SOIL MICROBIAL RESPONSES TO SIMULATED CLIMATE CHANGE DRIVERS

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Submitted in fulfilment of the academic requirements for the degree of Doctor of Philosophy (PhD) in the Discipline of Microbiology; School of Life Sciences; College of Agriculture, Engineering and Science at the University of KwaZulu-Natal, Durban.

As the candidate's supervisor, I have approved this thesis for submission.

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PREFACE

The experimental work described in this thesis was carried out in the School of Life Sciences, University of KwaZulu-Natal (Westville Campus), Durban, South Africa from September 2008 to December 2012, under the supervision of Professor B. Pillay and Professor A. O. Olaniran. Real Time PCR experiments (as described in Chapter Four) was conducted at the Institute for Evolution and Biodiversity, University of Münster, North Rhine Westphalia, Germany from November 2011 to June 2012, under the supervision of Professor J. Kurtz.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text.

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DECLARATION 2 - PUBLICATIONS

Details of contributions to publications that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication).

Publication 1

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Authors:
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A. O. Olaniran - Conceptualisation and editing of manuscript
B. Pillay - Conceptualisation and editing of manuscript

Publication 2

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Authors:
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A. O. Olaniran - Conceptualisation, Experimental design, Manuscript editing
B. Pillay - Conceptualisation, Experimental design, Manuscript editing

Publication 3

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ABSTRACT

Climate change is one of the biggest environmental challenges being experienced in the 21st century and is expected to continue to cause drastic alterations to the hydrological, biological and ecological ecosystems. Soil, the second largest carbon pool after the oceans, is a major factor in the global response towards climate change. The ability of soil to act as a sink or source of carbon as climate change increases can be influenced by soil microbial activity. Soil microbial activity is a key driver of terrestrial ecosystem functions and is extremely sensitive towards climate changes. Therefore, the main objective of this study was to investigate the effects of individual and/or interactive global change factors on soil microbial activity and diversity under elevated or ambient temperature incubations during the spring and summer seasons. This was accomplished by the addition of carbon dioxide (CO_2), methane (CH_4) or simulated rainfall to soil over a 60-day period using Screen Aided Carbon Dioxide Control experiments. Soil microbial dehydrogenase, urease, arylsulphatase and β-glucosidase activities were determined using standard enzyme assays over the 60-day period. In spring, the soil dehydrogenase and arylsulphatase activities increased by 28.07% and 28.48%, respectively, after the addition of elevated CO_2 under elevated temperature. Lower dehydrogenase activities were observed at day 60 for most plots during summer while β-glucosidase activity was unaffected by the addition of single or multiple global change drivers during spring. In summer however, all treatments resulted in 28.05 - 36.39% higher β-glucosidase activity by day 15, compared to day 0. Urease enzyme activity was higher during spring at both temperature conditions indicating that moisture limitation and temperature change constrained the urease enzyme production during the summer period. Neither the combination of elevated CO_2 and rainfall nor the combination of elevated CO_2, rainfall and methane induced substantial changes to the enzyme activities during both seasons, suggesting an antagonistic effect of the combination of these global change factors. However, differences observed from a combination of elevated CO_2 at higher temperature clearly reflect a potential for interaction that will affect soil enzymes and subsequent nutrient cycling. This study also investigated the changes in the soil bacterial RuBisCo gene (*cbbL)*, important for CO_2 fixation and the corresponding changes in soil organic carbon (SOC), upon exposure to various single or multiple global change drivers. Lowest *cbbL* gene copy numbers were observed during summer, while, during spring, the *cbbL* gene copy numbers increased (90.9 – 93.09%) by day 60 compared to day 0, under elevated temperatures. The combination of global change drivers did not result in a substantial variation in *cbbL* gene copy numbers

across seasons suggesting a counteractive effect of the factors, similar to changes in soil microbial enzyme activity. No direct correlation between changes in copy number and SOC was observed, although lower SOC in summer at elevated temperature did result in overall lower *cbbL* gene copies. Denaturing Gradient Gel Electrophoresis, used to investigate changes in soil microbial community structure, revealed seasonal variability changes in microbial diversity during spring. Soil moisture was a key factor in determining microbial responses during both seasons, with the elevated rainfall treatments able to counteract the adverse effects of elevated temperature during the spring season, with communities in these plots appearing more robust. Increased temperatures and lower soil moisture during the summer period had a negative effect on microbial diversity; however, sequence analysis of excised bands revealed the dominance of thermotolerant bacterial species. A combination of all the global change factors did not induce substantial change in community structure during spring at both temperature regimens. During summer at elevated temperature, growth of certain microbial species were inhibited by a combination of all the global change factors, highlighting the interactive effect between temperature, greenhouse gases and soil moisture. Furthermore, the loss of methanotrophic bacteria, (*Methylosinus* and *Methylocystis)* during both seasons can negatively impact greenhouse gas flux and consequently the carbon cycle at large. In this study, seasonal changes linked to variations in soil moisture, substrate availability and temperature strongly influenced soil microbial responses towards climate change. Considering that climate change is a multifactorial process, this study also clearly highlights the necessity for multi-factor global change studies, especially across different seasons in order to accurately predict the fate of soil ecosystem as climate changes continue to increase. Climate change studies often disregard microbial contributions and if carbon sequestration strategies are to be successful, we must fully understand microbial responses under various environmental conditions. An in depth understanding of factors that can lead to changes in soil microbial community activity and structure which influence nutrient and greenhouse gas cycling is essential towards enhancing knowledge of climate change mitigation strategies. Despite the drastic increases in greenhouse gases, temperature and/or rainfall simulated in the present study, it was evident that certain species of soil microorganisms were still able to survive and mediate biochemical activities that are beneficial to the community as a whole.

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CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Prevailing global climate change due to increased greenhouse gas emissions is currently being regarded as one of the most significant scientific and political challenges of the 21st century (Bardgett *et al*., 2008). The increased combustion of fossil fuels for energy has resulted in higher concentrations of atmospheric gases viz. carbon dioxide (CO_2, nitrous oxide and methane, which contribute to the greenhouse effect. These gases are the main causes of the current unprecedented increases in global warming (Vijaya Venkata Raman *et al*., 2012).

Terrestrial ecosystems function to absorb or release greenhouse gases such as CO_2 methane or nitrous oxide as well as to emit aerosols and their precursors (Heimann and Reichstein, 2008). Therefore, terrestrial ecosystems are closely associated with the climate system because of their crucial role in carbon cycling that occurs between the vegetation, the atmosphere and soils (Cao and Woodward, 1998). Microbial communities are responsible for approximately half of all global photosynthesis and almost all organic matter decomposition, nitrification, denitrification and methane production etc. (Schlesinger, 1997), and influence global emissions of the main greenhouse gases (Singh *et al*., 2010). The response of soil microbes to climate change mainly through their role in the decomposition of soil organic matter influences their effect on the carbon cycle.

Soil microbes can provide feedback directly to climate change by their contribution towards organic matter decomposition. Increased global warming is likely to accelerate heterotrophic

microbial activity, effectively resulting in increased efflux of CO_2 to the atmosphere (Davidson and Janssens, 2006; Bengston and Bengtsson 2007, Bradford *et al*., 2008; Pritchard, 2011; Suseela *et al*., 2012), thus creating a positive feedback on climate change. Alternatively, various environmental constraints such as the physical and chemical composition of organic matter can lead to decreased substrate availability necessary for microbial activity. This is likely to decrease microbial responses to increased global warming (Luo *et al*. 2001; Ågren and Bosatta 2002; Davidson and Janssens, 2006; Xu *et al*. 2012).

1.2 SCOPE OF THIS STUDY

It is evident that microbial processes have dominant roles in biogeochemical cycles and affect the global emissions of the main greenhouse gases (Singh *et al*., 2010). Therefore, understanding their response to climate change is critical towards interrogating and predicting carbon budgets. Despite the various reactions catalysed by the soil microbial community towards the maintenance of various atmospheric functions, there are significant gaps in the understanding of the survival and fate of microbes, and the processes they mediate, in response to climate change (King *et al*., 2001; Castro *et al*., 2010; Das *et al*., 2011). Ambiguity regarding microbial processes leads to unreliable model predictions of soil carbon feedbacks to climate change (Kirshbaum, 2006), resulting in frequent exclusions of microbial activities from climate change data projections. Determining the response of soil microbial communities and their roles in creating positive or negative feedbacks to the atmosphere, represents an important research challenge. Studies detailing the association between heterotrophic microbial respiration and various climate change variables viz. temperature, greenhouse gas concentrations etc., is seriously lacking, and demands intensive examination (Bardgett *et al*., 2008; Singh *et al*., 2010). Furthermore, the link between microbial community composition and ecosystem processes remain unresolved (Bradford *et al*., 2008).

The composition and function of soil microbial communitites can be affected by climate change (Gray *et al*., 2011) and previous studies on the effects of climate change on biological systems and soil microbes focused mainly on the effects of single global change drivers (Bardgett *et al*., 2008). However, realistic global change is effectively the result of various factors acting in concert (Shaw *et al*., 2002). In addition, it has been speculated that global change factors e.g. CO_2, warming, drought etc. may exert antagonistic or additive effects on soil microbes, and it is impossible to determine the outcome of interactive effects from single factor experiments (Gray *et al*., 2011). It is therefore crucial to investigate the effects of multiple global change drivers on the soil microbial community. Consequently, this knowledge will enable a concise understanding of soil microbial ecology that is paramount to effectively assess terrestrial carbon cycle-climate feedbacks (Bardgett *et al*., 2008).

Knowledge concerning the behaviour of soil microorganisms to combinations of global change drivers will provide a more realistic overview of expected microbial responses. Finally, the use of current molecular and genomic methodologies will provide a deeper insight into ecological functions catalysed by the soil microbes (Singh *et al*., 2010). Therefore, the main focus of this research was to determine the response of soil microbial populations and activities to global change simulations particularly, with regard to predicted climate changes in South Africa. The effects of individual and/ or combination of several factors expected to exacerbate the effects of climate change on soil microbial communities was also investigated. Finally, potential for soil carbon sequestration was determined through the quantification of microbial genes necessary for CO_2 fixation.

1.3 HYPOTHESIS

It is hypothesised that seasonal variations as well as elevated temperature, CO_2, methane and precipitation will negatively affect soil microbial activity and diversity. It is further hypothesised that global change drivers influences changes in the *cbbL* gene copy number that codes for the ribulose-1,5-bisphosphate carboxylase/ oxygenase enzyme (RuBisCo) by modifying the soil nutritional status.

1.4 OBJECTIVES

1.4.1 To determine the effects of simulated global change drivers viz. temperature, CO_2, methane and precipitation on soil microbial diversity and activity.

1.4.2 To evaluate the impact of global change drivers on soil bacterial RuBisCo genes.

1.5 AIMS

1.5.1 To construct Screen-aided carbon dioxide control experiments.

1.5.2 To alter the global change drivers; CO_2, methane and precipitation in soil to projected increments and determine their effects on the soil microbial activities under elevated and ambient temperature across the spring and summer seasons over time.

1.5.3 To assess the effects of the global change drivers on important soil microbial enzymes during spring and summer seasons over time.

1.5.4 To profile microbial diversity in the soil samples subjected to different global change drivers using Denaturing Gradient Gel Electrophoresis (DGGE) and to identify the dominant microorganisms in soil, during and after exposure to the global change drivers.

1.5.5 To determine changes in soil organic carbon and nutrients after exposure to global change drivers.

1.5.6 To quantify changes in the *cbbL* gene copy number during exposure to global change drivers using Real time PCR (qPCR).

1.6 KEY QUESTIONS TO BE ANSWERED

1.6.1 What are the effects of the different global change drivers on microbial activity?

1.6.2 What is the effect of seasonal variations and elevated temperature conditions on soil microbial activity and diversity?

1.6.3 Does a combination of global change drivers exert an antagonistic or synergistic effect on the soil microbial community?

1.6.4 How do the global change drivers affect bacterial *cbbL* gene copy numbers?

1.6.5 Does microbial community structure change over time as a result of exposure to the global change drivers?

1.6.6 What are the dominant microorganisms in soil that have been exposed to the global change drivers?

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CHAPTER TWO

'CLIMATE CHANGE AND SOIL MICROORGANISMS: THE 'CATCH 22' OF THE 21ST CENTURY'

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CLIMATE CHANGE AND SOIL MICROORGANISMS: THE 'CATCH 22' OF THE 21ST CENTURY

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Abstract

Climate change is one of the biggest environmental challenges being experienced in the 21st century and will continue to cause drastic alterations to the hydrological, biological and ecological ecosystems. Soil is the second largest carbon reservoir and is important for carbon cycling in terrestrial ecosystems. Climate change such as increases in temperature, changing precipitation patterns etc. will not only affect humans, society and the environment but may also affect the functioning of the soil ecosystem, either positively or negatively. Soil microbial activity is a key driver of terrestrial ecosystem functions and is extremely sensitive towards climate changes. The ability of soils to act as a sink or source of carbon as climate change increases can be influenced by soil microbial activity. Soil microorganisms may experience direct or indirect effects of climate change which can impact nutrient and biogeochemical cycling. In particular, soil methanogenic and methanotrophic bacteria are important mechanisms for methane (CH_4) production and consumption. An understanding of the response of soil microbial activity is imperative if soil is to be used effectively to mitigate the effects of climate change on the carbon cycle. This review summarises the main causes and effects of climate change, and emphasized the relevance of terrestrial ecosystems and soil microorganisms in regulating carbon cycle feedbacks.

Keywords: terrestrial carbon cycle feedbacks, soil microorganisms, climate change, greenhouse gas

CONTENTS

1.0 Introduction

Nearly a century ago, poet Robert Frost contemplated the downfall of mankind and imagined that the world would end in conditions that were either 'fiery hot' or 'icy cold' in his statement: *"Some say the world will end in fire, some say in ice. From what I've tasted of desire, I hold with those who favor fire. But if it had to perish twice, I think I know enough of hate, To say that for destruction; ice is also great. And would suffice"* (Frost 1920). Six hundred and fifty thousand years of history have revealed several changes in Earth's climate supporting these ideas. The abrupt conclusion of the last ice age about 7000 years ago (VijayaVenkataRaman et al. 2012) however, renders Robert Frosts hypothetical notions of an icy cold apocalypse obsolete. In the 21st century, scientific consensus is absolute and it is clear that *'ALL say the world will end in fire'*.

The term 'Climate Change' refers to a statistically significant variation in the typical state of the climate that usually prevails for extended time periods, often 10 years or longer. These variations are caused by changes in atmospheric composition resulting from prolonged anthropogenic activities, natural internal processes or external forces (VijayaVenkataRaman et al. 2012). Previously only investigated as a matter of scientific curiosity, global climate change is currently being regarded as one of the most significant scientific and political challenges of the 21st century (Bardgett et al. 2008). Multi-scale and multi-level global climatic changes are increasingly apparent across various fields and these changes are likely to have both positive and negative effects (Sutherst 2004; Nan et al. 2011). The impending dangers of climate change have set the international community an impossible challenge of curbing greenhouse gas emissions or facing the risks associated with

increased global temperatures (Poortinga et al. 2011). This review summarises the main causes and effects of climate change, and emphasized the relevance of terrestrial ecosystems and soil microorganisms in regulating carbon cycle feedbacks. Some important microbial groups that can affect the carbon cycle were also highlighted

1.1 Causes of climate change

While global change is not a new phenomenon and has been occurring naturally throughout Earth's history (King et al. 2001), there is considerable unanimity from the scientific community that human activities are the primary reason for the recent global climate change trends (Watson 2003). Human induced climate change has proven to be an extremely controversial issue mainly because the detection of minor changes in the average global temperature is generally masked by significant annual variability (Sutherst 2004). In 1996, a cohort of international scientists known as the Intergovernmental Panel on Climate Change (IPCC) was appointed to advise governments on developments in the science that underpins climate change. This panel, for the first time stated that, "the balance of scientific evidence suggests that human activities during the last century have begun to have a discernible effect on the world's climate causing it to warm" (Houghton et al. 1996). Some of the factors responsible for climate change include global warming, changes in atmospheric greenhouse gas concentration and aersosols or changes in natural processes such as volcanic eruptions and solar changes (Forster et al. 2007).

1.1.1 The Greenhouse Effect

Thermal equilibrium on Earth is maintained by a balance of the net incoming solar radiation obtained from the sun, with the infra-red radiation emitted back into space. To equalize this incoming solar radiation, Earth begins to emit long wave radiation, some of which is deflected and absorbed by the greenhouse gases that are resident in the atmosphere (Le Treut et al. 2007). Such gases include carbon dioxide (CO_2), methane (CH_4), nitrous oxide, dimethyl sulphide and water vapour with the amount of radiation that they are able to absorb dependent on their molecular structure (Huntingford et al. 2004). These gases are borne almost entirely from biological sources and function to trap heat, thereby warming the planet by the 'Natural Greenhouse Effect' (IPCC 2001; Kasting and Siefert 2002; CSIR 2005). Thus, the concentrations of the greenhouse gases significantly influences mean atmospheric temperatures (Huntingford et al. 2004) as well as any potential changes in the climate. Numerous chemical and biological processes can lead to changes in the composition and concentration of these important atmospheric gases (King et al. 2001).

1.1.2 Anthropogenic Greenhouse Effect

The most significant greenhouse gases are CO_2 and water vapour (Le Treut et al. 2007). Carbon dioxide is generally regarded as the most dominant greenhouse gas and is therefore frequently used as a proxy when referring to climate change issues and the anthropogenic greenhouse effect (Siohansi 2010). However, more than a century ago, Arrheinus identified CO_2 as a critical component of the atmosphere that regulates Earths temperatures (Arrheinus 1896; Mitchell et al. 1995). Therefore, higher average planetary temperatures would be expected as CO_2 concentrations are increased (Folland et al. 2001). This has been an unfortunate secondary effect of the industrial revolution which began about 150 years ago. During this period, enormous quantities of greenhouse gases, especially CO_2 were added to the atmosphere via human activities (VijayaVenkataRaman et al. 2012).

The primary source of CO_2 into the atmosphere is combustion of fossil fuels which include oil and coal. These substances are essential for electricity production and important for various industrial processes. Continual deforestation, burning of waste products and gas emission from sanitary landfills are also important contributors of elevated atmospheric CO_2 concentrations (Gomes et al. 2008). In addition, the global transport sector was responsible for 21% of fossil fuel-related CO_2 emissions in the year 2000 (Fuglestvedt et al. 2008). Globally, this anthropogenic greenhouse effect is dominated by 60%, 15% and 5% of CO_2, CH_4 and nitrous oxide gases, respectively (Rodhe 1990). The concentrations of these gases have increased exponentially since the industrial revolution (IPCC 2001) exacerbating the anthropogenic greenhouse effect. In fact, between 1750 and 2000, the atmospheric concentrations of CO_2, CH_4 and nitrous oxide had escalated by 31%, 151% and 17%, respectively and are steadily increasing annually by 0.5%, 1.1% and 0.3% respectively. Average global temperatures are expected to increase by approximately 1^o C by the year 2025 with the continued increases in greenhouse gas concentrations (IPCC 2001). It is apparent that mankind has drastically modified the chemical composition of the atmosphere with significant repercussions for the climate system (Le Treut et al. 2007).

