00
<i>Staphylococcus</i>	1.08	0.00	0.50	0.00
<i>Streptomyces</i>	0.50	0.00	5.45	0.00
<i>Actinobacteria (uncultured)</i>	0.40	0.00	2.60	0.00
<i>Nocardioides</i>	0.60	0.00	1.72	0.00
<i>Gaiella</i>	0.80	0.00	1.38	0.00
<i>Pseudonocardia</i>	0.50	0.00	1.34	0.00

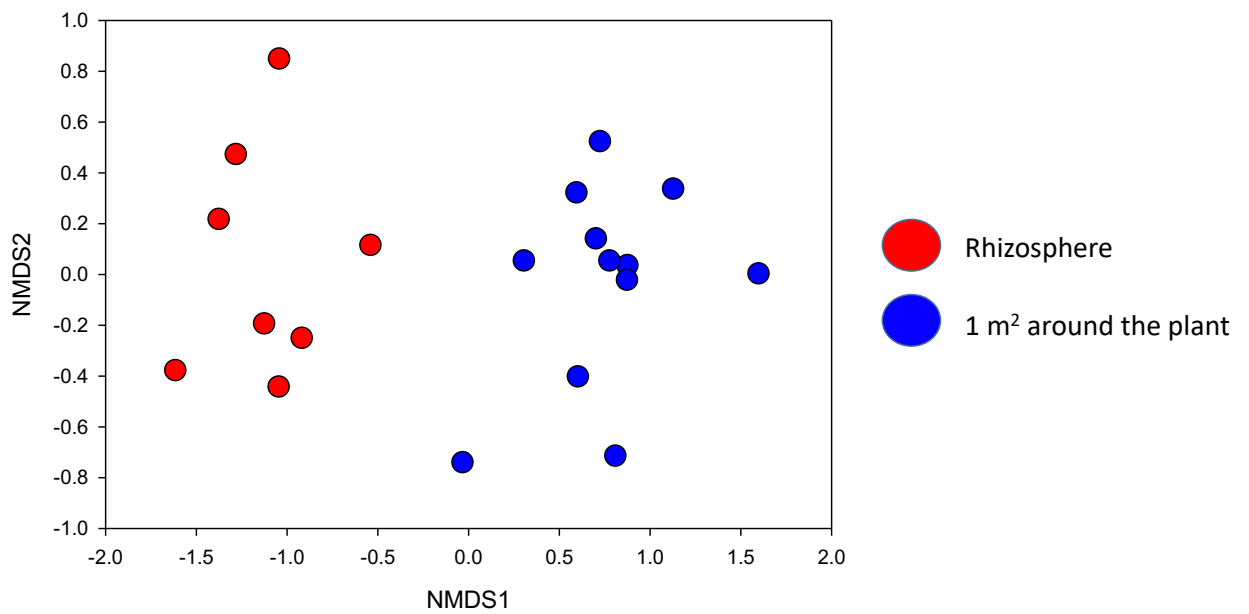


Figure 4.25: Non-metric multidimensional scaling plot (NMDS) of microbial communities present in the rhizosphere and bulk soil collected from around 1 m² of *Ipomoea longituba*. Plot is based on a Weighted Unifrac comparison and shows that the bacterial communities in the rhizosphere are different from the bacterial communities that exist in the bulk soil.

CHAPTER FIVE: DISCUSSION

5.1 Effects of moisture and oxygen on the biodegradation of starches

To interpret the gas chromatography results in this study, we needed to make a reasonable assumption that CO₂ production is a reliable proxy of metabolic activity by microbial cells as confirmed by scientific literature (Bruning et al. 2013; Williams et al. 2012; Solokhina et al. 2017). This assumption is reasonable because the amylases produced by microbes break down starch into monomers (e.g. glucose) and oligomers (e.g. maltose) (Xu, Yan, and Feng 2016). These end-products are utilized by microbes through cellular respiration to produce microbial biomass, CO₂ and H₂O (aerobic pathway). Alternatively, anaerobic pathways (which are less efficient) produce microbial biomass, CO₂, H₂O, respiratory end-products such as CH₄, H₂S or N₂, and fermentation products such as acetate in the absence of oxygen (Jurtshuk 1996).

Prokaryotes that are adapted to anoxic conditions rely on an anaerobic pathway to break down starch. In anaerobic respiration, a molecule other than oxygen is used as the terminal electron acceptor in the electron transport chain (Jurtshuk 1996). For instance, sulfate reducing bacteria and archaea use sulfate as a terminal electron acceptor, producing hydrogen sulfide (H₂S) as a by-product while some archaea (methanogens) use CO₂ as a terminal electron acceptor, producing methane as a by-product (Jurtshuk 1996). Then there is fermentation which occurs under anaerobic conditions and is carried out by bacteria and fungi. The main end products of starch fermentation are the short-chain fatty acids (e.g. propionate and acetate), lactic acid, ethanol, carbon dioxide and hydrogen (Reddy et al. 2008; Kalhan 2018). For example, amylolytic lactic acid bacteria have been shown to convert starch directly into lactic acid and are commercially used in food fermentation. Lactic acid bacteria consist of > 20 genera within the phylum *Firmicutes* and *Lactobacillus* is the most diverse of these genera (Reddy et al. 2008). However, a previous study

reported the optimization of the process for the thermophilic production of lactic acid by *Geobacillus stearothermophilus* from potato starch (Smerilli et al. 2015). Lactic acid bacteria grow slowly because of the low energy produced (Reddy et al. 2008). Because anaerobic pathways are less efficient compared to the aerobic pathway, they produce less energy and the rate of release of CO₂ molecules is slower (Jurtshuk 1996).

Therefore, using CO₂ as a proxy of metabolic activity is complicated by the production of other C-based products under anaerobic conditions, which makes comparison of oxic and anoxic incubations difficult. Nevertheless, the results indicate that wheat starch granules and *Ipomoea longituba* starch granules were highly susceptible to microbial degradation while potato starch was resistant to microbial breakdown, under both oxic and anoxic conditions. The relative susceptibility of the tested starches in the first experiment to microbial degradation can be stated as follows: wheat starch > corn starch > potato starch. This trend was true for the calcium-phosphate precipitated starches also as wheat starch precipitated with calcium-phosphate was most susceptible while calcium-phosphate precipitated potato starch was most resistant. The relative susceptibility of the native starches to biodegradation as obtained in the first experiment is not surprising because scientific literature confirms that the extent of enzymatic breakdown of different starch granules varies with the botanical source. Starches that possess the 'A' X-ray diffraction pattern are known to be more susceptible to biodegradation than starch granules exhibiting the 'B' or 'C' pattern (Planchot et al. 1995; Adejumo et al. 2013; Gallant et al. 1992). Wheat and corn are cereals and they exhibit the 'A' pattern while potato possesses the 'B' pattern, and this explains why wheat and corn were more susceptible to biodegradation than potato (Gallant et al. 1992).

The relative susceptibility of these starches to biodegradation could also be explained in terms of their granule size. Compared to large granules, smaller starch granules are known to be more susceptible to amylolysis because of their relatively large surface area to volume ratio making it easier for enzymes to attach themselves to the granule surface (Valk et al. 2015; Sarian et al. 2012). Potato starch has a larger granule size (5 – 100 μm in diameter) than starch granules from cereals like wheat (2 – 10 μm in diameter) or corn (2 – 30 μm in diameter) (Vamadevan and Bertoft 2015; Tester, Qi, and Karkalas 2006). In fact, research has quantified the relative susceptibility of commercial starches from various botanical sources to amylolysis and has shown that wheat > maize > pea > potato, reflecting the increase in granule size (Tester, Qi, and Karkalas 2006).

Comparing the cumulative CO_2 produced by the starches among the four incubatory conditions, the relative suitability of these conditions to biodegradation can be stated as follows: 4% water – oxic > 20% water – oxic > 20% water – anoxic > 4% water – anoxic. A proper carbon budget could have required measuring biomass and fermentation products, so this order is not certain. Oxygen seems to be the main factor influencing the microbial degradation of these starches because the two oxic groups produced much more CO_2 than the two anoxic groups. Water seemed to influence the biodegradation because the 4% water – oxic group produced more CO_2 than the 20% water – oxic group. One reason that could be given for this outcome is that the 20% water content was high for the artificial soil (this soil lacked a proper aggregate structure and therefore could have a low water holding capacity) and it created anoxic conditions similar to what is obtained in water-logged soils whereby water filled the soil pore spaces. The rate of oxygen diffusion into water and sediment is slow because gas diffusion is 10^4 times slower in water than in air (Greenway, Armstrong, and Colmer 2006). Therefore the oxygen in the soil pore spaces is

consumed by microbes and anaerobic conditions are created, reducing microbial activity (Greenway, Armstrong, and Colmer 2006; Neira et al. 2015).

Because it seemed that the 20% water content for the first experiment was too high, we lowered the water content to 12% in the second experiment. An effect of water was clearly visible in the second experiment as the 12% water – oxic group was the most suitable condition for the biodegradation of potato and *Ipomoea* starches. Furthermore, it is interesting to note that after about 30 days, potato starch began to degrade more rapidly in the 12% water – oxic condition as made evident by the release of CO₂ (see Figure 4.6). We did not observe a similar degradation of potato after 30 days in the 7% water – oxic condition as the CO₂ production curve for potato starch remained almost indistinguishable from the control curve. These results indicate that water improves microbial activity and the enzymatic biodegradation of these starches and scientific research has shown that the activity of enzymes increase with increasing water content as long as the water content remains below the saturation point (Rezaei, Jenab, and Temelli 2007; Páez et al. 2003).

The main purpose of including the starches precipitated with calcium phosphate in the first experiment was to see how calcium phosphate will affect the bioavailability of the starches to the soil microorganisms present to degrade them. Previous archaeological studies have shown starch granules to be preserved within the dental calculus and calcium phosphate is the mineralized matrix in dental calculus that prevents starch granules from salivary amylase attack (Power et al. 2014; Hardy et al. 2009; Radini et al. 2017). So we wanted to observe how calcium phosphate precipitate would protect the tested starches from soil microbial enzymes. From Figure 4.5, it can be seen that although some level of degradation occurred with the calcium phosphate-treated starches, it is less than that for the normal starches (with the exception of potato). Statistical

analysis showed that precipitation with calcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$) significantly reduced the bioavailability of starches to the microorganisms when considered across all incubation treatments and starches tested.

Finally, from the results chapter, we saw that the 4% water – anoxic condition produced the least CO_2 in the first experiment for the normal starches and the starches precipitated with calcium phosphate. Also, the 7% water – anoxic condition produced the least CO_2 in the second experiment. These results show that despite the exposure of these starches to enzymes produced by soil microbes, starch preservation can ultimately be improved under dry and anoxic conditions.