1.2 Impacts of climate change

Primarily, the hydrological, biological and ecological ecosystems will experience drastic alterations due to climate change. In addition, it is assumed that economy and sustainable development will be extremely vulnerable at regional, national and global levels (Nan et al. 2011). Therefore, it is assumed that developing countries will encounter strong negative impacts and socioeconomic systems will be adversely affected (Watson

2003). Several changes in climate including increases in temperature, extreme weather events, sea level rise and rainfall variability have already been observed. In southern Africa, in particular, it is predicted that warming will be greatest in the northern regions, and that the arid regions will be most vulnerable (CSIR 2005).

1.2.1 Impact of Climate change on human health

The observation that climate changes can impact human health has been recognised since the time of Hippocrates, thousands of years ago. In general, global changes such as population growth, urbanisation, land use change and depletion of freshwater resources are all known to contribute to the state of human health (Fig. 1). Considering that these changes are also closely linked to the progression of climate change, it is likely that the effects of climate change on human health can be magnified in the future (Haines et al. 2006). Directly, the effects of heat stress and flooding are anticipated. Changes in disease transmission and malnutrition as a result of competition for crops and water resources are expected indirect effects (VijayaVenkataRaman et al. 2012).

1.2.1.1 Heatwaves

Climatologists have predicted a greater incidence of heatwaves resulting from increased global temperatures and weather variability (McMichael and Haines 1997). Physiological, behavioural and technological responses will ultimately enable regional populations to acclimatize and adapt to local prevailing warmer climates. Extreme events such as heatwaves however, incur additional pressure and stress whereby a population adaptation limits are exceeded. While heatwaves are not a common occurrence, these temperature extremes can have direct immediate effects on mortality (Haines et al. 2006).

Epidemiological studies involving extreme temperatures conducted in Europe and North America reveal a positive relationship between mortality and heatwaves, particularly amongst elderly people (McGeehin and Mirabelli 2001; Basu and Samet 2002). The European heatwave in the summer of 2003 was responsible for the deaths of 21 000 to 35 000 people in five countries and was arguably one of the hottest years in Europe since 1500 (Luterbacher et al. 2004; Epstein 2006). Excess mortality that occurs due to heatwaves results from cardiovascular, cerebrovascular and respiratory diseases (Mcmichael et al. 2006).

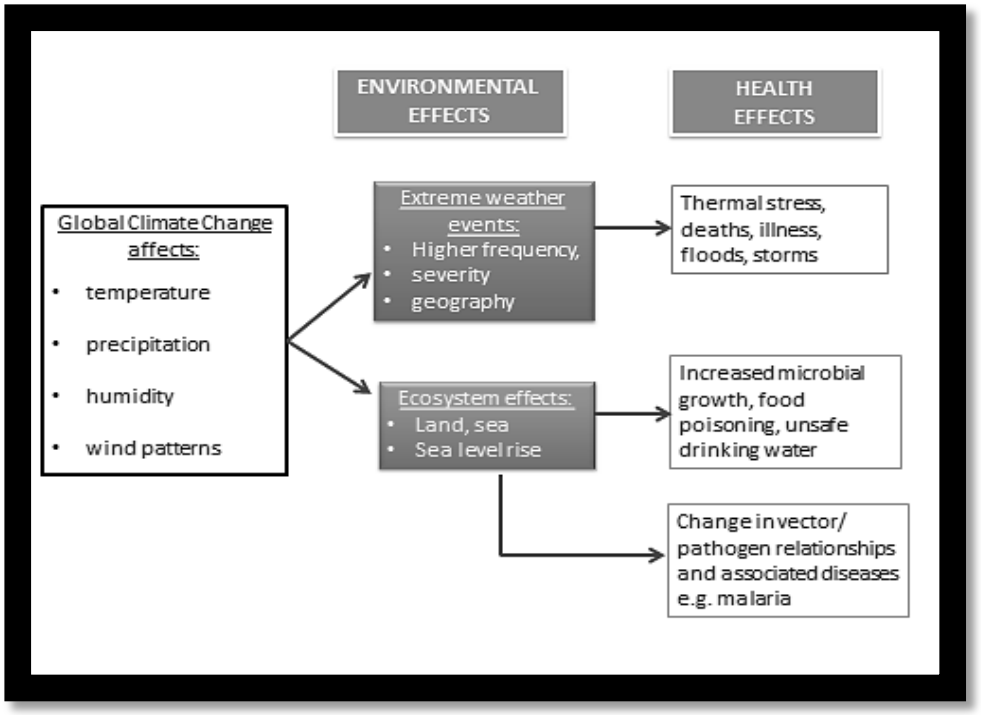

Fig. 1: Main pathway by which climate affects population health (Adapted from McMichael et al. 2006).

1.2.1.2 Floods, storms and rainfall

Amongst the various natural disasters observed worldwide, flooding is by far the most common (Greenough et al. 2001). Floods are high impact events that can severely damage physical infrastructure, impact human resilience and disrupt social organisation (Haines et al. 2006). Typically, floods may follow periods of extended heavy rainfall; although tidal surges and rapid snow melt have also been implicated as major causes of flooding (Hunter 2003). The most obvious threat of flooding to human health is drowning with most deaths from flooding resulting from rapid rise floods (French et al. 1983). The IPCC has projected a 40 cm increase in sea level by 2100, which is an alarming forecast considering that nearly 50% of the world's population lives within 60 km of the ocean. Considering population statistics, such a sea level rise would result in a two-fold increase (around 46 million individuals annually) in the number of people that would experience flooding (Baarse 1995).

Floods can also originate as a result of other extreme events such as tsunamis or hurricanes (Bariweni et al. 2012). One of the most devastating, 'most expensive' natural disasters in United States history, Hurricane Katrina resulted in over 1300 deaths and 2000 injuries, while the Indian Ocean tsunami lead to approximately 230 000 deaths in 2004 (Haines et al. 2006; Bariweni et al. 2012). Flooding also results in various other secondary effects, most importantly the increased frequency of disease clusters that usually occur soon after

flooding (Epstein 2006; McMichael et al. 2006), particularly an increase in diarrhoeal and respiratory diseases (Ahern et al. 2005). Twenty million people were affected by a devastating flood that occurred in Pakistan in 2010. Kirsch et al. (2012) surveyed 1769 households that experienced this flood and discovered that although immediate deaths and injuries were not common, 77% of respondents declared some type of flood related illness. It is also possible that excessive rainfall may ultimately contaminate waterways and drinking water supplies by allowing the dispersal of human sewage, animal wastes and toxic pollutants, thus increasing the frequency of water-borne diseases (Rose et al. 2000; Epstein et al. 2006).

1.2.1.3 Infectious disease

Social, economic, climatic and ecological conditions impact the transmission of infectious diseases (Weiss and McMichael 2004). In particular, climatic changes including those due to temperature, humidity, altered rainfall, soil moisture and sea level rise play a significant role in the spread of infectious diseases worldwide (Haines et al. 2006). Bacterial, protozoan and viral infectious agents together with associated vector organisms such as mosquitoes, ticks and sandflies do not harbour any thermostatic mechanisms. Thus, temperature variations significantly influence their reproduction and survival rates (Gubler et al. 2001; Kovats et al. 2001). Presumably, global climate change can either increase or decrease the frequency of vector-borne diseases. In addition, geographic location also influences the duration of the transmission (Haines et al. 2006). In particular, owing to the tropical climate, Africa is an ideal environment for the development of most major vector-borne diseases including malaria, schistosomiasis, onchocerciasis, trypanosomiasis, filariasis, leishmaniasis, plague, Rift Valley fever, yellow fever and tick-borne haemorrhagic fevers. New disease patterns are also likely to emerge because of the diversity of vector-species complexes in Africa that have the ability to redistribute themselves to new climate driven habitats (Githeko et al. 2000).

The El Nino Southern Oscillation cycle (ENSO) is a dominant factor in determining global weather patterns and is strongly associated with the development of infectious diseases (Haines et al. 2006). The ENSO pattern has been linked to malaria outbreaks in South Asia and South America as well as with cholera epidemics in Bangladesh (Kovats et al. 2003). It is believed that climate change may already be responsible for the transmission of infectious diseases (McMichael et al. 2006). In Sweden, disease-bearing ticks are migrating northward as winter temperatures have escalated over the years. An increase in the number of tick-borne (viral)

encephalitis cases has been reported over the last two decades and projections reveal that a similar trend will occur in Canada and the United States (Lindgren 1998; Lindgren et al. 2000; Epstein 2006).

1.2.2 Socio-economic impacts

Socio-economic effects are expected to occur as an indirect consequence of the various climate change driven effects. Naturally, many countries worldwide are interested in the positive and negative effects that may occur in the future due to climate change. These effects are likely to influence domestic and international policies, trading patterns, resource use, regional planning and the general welfare of many populations (Fischer et al. 2005). It has been presumed that developing countries will encounter stronger socio-economic effects in response to climate change due to various factors. Primarily, the use of agriculture features quite prominently as this practice is important in the maintenance of economies globally (Parry et al. 2004). A large proportion of the world's food is grown in the tropics. Since these are predominantly rainfed annual crops, climate variability is important in determining crop productivity (FAO 2002). Consequently, threats to food security are imminent and it is predicted that all societies will be susceptible to changes in the production, quality and supply of food (Slingo et al. 2005). In South Africa, climate change is anticipated to have a strong impact on agriculture due to higher rainfall variability and increased frequency of drought. These forecasts are disturbing considering that more than a million people are dependent on agriculture to sustain their livelihoods (Hassan 2006).

Accurately determining the impact of climate change on economic growth is an arduous task mainly because of a country's economic development and political capacity (Koubi et al. 2012), however it has been suggested that developing countries may be confronted with the problems of insufficient capital for adaptation measures (Fischer et al. 2005). Furthermore, the ability of a country or region to recover after the occurrence of an extreme weather event can be substantially altered for many years following the event. This is especially pronounced in the case of Hurricane Mitch in Central America in 1998 that triggered long term effects in the regional economies (Hidalgo and Alfaro 2012). Incidences of extreme events can also lead to repercussions that are coupled to economic activity which may include changes in incomes and terms of trade, increased inflation and demand and supply variations (Bergholt and Lujala 2012). One of the major socio-economic effects predicted and of grave concern is the hypothesis that non-democratic countries have an increased risk of civil conflict associated with a decline in economic conditions. It has been argued that increased competition among

groups in a region will occur more frequently and thus the possibility of conflict will be enhanced (Koubi et al. 2012).

1.2.3 Effect of climate change on hydrological systems

Earth's oceans are an important component in the global climate system covering approximately 72% of the planet. Primarily, the ocean is involved in reducing and/ or maintaining climate variability owing to its thermal inertia and heat capacity (Barnett et al. 2001). Studies detailing the effects of climate change on marine ecosystems are severely lacking, due to various factors including the magnitude and complexity of the ocean as well as complications that arise from obtaining measurements directly from marine environments (Hoegh-Guldberg and Bruno 2010). However, it has been observed during the last 10-15 years that sea water temperatures have experienced drastic changes. These alterations have occurred due to the disappearance of the sea ice cover in the Arctic, the increased melting of glaciers and the Greenland Ice cap, reduction in the volume of the Antarctic ice sheets as well as rise in sea levels. All these events have been attributed to climate change (IPCC 2007; Solomon et al. 2007; Hoegh-Guldberg and Bruno 2010).

The oceans routinely function as the 'planets heat sink' and it is estimated that approximately one-third of the CO_2 emissions resulting from human activities are absorbed by the ocean (Hoegh-Guldberg and Bruno 2010). Another important consequence of ongoing climate change is ocean acidification that results from increased dissolution of atmospheric CO_2 in sea water (IPCC 2007). This has led to the acidification of the surface layers of the ocean and a decrease of 0.02 pH units per decade has been observed for the last 30 years (Doney et al. 2009). Ocean acidification could seriously influence the capacity of marine and coastal systems to supply services to humans (Rodrigues et al. 2013). In addition, slower growth rates, change in disease susceptibility and a reduction in reproductive capabilities on a vast range of processes and species may also occur (Wernberg et al. 2011). A number of marine organisms live in close proximity to their thermal tolerance levels, thus negative effects on their reproduction, survival and performance are expected in response to elevated temperatures (Somero 2002; Hughes et al. 2003; Harley et al. 2006). Consequently, a range of undesirable secondary effects may occur. For example, changes in the composition and distribution of the microbial assemblages that comprise plankton (phytoplankton, bacteria, heterotrophic nanoflagellates and viruses) are anticipated and will impact the ecology and biogeochemistry of marine ecosystems. These indirect effects will have implications for

the exchange of carbon between the oceans and atmosphere by altering the efficiency with which biotic processes relocates carbon from the surface to the deep ocean via the biological pump (Evans et al. 2011).

1.3 Importance of terrestrial ecosystems in carbon cycle feedbacks

Carbon equilibrium on Earth is maintained by three reservoirs viz. the oceans, atmosphere and the terrestrial ecosystems which are in constant interaction with each other (Eswaran et al. 1993). Terrestrial ecosystems contain approximately three times more carbon than that of the atmosphere (Schimel, 1995) and are therefore a crucial component in carbon uptake and exchange. Terrestrial ecosystems function to absorb or release greenhouse gases such as carbon dioxide, methane or nitrous oxide as well as to emit aerosols and their precursors. Furthermore, the terrestrial biosphere is important for interchange of water and energy between the land surface and the atmosphere (Heimann and Reichstein 2008). Therefore, terrestrial ecosystems are closely associated with the climate system because of their crucial role in carbon cycling that occurs between the vegetation, the atmosphere and soils (Cao and Woodward 1998). Furthermore, these ecosystems are strongly impacted by climate and can also have significant effects on climate regimes (Yurova et al. 2010). Recently, it has been suggested that terrestrial ecosystem feedbacks can alter the Earth in a globally significant way (Heimann and Reichstein 2008). The effect of carbon cycle feedbacks on climate change remains unclear mainly due to a lack of detailed information about terrestrial ecosystem processes, especially under multifactorial global change conditions (Singh et al. 2010).

Carbon balance in a specific ecosystem at a specific point in time can be defined as the difference between carbon gain and loss. Terrestrial ecosystems acquire carbon via photosynthetic processes and emit CO_2 through autotrophic (plants and photosynthetic bacteria) and heterotrophic (fungi, animals and certain bacteria) respiration (Heimann and Reichstein 2008) and are considered as a significant carbon source or sink according to projections from CO_2 models (Raich and Potter 1995). Variations in terrestrial metabolism will prove to be valuable indicators of global change (Canadell et al. 2000). An extensive knowledge of the sensitivity of the terrestrial carbon balance to change in climate is a high priority as it is possible that fluxes in terrestrial carbon storage will influence the progression of climate change (Cox et al. 2000).

1.4 Soil: Sink or source of CO_2?

Soil is imperative for sustaining life on Earth and it is essential for the maintenance and support of plant growth from an agronomic perspective. In addition to its general ecological importance, interactions that occur in soil have global scale implications on the environment due to their role in carbon cycling. Soil is a three-dimensional; multiphase; multicomponent, non-isothermal biogeochemical system (Rockhold et al. 2004) and typically contain approximately 1500 Pg (Pg = petagram = 10^{15} g) of carbon. This value constitutes approximately twice the amount of CO_2 found in the atmosphere (Smith 2012), therefore, even relatively minor changes in this pool may have significant effects on atmospheric concentrations and the global carbon cycle (Belay-Tedla et al. 2009).

Soil or pedologic pool is composed of two components viz. the soil organic carbon and soil inorganic carbon pools. Typically, soil organic carbon concentrations are low in arid regions, high in temperate soils and the highest concentrations have been measured in peat or organic soils. It has been suggested that improving the quantity and quality of the soil organic pool will be beneficial in several ways, including increased biomass/ agronomic production, better water quality, reduction of sedimentation of reservoirs and waterways and most critically; global warming and climate change (Lal 2004a). Most climate change projections indicate that changes in soil carbon will inevitably range from small losses to moderate gains, while significant regional variation is apparent globally (Smith 2012). This soil organic carbon pool is positively influenced by the biodiversity of soil organisms and healthy soils contain a highly diverse soil biota which includes representatives of microorganisms (fungi, green algae and cyanobacteria) (Lal 2004a). Ecosystems that have a higher biodiversity of the soil biota are suggested to sequester more carbon than those with reduced biodiversity (Lal 2004a). Yuan et al. (2012) suggest that in terrestrial ecosystems, microbial autotrophy could be responsible for up to 4% of the total CO_2 fixed annually. Soil carbon sequestration has the ability to offset fossil fuel emissions by 0.4 to 1.2 Gt carbon/ year which is equivalent to 5-15% of the total global emissions (Lal 2004b). Thus, with the proper recommended management practices especially with agricultural soils, a drastic reduction in the atmospheric CO_2 concentrations can be achieved (Lal 2004a).

Root respiration and microbially mediated organic matter decomposition drives CO_2 production from soils (Davidson and Janssens 2006), therefore, net CO_2 flux from soils is usually directed towards the atmosphere (Raich and Potter 1995). Thus, steady state soils are considered as a source of CO_2 (Miltner et al. 2004). An

increase in soil respiration, resulting in the release of additional CO_2 to the atmosphere is forecast as global warming continues (Smith and Fang 2010), mainly because bacteria in soil are generally considered to be carbon limited. Under higher temperatures, nutrient availability would be enhanced thus alleviating soil microbial resource limitations, creating a substantial feedback to climate change (Rinnan et al. 2007). However, Bradford et al. (2008) indicated that the effects of global temperature rise on soil respiration rates may not be as significant as predicted after investigating the thermal adaptation of soil microbial respiration at elevated temperatures. This conclusion also concurred to other reports that suggest that soil microbial respiration will thermally adapt to higher temperatures, dampening the stimulatory effect of warming on soil respiration rates (Oechel et al. 2000; Luo et al. 2001; Reichstein et al. 2005; Davidson and Janssens 2006). Thus, in order to fully manage soil microbial communities to increase carbon sequestration and mitigate the effects of climate change, it is imperative to fully comprehend their ecological responses towards climate change (Singh et al. 2010).

1.5 Soil microbes as regulators of biogeochemical cycles and soil carbon

The microbial communities of bacteria, fungi and unicellular algae are at the smallest scales of life (Schimel 2004) and are essential for a variety of functions in all ecosystems (Table 1). Earth is made habitable mainly due to some of the essential functions that microorganisms catalyse which results in the production of oxygen. Therefore it is clear that the presence and activity of microorganisms are fundamental in determining the course of atmospheric evolution (Kasting and Siefert 2002) and consequently human life.

In particular, soil microbes are known to encompass the largest genetic diversity on Earth, with as much as 1.5 million fungal species and between 6000 – 50 000 bacterial species (Whitman et al. 1998; Curtis et al. 2002; Coleman and Whitman 2005). Soil microorganisms exert a dominant role in managing and mediating the various biogeochemical cycles that regulate Earths system (Schimel 2007 Torsvik et al. 2002; Mohanty et al. 2006; Falkowski et al. 2008). The biological fluxes of five of the six primary building blocks of life for all biological macromolecules (the elements: H, C, N, O, S and P) are directed by microbially catalysed reactions (Schlesinger 1997; Falkowski et al. 2008). Furthermore, microbial processes are also responsible for approximately half of all global photosynthesis and almost all organic matter decomposition, nitrification, denitrification and methane production etc. (Schlesinger 1997) and are thus key players in the carbon, nitrogen and oxygen cycles. Carbon exchange between the land and atmosphere is determined by soil microbial activities

and can be divided into two categories; viz. carbon and methane uptake or soil carbon loss via respiration and production of methane (Bardgett et al. 2008). Thus, it is apparent that these organisms can control ecosystem functioning mainly because of their contributions towards various biological functions and their roles in nutrient recycling (Bruce et al. 2000).

Table 1: Influence of soil microbes on the carbon, nitrogen and phosphorus cycles (adapted from van der Heijden et al. 2008).

	Microbes involved	**Estimated microbial contribution to ecosystem process**
Carbon cycle		
Plant productivity	Nitrogen fixing bacteria, mycorrhizal fungi	0-50%
	Microbial pathogens	ND[a]
Decomposition	Bacteria, fungi	Up to 100%
Nitrogen cycle		
Plant nitrogen acquisition		
Nitrogen fixation	Rhizobia, actinomycetes, free-living bacteria	
Soil uptake	Mycorrrhizal fungi	0 – 80%
Nitrogen loss		
Denitrification	Denitrifying bacteria and some fungi	Up to 60%
Phosphorus cycle		
Plant phosphorus acquisition	Mycorrhizal fungi, P-solubilising bacteria	0 – 90%

[a] ND – Not determined

Plant nutrient availability and organic matter decomposition are mediated by soil microorganisms; and are therefore central towards understanding terrestrial ecosystem responses to global climate change (Freeman et al. 2004). The role of microorganisms in organic matter decomposition under higher temperatures is especially critical as it is presumed that increased warming will amplify CO_2 efflux from soil to the atmosphere (Bardgett et al. 2008). Furthermore, Luo et al. (2001) suggested that the shift of the terrestrial biosphere from a carbon sink to a carbon source is dependent on soil microbial respiration and its sensitivity to long term warming trends. Carney et al. (2007) showed that microbial utilisation of soil organic matter increased under elevated CO_2 fertilization. Addition of elevated CO_2 had resulted in the change in composition of soil microbial communities and this has implications in converting soil carbon sinks into carbon sources (Carney et al. 2007).

Basically, the net effect of climate change on carbon budgets of terrestrial ecosystems is determined by the equilibrium between photosynthesis and respiration i.e. autotrophic root respiration and heterotrophic soil microbial respiration (Bardgett et al. 2008). Soil CO_2 concentrations are a few orders of magnitude higher than the overlying atmosphere (Sadowsky and Schortemeyer 1997) and controlled by various factors including temperature and moisture (Davidson et al. 1998; Feiziene et al. 2010). Consequently, the effects of climate change on soil microorganisms (Fig. 3) will be largely indirect (Sadowsky and Schortemeyer 1997; Bardgett et al. 2008), while direct effects are expected to be minimal (Dhillion et al. 1996).

1.5.1 Direct effects of climate change on soil microorganisms

The main direct effects of climate change on soil microbial community are presumed to occur due to changes in temperature and soil moisture content (Bardgett et al. 2008; Singh et al. 2010). These factors can determine changes in soil microbial communities and affect greenhouse gas emissions by either influencing the physiological capabilities of the existing microbial populations or by causing a transformation of the microbial community structure. Two scenarios have been hypothesized; in the first scenario, the functions of the existing microbial community are directly impacted by the global changes, however microbial community structure remains intact. Process rates (e.g. soil respiration, decomposition of organic matter etc.) may be altered but controls of these processes remain the same. In the second scenario, the basic control mechanisms of the processes will be altered due to microbial community structure shifts in response to global changes. This effectively translates into a 'new' microbial community with modified physiological capabilities (Singh et al. 2010).

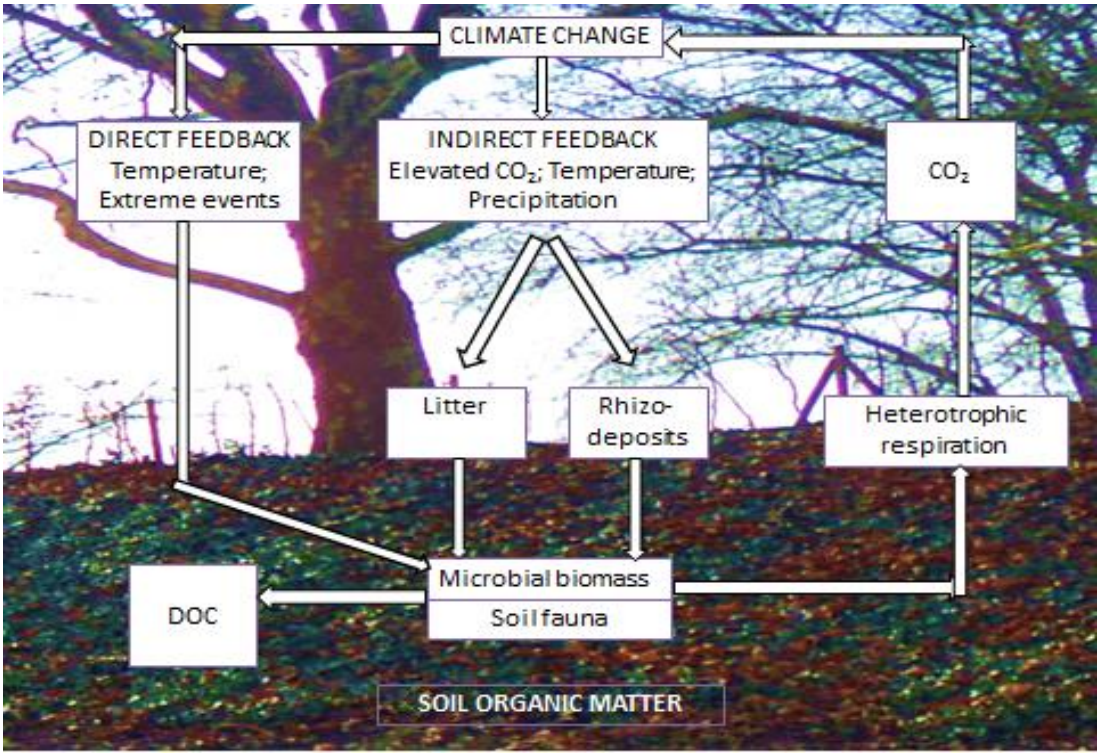

Fig 2: Direct and indirect effects of climate change on soil microbial communities and routes of feedback to global warming through carbon dioxide (adapted from Bardgett et al. 2008).

It has been established that soil microbial respiration and soil organic matter decomposition depend strongly on temperature (Kirschbaum 2006). If this temperature dependence is extrapolated into the long term, an increase in heterotrophic soil respiration rates will occur with the concurrent increase in global temperatures (Bradford et al. 2008). Overall, higher temperatures are expected to accelerate heterotrophic microbial activity, effectively resulting in greater CO_2 efflux to the atmosphere (Jenkinson et al. 1991; Hu et al. 1999; Davidson and Janssens 2006), creating a positive feedback on climate change (Cox et al. 2002). Some researchers have already indicated that increased temperatures due to climate change will inevitably lead to increased organic matter decomposition rates by soil microorganisms thus enhancing the release of CO_2 from soils and exacerbating climate changes (Subke et al. 2003; Knorr et al. 2005; Bengston, and Bengtsson 2007). Such conclusions are not surprising considering the inherent nature of biological reactions that generally proceed faster at higher temperatures. However, this concept has been contradicted in other studies which conclude that soil carbon will prove to be insensitive to increased temperatures (Luo et al. 2001; Ågren and Bosatta 2002; Xu et al. 2012). Thus it is clear that although a dearth of studies have been conducted in this area, the outcomes still remain debatable. Alternatively, various environmental constraints such as the physical and chemical composition of

organic matter, the presence of droughts/ floods and oxygen availability can lead to decreased substrate availability necessary for microbial utilisation. This is likely to decrease microbial responses to increased global warming (Davidson and Janssens 2006).

Another factor to consider is the quality and quantity of litter which is determined by plants but can have a direct effect on microbial community structure dynamics. Litter is the primary source of soil organic matter. However, it has been suggested that the addition of this substrate will encourage a 'priming' effect, which is the induced decomposition of certain soil organic matter fractions (Kuzyaka et al. 2000). Soil microorganisms are more prone to use labile carbon sources over complex carbon sources, ultimately resulting in a slower rate of litter decomposition. Consequently, this will generate lower respiration rates and CO_2 emissions, thereby enhancing carbon sequestration (Balser and Wixon 2009; Singh et al. 2010).

It has long been recognised that distinct microbial groups have specific optimal temperature ranges for activity and growth. There is considerable vagueness about the reactivity of different microbial species and groups towards temperature changes. It is possible that shifts in microbial community composition may occur due to higher temperatures as global warming progresses. These shifts may result in the reduction of soil organic carbon fluxes because of the loss of microbial groups already acclimatised to a particular set of environmental conditions (Kandeler et al. 1998; Monson et al. 2006). It still remains unclear whether changes in soil microbial respiration are as a result of shifts in microbial community composition and activity or the amount and quality of soil organic carbon (Harley et al. 2006; Bradford et al. 2008).

Soil moisture is another main driver of microbial community structure and organic matter decomposition (Bardgett et al. 2008; Singh et al. 2010). Soil microbes are known to encounter various physiological challenges as a result of physical disturbances. This is a consequence of altered micro-climates and change in resource availability. Directly, microbial communities are influenced by moisture levels as water is a necessity for microbial growth. Furthermore, the effect of moisture also contributes to changes in gas diffusion rates and the availability of oxygen (Singh et al. 2010). However, microbes can also experience pulse changes in the state of water, due to events that disturb soil structure such as drying, re-wetting and freeze thaw. Extreme events related to climate change including drought and freezing are also anticipated to exert strong controls on microbial activities (Schimel et al. 2007). Soil microorganisms that encounter moisture limitation are likely to produce

negative feedbacks on decomposition processes owing to prolonged drier conditions placing additional constraints on microbial growth and activity (Fierer and Schimel 2003; Singh et al. 2010). The impact of temperature and moisture changes is subject to differ between different ecosystems and regions. Therefore, it is critical to determine feedback responses of climate change at regional scales to avoid unreliable model predictions on a global scale (Kirschbaum 2006; Bardgett et al. 2008; Singh et al. 2010).

1.5.2 Indirect effects of climate change on soil microorganisms

Indirect effects of elevated CO_2 on soil microorganisms are likely to be mediated primarily by plants, which have a fundamental role in ecosystem responses to climate change (Dhillion et al. 1996). It is expected that changes in composition of rhizodeposits and root exudates may also cause an alteration in the amount, composition and functions of the soil microbial community (Blagodatskaya et al. 2010). Therefore, a complete understanding of plant responses such as root dynamics and exudates, litter production and decomposition will greatly improve knowledge of microbial responses (Freeman et al. 2004).

Two main mechanisms can be outlined regarding the indirect plant mediated effects on soil microbes and the associated climate change feedback routes. The first mechanism considers the indirect effects of elevated CO_2 concentrations on soil microbes due to higher photosynthesis rates. Higher CO_2 levels will modify the transfer of labile sugars, organic acid and amino acids from plant roots with the consequence of enhanced microbial growth and activity (Bardgett et al. 2008). Diaz et al. (1993) proposes that this can be problematic in the long term for plant growth as increased carbon efflux by roots can result in soil nitrogen immobilisation. Consequently, plants may experience nitrogen limitations and a negative feedback to plant growth will be created, restricting future plant growth (Diaz et al. 1993). Nitrogen limitations will also result in plant-microbial competition for available nitrogen placing constraints on microbial activity and subsequently microbially mediated soil decomposition processes (Hu et al. 2001). This indirectly favours carbon accumulation in soil ecosystems and these alterations can also have implications on the delicate soil carbon to nitrogen ratio. Higher soil carbon to nitrogen ratios have been shown to encourage higher fungal dominance and diversity (van den Heijden et al. 2008). Ecosystems with higher fungal abundance have lower soil respiration rates as fungi characteristically have high carbon assimilation efficiency (Six et al. 2006). The proliferation of mycorrhizal fungi will be advantageous as it can create a positive feedback to plant growth by assisting their host plants to cope with the higher nutrient demands, possibly also resulting in a positive feedback on plant growth (Gamper et al. 2005; Six et al. 2006).

Secondly, microbes may experience indirect effects of climate change through shifts in composition and diversity of vegetation. The distribution of plant species and functional groups will be affected by climate changes such as variations in temperature and precipitation (Prentice et al. 1992; Woodward et al. 2004). The uptake of CO_2 via photosynthesis can be influenced by changes in vegetation composition. These modifications of vegetation are able to strongly control carbon exchanges and changes in the soil physical environment can also be anticipated (Jackson et al. 1996). The principal mechanism by which climate-driven changes in vegetation affect soil microbial communities and their activities is through litter. The quality and quantity of plant litter entering the soil ecosystem will indirectly impact microbial metabolism. Furthermore, shifts in vegetation and composition can also result in competition for important nutrients between plant species, and between plants and soil microbes. These factors will have implications for nutrient cycles and soil carbon exchange, and therefore can also have consequences for terrestrial carbon cycle feedbacks (Bardgett et al. 2008).

1.6 Microbial production and consumption of methane: Implications for the carbon cycle

Activities within the soil ecosystem generate numerous trace gases including methane (CH_4), carbon monoxide (CO), nitrous oxide (N_20) and hydrogen (H_2), whose concentrations are determined by the chemical and biological activity exerted by a particular soil type. Thus, soils are able to function either as a sink or source of these trace gases and investigations into the mechanisms that control their flux is becoming increasingly important due to their involvement in enhancing the greenhouse effect and subsequently climate change (Conrad 1996; Hanson and Hanson 1996; Ritchie et al. 1997).

The chemical and biological processes that regulate CH_4 flux are of particular interest because CH_4 is 20 times more potent as a greenhouse gas than CO_2 (Matthews and Fung 1987; Wahlen 1993) and its concentration has reached record highs since the last 650 kyrs (Spahni et al. 2005). Furthermore, CO_2 is known to generate only 5% of global warming potential compared to CH_4 (Kim et al. 2012). The discovery of new sources of CH_4 (Walter et al. 2006) coupled with its nine to twelve year residence time in the atmosphere (Pearman and Fraser 1988) has fuelled concern and curiosity into the mechanisms that regulate atmospheric concentrations of CH_4. Higher atmospheric methane concentrations have resulted due to the differences between the sources and sinks of methane. Approximately 70% of global CH_4 is emitted by natural wetlands, tundra, rice paddies, ruminants and fossil fuel production and use, and these emissions are governed by both biological and chemical processes (Born et al. 1990; Ritchie et al. 1997). The effects of climate change can result in environmental modifications

that will impact microbial methane consumption and production rates in soils (Mosier et al. 2002; Davidson et al. 2004). Two groups of microorganisms, i.e. the methanogens (methane producing archaea) and the methanotrophs (methane oxidising bacteria) are involved in regulating soil exchange of CH_4 to the atmosphere (Aronson et al. 2013). The distribution of the methanogenic and methanotrophic organisms within soil ecosystems is dependent on oxygen, temperature, moisture and nutrient availability (Le Mer and Roger 2001). Bacterial activity is a controlling factor of global methane production and consumption, therefore knowledge of the organisms that contribute towards methane flux and their responses to short and long term global climate change is now imperative (Ritchie et al. 1997).

1.6.1 Methanogens

Discussions regarding microbially produced methane gas began almost two centuries ago after discoveries by Bechamp, Popoff, Tappeniner, Hoppe-Seyler, Sohngen and Omelianski (Barker 1956). Methanogenensis is the utilisation of carbon in CO_2 or other low molecular weight organic compounds as an electron acceptor for CH_4 production by methanogenic archaea (Mitsch and Gosselink 2007). Methanogenic microorganisms are solely responsible for methane production in natural wetlands and rice fields (Cicerone and Oremland 1988) and their activity in rice paddies generates approximately 38% of the total global average of CH_4 emitted annually (Chowdhury and Dick 2013). The methanogens represent a morphologically diverse group of anaerobic organisms that belong to the phylum Euryarchaeota, with over 26 genera and 60 species identified (Balch et al. 1979; Huttunen et al. 2003; Talbot et al. 2008; Lee et al. 2009). There are 5 trophic groups of methanogens which have been grouped according to their terminal electron donors i.e. H_2, CO_2, acetate, formate and methylated compounds (Hanson and Hanson 1996; Chowdhary and Dick 2013). In soil ecosystems, there are two major pathways of CH_4 production (Chowdhury and Dick 2013), which rely on two major physiological groups of methanogenic microorganisms i.e. the acetoclastic and hydrogenotrophic methanogens (Conrad 2007).

1.6.1.1 Acetoclastic methanogens

The acetotrophic or acetoclastic methanogens are responsible for the production of CH_4 and CO_2, via the conversion of acetate, and only two genera viz. *Methanosarcina* and *Methanosaeta* are known to catabolize acetate (Conrad 2007). *Methanosarcina* which belong to the Methanosarcinaceae family; exhibit diverse substrate versatility and are able to acquire energy via the oxidation of H_2 or by utilising methanol, methylamine or acetate; both mechanisms resulting in the formation of CH_4 (Balch et al. 1979). Unlike *Methanosarcina*,

Methanosaeta are specialists and demonstrate a strict preference for acetate as a substrate for methanogenesis (Smith and Ingram-Smith 2007). One of the main distinguishing features between these two groups of methanogens is their affinity for acetate. *Methanosaeta* are able to utilise lower threshold concentrations of acetate (7-70μM) compared to *Methanosarcina* (0.2-1.2mM), however *Methanosarcina* displays a higher growth rate in the presence of sufficiently high acetate concentrations (Jetten et al. 1992). Nevertheless, *Methanosaeta* are widely distributed in low acetate environments such as rice paddies and anaerobic waste digesters (Griffin et al. 1998; Grosskopf et al. 1998; Fey and Conrad 2000) and have therefore been described as 'the most predominant methane producer on Earth' (Smith and Ingram-Smith 2007). Approximately 70% of methane generated uses acetate as a precursor (Ritchie et al. 1997), thus information about the physiological activities of these methanogens are imperative. Recently, Godin et al. (2012) suggested that temperature may facilitate CH_4 production from acetate in boreal peatlands after the discovery of acetoclastic methanogens across a nutrient gradient. Furthermore, during the fall when CH_4 emission and production rates were higher, methanogen diversity indices were a significant predictor of CH_4 production (Godin et al. 2012). The authors concluded that the diversity of methanogenic organisms will impact biogeochemical cycling and CH_4 emissions in northern peatlands which constitute an important reservoir of global fixed carbon (Godin et al. 2012). Similarly, Kim et al. (2012) showed that bacterial communities were favoured over methanogens after a 3 year warming experiment in peat mires. These community changes can lead to imbalances between CO_2 and CH_4 in peatlands, which could transform regional carbon and nutrient cycles, thus studies incorporating global changes and methanogenic organisms are extremely necessary.

1.6.1.2 Hydrogenotrophic methanogens

The hydrogenotrophic methanogenic microorganisms use H_2 and CO_2 for the production of CH_4. Interestingly, *Methanosarcina* (1.6.1.2) can also be classified as a hydrogenotrophic methanogen based on its unique biochemical properties (Conrad 2007). The hydrogenotrophic methanogens include members of the Methanobacteriales order viz. *Methanobacterium* and *Methanobrevibacter* and the Methanomicrobiales order viz. *Methanoculleus* and *Methanospirillium*. All these organisms are frequently found in (but not limited to) rice field soil (Joulian et al. 1998; Adachi et al. 1999; Joulian et al. 2000). Hydrogenotrophic methanogens occur in similar numbers (10^6 per gram of soil) as the acetotrophic methanogens (Joulian et al. 1998). Similar to the acetotrophic methanogens (1.6.1.2), the reaction that yields CH_4 is catalysed by the methyl-CoM reductase (MCR) enzyme, the principal enzyme that is unique to methanogens (Conrad 2007). This enzyme is coded for

by the *mcr*A, *mcr*B and *mcr*G genes corresponding to the α, β and γ subunits of the MCR enzyme (Reeve 1992; Eggen 1994). Aronson et al. (2013) used real time quantitative PCR (qPCR) of the *mcr* gene to quantify the numbers of methanogens present in a pine forest foil. CH_4 release, soil moisture, soil carbon and higher nitrogen concentrations were related to a higher abundance of methanogenic organisms in a poorly drained soil. Soil moisture was considered the principal factor for the observed changes; and this can impact methane cycling microbes as climate change progresses (Aronson et al. 2013).