In the second experiment, it was expected that *Ipomoea longituba* would show similar level of CO_2 production to potato starch because they are both tubers, but the results proved otherwise. Figure 4.6 shows that the level of CO_2 production in *Ipomoea longituba* was much higher than that for potato in all the four incubatory conditions. Though the X-ray diffraction pattern of *Ipomoea longituba* is unknown as at the time of this writing, we expect it to possess an ‘A’ pattern because its level of CO_2 production is similar to what was obtained for wheat and corn starches in the first experiment. This expectation is backed up by the fact that there are other species of tubers in the *Ipomoea* genera that possesses the ‘A’ X-ray diffraction pattern. For example, several varieties of the sweet potato tuber named *Ipomoea batatas* exhibit the ‘A’ crystallographic pattern (Srichuwong et al. 2005; Cai and Wei 2013). However, *Ipomoea* is a rich cosmopolitan genus with hundreds of species and starch granule crystallinity is not controlled phylogenetically. Also, CO_2 production has no bearing on establishing crystallinity in scientific literature. Therefore, *Ipomoea longituba* may not possess the ‘A’ X-ray diffraction pattern as posited at the time of this writing.

An interesting outcome in the second experiment is the fact that although 2.2 millimoles of carbon was added to the soil as starch, the soil in the 12% water – oxic condition for the *Ipomoea*

longituba microcosms produced over 3.5 millimoles of carbon as CO₂. However, this outcome may be a good example of what is called the ‘soil priming effect’. Soil priming effect can be defined as the acceleration of the decomposition of native soil carbon by soil microbes due to the addition of fresh organic matter to the soil (Liu et al. 2020). Soil priming can also have a negative effect when fresh carbon is added to soil to reduce the microbial degradation of soil organic matter (Fontaine et al. 2004; Fontaine, Mariotti, and Abbadie 2003; Liu et al. 2020). However, the mechanisms by which soil priming occurs have remained elusive to researchers (Fontaine, Mariotti, and Abbadie 2003; Fontaine et al. 2004). Furthermore, one model that has been proposed to cause soil priming is called ‘microbial activation’ whereby the addition of new carbon substrate to the soil stimulates microbial metabolism and biomass increase at the initial stage and after depletion of the fresh carbon input, microbes begin to degrade the soil organic matter at a later stage (Liu et al. 2020). Microbial activation is what likely happened in the second experiment with *Ipomoea longituba* because, as proposed by the model, it is very likely that microbial respiration and activity were initially supported by the addition of the *Ipomoea longituba* starch to the soil but once the 2.2 millimoles of carbon added as starch were depleted, microbes began consuming the native soil organic matter to maintain their metabolic activity.

Since this soil priming effect occurred in the 12% water – oxic and not in the 7% water – oxic condition, we suggest that soil moisture is a major factor affecting soil priming. Indeed, several efforts in scientific literature to investigate the effect of soil moisture on the priming effect seem to suggest the need for adequate moisture for soil priming to take place (Bao et al. 2016). Studies have shown that decrease in soil microbial respiration or metabolism that could lead to the priming effect may be attributed to a decrease in soil moisture levels (Wang et al., 2016; Dijkstra and Cheng 2007). Also, the botanical source of the fresh carbon input or litter is known to

determine whether or not the soil priming effect would take place (Wang, Zeng, and Zhong 2016). In our experiment, the soil priming effect was evident only with *Ipomoea longituba* starch and not with the potato starch because CO₂ was still being produced from the potato starch when the second experiment was stopped and the experiment would need to have been left longer to know if there was a priming effect.

Beyond soil priming, another interesting outcome in the second experiment under the 12% water – oxic condition occurred in the potato starch microcosms. Figure 4.6 shows that before approximately 30 days, the CO₂ curve for potato starch was indistinguishable from the control curve but afterwards, potato starch began to degrade as shown by the increase in the amount of CO₂ produced and this increase levelled off after 60 days. This phenomenon did not occur in the 7% water – oxic condition so it is safe to conclude that the increase in the soil moisture content from 7% to 12% is responsible for this result. From scientific literature, we know that soil moisture is an important factor affecting the biodegradation of organic matter and that extreme conditions such as very wet or very dry soil moisture conditions significantly reduce the rate of biodegradation (Fichtner, Goersmeyer, and Stefan 2019). Generally, aerobic microbial activity increases with soil moisture content until a certain optimum point is reached. Beyond this optimum moisture content, the diffusion and availability of oxygen is much reduced such that the biodegradation condition becomes anaerobic (Miller and Johnson 1964; Parker and Larson 1962; Fichtner, Goersmeyer, and Stefan 2019). We take the 12% water – oxic condition to be an optimum condition whereby the moisture content is just sufficient for microbial activity, but the increased biodegradation of potato starch after 30 days is likely caused by an increase in the growth of particular microbial clades growing on the potato starch. Based on the 18S rRNA gene sequencing results, we suggest that one such microbial clade is the *Ascomycota* (fungi) as Figure 4.23 shows that the relative

abundance of *Ascomycota* growing on potato starch drastically increased from below 3% on day 6 to 73% on day 41, before it dropped to 42% on day 133. These data agree with the CO₂ production curve of potato starch in that the cumulative production of CO₂ increased from day 30 to day 60 and leveled off from day 60 to day 133 (see Figure 4.6).

5.2 Dominant bacterial taxa in the wheat, corn and potato microcosms (Experiment 1)

The 16S rRNA gene sequencing results in the first experiment showed that *Bacillus* was the most abundant taxon initially in most microcosms. The *Bacillus* genus is renowned for the production of α -amylase and many *Bacillus* species such as *Bacillus stearothermophilus*, *Bacillus subtilis*, *B. cereus*, *B. licheniformis* and *B. amyloliquefaciens* are known to produce thermostable amylases that have been widely used in the industry (Elmansy et al. 2018; Yao et al. 2019). Furthermore, several strains of *Bacillus* like *B. amyloliquefaciens*, *Bacillus* sp I-3 and *Bacillus licheniformis* ATCC 9945a have been shown to produce α -amylase capable of degrading raw native starch granules like the one used in this study (Božić et al. 2011; Goyal, Gupta, and Soni 2005; Demirkan et al. 2005). *Bacillus* species are common soil microbes and they are usually mesophilic and aerobic or facultative anaerobic rods capable of producing resistant endospores (Attitalla et al. 2018). Nonetheless, in most cases, the relative abundance of *Bacillus* decreased from day 0 to day 70 in our incubations so we cannot conclude that *Bacillus* was growing on the starch. The *Bacillus* might just be present in the initial soil as resistant endospores.

However, there were several times where the relative abundances of certain taxa superseded that of *Bacillus*. With wheat starch as an example, *Alkalibacterium* (belonging to the phylum *Firmicutes*) dominated the microbial community in the 4% water – anoxic condition on day 15. All species that constitute the *Alkalibacterium* genus exhibit both alkaliphilic and

halophilic properties and they normally produce lactic acid as the main product of glucose fermentation. The genus *Alkalibacterium* includes strains that are both obligate and facultative alkaliphiles. They are widely distributed and have the capacity to grow under extreme conditions e.g. high pH (Ishikawa et al. 2011; Yumoto, Hirota, and Nakajima 2014). The production of polysaccharide-degrading enzymes by *Alkalibacterium* has been documented in the scientific literature – for example, *Alkalibacterium* is known to produce xylanases and one study reported the cloning of a xylanase-encoding gene (*xynSL3*) from *Alkalibacterium* sp. SL3, an alkaliphilic strain isolated from the sediment of soda lake Dabusu (Wang et al. 2017). This same strain – the *Alkalibacterium* sp. SL3 was shown to possess an α -amylase-encoding gene (*amySL3*) of glycoside hydrolase (GH) family 13. The amylase-encoding gene (*amySL3*) was found to share a high degree of similarity with putative α -amylase-encoding genes from other species of the *Alkalibacterium* genus (Wang et al. 2019). Based on these research works, we do expect other strains of the genus *Alkalibacterium* to produce starch degrading enzymes of some kind, particularly α -amylases.

The genus *Streptomyces* was most dominant taxon growing on potato starch under the 4% water – oxic condition. This outcome is not surprising because α -amylases are produced by several species of *Streptomyces*. In fact, species of *Bacillus* and *Streptomyces* together with a few other genera in the phylum Actinomycetes (e.g. *Thermonospora* and *Thermoactinomyces*) are considered to be the most important sources of microbial amylase (Manivasagan et al. 2015), The scientific literature contains many studies that focused on the isolation and characterization of α -amylase as well as the cloning and characterization of α -amylase genes from *Streptomyces*. Starch degrading activity is widely distributed in species of *Streptomyces* and there are certain species that can attack and hydrolyze raw starch granules with the release of maltose as the end-product. Examples of *Streptomyces* strains that produce amylases include *Streptomyces* sp. SLBA-08, *S.*

avermitilis, *S. rochei* BTSS 1001 and *Streptomyces* strain A3 (Syed, Agasar, and Pandey 2009; Al-Dhabi et al. 2020). Recently, the scientific investigations focusing on the production of thermostable amylase from *Streptomyces* have gained attention because of the huge potential inherent in its commercialization (Kaneko, Ohno, and Ohisa 2005). α -Amylase from *Streptomyces hygrosopicus* SF-1081 is one of the most thermostable amylases that can hydrolyze raw starch granules and is used in the industry. Another *Streptomyces* strain that produces a raw starch digesting amylase is *S. thermocyaneoviolaceus* and several other *Streptomyces* strains that can digest raw starch granules are reported in scientific literature (Kaneko, Ohno, and Ohisa 2005; Hwang et al. 2013).

Promicromonospora was one of the consistent bacteria genera that appeared in virtually all the incubatory incubations for each of the tested starches with a peak average relative abundance of 18% in the 4% water – anoxic condition. The genus *Promicromonospora* belongs to the family *Promicromonosporaceae* and was first described by Krasilnikov in 1961 (Jiang et al. 2009). *Promicromonospora citrea* is the type species and it particularly produces extensively branched substrate mycelia that fragment into bacilliary or coccoid elements (Jin et al. 2018). As at 2018, there are 14 different species of the genus *Promicromonospora* that are published in scientific literature and these include *Promicromonospora soli* and *Promicromonospora callitridis* (Jin et al. 2018). Because it appeared in both the oxic and anoxic condition, it is safe to assume that many of the *Promicromonospora* strains in this experiment are facultative anaerobes. *Promicromonospora sukumoe* is another species that was first isolated from a soil sample collected at Sukumoe City, Kochi Prefecture in Japan (Takahashi et al. 1987). The type strain of *P. sukumoe* is the SK-2049 strain. *P. sukumoe* is filamentous and it grows very well on synthetic and organic media under aerobic conditions (Takahashi et al. 1987). *Promicromonospora* has also been shown

to be endophytic, living inside the roots of medicinal plants (Passari et al. 2017). There is paucity of information in scientific literature as regards the production of enzymes in *Promicromonospora* but a recent study reported the production of xylanase in *Promicromonospora* sp MARS (Kumar et al. 2011). To the best of our knowledge, there are no reports yet of studies investigating the production of amylase in *Promicromonospora*, hence this is an important area for future scientific efforts.