1.6.2 Methanotrophs

Methanotrophs represent one of the most abundant groups of free-living organisms and are frequently encountered in soil, sediment and freshwater (Whittenbury et al. 1970; Hanson and Hanson 1996). Despite initial reports as early as 1906; they still continue to add surprises to microbiologists in the 21st century (Hanson and Hanson 1996; Semrau et al. 2008). Aerobic methanotrophic bacteria are able to oxidise freshly produced CH_4 and atmospheric CH_4, and are therefore considered the largest biological methane sink (McDonald et al. 1999; Conrad 2007). Methane oxidation pathways via methanol, formaldehyde and formate have been documented in methanotrophic many years ago (Higgins et al. 1981). Biological methane oxidation by methanotrophs is responsible for significantly attenuating biogenic CH_4 emissions. In fact, biological oxidation removes more biogenic methane than chemical oxidation, before it is even emitted from the biosphere (McDonald et al. 1999; Conrad 2007). Kolb et al. (2005) showed that elevated CO_2 levels resulted in a reduction of methanotroph abundance by up to 70%, which also impacted CH_4 efflux.

The unique physiological characteristics of methanotrophs combined with their ubiquity in natural environments make them a promising target for global warming mitigation (Horz et al. 2005). Methanotrophic bacteria grow optimally at moderate pH and temperatures ranging between 20 - 35˚C, although methanotrophs belonging to extreme environments have been isolated (Trotsenko and Khmelenina 2002). These gram-negative bacteria are divided into two groups; Type I and Type II including members of the Methylococcaeceae (*Methylococcus, Methylomicrobuim; Methylomonas; Methylobacter*) and Methylocystaeae (*Methylocystis; Methylosinus; Methylocella*) families, respectively (Whittenbury et al. 1970; Conrad et al. 2007). Type I and Type II can be differentiated based on variations in arrangements of intracytoplasmic membranes; carbon assimilation pathways and phylogenetic affliliation (Higgins et al. 1981; Conrad 2007). Type I and Type II methanotrophs oxidise CH_4 via the assimilation of formaldehyde; with Type I methanotrophs utilising the ribulose

monophosphate pathway, while Type II demonstrates a preference for the serine pathway for CH_4 consumption (Higgins et al. 1981; Anthony 1986). Ecological niche specificity of both methanotroph types are affected by CH_4, O_2 and acetate concentrations; availability of nitrogen and copper as well as pH and temperature (Bender and Conrad 1995). While methanotrophic bacteria use CH_4 as their sole source of C for energy, they are still able to survive in environments that are CH_4 or O_2 deficient (Knief and Dunfield 2005). In a California upland grassland soil, increased precipitation and temperature decreased the number of Type II methanotrophic organisms in a simulated global change experiment (Horz et al. 2005). Methanotrophic activity is expected to increase as global temperatures increase, when other factors are non-limiting. The net effect of this response on global CH_4 oxidation / emissions is still unknown (Singh et al. 2010). Oxidation of CH_4 by aerobic methanotrophs is conducted by the methane monooxygenase enzyme (MMO) that requires molecular O_2 and results in methanol formation (Dalton 2005). Co-metabolism of a variety of other compounds is common, due to the low substrate specificity exhibited by this enzyme. This enzyme exists in two forms: a particulate membrane bound form (pMMO) and a soluble, cytoplasmic form (sMMO). pMMO is often found in methanotrophs that have sufficient copper for their growth and activity; while the sMMO is generally expressed when copper concentrations are low i. e. < 1µM (Lieberman and Rosenzweig 2004; Conrad 2007; Chowdhury and Dick 2013). Ritchie et al. (1997) suggests that only advantage that may be conferred to methanotrophs with sMMO would be the ability to tolerate copper deficient conditions.

A major research challenge still exists in investigating and comprehending the response of methanotrophs and methanogens to multifactorial climate change. Understanding the physiology and response of these organisms towards climate change is now being aided by the advent of sophisticated molecular biology techniques that can provide information on the large proportion of uncultivable organisms. It is apparent that both the methanogens and methanotrophs can present a successful alternative for the mitigation of atmospheric CH_4.

1.7 Conclusion

If carbon sequestration strategies are to be successful, we must fully understand microbial responses under various environmental conditions. Recently, Garcia-Pichel et al. (2013) reported that temperature change may be responsible for the distribution of ecologically significant topsoil bacteria across continents. These new findings suggest that researchers have barely scratched the surface with regards to our understanding of microbial responses towards climate change. Considering that microbes have been able to withstand a variety of environmental factors since the origin of life on Earth, it is reasonable to assume that they will continue to persist and drive important processes that sustain habitability on this planet. Another cause for concern is the continued destruction of the planets natural resources by mankind and disregard for the environment and how this will impact future generations. Certainly, since the time of Alexander Flemming and the discovery of penicillin, man has been intelligent enough to understand the importance of microorganisms. Through continued research and in depth study of microbial activity, man's intelligence will extend towards harnessing the amazing potential of microorganisms as bio-engineers of the climate system and administrators of our environment.

1.8. References

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CHAPTER THREE

RESPONSE OF SOIL MICROBIAL ENZYME ACTIVITIES TO SINGLE AND MULTIFACTOR CLIMATE CHANGE SIMULATIONS

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Response of soil microbial enzyme activities to single and multifactor climate change simulations

(Running title: The effects of climate change on soil enzymes)

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ABSTRACT

Soil, the second largest carbon pool after the oceans; is a major factor in the global response to climate change. Climate change such as increases in temperature, changing precipitation patterns etc. will not only affect humans but may also affect the functioning of the soil ecosystem. The ability of soils to act as a sink or source of carbon as climate change increases can be influenced by soil microbial activity. In this study, the individual and/or interactive effects of elevated carbon dioxide (CO_2), methane, precipitation and temperature on soil microbial enzyme activity were investigated under elevated and ambient temperature conditions. The dehydrogenase, urease, arylsulphatase and β-glucosidase activities were determined over a 60 day period during the summer and spring seasons using standard enzyme assays. In spring, the soil dehydrogenase and arylsulphatase activities increased by 28.07% and 28.48%, respectively, after the addition of elevated CO_2 under elevated temperature. Lower dehydrogenase activities was observed at day 60 for most plots during summer while β-glucosidase activity was unaffected by the addition of single or multiple global change drivers during spring. In summer however, all treatments resulted in 28.05 - 36.39% higher β-glucosidase activity by day 15, compared to day 0. Urease enzyme activity was higher during spring at both temperature conditions indicating that moisture limitation and temperature change constrained the urease enzyme production during the summer period. Neither the combination of elevated CO_2 with rainfall nor the combination of elevated CO_2, rainfall and methane induced substantial changes to the enzyme activities during both seasons, suggesting an antagonistic effect of the combination of the global change factors. However, differences observed after a combination of elevated CO_2 at higher temperature clearly reflects a potential for interaction that will affect soil enzymes and subsequent nutrient cycling. Furthermore, it was apparent that seasonal changes linked to alterations in soil moisture, substrate availability and temperature can also affect soil enzymes and nutrients. Considering that

climate change is multifactorial, this study clearly highlights the necessity for multi-factor global change studies, especially across different seasons to successfully predict the fate of soil ecosystem as climate changes continue to increase.

Keywords: multifactorial global change; soil enzymes, soil nutrients

1. INTRODUCTION

The momentum of the current global climate change crisis has increased exponentially in recent times with the Earth's climate system being the subject of much debate. Rapid and extreme climate changes have been been attributed to human induced activities resulting in increased concentrations of atmospheric greenhouse gases such as carbon dioxide (CO_2) and methane (CH_4). Considering that CO_2 concentrations are expected to increase to 500 - 1000 ppm by the year 2100 (IPCC, 2007), strategies that will aid in decreasing atmospheric CO_2 concentrations, thereby mitigating the effects of climate change are now dominating scientific research globally. Changes in terrestrial carbon storage could dramatically alter the progression of ongoing climate change (Cox et al. 2000). Soil is responsible for the second largest carbon input to the atmosphere (Schlesinger and Andrews, 2000), with a global carbon pool of 2500 Gigatons (Lal, 2004). A 10% increase in soil organic carbon would effectively be equivalent to anthropogenic CO_2 emissions over a 30 year period (Kirschbaum, 2000). However, soil can also buffer atmospheric CO_2 concentrations and ultimately can function as either a sink for or source of carbon as climate changes continue to increase (Uvarov et al. 2006; Dungait et al. 2012), thus much effort has been directed towards investigating the properties that will enable their use in the mitigation of rising atmospheric CO_2 (Lal, 2008).

The soil microbial community is a fundamental component of the terrestrial carbon ecosystem and is central to many crucial functions, including the recycling of nutrients and the decomposition of organic matter (Schimel, 1995). An increase in the average global temperature is expected to accelerate heterotrophic microbial activity, effectively resulting in an increased efflux of CO_2 to the atmosphere (Jenkinson et al. 1991; Subke et al. 2003; Knorr et al. 2005; Bengston and Bengtsson, 2007), thus creating a positive feedback on climate change (Cox et al. 2000). Alternatively, various environmental constraints such as the physical and chemical composition of organic matter can lead to decreased substrate availability necessary for microbial attack, diminishing microbial responses to increased global warming (Luo et al. 2001; Ågren and Bosatta. 2002; Davidson and Janssens, 2006; Xu et al. 2012).

The Intergovernmental Panel on Climate Change has projected a temperature increase between 1.1 to 6.4° C by the end of the 21st century (IPCC, 2007). Significantly higher increases in temperature in projected warming estimates have been observed in climate change simulation studies that include carbon cycle feedbacks (Füssel, 2009). In fact, Scheffer et al. (2006) suggested that in the next century warming will increase by an additional 15 – 78%, if biological carbon cycle feedbacks are integrated into existing climate change models. Thus, it has become necessary to address the effects of elevated temperatures in combination with other global change drivers in order to obtain a better understanding of soil ecosystem responses. Single factor experiments have proven to be valuable as they have revealed the foundations of soil microbial response to climate changes however, there still remains the need for investigations of microbial activity with regard to multiple, climate change factors (Carmona-Monero et al. 2005) and seasonal variability conditions. In addition, it has been speculated that global change factors e.g. CO_2, warming, drought, etc., may exert antagonistic or additive effects on soil and its properties. Consequently, effects of climate change and/ or future predictions for terrestrial carbon - cycle feedbacks seem to be based on hypothetical or presumptive information rather than observed evidence from simulation experiments (at field-scale) due to lack of knowledge in this area (Kampichler et al. 1998).

Soil enzyme activity profiles can be used as indices of soil fertility and are valuable indicators of biological activity because biochemical reactions are catalysed by these enzymes (Verchot and Borelli, 2005; Baldrian, 2009). As a result, the measurement of soil enzyme activity has become widespread, especially for investigating soil microbial responses in climate change experiments (Henry, 2012). It has also been acknowledged that soil microbial enzymes are amongst the fastest reacting components to external disturbances, particularly in the soil ecosystem (Vepsalainen et al. 2001). Furthermore, extracellular enzyme production is directly correlated with microbial status (Aon et al. 2001) and thus provides reliable evidence for the estimation of microbial metabolic activities and nutritional demands. Several authors have reported that temperature is one of the main factors that drive soil enzyme activities in climate change studies across several ecosystems. (Koch et al. 2007; Allison et al. 2010; Wallenstein et al.2011; Stone et al. 2012). However, many of these studies have failed to produce distinct trends. Similarly, Freeman et al. (2004) reviewed the effects of elevated CO_2 on soil enzyme activities and revealed that while it is imperative to understand the responses of soil enzymes towards climate changes, conflicting results have been reported. These results have been attributed to several factors including modifications to vegetation type, litterfall and seasonal changes.

Knowledge of the fate and activity of soil enzymes in response to a combination of climate change drivers is limited. Furthermore, to the best of our knowledge, the effect of seasonal variability changes on the soil microbial activities and their response to multiple experimental climate change drivers is lacking or limited, despite its importance towards understanding and properly assessing terrestrial carbon cycle feedbacks. Therefore, this study was undertaken to determine the individual and interactive effect of multiple climate change drivers i.e. temperature, elevated CO_2, elevated CH_4 and elevated rainfall on soil microbial activities during spring and summer seasons using soil enzymes viz. dehydrogenase, urease, arylsulphatase and β-glucosidase as indices.

2. MATERIALS AND METHODS

2.1 Site description and experimental design

Sampling below the zone of short term agricultural influence with sampling depth of up to 60cm for perennial vegetation has been suggested (Carter and Gregorich, 2008), thus sandy loam soil from the top 0-50 cm was collected from an area adjacent to the University of KwaZulu Natal (Westville) which is situated within an environmental conservancy. The area is dominated by flowering plants such as *Tecomaria capensis, Strelitzia reginae, Plumbago auriculata and Aloe succotrina* ; while alien invasive species such as *Leucaena leucocephala, Lantana camara, Schinus terebinthifolius, Chromolaena odorata and Syringa vulgaris* are also frequently encountered (http://conservancy.ukzn.ac.za/IndigenousPlanting.aspx). The climate change experiments were started within 4 days of soil collection due to the complex experimental setup. Soil was stored at ambient environmental temperature (identical to the area from which soil was collected) in polyethylene bags that had holes to allow for aeration up until it was used for the experiments. The individual and/or interactive effects of elevated carbon dioxide (CO_2), methane (CH_4), precipitation and temperature were investigated using constructed modified Screen-Aided-Carbon-Dioxide-Control (Leadley et al. 1997) in two separate greenhouses (E and A) situated at the University of KwaZulu-Natal (Westville). Screen Aided Carbon Dioxide Control is recommended as the most suitable method for determining the effects of elevated CO_2 levels for short stature vegetation and is useful as it combines the advantages of Open Top Chambers and Free Air Carbon Dioxide Enrichment experiments (Leadley et al., 1997; Macháčová, 2010). Briefly, 1 m x 0.5 m polycarbonate screens were attached to stainless steel tables in the greenhouses and were arranged to achieve 1m x $1m^2$ plots within

which the climate change simulations were conducted (Fig 1). The base of each plot was then lined with gas tubing (Afrox, South Africa) which had 10 mm holes drilled every 10 cm apart and a depth of 1.5cm.

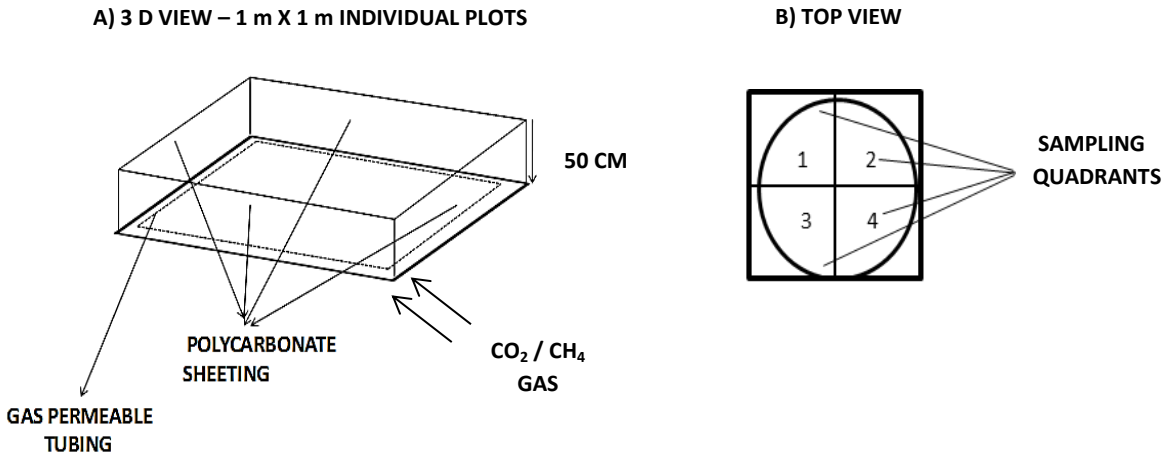


Fig 1: Experimental set-up used for investigating the effects of global change drivers. A – illustrates the 3D view of individual experimental plots covered at sides and base with polycarbonate sheeting, while B illustrates the top view with 4 sampling quadrants.

Sandy loam soil (25 kg) was then added to all the plots within the greenhouses. Greenhouse A corresponded to ambient environmental temperature, while Greenhouse E had temperatures approximately 5 – 10 ˚C higher than Greenhouse A (sunlight and humidity dependent). These experiments were conducted during spring and summer (September 2011- March 2012). Simulations of elevated greenhouse gas conditions was done once daily addition of 425 ppm CO_2 and 2.6 ppm CH_4 gas (Afrox, South Africa) to the soil at a flow rate of 10 litres per min for 5 min. The CO_2 and CH_4 concentrations used represent atmospheric concentrations plus a 10% increment (IPCC, 2001). Changes in rainfall were simulated weekly by addition of synthetic groundwater (Klier et al. 1999), based on the rainfall recorded for the relevant period in the previous year as per data from the South African Weather Service (72.6 and 28.4 mm for spring and summer, respectively) and increased by 10%. The effects of the following global change drivers were investigated: Carbon dioxide (CO_2); carbon dioxide and rainfall (CO_2R); carbon dioxide and methane (CO_2CH_4); carbon dioxide, methane and rainfall (CO_2CH_4R) and rainfall (R).Control plots that did not receive any treatment were also set up in both greenhouses (CTRL). At each sampling time, each experimental plot was divided into four quadrants and four soil samples were collected from each quadrant. Subsequently, these four samples from each quadrant were then homogenised. A single homogenised sample was then used from each quadrant (n = 4) to provide 4 replicates per plot.

2.2 Dehydrogenase activity

Dehydrogenase enzyme activity was determined according to a modified method of Von Mersi and Schinner (1991). One millilitre of 9.8 mM 2-(*p*-iodophenyl)-3-(*p* nitrophenyl)-5-phenyl tetrazolium chloride (Sigma) and 0.75 ml Tris buffer (1 M, pH= 7.0) was added to 1 g of soil in test tubes, mixed thoroughly and then incubated at 40 ºC for 2 h. Post-incubation, samples were mixed with 5 ml extracting solution (N, N-dimethylformamide and ethanol, ratio = 1:1; Merck), followed by a second incubation in the dark at room temperature for an 1 h, with vigorous shaking every 20 min. The solution was then filtered (Whatman No.1) and the resultant iodonitrotetrazolium chloride (INTF) were spectrophotometrically determined at 464 nm. Autoclaved soil was used as a negative control and extracting solution was used as the blank. INTF concentrations and enzyme activity was calculated from a regression equation derived from a standard curve of known INTF (Sigma) concentrations.

2.3 Urease activity

Urease assay was conducted according to a modified protocol of Kandeler and Gerber (1988). Briefly, 2.5 g of soil was mixed with 1.25 ml urea solution (80 mM), 10 ml borate buffer (pH 10.0) and incubated at 37 ºC for 2 h. Subsequently, 15 ml KCl solution was added and soil was incubated for 30 min at 37 ºC on an orbital shaker at 100 rpm. All samples were then filtered (Whatman No.1). In order to determine ammonium concentrations, 1 ml of the clear filtrate was dispensed into glass test tubes, followed by the addition of 9 ml of distilled water, 5ml sodium salicylate/NaOH solution and 2 ml sodium dichloroisocyanide solution. This was vigourously vortexed and allowed to stand for 30 min prior to absorbance measurement at 690 nm. Urease activity was calculated from a regression equation derived from standard curve generated using a range of ammonium chloride concentrations.

2.4 β- Glucosidase and Arylsulphatase activity

These enzyme activities were determined using the method of Alef and Nannipieri (1995), using either 25 mM *p*-nitrophenyl-β-D-glucopyranoside (Sigma) or *p*-nitrophenyl-sulphate (Sigma) for the assay of β- glucosidase and arylsulphatase activity, respectively**.** A standard curve for both assays was plotted using a range of *p*-nitrophenol (Sigma) concentrations as described by Taylor et al. (2002) to determine enzyme activity.

2.5 Soil nutrient analysis

All soil samples were analysed by Soil Fertility and Analytical Services at the KwaZulu-Natal Department of Agriculture, Environmental Affairs and Rural Development. Phosphorus (P), Potassium (K), Zinc (Zn),Copper (Cu) and Manganese (Mn) were determined using atomic absorption spectroscopic method after extraction with Ambic-2 extracting solution (Murphy and Riley, 1962; Manson and Roberts, 2000). Calcium (Ca) and Magnesium (Mg) were determined by atomic absorption spectroscopy after incubation of soil with 1 M KCl and subsequent filtration with 0.0356 M $SrCl_2$ (Manson and Roberts, 2000). Soil pH was determined using a pH meter after incubation with 1 M KCl (Mason and Roberts, 2000). Nitrogen was determined using the Automated Dumas combustion method using a LECO CNS 2000 (LecoCorp, Michigan, USA, Matejovic, 1996). Total organic carbon was determined by the Walkley-Black method (Allison, 1965).

2.6 Statistical Analysis

Data was analysed with PASW 18 Statistics Processor. Differences amongst treatments were evaluated using Repeated Measures ANOVA incorporating the GLM procedure. Bonferronii adjustment for post hoc analysis was also conducted. T-test was also used to determine significance between treatments at specific times, with $p < 0.05$ being considered significant.

3. RESULTS

3.1 Soil dehydrogenase enzyme activity in response to climate change drivers

Dehydrogenase activity was positively affected by the increased greenhouse gas concentrations at both elevated and ambient temperatures, with higher values observed in most experimental plots during spring after 15 days (Fig. 2). Dehydrogenase enzyme activities were substantially higher in plots that received elevated CO_2 at elevated temperature (Fig. 2a), and this was 27.06 % greater than values observed in the control plots. The experimental plots that received a combination of greenhouse gases; CO_2CH_4 and CO_2CH_4R displayed similar trends of dehydrogenase activity throughout the sampling period, with the highest enzyme levels also recorded at day 15. By day 25, all treatments induced considerably lower dehydrogenase values (compared to day 15) including the control indicating that the treatments were not responsible for treduction in enzyme activity during this period. At day 40, only the CO_2 and control plots showed higher dehydrogenase enzyme levels compared to day 25, but these were not statistically significant. Overall, lower dehydrogenase enzyme activities were observed during spring at elevated temperature conditions however, elevated rainfall simulations resulted in the

most significant loss of dehydrogenase enzyme activity by day 60 compared to day 0 (p = 0.012).The lower temperatures observed under the ambient temperature incubations did not drastically alter the pattern of dehydrogenase enzyme activity during spring (Fig. 2b) compared to elevated temperature experiments. A similar effect characterised by increased dehydrogenase enzyme activity at day 15 was observed in all plots. The CO_2R treatment resulted in the most significant (p= 0.016) increase in dehydrogenase enzyme activity at

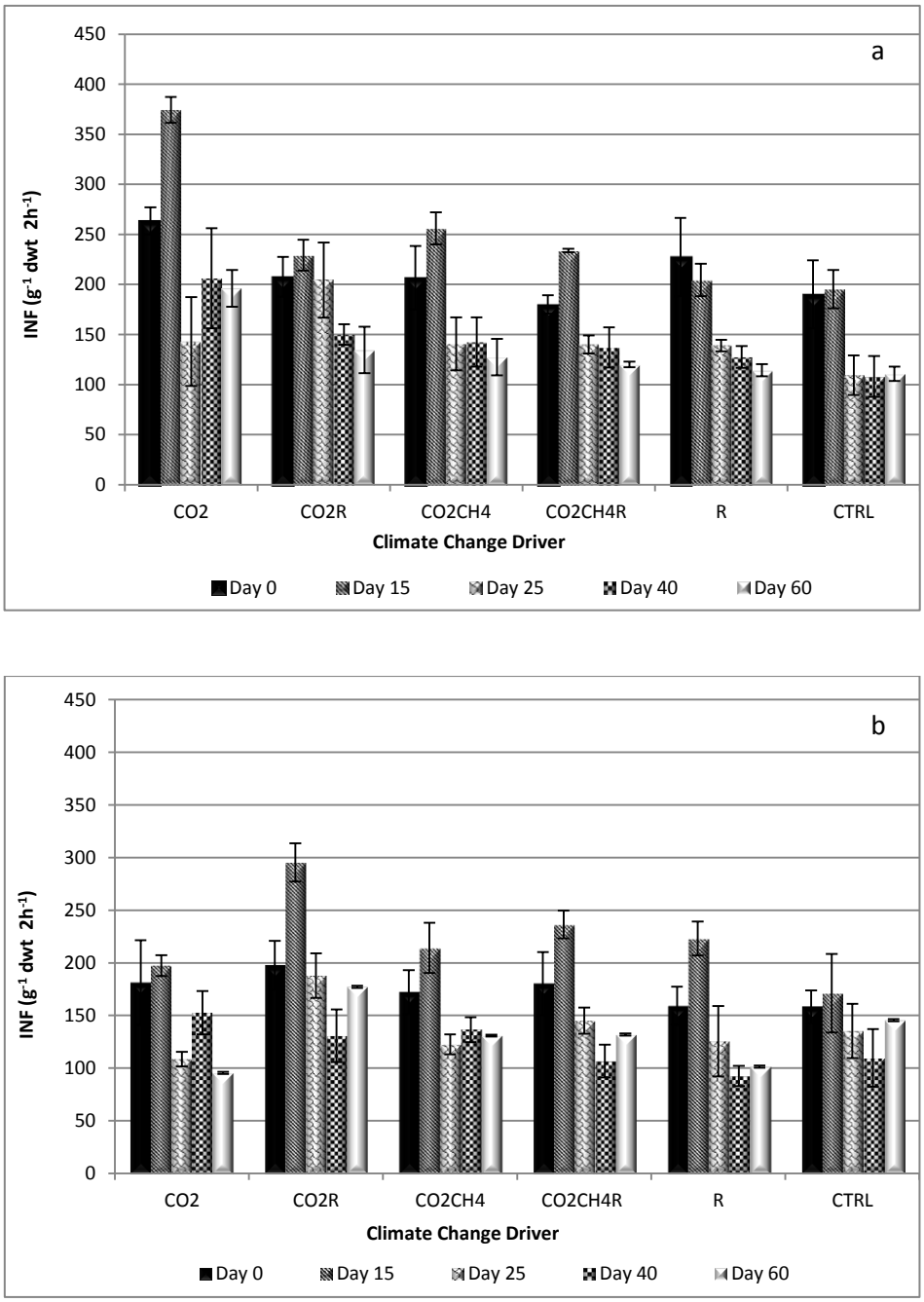
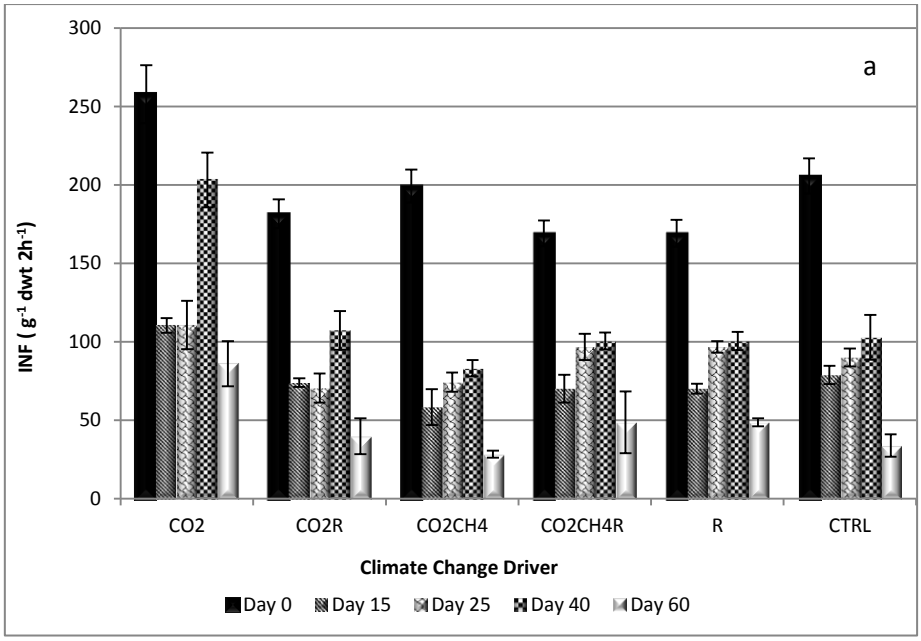


Fig 2: Effect of simulated climate conditions on dehydrogenase activity at a) elevated temperature and b) ambient temperature during spring

day 15 compared to the start of the experiment, while the plots simulated with higher rainfall and CO_2CH_4R conditions showed 28.66% and 23.77% greater dehydrogenase enzyme activities compared to day 0, respectively. At day 40, only plots that obtained CO_2 and the combination CO_2CH_4 displayed increased dehydrogenase enzyme activities compared to other experimental plots, but only the change observed in the CO_2 plot was significant ($p = 0.016$).

In contrast to results obtained during the spring season, substantially lower dehydrogenase enzyme activity was observed within 15 days of incubation under elevated temperature incubation (Fig. 3a) including the control, indicating the negative effect of higher temperature on soil microbial activity. Soil that received CO_2CH_4; CO_2CH_4R; R and the control displayed increased activity from day 15 to day 40. Plots that were enriched with CO_2 and/ CO_2R showed a reduction in dehydrogenase activities between days 15 and 25, however these were not statistically significant. Similar to the effect observed under elevated temperature during spring, the CO_2 plot showed significantly ($p = 0.0004$) higher dehydrogenase enzyme levels by day 40 compared to day 25, while the addition of CO_2R resulted in a 52.16% decrease in dehydrogenase enzyme activity at day 40 compared to day 0. It was apparent that a combination of temperature and CO_2 produced a more significant change in dehydrogenase enzyme levels compared to plots that investigated the interactive effects of multiple global change drivers.

Lower dehydrogenase activity was measured for all treatments at ambient temperature during summer (Fig. 3b) after 15 days. Comparatively, similar enzyme activities were obtained in the CO_2CH_4; CO_2CH_4R and R plots indicating that lower temperatures did not substantially alter the effect these treatments had on dehydrogenase enzyme values. These plots (CO_2CH_4; CO_2CH_4R and R plots) including the control were characterised by a reduction in dehydrogenase enzyme activity from days 25 to 60, with the CO_2CH_4 enriched plots displaying the most significant ($p = 0.001$) reduction in dehydrogenase enzyme values at day 60. Similar to the trend at elevated temperatures, soil that was fertilised with CO_2 and/ CO_2R showed significantly ($p > 0.05$) higher dehydrogenase enzyme levels at day 40 compared to other plots.



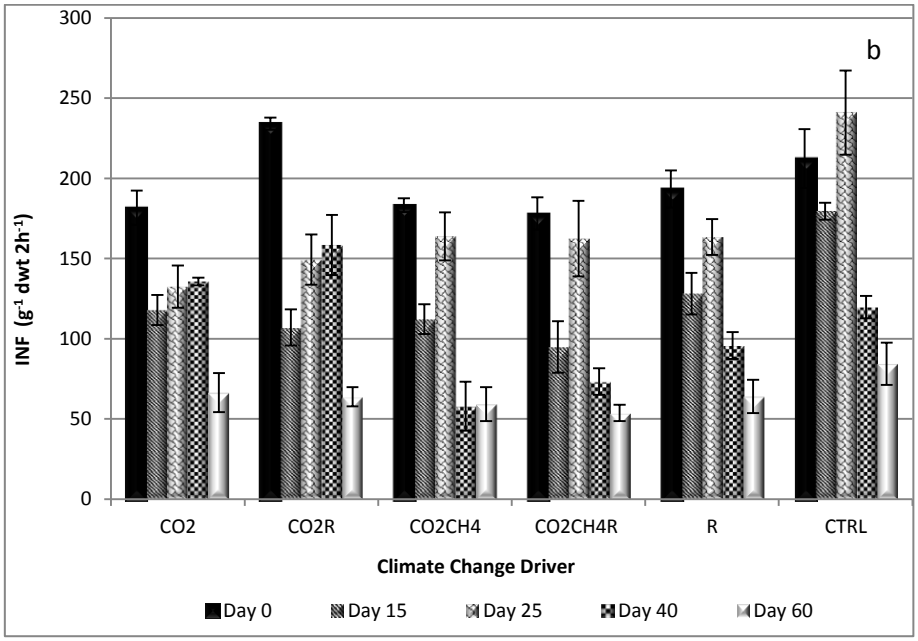


Fig 3: Effect of simulated climate conditions on dehydrogenase activity at a) elevated temperature and b) ambient temperature during summer

3.2 β- glucosidase enzyme activity in response to climate change drivers

β – glucosidase enzyme activity did not show any consistent trends under elevated temperature conditions during spring (Fig. 4a). CO_2 addition resulted in a 15.40% higher enzyme activity compared to other treatments by day 15. A combination of global change drivers; CO_2CH_4R demonstrated similar trends and enzyme values as observed in the CO_2CH_4 experiments suggesting that the additional rainfall did not affect β – glucosidase enzyme activities considerably during the 60 day period. At ambient temperatures during the spring (Fig. 4b), similar trends compared to the elevated temperature experiments was observed. However by day 15, soil that received only CO_2 or R showed significant ($p > 0.05$) reduction in enzyme activities with values that were 16.57% and 9% less than that compared to day 0. As with observations at elevated temperature, substantially lower enzyme levels were measured at day 40 for all treatments. However, by day 60, enzyme levels for all treatments (including the control) at both elevated and ambient temperatures returned to levels that were similar to values at day 0.

Soil incubated at elevated temperatures during the summer period showed higher β –glucosidase enzyme activities after 15 days due to the climate change drivers at elevated (Fig. 5a) and ambient (Fig. 5b) temperatures. By day 15, β – glucosidase activity in the CO_2 and CO_2R treated plots increased by 33.69% and 35.42%, respectively (Fig. 5a). Changes in rainfall affected this enzyme activity most significantly ($p = 0.003$) at this sampling time implying that moisture content clearly contributed towards increases observed in the CO_2R plot. A decrease in temperature from day 15 to day 25 (38° C to 35 °C) lead to lower β – glucosidase enzyme activity observed for all treatments. Between days 40 and 60, all plots besides the elevated CO_2 and elevated CO_2R treated plot continued to show increases in enzyme levels, however only the CO_2R plot exhibited a significant change ($p = 0.004$). A similar trend of fluctuating β – glucosidase enzyme activity was observed at ambient temperature when soil was exposed to increased greenhouse gases and/or rainfall (Fig. 5b) during summer. Similar to the trends observed at elevated temperature, significantly ($p > 0.05$) higher β – glucosidase enzyme levels were detected after 15 days. At day 15, CO_2 fertilisation lead to a 31.82 % increase in enzyme levels compared to the control. All experimental plots were characterised by increased enzyme activity from day 25 to day 60, and this was highly significant ($p = 0.001$) for the plots that contained additional rainfall. Overall, β – glucosidase enzyme activities were not strongly affected by a combination of global change factors; with the CO_2CH_4 and CO_2CH_4R treated plots displaying similar trends compared to the control plots for the duration of the experiment. Overall, higher β – glucosidase enzyme activity was noted in all plots at the end of the sampling period for both seasons.

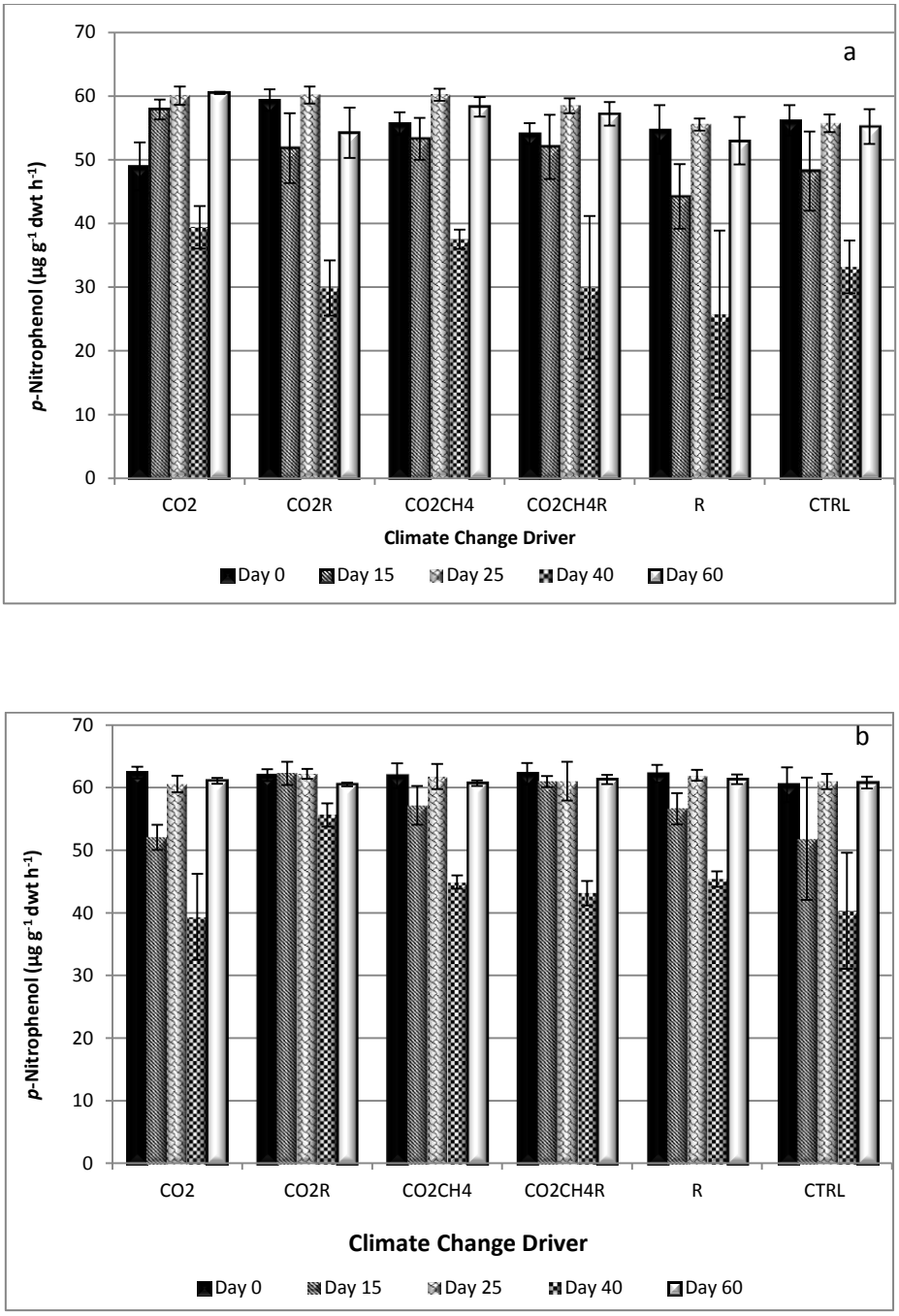
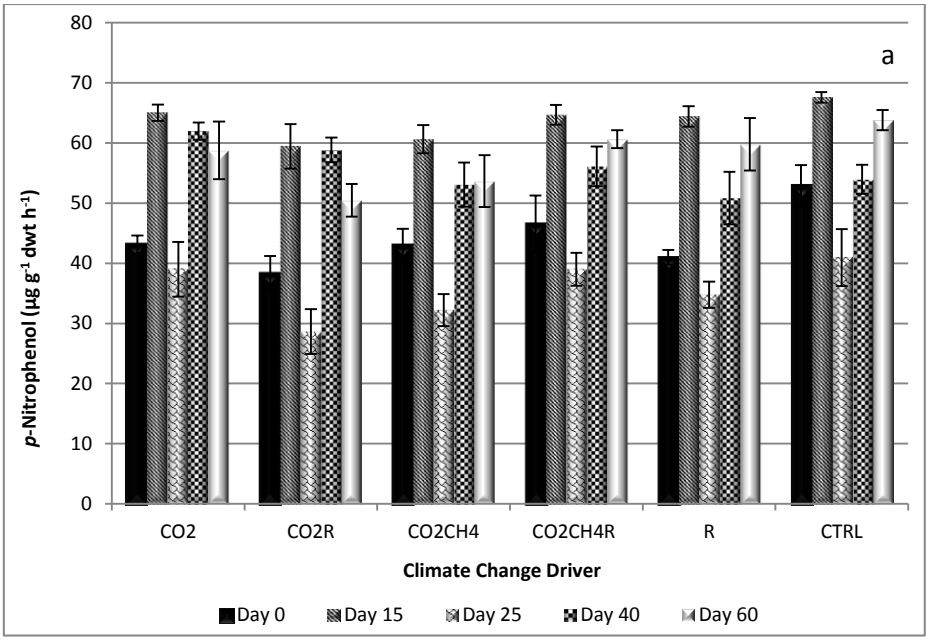