In the calcium-phosphate precipitated wheat starch, it was interesting to find a bacterium of the genus *Sinomonas* growing in the 4% water – oxic and 4% water – anoxic conditions at both days 15 and 70. *Sinomonas* also dominated the microbial community in the calcium-phosphate treated corn starch at day 15 under the 4% water – anoxic condition. We did not find *Sinomonas* at a dominant level under the 20% moisture content, implying that the elevated water content was not conducive to its growth. The genus *Sinomonas* belongs to the family *Micrococcaceae* under the *Actinobacteria* phylum (Rao, Xiao, and Li 2018; Zhou et al. 2009). As of 2019, there are ten species in the *Sinomonas* genus that have been isolated from different niches like mangrove soil, forest soil, rhizosphere soil, volcanic rock and surface of weathered biotite (Fu et al. 2019; Rao, Xiao, and Li 2018). Out of the ten species, only one has been recorded to produce amylase. One study reported a strain of *Sinomonas humi* (*S. humi* MUSC 117^T) that was isolated from mangrove soil to exhibit amylolytic activity for starch hydrolysis (Lee et al. 2015). The capacity of some of the other species of *Sinomonas* to produce amylase has not yet been determined (Rao, Xiao, and Li 2018).

Pseudomonas was one other bacterial genus that dominated the microbial community in the 20% water – oxic condition for wheat starch precipitated with calcium phosphate at day 15. (see Figure 4.11) and for wheat starch at day 70 (see Figure 4.8). The *Pseudomonas* genus (phylum

Proteobacteria, class *Gammaproteobacteria*) consists of many species that are ubiquitous in both soil and water (Khannous et al. 2014). Also, the *Pseudomonas* genus is famous for their high genetic and physiological diversity (Zhang and Zeng 2011). They have high ecological significance, playing essential roles in carbon and nitrogen nutrient cycling. In recent years, *Pseudomonas* strains have been the focus of several scientific investigations because of their industrial importance as they are metabolically versatile with the ability to use a wide range of simple and complex organic compounds. They are also well known for their ability to produce and secrete many useful extracellular enzymes that includes lipase and amylase (Zhang and Zeng 2011; Cladera et al. 2004; Khannous et al. 2014). In a recent publication that reported the study of the hydrolysis of starch granules by bacterial and fungal attack, *Pseudomonas* was shown to dominate the microbial community that formed the microbiome of the African potato (*Hypoxis* spp) at days 7, 14 and 30 of the experiment when DNA sampling occurred. At 90 days, *Pseudomonas* was replaced by *Azospirillum* as the most dominant bacteria (Mercader et al. 2018). Several strains of *Pseudomonas* have been shown to produce amylase. For instance, *Pseudomonas stutzeri* AS22 was evaluated by a recent study to produce a α -amylase, and it was noted that amylolytic activity is one of the phenotypic characteristics of *Pseudomonas stutzeri* strains (Maalej et al. 2014; Lalucat et al. 2006). Psychrotolerant *Pseudomonas stutzeri* 7193 strain isolated from deep sea sediment at Prydz Bay, Antarctic was found to possess α -amylase gene which was cloned and expressed in *Escherichia coli* (Zhang and Zeng 2011). Another study reported the high amylase activity of the strain *Pseudomonas luteola* C2 (Khannous et al. 2014).

Other dominant bacterial genera growing in the calcium-precipitated corn starch included *Alkaliphilus* (20% water – anoxic), *Planococcus* (20% water – oxic) and *Paenibacillus* (20% water – anoxic). There are no reports in scientific literature on the production of amylase by bacterial

strains in the *Alkaliphilus* and *Planococcus* genera. However, there are several species in the *Paenibacillus* genus whose amylolytic activities have been reported. *Paenibacillus* species were initially included in the *Bacillus* genus until 1993 when it was named a novel genus and its strains produce a variety of enzymes that include amylase among many others with potential uses in the industrial process manufacturing for detergents, food, textiles and biofuels (Grady et al. 2016). For instance, one study explored the potential of *Paenibacillus amylolyticus* for the production of thermostable α -amylase that would be suitable for industrial applications (Ikram-Ul-Haq et al. 2012). Another study identified and characterized the α -amylase encoding gene called *amyI* from the genomic DNA library of *Paenibacillus* sp (Rajesh et al. 2013). It was discovered that this amylase-encoding gene (*amyI*) shared sequence identity with α -amylase-encoding genes from *Bacillus* sp and the amylase was shown to be highly stable at a broad range of pH and temperature values which underscores its importance for industrial applications (Rajesh et al. 2013).

One reason that could be given for the separation of wheat starch-degrading versus control microbial communities in the NMDS plot shown in Figure 4.15 is that in the control microcosms, *Bacillus* dominated the microbial community whereas in the wheat starch microcosms, other clades like *Alkalibacterium*, *Pseudomonas*, *Exiguobacterium* and *Lachnospiraceae* took over in the four different incubatory conditions tested. As a result, the microbial communities in the wheat starch microcosms were different from the microbial communities in the control microcosms where no starch was added. This observation was expected because wheat starch was the most susceptible to enzymatic degradation by microbes compared to the other two starches that were tested. In addition, from the NMDS plot shown in Figure 4.16, it can be seen that microbial communities differentiated based on water content. This outcome is not surprising because we expect that each of the moisture content levels will be suitable to certain kinds of microbes, so

some form of differentiation is expected. Figure 4.3 already showed us that the most suitable moisture content level for the enzymatic degradation of all the starches tested was the 4% water – oxic condition followed closely by the 20% water – oxic condition. We did not observe the differentiation of microbial communities based on the oxygen level i.e. under oxic or anoxic conditions. This is probably due to the fact that most of the clades like *Bacillus* and *Streptomyces* that dominated the prokaryotic communities consist of strains that can carry out their metabolism under aerobic and anaerobic conditions which means that they are facultative anaerobes.

5.3 Dominant bacterial taxa in the *Ipomoea longituba* and potato microcosms

The second experiment showed a high initial level of *Bacillus* sp in the *Ipomoea longituba* microcosms under all the incubatory conditions that were tested. The average relative abundance of *Bacillus* was highest in day 6 (the initial measurement after adding the starch, since the day 0 value was taken before adding starch) and it decreases as the days went by. For instance, the relative abundance was as high as 70% on day 6 under the 7% water – anoxic condition and as low as 15% on day 133 under the 12% water – anoxic condition. In fact, the relative abundance of *Nitrospirillum* superseded that of *Bacillus* on that same day 133 under the 12% water – anoxic condition.

Again, as in the first experiment, we cannot conclude that *Bacillus* was actually degrading and growing on the *Ipomoea longituba* starch. Since the samples for day 0 were taken before starch was added to the microcosms, it seemed that *Bacillus* actually came from the *Ipomoea longituba* starch itself. The *Ipomoea longituba* starch, which was dried and prepared in the field and not sterilized before use and may have been contaminated with *Bacillus* spores. To confirm this, we conducted a third experiment whereby the starch was added to the microcosms on day 0 and the

microcosms were then sampled for DNA analysis at day 0, day 1, day 3 and day 6. A gradual increase in the relative abundance of *Bacillus* from day 0 to day 6 would indicate that *Bacillus* actually came from the soil and that it has a high affinity for *Ipomoea longituba*. Conversely, a high abundance of *Bacillus* at day 0 would indicate that it came from the *Ipomoea longituba* starch and not from the soil. However, due to coronavirus pandemic, efforts to sequence the samples from this experiment have been thwarted because the sequencing center at the Centre for Health Genomics and Informatics, University of Calgary has been shut down due to efforts to contain the spread of the virus.

On potato starch in the second experiment across the four incubatory conditions, *Streptomyces* was one of the dominant clades. It is important to note that in the first experiment, *Streptomyces* also dominated the microbial community growing on potato starch in the 4% water – oxic condition at day 70 (see Figure 4.10). Based on the results of these two experiments, we conclude that the genus *Streptomyces* has a strong affinity for potato starch under low moisture and aerobic conditions. Even though *Streptomyces* was the most dominant genus in the 12% water – oxic condition at day 41, it is very likely that *Streptomyces* was not responsible for the huge spike in CO₂ production in potato starch under the 12% water – oxic condition as the relative abundance of *Streptomyces* increased from 6% at day 14 to just 13% at day 41 which is not a drastic increase. If *Streptomyces* played a major role in the degradation of potato starch under that condition, a huge increase in its relative abundance should have been observed in the second experiment.

Nitriliruptor and *Euzebya* were two of the most consistent bacteria genera that appeared in all the incubatory conditions for potato starch. *Nitriliruptor* and *Euzebya* had a peak average relative abundance of 9% and 10% respectively in the 7% water – oxic condition. According to

the List of Prokaryotic names with Standing in Nomenclature (LPSN), the genus *Nitriliruptor* has one species named *Nitriliruptor alkaliphilus*. *Nitriliruptor alkaliphilus* was first isolated from soda lake sediments. The type strain of the type species *Nitriliruptor alkaliphilus* is ANL-iso2^T (Sorokin et al. 2009) in addition to several unclassified and uncultured strains (Stackebrandt and Otten 2014). Also, according to LPSN, the genus *Euzebya* has two species namely *Euzebya tangerina* and *Euzebya rosea*. *Nitriliruptor* and *Euzebya* are closely related in that they belong to the same class called Nitriliruptoria, in the phylum *Actinobacteria*. There is no information in scientific literature concerning the ability of *Nitriliruptor* and *Euzebya* to produce starch degrading enzymes but in the context of our second experiment, they seem capable of producing amylases since they are among the most dominant microorganisms and they appear consistently throughout all the four incubatory conditions and days of DNA sampling. However, more research efforts are needed to confirm the production of amylolytic enzymes by *Nitriliruptor* and *Euzebya*.

According to the NMDS plot in Figure 4.20, bacterial community structure in the *Ipomoea longituba* microcosms are different from those of potato starch and control microcosms. This differentiation is likely partly due to the high relative abundance of *Bacillus* in the *Ipomoea longituba* microcosms. As revealed by the CO₂ production curves, not much degradation was occurring in the potato starch microcosms in all the incubatory conditions (with the exception of the 12% water – oxic condition). Hence the overall bacterial community structure in the potato starch microcosms was similar to what was obtained in the control microcosms where no starch was added. In addition, we find in another NMDS plot in Figure 4.21 that the bacterial communities in the potato and *Ipomoea longituba* microcosms changed over time in the 7% water – oxic condition. It was expected that bacterial genera with a high starch hydrolyzing capacity would dominate at the beginning of the experiment when the starch input was new and that the

bacterial community when the starch input would have been depleted at the end of the experiment would be different from the bacterial community at the beginning of the experiment. However, we found this ‘day effect’ only in the 7% water – oxic condition and no explanations can be offered as to why this day effect did not occur in the three other incubatory conditions.

5.4 Dominant eukaryotic taxa in the *Ipomoea longituba* and potato microcosms

The fungi phylum *Ascomycota* appeared to grow on *Ipomoea* and potato starch. On potato starch its relative abundance jumped from below 3% on day 6 to 14% on day 41, although its relative abundance decreased to 3% on day 133. One reason that could be given for the decrease in the relative abundance of *Ascomycota* on day 133 is that nematodes might have been feeding on the fungi, because the relative abundance of phylum *Nematoda* increased from 20% on day 41 to 29% on day 133. In the 12% water – oxic condition, the ‘nematode effect’ was more pronounced as the relative abundance of *Nematoda* increased from below 3% on day 41 to 24% on day 133 while the relative abundance of *Ascomycota* decreased from 73% on day 41 to 42% on day 133. The group of nematodes that feed on fungi are called fungivores and their effects on soil nitrogen mineralization and the suppression of plant diseases caused by fungi have been well documented in scientific literature (Lagerlöf et al. 2011; Hasna et al. 2007).