Fig 4: Effect of simulated climate conditions on β glucosidase activity at a) elevated temperature and b) ambient temperature during spring



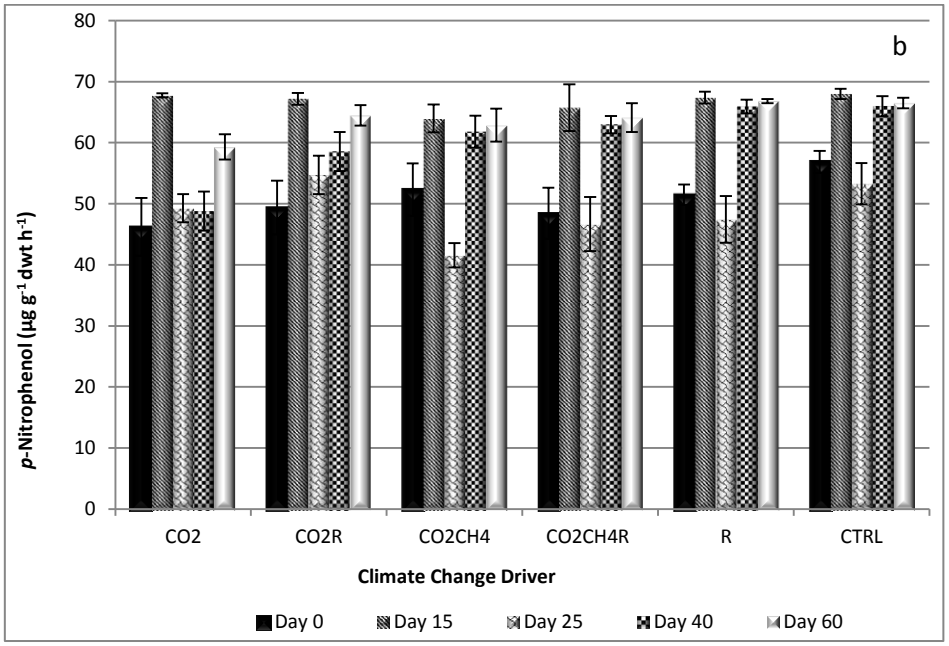


Fig 5: Effect of simulated climate conditions on β glucosidase activity at a) elevated temperature and b) ambient temperature during summer

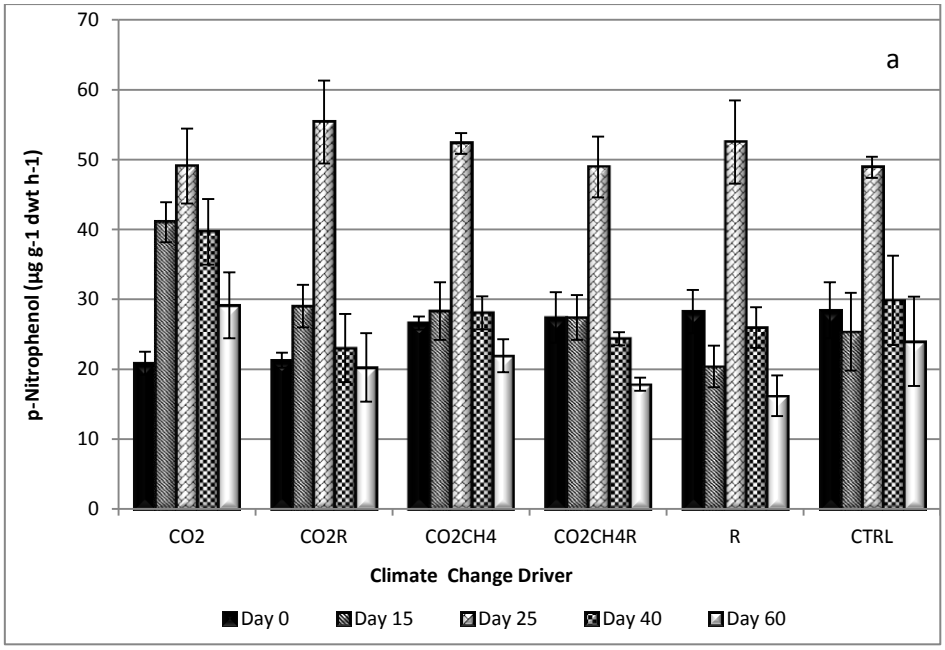
3.3 Arylsulphatase enzyme activity in soils exposed to climate change drivers

Arylsulphatase activity increased progressively by day 25 (Fig. 6a) in all plots that obtained CO_2, with an increase of 135.31% and 160.05% observed for the CO_2 and CO_2R treated plots, respectively. Subsequently, substantially lower arylsulphatase enzyme activity was detected by day 25 and continued to decrease in all plots till day 60. It was also noted that the CO_2CH_4 and CO_2CH_4R treated plots displayed similar trends and values but differed from the plots that were treated with either CO_2 or rainfall. However, by day 60 most experimental plots showed values similar to those observed at day 0, with the exception of the CO_2 plot that exhibited 39.81% higher arylsulphatase enzyme activity. Under ambient temperature incubation (Fig 6b), all other experimental plots except soil that received CH_4 (in combination with either CO_2 and/ or rainfall) displayed decreased arylsulphatase activity by day 15. This was followed by higher enzyme values by day 25, although these were not considerably different compared to the control. The interactive effects of the global change factors on arylsulphatase enzyme activity was evident at ambient temperature in the CO_2CH_4 and CO_2CH_4R experimental plots at day 25 and was 30.63% and 19.54% higher respectively, compared to day 0. Similar to the trend that occurred under elevated temperature, a net decrease in enzyme activity was noted by the end of the sampling period.

No obvious trends were established with regard to arylsulphatase enzyme activity during summer under either elevated (Fig. 7a) or ambient temperature (Fig. 7b) conditions and all plots were characterised by fluctuating enzyme activity from throughout the experimental period. Furthermore, the treatments did not exert strong changes in enzyme activity compared to the control, with the CO_2 and CO_2CH_4R plots showing a 5.90% and 1.98% greater arylsulphatase enzyme activity at elevated temperature, respectively by day 60 compared to day 40. Similar trends to those detected under higher temperature were observed for the arylsulphatase enzyme at ambient temperatures. By day 60, however, an overall increase in activity was observed. The effect of CO_2 addition was not pronounced as these plots had enzyme activity values close to those detected in the control.

3.4 Soil urease enzyme activity in response to climate change drivers

Urease enzyme activity during the spring and summer seasons are represented in Fig. 8 and Fig. 9, respectively. During spring under elevated temperatures, all treatments resulted in lower urease enzyme activity by day 15 (Fig. 8a), except plots treated with a combination of factors viz. CO_2CH_4R, where higher enzyme activity was measured compared to that observed at the beginning of the study. The urease enzyme activity was strongly



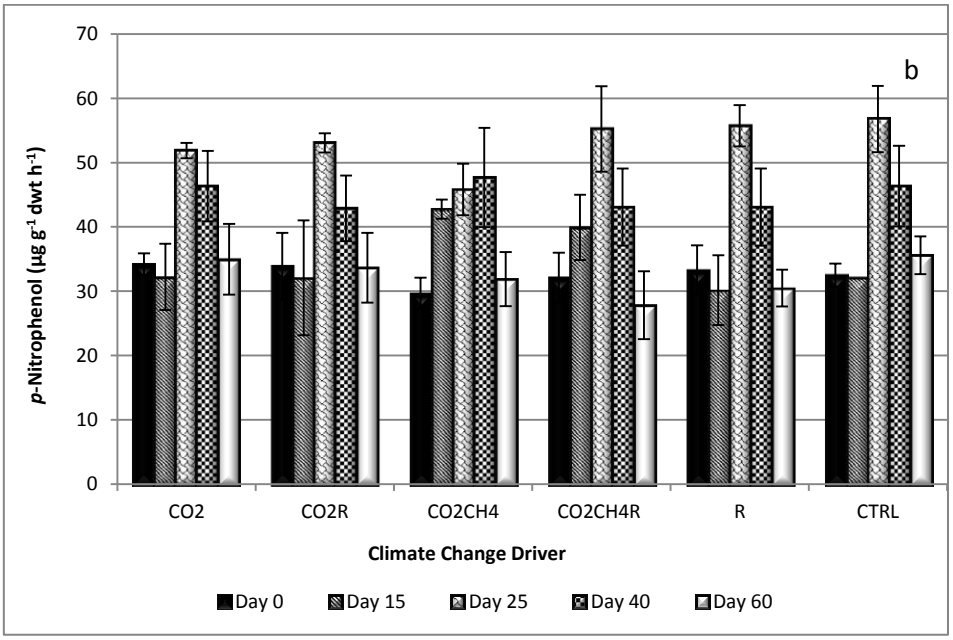
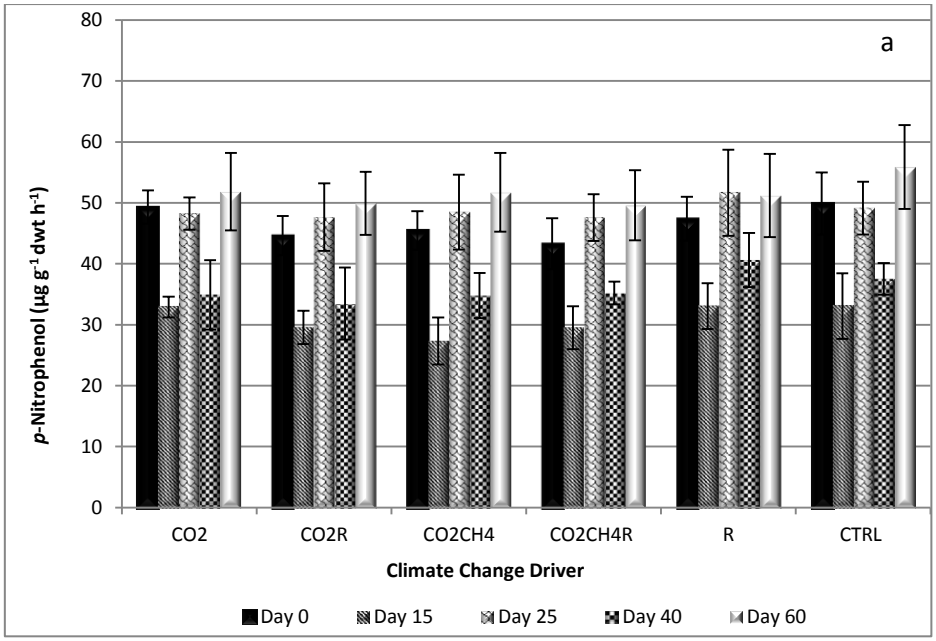


Fig 6: Effect of simulated climate conditions on arylsulphatase activity at a) elevated temperature and b) ambient temperature during spring



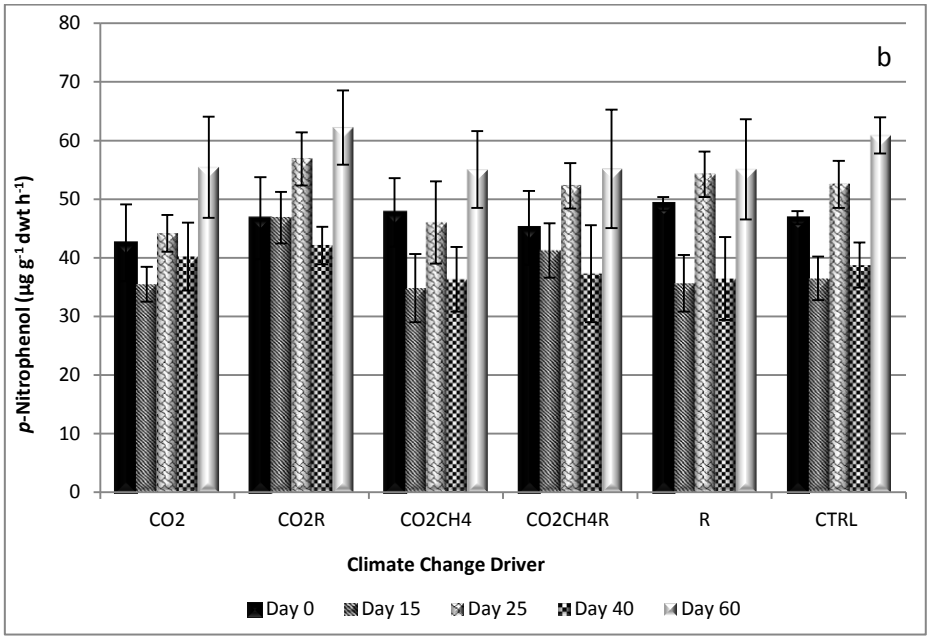
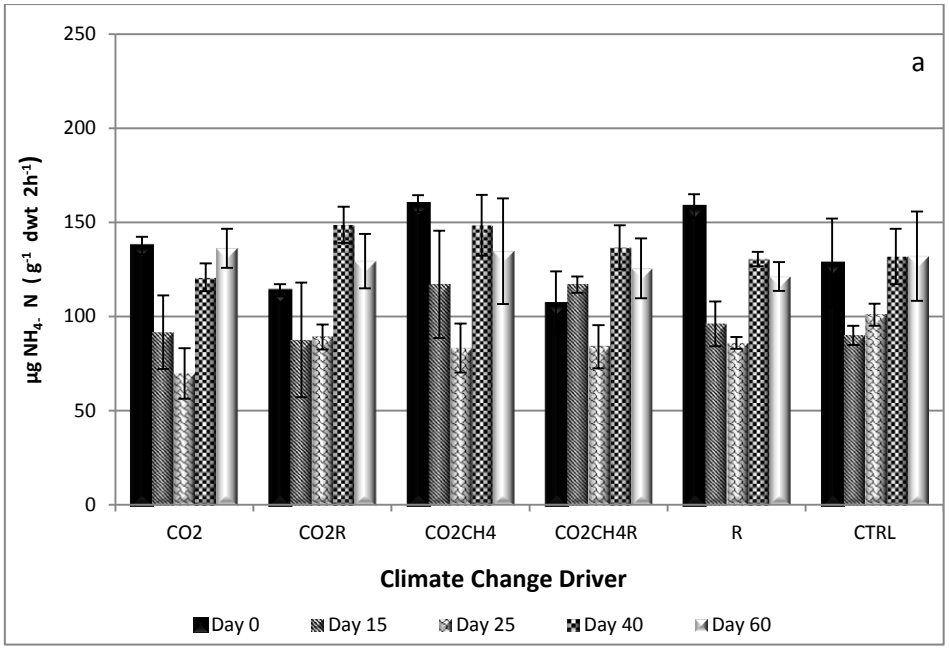


Fig 7: Effect of simulated climate conditions on arylsulphatase activity at a) elevated temperature and b) ambient temperature during summer

affected by the CO_2, CO_2CH_4 and excess rainfall treatments after 25 days, with a gradual reduction in enzyme activities by 99.42%, 47.96% and 45.82%, respectively from day 0 to day 25. By day 60, only the CO_2CH_4R and CO_2R treated soil showed higher urease enzyme activity with respect to those obtained at the start of the experiment; however these were not statistically significant with *p* values of 0.678 and 0.55, respectively. Neither the CO_2 treated nor the control plots showed much difference in urease activity by day 60 and also displayed similar enzyme profiles during the course of the experiment. Similar trends were apparent under ambient temperature conditions (Fig. 8b) compared to elevated temperatures. During spring, under ambient temperatures, the addition of CO_2, CO_2CH_4R and rainfall induced a progressive reduction in urease enzyme activity from day 0 to day 25. Between days 15 and 25, the CO_2R, CO_2CH_4 and control plot had similar profiles with high activities, however these were not significant ($p = 0.05$). By day 40, all plots showed substantially higher urease enzyme activity, with the CO_2 and rainfall treatments exhibiting a 42.34% and 33.89% difference, respectively compared to day 25. At the end of the sampling period, all plots displayed lower urease enzyme activity, with the exception of the CO_2CH_4 treated plots which had values similar to those at day 0.

In contrast to patterns observed during spring, urease enzyme activity increased after 15 days of treatment in all plots, with the addition of CO_2 and CO_2R showing considerable differences of 35.92% and 34.16%, respectively. In the control plots and the CO_2 and CO_2CH_4 treated soil higher urease enzyme values were evident from day 25 until day 60 (Fig. 9a). This was highly significant ($p = 0.02$) in the control plot that differed by 69.24% (by day 60), suggesting that the CO_2 and CO_2CH_4 treatments were not as strongly affected by the temperature change during this period. However, at the end of the experimental period urease activity responded positively with greater values obtained by day 60.

Under ambient temperature during summer (Fig. 9 b), with the exception of the CO_2CH_4 and rainfall applications, all other treatments resulted in higher urease enzyme activities by day 15. The addition of CO_2 and CO_2R lead to 54.92% and 80.90% increase in urease enzyme values, respectively after 15 days. Neither rainfall nor CO_2CH_4 addition increased soil urease activity between days 0 to day 25. However, a combination of factors i.e. CO_2CH_4R continued to produce higher enzyme activity from day 0 to day 25, with a 23.59% higher urease activity observed by day 25. Plots that were supplemented with rainfall exhibited decreased urease enzyme levels from day 0 to day 40, indicating the significant effect of moisture in regulating enzyme production ($p =$



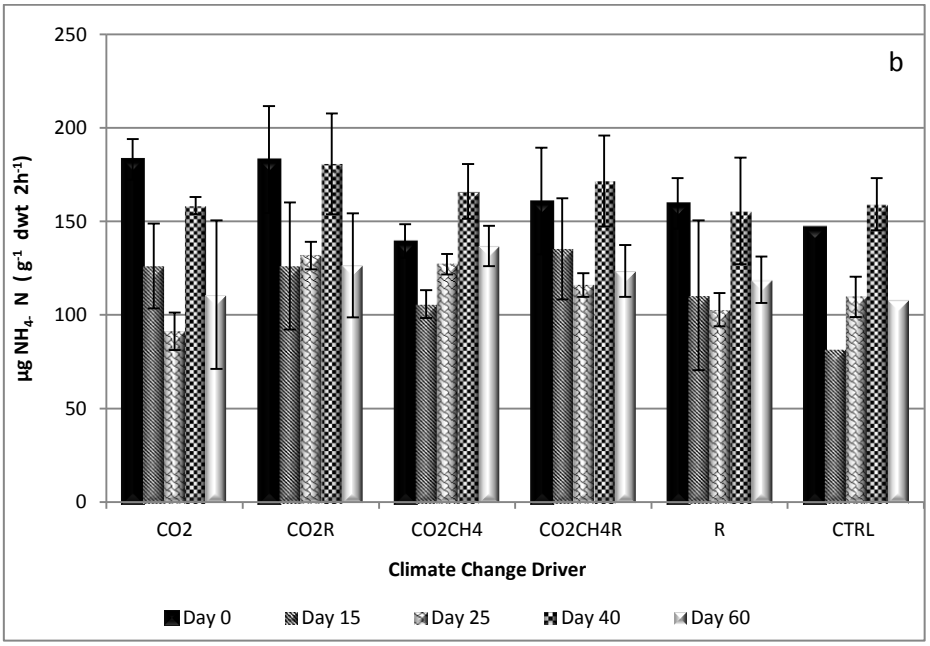
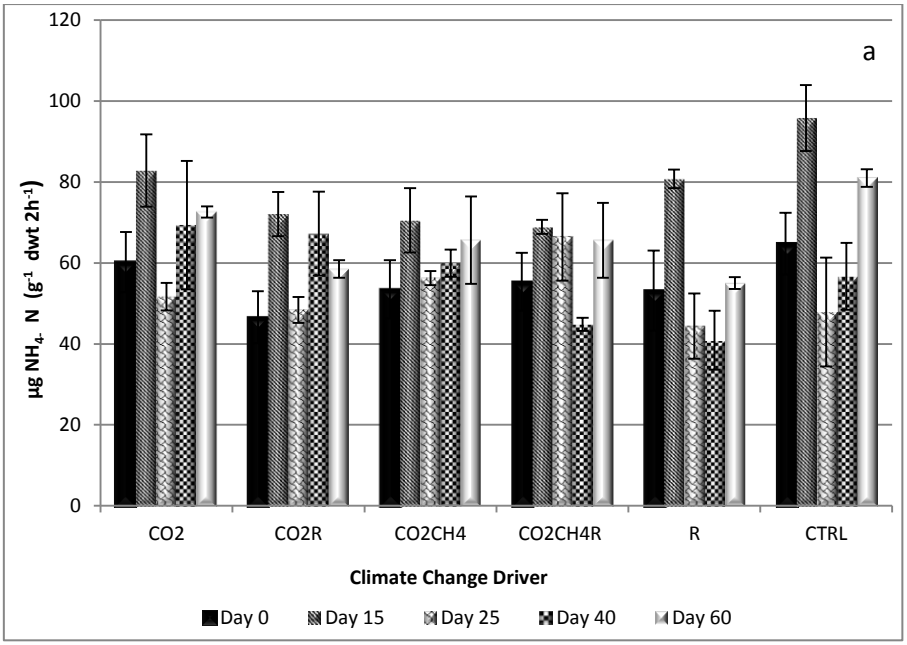


Fig 8: Effect of simulated climate conditions on urease activity at a) elevated temperature and b) ambient temperature during spring



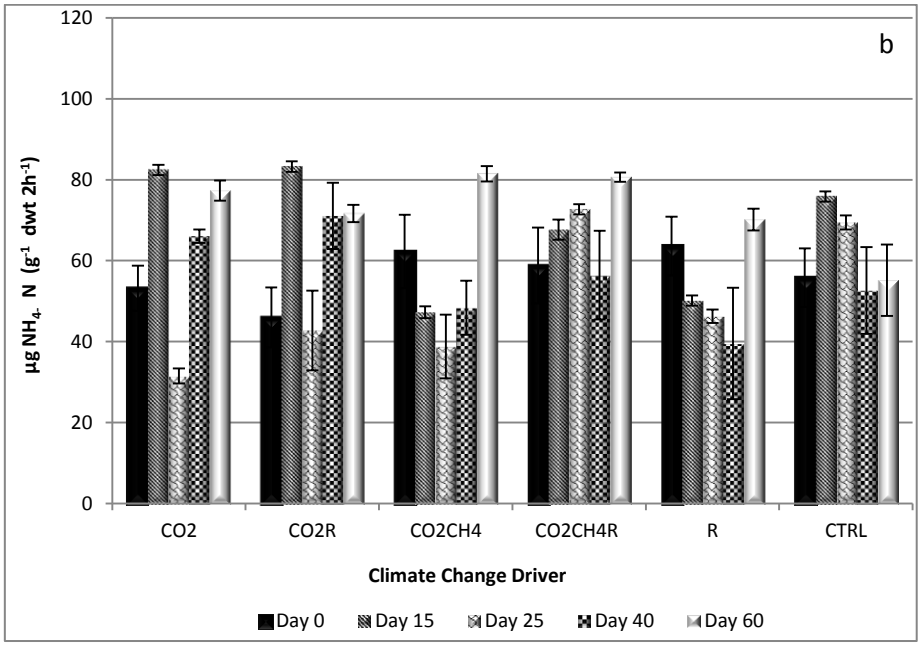


Fig 9: Effect of simulated climate conditions on urease activity at a) elevated temperature and b) ambient temperature during summer

0.02). With the exception of the control plot, higher urease values were noted by day 60 demonstrating that the climate change drivers positively affected urease enzyme activity even under ambient temperature conditions.

3.5 Changes in soil nutrients and organic carbon after exposure to climate change drivers during spring

Phosphorus (P) and potassium (K) concentration increased in all experimental plots during spring at elevated temperature (Table 1). In plots that received CO_2 only, a progressive increase of these nutrients from day 0 till day 60 was observed, with 65 mg/L and 446 mg/L for P and K concentration obtained, respectively by day 60. Similarly, higher Calcium (Ca) and magnesium (Mg) levels were recorded in soil that received the CO_2 treatment. All treatments resulted in lower zinc (Zn) values (relative to day 0), and was substantially lower in the CO_2R and control soils with 10.6 mg/L and 9.7 mg/L obtained, respectively by day 60. Manganese (Mn) was positively affected by the higher temperatures and climate change drivers with a 3-fold increase in concentration day 25 and day 60 compared to day 0. Copper (Cu) increased by day 25 and was reduced in all samples by day 60, with the exception of the CO_2R treated plot which showed gradually higher Cu values of 3.4 mg/L and 5.7 mg/L at day 25 and day 60, respectively. Generally, pH was higher in all plots by day 60. The addition of CO_2 under elevated temperature resulted in pH change from day 6.3 to 7.1 at day 60. Nitrogen values remained the same in the control between days 25 and 60. During spring, soil in the CO_2 and CO_2R treated plots incubated under ambient temperatures (Table 2), displayed higher P concentration of 53 mg/L and 58mg/L respectively by day 60, while the concentration remained the same in the days 25 and 60. K was negatively affected by the addition of CO_2 and CO_2CH_4R to the soil and decreased by 5.611 % and 23.74 %, respectively, compared to day 60. Ca, Mg and Zn values increased by day 25 and then decreased by day 60 in most plots. In contrast to the trend observed at elevated temperatures however, the lowest value of 1072 mg/L was recorded for Ca in the plot that was supplied with CO_2 only at day 60, while the control plot showed progressively lower Mg from day 0 to day 60. The CO_2CH_4R treatment resulted in higher Zn values from day 0 to day 60, while the addition of rainfall resulted in a 75.52% reduction in Zn concentration by day 60. Mn concentrations were elevated in all plots, and by day 60 reflected 21 mg/L in the soil that was enriched with CO_2 only which was 3-fold greater than day 0. Higher pH values were observed in the CO_2R, CO_2CH_4 and rainfall treated plots from day 0 through to day 60, while the addition of CO_2CH_4R resulted in the most acidic conditions of pH 6.27 at day 60. The highest nitrogen concentration was observed in the CO_2R plot at day 25 and was 36.66 % higher than that recorded at day 0. At day 60, the highest soil organic carbon values were observed for the CO_2R treated plots under elevated temperature (Fig. 10). Plots that received CO_2CH_4, CO_2CH_4R and R showed similar values for both elevated and ambient temperature and ranged from 2.3 to 2.5 % $SOCg^{-1}$.

Table 1: The effect of elevated greenhouse gases and rainfall on soil nutritional quality at elevated temperature during spring

	P(mg/L)		K(mg/L)		Ca(mg/L)		Mg(mg/L)		Zn(mg/L)		Mn(mg/L)		Cu(mg/L)		pH(KCl)		N(%)	
Time (Days)	25	60	25	60	25	60	25	60	25	60	25	60	25	60	25	60	25	60
CO_2	62	65	418	446	1326	1468	443	455	8	11.7	23	23	3.6	2.3	6.6	7.1	0.22	0.33
CO_2R	38	57	295	365	1170	1351	387	418	11.9	10.6	25	30	3.4	5.7	6.24	6.94	0.27	0.3
CO_2CH_4	36	47	348	353	1131	1256	380	412	10.8	14	22	28	5.2	2.9	6.29	6.55	0.27	0.3
CO_2CH_4R	36	47	314	358	1155	1172	375	397	9.3	11.7	18	32	2.7	2.1	6.27	6.54	0.27	0.3
R	39	50	299	341	1533	1357	485	422	12.3	12.2	20	29	3.7	2.2	6.26	6.49	0.28	0.29
CTRL	29	39	304	354	1566	1291	477	406	11	9.7	21	29	4	1.9	6.25	6.43	0.35	0.35

Table 2: The effect of elevated greenhouse gases and rainfall on soil nutritional quality at ambient temperature during spring

	P(mg/L)		K(mg/L)		Ca(mg/L)		Mg(mg/L)		Zn(mg/L)		Mn(mg/L)		Cu(mg/L)		pH(KCl)		N(%)	
Time (Days)	25	60	25	60	25	60	25	60	25	60	25	60	25	60	25	60	25	60
CO_2	45	53	331	328	1721	1072	549	328	12.5	14.9	13	21	3.6	2.2	6.4	6.38	0.27	0.31
CO_2R	53	58	373	371	1773	1199	519	377	11.9	17.2	20	16	3.6	2	6.56	6.71	0.41	0.28
CO_2CH_4	40	43	279	319	1800	1139	529	309	22.6	19.6	14	20	3.7	2.2	6.36	6.37	0.29	0.28
CO_2CH_4R	53	37	344	265	1612	1176	550	301	20.4	22	14	14	2.8	1.9	6.56	6.27	0.31	0.3
R	43	46	320	349	1355	1119	418	308	5.1	8	18	19	3.1	2.8	6.54	6.63	0.28	0.27
CTRL	35	35	308	291	1248	1244	399	392	25.6	5	15	20	2.6	2.5	6.44	6.41	0.3	0.28

3.6 Changes in soil nutrients after exposure to climate change drivers during summer

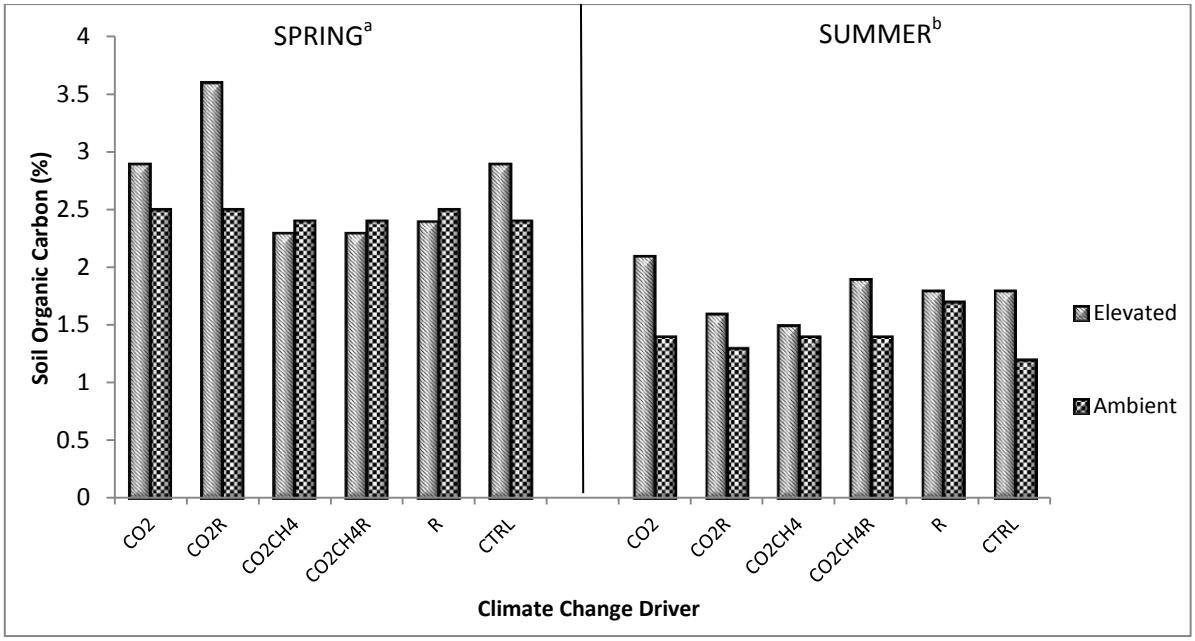
P was considerably lower in soil during the summer season (Table 3 and Table 4). Lower P values were measured in the samples from the CO_2, CO_2CH_4R and rainfall treatments (Table 3) between days 25 and 60; however by day 60 the control showed the lowest value which was approximately 2-fold less than that at day 0. K was most negatively affected by a combination of factors viz. CO_2CH_4R and was reduced by 39.27 % at day 60, compared to day 0. The treatments in combination with the elevated temperature resulted in considerably lower Ca values by 25, however by day 60 these values had increased by approximately 3-fold in all plots. By day 60, Mg and Mn concentrations were higher compared to day 0, and were 14.66 % and 68.63% greater, respectively by day 60 in the control plot. Interestingly, CO_2, CO_2R and CO_2CH_4 treatments exhibited the same pattern of change for Cu and pH, while N did not show much variation between treatments throughout the experimental period. Incubations of soil at ambient temperatures during the summer resulted in much lower P concentrations compared to elevated temperature (Table 4). However, in contrast P concentrations in the control were higher at ambient temperatures. The CO_2, CO_2R and CO_2CH_4R treated plots resulted in gradually lower K values until day 60, the lowest observed in the CO_2CH_4R which was 39.58 mg/L less than that observed at day 0. Consequently, this combination of factors viz. CO_2CH_4R also exhibited the highest Ca concentration of 1017 mg/L after 60 days, 8% greater than day 0. However all plots showed lower Ca values at the end of the sampling period. Mg levels were positively affected by the ambient temperature incubations, with all plots displaying higher Mg values by day 60. All samples showed gradually higher Zn concentrations from day 0 through to day 60, with the exception of the plot that received CO_2, which showed a 12.32% lower Ca concentration at day 60 compared to day 25. An increase in Mn concentrations was evident in all plots from day 25 to day 60, with the addition of rainfall showing almost a 2-fold increase by the end of the sampling period. Cu decreased in all samples by day 60, with the rainfall and the CO_2CH_4 treated plot exhibiting 16.12 % lower concentrations compared to day 0. At ambient temperatures, pH decreased in all plots by day 60, and pH was most acidic (5.41) in the plot that received CO_2 by day 25. N did not change substantially amongst all treatments, with the greatest value observed at day 60 in the CO_2CH_4R treatment. SOC concentrations were lower during summer compared to spring at both temperature regimens (Fig. 10). Plots that received R and the CTRL plot showed similar values of 1. 8% SOC g^{-1}. CO_2, CO_2CH_4 and CO_2CH_4R treated plots displayed similar values at day 60 under ambient temperatures of 1.4 % SOC g^{-1}.

Table 3: The effect of elevated greenhouse gases and rainfall on soil nutritional quality at elevated temperature during summer

	P(mg/L)		K(mg/L)		Ca(mg/L)		Mg(mg/L)		Zn(mg/L)		Mn(mg/L)		Cu(mg/L)		pH(KCl)		N(%)	
Time (Days)	25	60	25	60	25	60	25	60	25	60	25	60	25	60	25	60	25	60
CO_2	14	9	151	154	1101	1060	340	356	6.3	5.8	15	17	3.4	3	5.79	5.68	0.24	0.28
CO_2R	6	7	135	137	1028	919	315	310	5.1	6.2	14	13	3.7	3.6	5.73	5.58	0.29	0.21
CO_2CH_4	6	8	140	151	1016	1022	329	343	5.6	5.9	16	14	4.2	3.3	5.69	5.66	0.23	0.22
CO_2CH_4R	11	8	142	111	1045	978	378	329	5.5	5.7	15	13	3.7	2.6	5.6	5.62	0.2	0.25
R	12	9	118	119	1076	1020	380	343	5.3	6.2	16	17	3.3	2.7	5.64	5.7	0.22	0.25
CTRL	4	5	140	133	1072	1067	398	354	4.7	4.7	17	22	2.9	2.4	5.61	5.67	0.21	0.21

Table 4: The effect of elevated greenhouse gases and rainfall on soil nutritional quality at ambient temperature during summer

	P(mg/L)		K(mg/L)		Ca(mg/L)		Mg(mg/L)		Zn(mg/L)		Mn(mg/L)		Cu(mg/L)		pH(KCl)		N(%)	
Time (Days)	25	60	25	60	25	60	25	60	25	60	25	60	25	60	25	60	25	60
CO_2	6	5	152	121	851	806	348	308	7.3	6.4	5	8	3.6	2.8	5.36	5.41	0.21	0.21
CO_2R	6	6	154	140	976	942	376	334	7.4	13.3	5	9	2.9	2.7	5.54	5.62	0.23	0.2
CO_2CH_4	4	6	165	192	900	887	365	315	8.1	11	5	10	4	2.6	5.5	5.54	0.2	0.22
CO_2CH_4R	7	9	131	115	1055	1017	347	333	6.7	7.7	5	11	3.1	2.4	5.66	5.74	0.22	0.25
R	7	7	181	172	873	839	331	314	29.5	64	8	12	5.1	2.6	5.51	5.65	0.2	0.23
CTRL	6	10	194	188	933	912	339	322	29	32	7	11	3.6	4.2	5.57	5.7	0.2	0.21



[a] initial SOC = 2.55; [b] initial SOC = 1.57

Figure 10: The effect of elevated greenhouse gases and rainfall on soil organic carbon at Day 60 during spring and summer

4. DISCUSSION

A variety of techniques have been employed in global change studies to simulate warming, including growth chambers, open top chambers, greenhouses, retractable passive warming curtains, heated coils or infrared heaters (Shen and Harte, 2000) and each technique suffers its own inherent advantages and disadvantages (Henry, 2012). However, a greenhouse is used in this study to enable a natural change in temperature and account for differences that occur during nocturnal and diurnal daily cycles. Furthermore, the formation of artefacts associated with a constant elevated temperature that can occur in many simulation studies, especially in the laboratory environment was greatly reduced.