Fungivorous nematodes possess a stylet and they consume the mycelia of many different species of beneficial and pathogenic fungi in the soil (Chen and Ferris 2000). The most common genera of fungivorous nematodes found in agricultural soils include *Aphelenchoides*, *Aphelenchus*, *Tylenchus* and *Filenchus* (Lagerlöf et al. 2011; Hasna et al. 2007; Bae and Knudsen 2001). One study investigated the fungal feeding habits of isolates in the nematode genus *Filenchus* and found out that *Filenchus* species reproduce by feeding on fungi in the soil and confirmed that fungal

feeding is a usual habit in this genus (Okada, Harada, and Kadota 2005). From the 18S rRNA gene sequencing in this experiment, several fungivorous nematodes such as *Aphelenchus avenae*, *Aphelenchoides* spp and *Filenchus* sp can be identified in our soil, which supports our presumption that the nematodes in this experiment were actually feeding on the fungi and that this led to a decline in the relative abundance of the fungi phylum *Ascomycota* on day 133. For the *Ipomoea longituba* starch, the ‘nematode effect’ can also be seen in the 7% water – oxic condition and the 12% water – oxic condition. For instance, in the 12% water – oxic condition, the relative abundance of nematodes increased drastically from 7% on day 41 to 40% on day 133 while that of fungi decreased from 36% on day 41 to 24% on day 133 (see Figure 4.22).

Streptophyta dominated the *Ipomoea longituba* microcosms on day 6 in the 7% water – oxic condition and the 12% water – oxic condition. Although the 18S rRNA gene sequencing identified that the phylum *Streptophyta* was composed of the species *Asclepias syriaca*, we believe that it was the *Ipomoea longituba* starch that was added to the soil that made up the phylum *Streptophyta*. Both *Ipomoea longituba* and *Asclepias syriaca* are classified under the phylum *Streptophyta*. The fact that *Streptophyta* did not appear in the potato microcosms confirms our assumption. Moreover, from Figure 4.22, it appears that the fungi were feeding on the *Streptophyta* (which we assume is *Ipomoea longituba* starch) because as the relative abundance of *Streptophyta* decreased from day 6 to day 133, the relative abundance of *Ascomycota* increased from 2% on day 6 to 59% on day 41.

The average relative abundance of the fungi *Ascomycota* was at its peak in the potato starch microcosms under the 12% water – oxic condition at day 41. From a relative abundance that was lower than 3% on day 6, *Ascomycota* increased in relative abundance to 73% on day 41 (see Figure 4.23). This dramatic increase in relative abundance on day 41 coincided with the increase in CO₂

production after approximately 30 days under the 12% water – oxic condition in the potato starch microcosms as shown in Figure 4.6. From all indications, we believe that the increase in the degradation of potato starch under the 12% water – oxic condition from day 30 to day 60 was caused in part by fungal strains in the phylum *Ascomycota*.

Various fungal species belonging to the phylum *Ascomycota* have been known to produce amylolytic enzymes in scientific literature. *Aspergillus niger* is one of such species and it is the most commonly used fungi for industrial amylase production because of its high acid tolerance and thermostability (Monga et al. 2011). One study described the isolation of amylase from a *Preussia minima* strain of endophytic origin (Zaferanloo et al. 2014). Another study documented the production of α -amylase and glucoamylase from a strain of *Trichoderma* sp capable of degrading raw sorghum starch (Pancheco-Chávez et al. 2004), while one study reported the activity of extracellular enzymes (amylase inclusive) produced by *Metarhizium anisopliae* (Mustafa and Kaur 2009).

CHAPTER 6: CONCLUSIONS

This study was aimed at investigating the effects of oxygen and soil moisture content on the biodegradation of a starch with ethnobotanical interest named *Ipomoea longituba* (collected from Oldupai Gorge) and also commercial starches wheat, potato and corn by microorganisms. To the best of our knowledge, this study is the first to explore the implications of experimental taphonomy investigations on the enzymatic degradation of starch by microbial communities over time under different oxygen and soil moisture levels. In addition, it is important to note that there is paucity of information in scientific literature on the effect of anoxia on the enzymatic degradation of starch by microbes in the soil *in vitro* and this thesis attempts to fill these knowledge gaps. To achieve this, two experiments were conducted, and four different incubatory conditions were created by combining oxic and anoxic conditions together with low and high soil moisture levels. For the first experiment, three of the most commercially available starches namely corn, wheat and potato were tested while for the second experiment, potato starch and starch from a tuber plant (*Ipomoea longituba*) were used.

In this research work, the two experiments mentioned above were carried out in serum bottles. The first experiment utilized nanospheres (artificial soil), clay and natural soil from Oldupai Gorge in northern Tanzania as the inoculum. The artificial soil was sterilized before use; therefore, the microorganisms came from the natural soil. The second experiment used only natural soil from Oldupai Gorge in Tanzania. The microcosms formed from these soil-starch incubations in the two experiments were sampled almost every week to observe the metabolic activity of the soil microbes using gas chromatography and also to identify the dominant microbial clades that were degrading the starch granules using 16S and 18S rRNA gene analysis.

Gas chromatography results for the first experiment indicate that wheat starch was by far the most susceptible to microbial degradation, followed by corn starch. Potato starch produced little carbon dioxide and was the most resistant to microbial breakdown. The effect of oxygen in the first experiment was clearly noted as the microbial degradation of the starches (measured as CO₂ production) was highest under the low moisture level and oxic incubatory condition named the 4% water – oxic condition. The anoxic incubatory conditions were the least suitable condition for the enzymatic breakdown of the starches as it produced the least CO₂. For the second experiment, *Ipomoea longituba* starch was highly susceptible to microbial degradation while potato starch was highly recalcitrant as usual based on the gas chromatography analysis that calculated the rate of CO₂ produced after about 40 days. The effect of moisture on starch biodegradation was observed in the second experiment as microcosms under a higher moisture level (12% water – oxic condition) produced more CO₂ compared to microcosms under a lower moisture level. Results from these two experiments indicate that both oxygen and moisture are important factors that affect the biodegradation of starch by microbial communities in the soil and that starch preservation is more likely to occur under dry and anoxic conditions.

The 16S rRNA gene sequencing results for the first experiment showed that *Bacillus* was the most abundant taxon at the genus level initially in most microcosms. However, in most cases, the relative abundance of *Bacillus* decreased as we go from day 0 to day 70 so we cannot conclude that *Bacillus* was growing on the starch. The *Bacillus* might just be present in the soil as resistant endospores. There are several other bacteria whose relative abundances increased over time in the first experiment and indicated they were growing on the tested starches. With wheat starch as an example, *Alkalibacterium* dominated the microbial community in the 4% water – anoxic condition. The genus *Streptomyces* replaced *Bacillus* as the most dominant taxon growing on potato starch

under the 4% water – oxic condition. Also, *Promicromonospora* was one of the consistent bacteria genera that appeared in almost all the incubatory incubations for each of the tested starches in the first experiment. For the calcium phosphate precipitated starches, *Sinomonas* dominated the microbial community in wheat starch microcosms (4% water – oxic and 4% water anoxic conditions) and corn starch (4% water – oxic condition). *Pseudomonas* was one other bacterial genus that dominated the microbial community in the 20% water – oxic condition for wheat starch precipitated with calcium phosphate.

The second experiment showed a high initial level of *Bacillus* in the *Ipomoea longituba* microcosms under all the incubatory conditions that were tested, and it seemed to decrease as the days went by like in the first experiment. Some of the genera with fold increases greater than 2 in *Ipomoea longituba* include *Geobacillus*, *Planomicrobium*, *Anaerobacillus* and *Glycomyces* among several others (see Supplementary Table 4). For potato starch, the dominant taxa on certain days of incubation include *Nitriliruptor*, *Euzebya*, *Streptomyces*, *Glycomyces* and *Nocardioopsis*. In fact, the most dominant clade in the 7% water – oxic condition was *Streptomyces*. It is noteworthy that in the first experiment, *Streptomyces* also dominated the microbial community growing on potato starch in the 4% water – oxic condition. Based on these two experiments, we conclude that the genus *Streptomyces* has a strong affinity for potato starch under low moisture and aerobic conditions. For the 18S rRNA sequencing, it seemed that the fungi phylum *Ascomycota* grew on both *Ipomoea longituba* and potato starch because its average relative abundance increased from day 6 to day 41, and the depletion of starch in the microcosms probably caused its relative abundance to decline on day 133. However, the decline of *Ascomycota* might have been caused by nematodes that feed on fungi called fungivores. These fungivores might have been responsible for the decline of the fungi *Ascomycota* in the 12% water – oxic condition because the relative

abundance of nematodes that could have been fungivores increased drastically in this same incubatory condition.

Based on the results of the first experiment and our statistical analysis, we can conclude that calcium phosphate does decrease the bioavailability of the tested starches to soil microbes and this experimental outcome corroborate previous archaeological studies that have shown starch granules to be preserved within dental calculus (which is dental plaque + calcium phosphate) (Power et al. 2014; Hardy et al. 2009; Radini et al. 2017).

In conclusion, starch degradation rates are significantly affected by starch type, soil moisture content, oxygen and calcium phosphate. Starch degradation involves both bacteria and fungi and important phyla that were involved in starch breakdown include *Ascomycota*, *Firmicutes*, *Proteobacteria* and *Actinobacteria*. In addition, from the preliminary results on the microbial communities living in the rhizosphere of *Ipomoea longituba* and bulk soil, we can conclude that the microbiome of the rhizosphere of *Ipomoea longituba* is very different from the microbiome of the surrounding bulk soil that was collected from about 1 m² of the plant.

6.1 Future Directions

In the future, we would like to determine the X-ray diffraction pattern for *Ipomoea longituba*. There is the possibility that *Ipomoea longituba* might possess an 'A' pattern because its level of CO₂ production was similar to that obtained for wheat and corn starches which are both cereals. But *Ipomoea longituba* is a tuber and in the second experiment it was expected that *Ipomoea longituba* would show similar level of CO₂ production to potato starch because they are both tubers, but the results showed that the level of CO₂ production in *Ipomoea longituba* was much higher than that for potato in all four incubatory conditions. Other species of tubers in the

Ipomoea genera possesses the 'A' X-ray crystallographic pattern e.g. *Ipomoea batatas* (sweet potato). However, *Ipomoea longituba* may not possess the 'A' X-ray diffraction pattern because starch granule crystallinity is not controlled phylogenetically.

Second, microscopic analysis has to be carried out on the starch granules that were degraded in the two experiments conducted in this thesis. During these experiments, aliquots of the soil-starch incubations were stored in ethanol in order to preserve the starch granules at the date of aliquot collection so that the microscopic analysis can be done at a later date. This is important in order to determine the morphological conditions of the starch granules after biodegradation by soil microorganisms (i.e. how the starch granules look after biodegradation). Microbial degradation can cause several types of alterations to starch granules that affect the physical characteristics of these granules like loss of birefringence, partial gelatinization, hilum implosion, etc. These impacts will be viewed and observed under the light microscope. This is important because it will enable archaeologists to be able to differentiate starch granules that have been damaged by microorganisms from those that have been modified by other processes such as cooking.

Third, stable isotope probing (SIP) could be used to link microbial identity to function. SIP is a procedure that can be used to identify microbes in environmental samples that use a certain growth substrate (Dumont and Murrell 2005). The two experiments already conducted in this thesis could be followed by a SIP experiment whereby we can efficiently identify the bacteria species or strains actually undertaking the starch degradation and link these species or strains to their function in the microbial community. One SIP experiment that we could do is to add stable isotope (^{13}C) labeled native potato, wheat and corn starches to the microcosms. The aim of the experiment would be to identify bacteria that assimilate the ^{13}C labeled potato, wheat and corn

starches under different incubatory conditions. The ‘heavy’ isotope labeled ^{13}C DNA or RNA from bacterial species that have metabolized the starch can then be separated from the ‘light’ unlabeled ^{12}C DNA or RNA from bacterial species that are not growing on the starch by density gradient centrifugation (Dumont and Murrell 2005; Herrmann et al. 2018). Furthermore, when combined with gas chromatography-mass spectrometry (GC-MS), SIP analyses can enable us to identify the metabolic by-products of starch degradation by the microbes which could be short chain fatty acids (SCFA), lactic acid and glucose under both oxic and anoxic conditions (Dumont and Murrell 2005; Herrmann et al. 2018). When combined with metagenomics, SIP holds a huge potential for uncovering the bacterial and fungal strains and enzymes such as amylases that mainly degrade starch in microbial communities present in soils. Moreover, we propose that starches from other botanical sources should be included in future experiments to determine their relative susceptibilities to enzymatic degradation. Also, the *Ipomoea longituba* starch that was used in the second experiment could be sterilized to ensure that resistant *Bacillus* spores are destroyed so that all microbes would come from the soil only.

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Appendix

Supplementary Table 1: Genera or Operational taxonomic units with mean fold increases ≥ 2 in all four treatment groups (A-D) for corn starch in Experiment 1 when comparing day 0 to days 15 and 70. "Uncultured" indicates that the genus is undescribed and can only be identified to a higher taxonomic level. Relative abundance is a decimal ratio and the mean fold increase was calculated by taking the average of the fold increase for the two replicates.

(A) 4% water – anoxic

<i>Genus or OTU</i>	Abundance D0	Abundance D0	Abundance D15	Abundance D15	Abundance D70	Abundance D70	MEAN FOLD INCREASE (D15/D0 + D15/D0)/2	MEAN FOLD INCREASE (D70/D0 + D70/D0)/2
<i>Alkalibacterium</i>	0.0131	0.0035	0.1254	0.0168	0.1027	0.0036	7.1902	4.4258
<i>Promicromonospora</i>	0.0324	0.0170	0.1242	0.0991	0.1984	0.1519	4.8282	7.5238
<i>Actinotalea</i>	0.0018	0.0010	0.0042	0.0037	0.0075	0.0070	3.0957	5.7155

(B) 4% water – oxic

<i>Genus or OTU</i>	Abundance D0	Abundance D0	Abundance D15	Abundance D15	Abundance D70	Abundance D70	MEAN FOLD INCREASE (D15/D0 + D15/D0)/2	MEAN FOLD INCREASE (D70/D0 + D70/D0)/2
<i>Pleomorphomonas</i>	0.0001	0.0000	0.0029	0.0001	0.0004	0.0001	26.5954	5.1746
<i>Rhizobiales (uncultured)</i>	0.0003	0.0001	0.0014	0.0020	0.0008	0.0012	9.0506	5.2196
<i>Pilimelia</i>	0.0001	0.0004	0.0014	0.0016	0.0007	0.0016	7.8302	5.0599
<i>Pelagibacterium</i>	0.0295	0.0150	0.0810	0.1808	0.0501	0.1171	7.4095	4.7592
<i>Nocardiosis</i>	0.0031	0.0010	0.0057	0.0116	0.0260	0.0470	6.7411	27.7998
<i>Fronidhabitans</i>	0.0002	0.0002	0.0013	0.0009	0.0005	0.0005	6.5617	2.6250
<i>Pseudaminobacter</i>	0.0079	0.0022	0.0055	0.0202	0.0119	0.0202	4.9708	5.3777
<i>Lysobacter</i>	0.0003	0.0002	0.0023	0.0001	0.0016	0.0002	4.3949	3.4151
<i>Nafulsella</i>	0.0088	0.0229	0.0242	0.1316	0.0244	0.0572	4.2452	2.6335
<i>Streptomyces</i>	0.0039	0.0317	0.0272	0.0458	0.1414	0.1569	4.2411	20.7547
<i>Actinotalea</i>	0.0010	0.0008	0.0033	0.0013	0.0025	0.0012	2.3683	1.9446
<i>Dermatophilaceae (uncultured)</i>	0.0011	0.0023	0.0034	0.0040	0.0034	0.0024	2.3446	2.0170
<i>Cytophagales (uncultured)</i>	0.0010	0.0024	0.0016	0.0064	0.0062	0.0046	2.1571	4.0882

(C) 20% water – anoxic

<i>Genus or OTU</i>	Abundance D0	Abundance D0	Abundance D15	Abundance D15	Abundance D70	Abundance D70	MEAN FOLD INCREASE (D15/D0 + D15/D0)/2	MEAN FOLD INCREASE (D70/D0 + D70/D0)/2
<i>Lachnospiraceae (uncultured)</i>	0.0008	0.0003	0.0022	0.1606	0.0006	0.0855	232.3205	123.3146
<i>Clostridiales (uncultured)</i>	0.0001	0.0002	0.0086	0.0004	0.0308	0.0007	38.2720	134.9917
<i>Salinibacterium</i>	0.0004	0.0005	0.0190	0.0002	0.0341	0.0006	23.2814	41.9841
<i>Cytophagales (uncultured)</i>	0.0001	0.0004	0.0043	0.0000	0.0142	0.0004	18.6834	61.9979
<i>Candidate division TM7 (uncultured)</i>	0.0006	0.0006	0.0016	0.0186	0.0053	0.0049	16.5577	8.4038
<i>Alkalibacterium</i>	0.0003	0.0002	0.0013	0.0063	0.0022	0.0025	15.4721	8.6432
<i>Paenibacillus</i>	0.0015	0.0026	0.0168	0.0081	0.0204	0.0032	7.2414	7.5086
<i>Gemmatimonadetes (uncultured)</i>	0.0005	0.0025	0.0054	0.0002	0.0050	0.0001	5.3489	4.8916
<i>Promicromonospora</i>	0.0159	0.0139	0.1064	0.0522	0.0750	0.0588	5.2130	4.4659
<i>Actinotalea</i>	0.0006	0.0005	0.0040	0.0019	0.0053	0.0044	5.1639	8.5696

(D) 20% water – oxic

<i>Genus or OTU</i>	Abundance D0	Abundance D0	Abundance D15	Abundance D15	Abundance D70	Abundance D70	MEAN FOLD INCREASE (D15/D0 + D15/D0)/2	MEAN FOLD INCREASE (D70/D0 + D70/D0)/2
<i>Alkalibacterium</i>	0.0001	0.0003	0.0045	0.0392	0.0001	0.0036	87.2232	6.9516
<i>Pontibacter</i>	0.0003	0.0003	0.0124	0.0062	0.0045	0.0002	33.0583	8.9758
<i>Rhodopirellula</i>	0.0002	0.0000	0.0051	0.0001	0.0061	0.0033	14.0934	66.0074
<i>Salinibacterium</i>	0.0013	0.0004	0.0168	0.0045	0.0334	0.0091	12.2475	24.5223
<i>Pelagibacterium</i>	0.0086	0.0043	0.0660	0.0670	0.1111	0.0349	11.6221	10.5241
<i>Devosia</i>	0.0023	0.0005	0.0247	0.0026	0.0331	0.0090	8.1792	16.9540
<i>Firmicutes (uncultured)</i>	0.0058	0.0004	0.0051	0.0050	0.0016	0.0031	7.2700	4.3455
<i>Anaeromyxobacter</i>	0.0003	0.0003	0.0024	0.0015	0.0052	0.0004	5.7999	8.6642
<i>Paenibacillus</i>	0.0036	0.0023	0.0203	0.0100	0.0444	0.0060	4.9645	7.4890
<i>Pseudaminobacter</i>	0.0059	0.0013	0.0125	0.0062	0.0139	0.0028	3.5070	2.2814
<i>Roseomonas</i>	0.0006	0.0007	0.0030	0.0008	0.0016	0.0054	2.9488	5.1926

Supplementary Table 2: Genera or Operational taxonomic units with mean fold increases ≥ 2 in all four treatment groups (A-D) for wheat starch in Experiment 1 when comparing day 0 to days 15 and 70. "Uncultured" indicates that the genus is undescribed and can only be identified to a higher taxonomic level. Relative abundance is a decimal ratio and the mean fold increase was calculated by taking the average of the fold increase for the two replicates.

(A) 4% water – anoxic

<i>Genus or OTU</i>	Abundance D0	Abundance D0	Abundance D15	Abundance D15	Abundance D70	Abundance D70	MEAN FOLD INCREASE (D15/D0 + D15/D0)/2	MEAN FOLD INCREASE (D70/D0 + D70/D0)/2
<i>Alkaliphilus</i>	0.0025	0.0001	0.0366	0.0362	0.0164	0.0064	327.0497	59.6917
<i>Alkalibacterium</i>	0.0131	0.0035	0.3573	0.3564	0.2780	0.0014	64.9492	10.7776
<i>Georgenia</i>	0.0048	0.0019	0.0643	0.0705	0.0517	0.0323	24.8410	13.6916
<i>Exiguobacterium</i>	0.0013	0.0013	0.0186	0.0173	0.0152	0.0056	13.6193	7.9517
<i>Actinotalea</i>	0.0018	0.0010	0.0059	0.0062	0.0042	0.0027	4.8436	2.5692
<i>Sinomonas</i>	0.0168	0.0175	0.0508	0.0524	0.1510	0.0018	3.0124	4.5573
<i>Kocuria</i>	0.0006	0.0007	0.0015	0.0024	0.0038	0.0000	2.9517	3.0608

(B) 4% water – oxic

<i>Genus or OTU</i>	Abundance D0	Abundance D0	Abundance D15	Abundance D15	Abundance D70	Abundance D70	MEAN FOLD INCREASE (D15/D0 + D15/D0)/2	MEAN FOLD INCREASE (D70/D0 + D70/D0)/2
<i>Alkalibacterium</i>	0.0002	0.0001	0.0071	0.0018	0.0035	0.0137	29.4492	62.3864
<i>Nesterenkonia</i>	0.0000	0.0003	0.0015	0.0019	0.0004	0.0019	26.3844	10.0990
<i>Escherichia-Shigella</i>	0.0001	0.0001	0.0015	0.0008	0.0004	0.0010	14.8960	6.4268
<i>Nocardiopsis</i>	0.0031	0.0010	0.0546	0.0011	0.0905	0.0010	9.3095	14.9569
<i>Corynebacteriales (uncultured)</i>	0.0005	0.0003	0.0012	0.0043	0.0064	0.0198	7.7313	36.4297
<i>Micrococcaceae (uncultured)</i>	0.0001	0.0003	0.0015	0.0005	0.0005	0.0031	6.8610	7.0591
<i>Streptomyces</i>	0.0039	0.0317	0.0428	0.0071	0.0623	0.0131	5.6499	8.2653

<i>Firmicutes (uncultured)</i>	0.0284	0.0411	0.1742	0.1558	0.1530	0.1276	4.9652	4.2494
<i>Georgenia</i>	0.0020	0.0017	0.0090	0.0069	0.0206	0.0264	4.2579	12.8457
<i>Sinomonas</i>	0.0196	0.0095	0.0650	0.0486	0.0192	0.0671	4.2110	4.0147
<i>Kocuria</i>	0.0006	0.0004	0.0021	0.0013	0.0008	0.0021	3.5413	3.5050

(C) 20% water – anoxic

<i>Genus or OTU</i>	Abundance D0	Abundance D0	Abundance D15	Abundance D15	Abundance D70	Abundance D70	MEAN FOLD INCREASE (D15/D0 + D15/D0)/2	MEAN FOLD INCREASE (D70/D0 + D70/D0)/2
<i>Lachnospiraceae (Incertae Sedis)</i>	0.0001	0.0001	0.0461	0.0170	0.0819	0.0251	346.1374	571.8799
<i>Anaerovirgula</i>	0.0001	0.0001	0.0145	0.0398	0.0182	0.0255	283.7573	215.7382
<i>Peptostreptococcaceae (Incertae Sedis)</i>	0.0000	0.0000	0.0057	0.0031	0.0081	0.0020	226.6113	282.2397
<i>Rhodospirillaceae (uncultured)</i>	0.0002	0.0003	0.0591	0.0002	0.0663	0.0609	149.8703	255.2090
<i>Lachnospiraceae (uncultured)</i>	0.0008	0.0003	0.0259	0.0728	0.0296	0.0273	121.4207	58.4063
<i>Exiguobacterium</i>	0.0039	0.0025	0.1659	0.2144	0.1516	0.0183	64.0323	23.3034
<i>Anaerobranca</i>	0.0003	0.0001	0.0056	0.0142	0.0046	0.0164	58.9473	64.9490
<i>Rhizobium</i>	0.0004	0.0002	0.0411	0.0001	0.0280	0.0208	52.2025	80.1771
<i>Christensenellaceae (uncultured)</i>	0.0001	0.0001	0.0095	0.0001	0.0066	0.0004	48.4422	37.2448
<i>Vibrio</i>	0.0001	0.0000	0.0040	0.0010	0.0049	0.0011	47.6495	56.9582
<i>Alkaliphilus</i>	0.0015	0.0033	0.0348	0.0701	0.0323	0.0746	22.4672	22.3188
<i>Georgenia</i>	0.0002	0.0002	0.0039	0.0013	0.0111	0.0093	12.3936	48.9930
<i>Peptostreptococcaceae (uncultured)</i>	0.0001	0.0004	0.0025	0.0003	0.0082	0.0048	11.2786	41.3060

(D) 20% water – oxic

<i>Genus or OTU</i>	Abundance D0	Abundance D0	Abundance D70	Abundance D70	FOLD INCREASE D70/D0	FOLD INCREASE D70/D0	MEAN FOLD INCREASE (D70/D0 + D70/D0)/2
<i>Xanthomonadaceae (uncultured)</i>	0.0066	0.0000	0.0005	0.0010	0.0740	63.4401	31.7571
<i>Pseudomonas</i>	0.0175	0.0211	0.2534	0.1436	14.5188	6.8078	10.6633
<i>Devosia</i>	0.0023	0.0005	0.0074	0.0074	3.2071	15.9012	9.5542
<i>Mongoliicoccus</i>	0.0102	0.0009	0.0279	0.0065	2.7230	7.1095	4.9163
<i>Pelagibacterium</i>	0.0086	0.0043	0.0243	0.0193	2.8368	4.4724	3.6546
<i>Pseudaminobacter</i>	0.0059	0.0013	0.0033	0.0074	0.5564	5.7823	3.1694
<i>Geobacillus</i>	0.0033	0.0019	0.0104	0.0040	3.2036	2.1524	2.6780
<i>Acidimicrobiales (Candidatus Microthrix)</i>	0.0022	0.0016	0.0018	0.0066	0.8091	4.1452	2.4772
<i>Paenibacillus</i>	0.0036	0.0023	0.0051	0.0059	1.4199	2.5019	1.9609

Supplementary Table 3: Genera or Operational taxonomic units with mean fold increases ≥ 2 in all four treatment groups (A-D) for potato starch in Experiment 1 when comparing day 0 to days 15 and 70. "Uncultured" indicates that the genus is undescribed and can only be identified to a higher taxonomic level. Relative abundance is a decimal ratio and the mean fold increase was calculated by taking the average of the fold increase for the two replicates.

(A) 4%water – anoxic

<i>Genus or OTU</i>	Abundance D0	Abundance D0	Abundance D15	Abundance D15	Abundance D70	Abundance D70	MEAN FOLD INCREASE (D15/D0 + D15/D0)/2	MEAN FOLD INCREASE (D70/D0 + D70/D0)/2
<i>Anaerobacillus</i>	0.0064	0.0058	0.0073	0.0372	0.0118	0.0148	3.7600	2.1921
<i>Bacillus</i>	0.2774	0.2620	0.4911	0.5701	0.4840	0.4802	1.9732	1.7888
<i>Promicromonospora</i>	0.0324	0.0170	0.0597	0.0343	0.1444	0.0632	1.9302	4.0838
<i>Actinotalea</i>	0.0018	0.0010	0.0019	0.0019	0.0046	0.0026	1.5206	2.6325

(B) 4%water – oxic

<i>Genus or OTU</i>	Abundance D0	Abundance D0	Abundance D15	Abundance D15	Abundance D70	Abundance D70	MEAN FOLD INCREASE (D15/D0 + D15/D0)/2	MEAN FOLD INCREASE (D70/D0 + D70/D0)/2
<i>Isoptericola</i>	0.00006	0.00003	0.00200	0.00025	0.00111	0.00036	20.80709	15.66254
<i>Streptomyces</i>	0.00387	0.03168	0.10687	0.16111	0.22773	0.28190	16.36118	33.89613
<i>Nocardiopsis</i>	0.00312	0.00099	0.01683	0.01401	0.05390	0.09057	9.73833	54.15144
<i>Promicromonospora</i>	0.02230	0.01833	0.14961	0.03845	0.07423	0.04063	4.40253	2.77216
<i>Actinotalea</i>	0.00102	0.00085	0.00414	0.00248	0.00239	0.00251	3.49286	2.64805
<i>Cytophagales (uncultured)</i>	0.00099	0.00237	0.00307	0.00571	0.01064	0.01307	2.75766	8.13782
<i>Gemmatimonadetes (uncultured)</i>	0.00773	0.00245	0.00160	0.01228	0.01254	0.01253	2.61310	3.37177

(C) 20%water – anoxic

<i>Genus or OTU</i>	Abundance D0	Abundance D0	Abundance D15	Abundance D15	Abundance D70	Abundance D70	MEAN FOLD INCREASE (D15/D0 + D15/D0)/2	MEAN FOLD INCREASE (D70/D0 + D70/D0)/2
<i>Anaerobranca</i>	0.0003	0.0001	0.0016	0.0094	0.0002	0.0076	35.4299	26.6288
<i>Aquamicrobium</i>	0.0002	0.0025	0.0057	0.0005	0.0032	0.0009	11.7268	6.5671
<i>Chromatiales (uncultured)</i>	0.0001	0.0001	0.0016	0.0005	0.0009	0.0017	11.1893	11.0618
<i>Promicromonospora</i>	0.0159	0.0139	0.1050	0.1045	0.0621	0.0586	7.0502	4.0535
<i>Alkaliphilus</i>	0.0015	0.0033	0.0026	0.0327	0.0053	0.0111	5.8563	3.5047
<i>Devosia</i>	0.0011	0.0044	0.0131	0.0008	0.0201	0.0039	5.8376	9.2729
<i>Actinotalea</i>	0.0006	0.0005	0.0031	0.0029	0.0049	0.0038	5.3295	7.6614
<i>Candidate division TM7 (uncultured)</i>	0.0006	0.0006	0.0049	0.0011	0.0030	0.0043	4.9680	6.0165
<i>Anaerobacillus</i>	0.0093	0.0144	0.0036	0.1302	0.0063	0.0966	4.7232	3.7005
<i>Opitutae (uncultured)</i>	0.0021	0.0012	0.0114	0.0009	0.0041	0.0029	3.0673	2.2112
<i>Pelagibacterium</i>	0.0060	0.0057	0.0338	0.0010	0.0147	0.0466	2.9045	5.3018
<i>Salinibacterium</i>	0.0004	0.0005	0.0022	0.0002	0.0139	0.0010	2.8162	17.7991

(D) 20%water – oxic

<i>Genus or OTU</i>	Abundance D0	Abundance D0	Abundance D15	Abundance D15	Abundance D70	Abundance D70	MEAN FOLD INCREASE (D15/D0 + D15/D0)/2	MEAN FOLD INCREASE (D70/D0 + D70/D0)/2
<i>Cesiribacter</i>	0.0001	0.0000	0.1024	0.0080	0.0056	0.0013	754.7389	68.2102
<i>Sphingomonadales (uncultured)</i>	0.0004	0.0000	0.0019	0.0062	0.0106	0.0050	189.3635	164.5572
<i>Alkaliphilus</i>	0.0001	0.0001	0.0112	0.0069	0.0140	0.0238	111.1708	189.1299
<i>Rhodopirellula</i>	0.0002	0.0000	0.0015	0.0045	0.0042	0.0033	71.5253	61.2632
<i>Xanthomonadaceae (uncultured)</i>	0.0066	0.0000	0.0018	0.0019	0.0047	0.0162	56.1957	489.2722
<i>Peptostreptococcaceae (uncultured)</i>	0.0001	0.0000	0.0017	0.0013	0.0007	0.0003	52.8527	15.8606
<i>Lachnospiraceae (uncultured)</i>	0.0001	0.0004	0.0090	0.0072	0.0159	0.0051	45.1187	69.1718
<i>Rhizobiales (uncultured)</i>	0.0002	0.0000	0.0036	0.0011	0.0022	0.0011	42.0616	39.1525
<i>Firmicutes (uncultured)</i>	0.0058	0.0004	0.0049	0.0291	0.0033	0.0408	40.4542	56.4553
<i>Opitutus</i>	0.0001	0.0000	0.0032	0.0010	0.0087	0.0067	40.2725	170.1201
<i>Rhizobium</i>	0.0003	0.0005	0.0195	0.0012	0.0046	0.0020	39.1765	10.8290
<i>Pseudospirillum</i>	0.0008	0.0000	0.0017	0.0012	0.0031	0.0040	36.1341	122.6036
<i>Pontibacter</i>	0.0003	0.0003	0.0082	0.0083	0.0025	0.0003	27.8814	5.2865
<i>Devosia</i>	0.0023	0.0005	0.0047	0.0166	0.0176	0.0273	18.9488	33.3546
<i>Massilia</i>	0.0001	0.0002	0.0023	0.0031	0.0005	0.0046	18.3041	16.0773
<i>Mongoliicoccus</i>	0.0102	0.0009	0.0189	0.0277	0.0064	0.0176	16.1694	9.9939
<i>Paenibacillus</i>	0.0036	0.0023	0.0468	0.0409	0.0417	0.0416	15.2575	14.6895
<i>Salinibacterium</i>	0.0013	0.0004	0.0040	0.0103	0.0139	0.0049	14.5954	11.5736
<i>Chthoniobacter</i>	0.0004	0.0002	0.0034	0.0015	0.0023	0.0009	7.1689	4.6804
<i>Ensifer</i>	0.0015	0.0003	0.0021	0.0031	0.0047	0.0012	6.5367	3.8304
<i>Roseomonas</i>	0.0006	0.0007	0.0047	0.0040	0.0046	0.0022	6.5360	5.1728
<i>Rubellimicrobium</i>	0.0003	0.0001	0.0027	0.0005	0.0007	0.0007	6.5174	3.9438
<i>Candidate division (uncultured)</i>	0.0008	0.0006	0.0053	0.0019	0.0284	0.0011	4.8851	18.0217
<i>Rhodospirillaceae (uncultured)</i>	0.0009	0.0003	0.0055	0.0007	0.0074	0.0025	4.1786	8.2069

Supplementary Table 4: Most responsive genera (with fold increases ≥ 2) across all four incubatory conditions (A-D) for *Ipomoea longituba* starch in Experiment 2, when comparing day 0 to days 41 and 133. "Uncultured" indicates that the genus is undescribed and can only be identified to a higher taxonomic level. Relative abundance is a decimal ratio and the mean fold increase was calculated by taking the average of the fold increase for the three replicates.

(A) 7%water – oxic

<i>Genus or OTU</i>	Abundance D6	Abundance D6	Abundance D6	Abundance D41	Abundance D41	Abundance D41	Abundance D133	Abundance D133	Abundance D133	MEAN FOLD INCREASE (D41/D6 + D41/D6 + D41/D6)/3	MEAN FOLD INCREASE (D133/D6 + D133/D6 + D133/D6)/3
<i>Dermatophilaceae (uncultured)</i>	0.0005	0.0003	0.0006	0.0035	0.0055	0.0039	0.0082	0.0172	0.0098	10.6890	29.9635
<i>Geobacillus</i>	0.0009	0.0005	0.0014	0.0056	0.0065	0.0063	0.0133	0.0172	0.0122	8.2658	20.2805
<i>Bacillales (Incertae Sedis)</i>	0.0004	0.0002	0.0003	0.0011	0.0017	0.0016	0.0021	0.0021	0.0023	6.4126	9.0224
<i>Lysinibacillus</i>	0.0001	0.0003	0.0003	0.0012	0.0014	0.0014	0.0028	0.0050	0.0030	5.8105	15.0780
<i>Acidimicrobiales (uncultured)</i>	0.0019	0.0006	0.0037	0.0053	0.0059	0.0043	0.0032	0.0071	0.0052	4.4790	4.8396

<i>Pseudonocardia</i>	0.0006	0.0003	0.0014	0.0020	0.0029	0.0008	0.0012	0.0013	0.0009	4.3734	2.2234
<i>Nitriliruptor</i>	0.0105	0.0140	0.0158	0.0457	0.0408	0.0454	0.1007	0.0917	0.0929	3.3717	7.3281
<i>Geodermatophilus</i>	0.0004	0.0005	0.0009	0.0013	0.0015	0.0019	0.0012	0.0010	0.0007	2.9339	2.0009
<i>Solirubrobacterales (uncultured)</i>	0.0049	0.0042	0.0043	0.0124	0.0130	0.0098	0.0169	0.0199	0.0169	2.6346	4.0451
<i>Planomicrobium</i>	0.0011	0.0016	0.0011	0.0031	0.0030	0.0030	0.0052	0.0057	0.0040	2.4633	3.9620
<i>Planococcaceae (Incertae Sedis)</i>	0.0007	0.0005	0.0014	0.0019	0.0012	0.0017	0.0023	0.0028	0.0022	2.1731	3.5661
<i>Propionibacteriaceae (uncultured)</i>	0.0018	0.0011	0.0037	0.0031	0.0043	0.0030	0.0047	0.0100	0.0076	2.1414	4.6005

(B) 7%water – anoxic

<i>Genus or OTU</i>	Abundance D6	Abundance D6	Abundance D6	Abundance D41	Abundance D41	Abundance D41	Abundance D133	Abundance D133	Abundance D133	MEAN FOLD INCREASE (D41/D6 + D41/D6 + D41/D6)/3	MEAN FOLD INCREASE (D133/D6 + D133/D6 + D133/D6)/3
<i>Planomicrobium</i>	0.0002	0.0002	0.0002	0.0029	0.0021	0.0027	0.0052	0.0057	0.0040	12.4158	23.8246
<i>Geobacillus</i>	0.0014	0.0012	0.0008	0.0092	0.0040	0.0088	0.0133	0.0172	0.0122	6.9895	13.0722
<i>Dermatophilaceae (uncultured)</i>	0.0005	0.0018	0.0010	0.0062	0.0034	0.0054	0.0082	0.0172	0.0098	6.4373	11.7113
<i>Propionibacteriaceae (uncultured)</i>	0.0052	0.0044	0.0080	0.0328	0.0364	0.0338	0.0047	0.0100	0.0076	6.3017	1.3832
<i>Acidimicrobiaceae (uncultured)</i>	0.0004	0.0006	0.0004	0.0030	0.0027	0.0016	0.0005	0.0012	0.0009	5.0326	1.8116
<i>Lamia</i>	0.0006	0.0004	0.0004	0.0019	0.0015	0.0019	0.0005	0.0015	0.0008	3.9902	2.2261
<i>Bacillales (Incertae Sedis)</i>	0.0010	0.0009	0.0010	0.0030	0.0028	0.0029	0.0021	0.0021	0.0023	3.0195	2.2380
<i>Rhodospirillales (uncultured)</i>	0.0004	0.0003	0.0010	0.0015	0.0009	0.0013	0.0010	0.0011	0.0007	2.9121	2.4183
<i>Nitriliruptor</i>	0.0266	0.0220	0.0220	0.0679	0.0638	0.0668	0.1007	0.0917	0.0929	2.8328	4.0651
<i>Acidimicrobiales (uncultured)</i>	0.0016	0.0014	0.0021	0.0037	0.0041	0.0047	0.0032	0.0071	0.0052	2.5104	3.1891

(C) 12%water – anoxic

<i>Genus or OTU</i>	Abundance D6	Abundance D6	Abundance D41	Abundance D41	Abundance D133	Abundance D133	MEAN FOLD INCREASE (D41/D6 + D41/D6)/2	MEAN FOLD INCREASE (D133/D6 + D133/D6)/2
<i>Anaerobacillus</i>	0.0012	0.0014	0.0690	0.0233	0.0472	0.0289	36.9130	29.8812
<i>Cyclobacterium</i>	0.0001	0.0003	0.0036	0.0073	0.0319	0.0213	34.2003	217.1239
<i>Halomonas</i>	0.0003	0.0001	0.0016	0.0024	0.0003	0.0008	21.4608	6.9229
<i>Pelagibacterium</i>	0.0002	0.0001	0.0022	0.0030	0.0013	0.0011	16.6678	7.1153
<i>Dermatophilaceae (uncultured)</i>	0.0007	0.0003	0.0078	0.0066	0.0036	0.0033	16.2193	7.8240
<i>Mongoliicoccus</i>	0.0004	0.0007	0.0067	0.0053	0.0062	0.0061	13.0338	12.8559
<i>Propionibacteriaceae (uncultured)</i>	0.0065	0.0063	0.0660	0.0808	0.0742	0.1007	11.4934	13.7032
<i>Planomicrobium</i>	0.0005	0.0007	0.0040	0.0039	0.0010	0.0013	7.1600	2.0425
<i>Lysinibacillus</i>	0.0004	0.0008	0.0033	0.0041	0.0019	0.0017	6.9412	3.6328
<i>Lamia</i>	0.0007	0.0004	0.0028	0.0034	0.0059	0.0091	5.8413	14.3496
<i>Myceligenerans</i>	0.0005	0.0006	0.0016	0.0047	0.0016	0.0094	5.7951	9.8596
<i>Isoptricola</i>	0.0006	0.0003	0.0012	0.0031	0.0028	0.0071	5.7520	13.4336
<i>Cellulosimicrobium</i>	0.0000	0.0001	0.0002	0.0003	0.0003	0.0003	5.7353	7.8145
<i>Geobacillus</i>	0.0010	0.0012	0.0069	0.0055	0.0041	0.0027	5.5570	3.0690
<i>Pseudaminobacter</i>	0.0028	0.0030	0.0161	0.0137	0.0262	0.0190	5.2162	7.9347
<i>Bacillales (Incertae Sedis)</i>	0.0008	0.0003	0.0024	0.0024	0.0010	0.0011	5.1919	2.3022
<i>Phyllobacteriaceae (uncultured)</i>	0.0001	0.0002	0.0007	0.0007	0.0008	0.0009	4.8152	5.7818

<i>Nitriliruptor</i>	0.0266	0.0157	0.1052	0.0875	0.1945	0.1386	4.7632	8.0705
<i>Streptomyces</i>	0.0151	0.0234	0.0855	0.0665	0.0873	0.0801	4.2465	4.5969
<i>Nocardiopsis</i>	0.0008	0.0015	0.0031	0.0053	0.0059	0.0084	3.7754	6.5719
<i>Nitratireductor</i>	0.0011	0.0011	0.0037	0.0043	0.0068	0.0052	3.6810	5.4309
<i>Promicromonospora</i>	0.0038	0.0028	0.0070	0.0121	0.0068	0.0109	3.0956	2.8449
<i>Thermomicrobia (uncultured)</i>	0.0007	0.0008	0.0018	0.0024	0.0026	0.0032	2.8859	3.9712
<i>Mesorhizobium</i>	0.0005	0.0004	0.0008	0.0011	0.0012	0.0022	2.1567	3.7213

(D) 12% water – oxic

<i>Genus or OTU</i>	Abundance D6	Abundance D6	Abundance D6	Abundance D41	Abundance D41	Abundance D41	Abundance D133	Abundance D133	Abundance D133	MEAN FOLD INCREASE (D41/D6 + D41/D6 + D41/D6)/3	MEAN FOLD INCREASE (D133/D6 + D133/D6 + D133/D6)/3
<i>Glycomyces</i>	0.0031	0.0052	0.0056	0.0273	0.0280	0.0428	0.0031	0.0288	0.0626	7.2939	5.8757
<i>Myceligenans</i>	0.0036	0.0037	0.0048	0.0137	0.0144	0.0238	0.0026	0.0099	0.0182	4.2317	2.4088
<i>Cellvibrio</i>	0.0007	0.0003	0.0002	0.0019	0.0025	0.0002	0.0010	0.0012	0.0006	3.7551	2.6609
<i>Rhodospirillaceae (uncultured)</i>	0.0005	0.0014	0.0005	0.0029	0.0031	0.0016	0.0020	0.0036	0.0034	3.7440	4.5940
<i>Thermomicrobia (uncultured)</i>	0.0003	0.0006	0.0007	0.0018	0.0020	0.0008	0.0020	0.0016	0.0014	3.4926	3.8503
<i>Candidatus Alysiosphaera</i>	0.0005	0.0017	0.0009	0.0028	0.0024	0.0014	0.0046	0.0030	0.0032	3.0772	5.1617
<i>Jiangella</i>	0.0005	0.0010	0.0014	0.0023	0.0022	0.0023	0.0015	0.0035	0.0025	2.7436	2.7759
<i>Steroidobacter</i>	0.0005	0.0007	0.0006	0.0019	0.0026	0.0004	0.0026	0.0012	0.0005	2.6356	2.5588
<i>Gemmatimonadetes (uncultured)</i>	0.0011	0.0014	0.0013	0.0035	0.0038	0.0023	0.0010	0.0085	0.0049	2.5728	3.6209
<i>Gemmatimonadetes (uncultured)</i>	0.0027	0.0031	0.0025	0.0067	0.0062	0.0060	0.0066	0.0096	0.0088	2.2787	3.0108
<i>Planctomyces</i>	0.0021	0.0029	0.0030	0.0079	0.0066	0.0022	0.0102	0.0160	0.0125	2.2604	4.8690
<i>Acidimicrobiaceae (uncultured)</i>	0.0012	0.0019	0.0005	0.0024	0.0018	0.0017	0.0015	0.0037	0.0034	2.1558	3.4322
<i>Tepidamorphus</i>	0.0014	0.0024	0.0023	0.0041	0.0040	0.0038	0.0056	0.0065	0.0046	2.1333	2.9739

Supplementary Table 5: Most responsive genera (with fold increases ≥ 2) across all four incubatory conditions (A-D) for potato starch in Experiment 2, when comparing day 0 to days 41 and 133. "Uncultured" indicates that the genus is undescribed and can only be identified to a higher taxonomic level. Relative abundance is a decimal ratio and the mean fold increase was calculated by taking the average of the fold increase for the three replicates.

(A) 7% water – anoxic

<i>Genus or OTU</i>	Abundance D6	Abundance D6	Abundance D6	Abundance D41	Abundance D41	Abundance D41	Abundance D133	Abundance D133	Abundance D133	MEAN FOLD INCREASE (D41/D6 + D41/D6 + D41/D6)/3	MEAN FOLD INCREASE (D133/D6 + D133/D6 + D133/D6)/3
<i>Gemmatimonadetes (uncultured)</i>	0.0111	0.0011	0.0016	0.0156	0.0041	0.0050	0.0116	0.0031	0.0033	2.7051	1.9426
<i>Acidimicrobiaceae (uncultured)</i>	0.0020	0.0014	0.0006	0.0023	0.0027	0.0023	0.0017	0.0011	0.0015	2.2334	1.3592
<i>Lamia</i>	0.0019	0.0011	0.0008	0.0026	0.0018	0.0026	0.0041	0.0009	0.0022	2.0334	1.8620
<i>Anaerobacillus</i>	0.0041	0.0011	0.0035	0.0022	0.0043	0.0061	0.0012	0.0030	0.0030	2.0323	1.2672
<i>Pseudonocardia</i>	0.0020	0.0081	0.0039	0.0039	0.0092	0.0102	0.0038	0.0106	0.0077	1.9043	1.7368
<i>Nitriliruptor</i>	0.0651	0.0163	0.0144	0.0872	0.0205	0.0302	0.1348	0.0406	0.0386	1.5669	2.4171
<i>Propionibacteriaceae (uncultured)</i>	0.0089	0.0011	0.0023	0.0070	0.0021	0.0040	0.0088	0.0032	0.0036	1.4669	1.8186
<i>Acidimicrobiales (uncultured)</i>	0.0066	0.0132	0.0158	0.0103	0.0232	0.0169	0.0164	0.0202	0.0203	1.4646	1.7619

(B) 7%water – oxic

<i>Genus or OTU</i>	Abundance D6	Abundance D6	Abundance D6	Abundance D41	Abundance D41	Abundance D41	Abundance D133	Abundance D133	Abundance D133	MEAN FOLD INCREASE (D41/D6 + D41/D6 + D41/D6)/3	MEAN FOLD INCREASE (D133/D6 + D133/D6 + D133/D6)/3
<i>Haloglycomyces</i>	0.0002	0.0007	0.0004	0.0048	0.0064	0.0053	0.0232	0.0136	0.0072	16.5356	54.0929
<i>Cytophagia (uncultured)</i>	0.0006	0.0009	0.0008	0.0061	0.0080	0.0082	0.0066	0.0013	0.0031	9.8185	5.5340
<i>Streptomyces</i>	0.0198	0.0169	0.0184	0.1255	0.1098	0.1442	0.2950	0.2993	0.2680	6.9025	15.7486
<i>Geobacillus</i>	0.0003	0.0004	0.0004	0.0015	0.0017	0.0023	0.0020	0.0021	0.0027	5.0340	6.3571
<i>Nocardiopsis</i>	0.0056	0.0090	0.0071	0.0344	0.0368	0.0264	0.0289	0.0618	0.0255	4.6401	5.1967
<i>Nocardiopsaceae (uncultured)</i>	0.0088	0.0054	0.0103	0.0082	0.0291	0.0137	0.0200	0.0253	0.0164	2.5626	2.8620
<i>Actinomadura</i>	0.0004	0.0006	0.0006	0.0013	0.0017	0.0006	0.0007	0.0003	0.0022	2.3810	1.9645
<i>uncultured actinobacterium</i>	0.0012	0.0009	0.0013	0.0026	0.0019	0.0028	0.0069	0.0035	0.0042	2.1570	4.3262
<i>Arthrobacter</i>	0.0024	0.0017	0.0018	0.0027	0.0030	0.0035	0.0027	0.0043	0.0027	1.6165	1.7144
<i>Propionibacteriaceae (uncultured)</i>	0.0091	0.0061	0.0083	0.0110	0.0128	0.0109	0.0255	0.0397	0.0418	1.5403	4.7813

(C) 12%water – anoxic

<i>Genus or OTU</i>	Abundance D6	Abundance D6	Abundance D6	Abundance D41	Abundance D41	Abundance D41	Abundance D133	Abundance D133	Abundance D133	MEAN FOLD INCREASE (D41/D6 + D41/D6 + D41/D6)/3	MEAN FOLD INCREASE (D133/D6 + D133/D6 + D133/D6)/3
<i>Gemmatimonadetes (uncultured)</i>	0.0014	0.0001	0.0013	0.0023	0.0010	0.0034	0.0018	0.0016	0.0028	3.6642	4.8831
<i>Mongoliicoccus</i>	0.0010	0.0115	0.0057	0.0063	0.0172	0.0097	0.0028	0.0112	0.0052	3.1181	1.5524
<i>Gemmatimonas</i>	0.0004	0.0004	0.0005	0.0022	0.0008	0.0012	0.0021	0.0016	0.0017	3.0897	4.0524
<i>Lamia</i>	0.0010	0.0011	0.0011	0.0024	0.0029	0.0036	0.0047	0.0052	0.0033	2.7220	4.0365
<i>Mesorhizobium</i>	0.0040	0.0055	0.0064	0.0089	0.0177	0.0119	0.0075	0.0236	0.0069	2.4363	2.4103
<i>Nocardiopsis</i>	0.0008	0.0023	0.0019	0.0031	0.0033	0.0034	0.0159	0.0047	0.0274	2.3647	12.1765
<i>Aureimonas</i>	0.0025	0.0023	0.0024	0.0067	0.0055	0.0046	0.0095	0.0065	0.0035	2.3635	2.7285
<i>Gemmatimonadetes (uncultured)</i>	0.0038	0.0028	0.0069	0.0062	0.0102	0.0089	0.0048	0.0104	0.0037	2.1705	1.8194
<i>Nitriliruptor</i>	0.0151	0.0354	0.0350	0.0398	0.0518	0.0716	0.0490	0.0779	0.0691	2.0454	2.4715
<i>Gemmatimonadetes (uncultured)</i>	0.0016	0.0028	0.0030	0.0035	0.0037	0.0079	0.0020	0.0061	0.0058	2.0252	1.7704
<i>Rhodospirillales (uncultured)</i>	0.0018	0.0017	0.0017	0.0032	0.0030	0.0038	0.0032	0.0035	0.0031	1.9218	1.8707
<i>Propionibacteriaceae (uncultured)</i>	0.0040	0.0070	0.0079	0.0069	0.0096	0.0162	0.0242	0.0158	0.0554	1.7164	5.0937
<i>Planctomyces</i>	0.0051	0.0027	0.0069	0.0070	0.0061	0.0101	0.0076	0.0100	0.0107	1.6971	2.2460
<i>Jiangella</i>	0.0011	0.0017	0.0007	0.0021	0.0010	0.0018	0.0027	0.0006	0.0015	1.6801	1.6452

(D) 12%water – oxic

Genus	Abundance D6	Abundance D6	Abundance D6	Abundance D41	Abundance D41	Abundance D41	Abundance D133	Abundance D133	Abundance D133	MEAN FOLD INCREASE (D41/D6 + D41/D6 + D41/D6)/3	MEAN FOLD INCREASE (D133/D6 + D133/D6 + D133/D6)/3
<i>Isoptericola</i>	0.0004	0.0007	0.0003	0.0339	0.0341	0.0343	0.1579	0.0002	0.0897	86.4045	236.9465
<i>Phyllobacteriaceae (uncultured)</i>	0.0007	0.0006	0.0002	0.0060	0.0051	0.0073	0.0235	0.0013	0.0131	18.8078	35.1377
<i>Acidithiobacillales (uncultured)</i>	0.0003	0.0001	0.0007	0.0021	0.0020	0.0020	0.0012	0.0005	0.0029	13.8590	5.4875
<i>Luteimonas</i>	0.0001	0.0004	0.0003	0.0016	0.0021	0.0012	0.0203	0.0337	0.0091	7.7855	96.5365
<i>Glycomyces</i>	0.0048	0.0053	0.0080	0.0323	0.0330	0.0264	0.0183	0.0176	0.0396	5.4386	4.0374
<i>Myceligenans</i>	0.0018	0.0022	0.0016	0.0053	0.0051	0.0067	0.0304	0.0012	0.0311	3.1770	12.5126
<i>Promicromonospora</i>	0.0025	0.0030	0.0025	0.0109	0.0057	0.0060	0.0620	0.0012	0.0345	2.8711	12.9518
<i>Pelagibacterium</i>	0.0008	0.0051	0.0014	0.0045	0.0022	0.0029	0.0051	0.0229	0.0059	2.6040	4.8722
<i>Nocardiopsis</i>	0.0042	0.0027	0.0039	0.0088	0.0061	0.0073	0.0058	0.0012	0.0094	2.0755	1.4135
<i>Propionibacteriaceae (uncultured)</i>	0.0122	0.0070	0.0109	0.0146	0.0161	0.0142	0.0109	0.0042	0.0257	1.5975	1.2838
<i>Rhodospirillaceae (uncultured)</i>	0.0020	0.0022	0.0028	0.0029	0.0026	0.0058	0.0152	0.0033	0.0129	1.5803	4.5867
<i>Gemmatimonadetes (uncultured)</i>	0.0100	0.0109	0.0096	0.0144	0.0185	0.0144	0.0074	0.0270	0.0126	1.5449	1.5092