Dehydrogenases are known to exist only in viable cells and is exclusively intracellular in origin (Quilchano and Maraňòn, 2002). It is therefore considered as a good indicator of overall microbial activity as it is responsible for transferring protons and electrons from substrates to acceptors and is directly linked to the respiratory chain of microorganisms (Brzezinåska et al. 1998). It is imperative for the oxidation of soil organic matter and assists in determining the ability of soil to support biochemical processes that are vital for the maintenance of soil health and fertility (Kumar Das and Varma, 2011). For both seasons and under both temperature regimens,

dehydrogenase enzyme levels were negatively affected by the end of the sampling period, however, higher enzyme activity levels were detected during the spring season compared to summer. This can be ascribed to the presence and quality of the substrates available for use by the microbial community since soil microbial activity is determined by the quality and quantity of easily decomposable substrates (Friedel et al. 1996; Klose et al. 1999). Soil nutritional content was much lower during the summer period than those measured for corresponding plots during the spring further emphasising the effect of substrates on soil dehydrogenase enzyme activity. Variations in dehydrogenase enzyme activity have been linked to soil texture, pH and nutrient availability. Quilchano and Maranon (2002) found a positive correlation between soil dehydrogenase activity and soil pH, Ca, Mg and K. Similarly, changes in dehydrogenase activity in the present study are also related to changes in soil nutrients and pH suggesting that the soil microorganisms became nutrient limited under the global change treatments particularly during the summer seasons. Interestingly, however, under elevated temperature during both the spring and summer seasons, plots that were enriched with CO_2 demonstrated higher dehydrogenase activities compared to the controls, indicating that a combination of CO_2 and temperature exerts a strong influence on soil dehydrogenase activity. This effect was most pronounced at elevated temperatures during the spring season. This relationship was not observed under ambient temperature conditions for both seasons, thus these results indicate an interactive effect of temperature and CO_2. Higher temperatures result in further increases in soil CO_2 concentrations thus promoting the growth of 'capnophilic' microorganisms which are microbes that require high CO_2 levels for their growth and are widespread in soil and water (Piterinaa et al. 2012, Ueda et al. 2008). It has been suggested that higher CO_2 levels may either trigger spore germination or proliferation of dormant cells amongst this group of microorganisms (Ueda et al. 2008) explaining the higher dehydrogenase enzyme activities observed in soils exposed to a combination of elevated temperature and CO_2.

The β-glucosidase enzyme is responsible for the degradation of labile cellulose and a variety of other carbohydrates (Deng and Tabatai, 1996) and this enzyme activity is therefore essential for organic matter degradation and release nutrients to assist subsequent microbial enzyme activities (Sardans et al. 2008). This enzyme is routinely used as a soil quality indicator as it is frequently linked to organic matter turnover rates (Bandick and Dick 1999; Ndiaye et al. 2000). In the current study, changes in β-glucosidase enzyme levels in response to the global change drivers were driven primarily by seasonal differences i.e. variations in temperature and moisture, temperatures were higher in summer, while soil moisture was higher in spring. Higher temperatures during summer stimulated enzyme production at both elevated and ambient temperature

conditions. Baldrian et al. (2013) also reported significant seasonal variations in β-glucosidase activity which was more pronounced in the summer periods. Although Wallenstein et al. (2009) indicated that temperature was the main factor driving β-glucosidase activity; the findings in the present study can also be attributed to soil macronutrient concentrations that also regulate extracellular enzyme production. In summer, higher β-glucosidase enzyme activities were observed for both temperature regimens in response to lower soil nutrient concentrations. Extracellular enzymes are the principal means by which soil microorganisms are able to derive benefit from biologically unavailable carbon and nutrients in soil organic matter (Sinsabaugh, 1994). The production of extracellular enzymes is both carbon and energy intensive, thus microbes will only produce them if they are to derive any benefit from the reactions these enzymes catalyse. Similar to the dehydrogenase enzyme, a substantial effect on β-glucosidase enzyme levels were observed in plots that received CO_2, under elevated temperature during spring. However, under ambient temperature there was a lack of any observable trend, with the net effect of treatments resulting in values that were similar to that detected at the beginning of the study. Gutknecht et al. (2010) observed an interactive effect of elevated CO_2 with increased warming on soil β-glucosidase enzyme activity; however, these workers were unable to distinguish an effect produced by warming alone, in contrast to the present study. It is generally accepted that elevated temperature will lead to an increase in the size of the substrate pool available for utilisation by the soil microbial community, while elevated CO_2 indirectly affects soil microbial activity by a change in resource allocation. However, fluctuations in seasonal climate will also have a considerable effect on soil enzyme activities, and could camouflage any treatment effects (Steinweg and Wallenstein, 2010) as was observed with β-glucosidase activity during spring. Das et al. (2011) found a significant increase in β-glucosidase enzyme activity after 20 days of elevated CO_2, at 3 different temperature regimens (25 ºC; 35 ºC or 45 ºC). These authors speculated that this was due to enhanced labile C input as a result of the elevated CO_2 conditions. Furthermore, Das et al. (2011) reported a significant relationship between the β-glucosidase enzyme activity, temperature and different moisture conditions. A similar effect was observed in the present study with the variations in moisture and temperature accountable for the observed β-glucosidase enzyme activity across seasons.This data was also supported by Sardans et al. (2008) who found a positive correlation between soil moisture content and β-glucosidase enzyme activity during spring in a Mediterranean shrubland exposed to prolonged warming conditions.

Arylsulphatase enzymes are responsible for the hydrolysis of sulphate esters and are commonly found in the soil environment. They are widespread in nature and generally secreted by bacteria into the external environment in

response to sulphur limitation (Tabatai and Bremner, 1970; Alef and Nannipieri, 1995). The activities of this enzyme were inconsistent over the sampling period for both seasons at both temperature conditions. In addition, by the end of the sampling period, for most plots, values did not vary substantially from those measured at the beginning of the experiment, similar to the β-glucosidase enzyme during spring. There can be several explanations for these trends. Firstly, seasonal differences amplified as temperature and moisture variations would have certainly affected enzyme production. Fekete et al. (2011) discovered a correlation between arylsulphatase activity and soil moisture further highlighting the importance of soil moisture in regulating enzyme synthesis. Secondly, higher arylsulphatase enzyme activity observed at the end of summer could be related to the nutritional requirements of the soil microbiota (Saa et al 1993) or presence of more sulphate compounds present in the soil available for use after the seasonal litterfall changes (Moscatelli et al. 2005, Chrost, 1991). Moscatelli et al. (2005) also proposes that higher arylsulphatase enzyme activity indirectly indicates the presence of a larger fungal population as the substrates necessary for the arylsulphatase enzyme are esther sulphates which are only present in fungal cells. Thirdly, Appiah and Ahenkorah (1989) suggested that a change in arylsulphatase enzyme activity can result due to complexity of soil organic colloids. Soil aggregation can control a range of the soil's physical, chemical, biological and agricultural properties (Rillig et al. 1999), while soil organic matter may become less available for microbial decomposition due to occlusion in soil aggregates (Rovira and Vallejo, 2003). Elevated CO_2 has been shown to increase soil aggregation caused by increased root growth (Rogers, 1998) and can also lead to the formation of bacterial extracellular polysaccharides or glomalin glycoproteins from arbuscular mycorrhizal fungi. These by-products of microbial activity surround mineral particles forming an organo-mineral sheath around the cells, an additional indirect effect of elevated CO_2 (Rillig et al. 1999). In this regard, microorganisms and their enzymes will be attached to the soil colloids and in close proximity to available nutrients and will not need to increase production of their enzymes. Furthermore, Al-Khafaji and Tabatai (1979) showed that arylsulphatase enzyme production can be inhibited by trace elements including Cu which decreased during summer when arylsulphatase activity increased by the end of the sampling period.

The urease enzyme catalyses the hydrolysis of urea to CO_2 and ammonia. These microbial products are not usually susceptible to environmental degradation and are capable of accumulating in cell free form ultimately releasing $N\text{-}NH_4$ through urea hydrolysis (Zantua and Bremner, 1977). Urease activity was reduced by day 25 under elevated and ambient temperature during the spring season. Considering that a similar trend was evident

in the control, it was clear that the observed changes across treatments during the spring period were not related to the differences between the elevated and ambient temperature experiments during spring. However, much lower urease enzyme activities were detected in summer indicating that variations in soil properties due to seasonal change have a greater effect on this enzyme than the other enzymes tested. Tscherko et al. (2001) also reported weak responses of the urease enzyme in response to elevated temperature and higher soil moisture, which is consistent with the results obtained for the present study during spring. The effect of elevated precipitation was simulated manually, and the repeated cycle of drying and rewetting can also be responsible for the observed variations in activity during summer. Soil microbes are known to encounter various physiological challenges as a result of physical disturbances and can also experience pulse changes in the state of water, due to events that disturb soil structure such as drying, re-wetting and freeze thaw (Schimel et al. 2007). Durban is located in a coastal region in KwaZulu-Natal, characterised by moderate rainfall and high levels of humidity, most notably during the summer season. This change in climate inevitably leads to a change in soil characteristics, generally resulting in dehydrated soils with poor nutritional status in turn causing soil microorganisms to experience moisture and nutrient limitations under such conditions. In contrast to the present study, in a Mediterranean shrubland, the greatest increases in urease enzyme activity were observed when the temperatures were lower i.e. a negative correlation between soil urease activity and temperature was observed in summer. It was suggested that while warming treatments increased urease activity, this was only significant when the natural soil water content was higher as well (Sardans et al. 2008). Tscherko et al. (2001) reported that a combination of elevated CO_2 and elevated temperature increased urease activity in a weedy model ecosystem, while Ebersberger et al. (2003) found higher urease enzyme production in response to elevated CO_2 in a calcareous grassland. Similarly, a combination of higher temperatures during summer in with elevated CO_2 or other CO_2 treatments including CO_2 also resulted in higher urease enzyme values during both elevated and ambient temperatures during spring. This observation further highlights the interaction of temperature and CO_2 as observed for the dehydrogenase and β-glucosidase enzyme and demands further investigation.

Ecosystem functioning is dependent on the nutrient cycling between soil and plants (Rodin et al. 1967) and is therefore essential for plant survival. The completion of a plants life cycle is influenced by 16 essential nutrients (Chesworth, 2008), which are categorised as macronutrients or micronutrients depending on the amount of the nutrient required by the plant (Allaway, 1975). Micronutrients (Zn, Fe, Mn, Cu, Mo, B and Cl) are generally only required in small amounts and usually are limited to 0.05% dry mass. Alternatively, macronutrients (C, H,

O, N, P, K, Ca, Mg, S) are necessary in larger amounts and comprise more than 0.1% dry mass (Lukac et al. 2010). During spring higher concentrations of P, K, Ca and Mg were observed after 60 days in the plots that received CO_2 when incubated under elevated temperature. This trend was not observed under ambient temperature conditions, suggesting that the interaction of CO_2 and temperature had resulted in the change in these macronutrients. Consequently, CO_2 addition also resulted in higher β – glucosidase and arylsulphatase enzyme activity during spring under the elevated temperatures. It is possible that the interaction of these factors can lead to changes in soil processes that result in enhanced soil fertility such as modification of the composition of soil organic matter as previously reported (Reynaldo et al. 2012). During summer, at both elevated and ambient temperature, soil nutrients were much lower than those observed during spring. This was most evident in plots that were supplied with a combination of factors viz. CO_2R, CO_2CH_4 or CO_2CH_4R and can be linked to the changes in moisture and temperature due to temporal variation of the soil samples collected before commencing the experiment. Similar to some trends observed for the enzyme activities, there were also fluctuations in several of the soil nutrients evaluated for both seasons at both temperature regimens. It is imperative to note however, that these nutrients are subject to different control mechanisms i.e. P and K are impacted by both geological and biological processes, while K, Ca, Mg and other micronutrients are primarily controlled by geological cycles (Wood et al. 2006; Watanabe et al. 2007). There was no distinct relationship between soil enzyme activities and soil organic carbon for both spring and summer by day 60. The lower SOC values observed during summer was probably linked to the changes in litter quality and quantity according to seasonal variation (Kirschbaum, 1995). Other studies have linked changes in SOC to the temperature dependant shifts that occur in the active microbial community which causes a subsequent change in the carbon pool (Andrews et al. 2000). Higher temperatures can modify chemical reaction kinetics or microbially mediated processes such as decomposition (Reynaldo et al. 2012), thus affecting nutrient conditions in the environment. Furthermore, higher temperatures have been reported to lead to faster nutrient cycling through alterations in soil respiration (Bond-Lamberty and Thomson, 2010), litter decomposition (Shaw and Harte, 2001), and water content (Zhang et al. 2012). Although litter was not added into the experimental plots, decomposition of pre-existing plant litter will reflect changes in the soil nutrient concentrations as well. Precipitation changes, atmospheric deposition combined with hydrological patterns also has great potential to affect nutrient dynamics, soil organic carbon and transport mechanisms (Matthews et al. 2007; Heimann and Reichstein, 2008). Korner (2000) suggests that multiple sampling over long periods is necessary due to the variations in soil parameters as a result of different stages of decomposition in soil. Thus, further studies encompassing longer incubation times

for soil under the influence of a combination of global change drivers will add a greater depth of understanding of the influence of a combination of global change drivers on soil enzymes.

In conclusion, the results from the current study clearly demonstrate that temperature changes in combination with other global change drivers produce data that are different when these drivers are investigated individually. Furthermore, it is evident that soil microbial enzymes respond differently during different seasons and these variations must be taken into account. In addition, the soil ecosystem is extremely sensitive towards disturbances such as that which occurred during the experimental setup which was unavoidable. Although controls were used and data are interpreted relative to changes that occurred in the control, these results must still be interpreted with caution. If carbon sequestration is to be considered a viable and sustainable option for mitigating global climate change, it is essential to understand the underpinnings of microbial activity in various contexts. This includes instituting multi-factor global change experiments such as the present study to effectively characterise how soil microbes respond either by changing their activities or by altering community composition. Experiments investigating these mechanisms will provide a more realistic view of the projected changes to the soil ecosystem in order to effectively assess terrestrial carbon feedbacks.

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CHAPTER FOUR

‘RED-LIKE’ SOIL BACTERIAL RUBISCO GENE COPY NUMBER (*cbbL*) IN RESPONSE TO SINGLE/MULTIPLE GLOBAL CHANGE DRIVERS AND SEASONAL CHANGE

This chapter has been submitted in the current format to ‘Soil Science’

'RED-LIKE' SOIL BACTERIAL RUBISCO GENE COPY NUMBER (*cbbL*) IN RESPONSE TO SINGLE/MULTIPLE GLOBAL CHANGE DRIVERS AND SEASONAL CHANGE

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Abstract

The search for novel methods for soil carbon sequestration strategies continue to dominate soil ecology research and microbial community functional gene profiling will support these efforts. This study investigated the changes in soil bacterial RuBisCo gene (*cbbL)* and the corresponding changes in soil organic carbon (SOC) upon exposure to various single or multiple global change drivers (viz. carbon dioxide, methane and rainfall) under elevated and ambient temperature during the spring and summer. Lowest *cbbL* gene copy numbers were observed during summer, while during spring the *cbbL* gene copy numbers increased (90.9 – 93.09%) by day 60 compared to day 0, under elevated temperatures. The combination of global change drivers did not result in a substantial variation in gene copy numbers across seasons suggesting a counteracting effect of the factors. No direct correlation between changes in copy number and SOC was observed, although lower SOC in summer at elevated temperature did result in overall lower *cbbL* gene copies. Results from this study indicate a general increase in *cbbL* gene copy numbers that could ultimately result in the net CO_2 fixation potential of soils and consequently on terrestrial C cycling.

Keywords: qPCR, Rubisco enzyme, *cbbL* gene, global change drivers, soil organic carbon

1. INTRODUCTION

Progressive global warming as a consequence of the rise in carbon dioxide (CO_2) and other greenhouse gas concentrations (IPCC, 2007) has prompted environmental, social, political and economic concerns, worldwide. Climate change models and simulation studies have predicted an acceleration of this global warming phenomenon due to carbon-cycle feedbacks (Fussel, 2009; Scheffer et al., 2006; Cox et al., 2000). Within the global carbon (C) cycle, the natural fluxes of CO_2 to and from the atmosphere, oceans and land are several-fold higher than annual CO_2 emissions related to the combustion of fossil fuels (Singh et al., 2010). In particular, soil is considered as a major biological sink or source of C and is thus critical to C cycling in terrestrial ecosystems (Uvarov *et al.*, 2006). It has been estimated that soil contains up to 1500 Pg (1Pg = 10^{15}g) of organic C (Amundson, 2001) and even minor variations of this soil C pool can lead to significant effects on atmospheric C

concentrations and the C cycle at large (Belay-Tedla et al., 2009). CO_2 fixation and respiration processes occur in nearly all soil microenvironments and are mediated by the soil microbial community. Ultimately, whether soils are able to act as a sink or source for carbon is influenced by which of these two processes is dominant (Miltner et al., 2004). It has been long recognised and well established that temperature exerts a strong control on microbial activity. The enhanced mobilization of microbial carbon and degradation of soil organic carbon (SOC) leading to increased CO_2 outputs into the atmosphere is projected as global environmental temperatures continue to rise (Subke et al., 2003). Therefore, an in-depth understanding of the regulation of physiological activities of the soil microbial community in response to various climatic changes is now imperative. Also, fossil fuel emissions can be offset by 0.4 to 1.2 Gt carbon yr^{-1} if soil carbon sequestration strategies are employed (Lal, 2004) and microbial activities will exert positive or negative effects on this process.

The principal mechanism of autotrophic CO_2 fixation in nature is via the reductive pentose phosphate pathway commonly known as the Calvin Benson Basham (CBB) cycle (Berg, 2011). The attachment of CO_2 to the acceptor molecule ribulose-1,5-bisphosphate (RuBP) resulting in the formation of two molecules of 3-phosphoglycerate is catalysed by the RuBP (EC 4.1.1.39, RubisCo) carboxylase/ oxygenase enzyme (Anderson and Backlund, 2008). This enzyme is fundamental in the removal and sequestration of environmental CO_2 as it is responsible for catalysing the reactions that involve the reduction of CO_2 to organic C (Tabita et al., 2008). RubisCo enzymes are grouped into four forms (I, II, III and IV) and these forms are characterised based on differences in structure, catalytic activity and oxygen sensitivity (Tabita, 1999). Typically, Form I RubisCo enzymes commonly occur in soil and have been detected in plants, algae, cyanobacteria and autotrophic bacteria (Tcherkez et al., 2006). The large subunit of the Form I RubisCo enzyme is coded for by the *cbbL* gene (Miltner et al., 2005). Form I RuBisCo can be further subdivided into two major types: 'green-like' and 'red-like' based on phylogenetic analysis of *cbbL* sequences (Watson and Tabita, 1997).

Environmental microbiological studies have benefitted tremendously from the advent of several molecular genetic techniques that have enabled analysis of soil microbial communities in complex

environmental samples (Sinigalliano et al., 2001). Tools such as Denaturing Gradient Gel Electrophoresis (DGGE) or Terminal Restriction Fragment Length Polymorphisms (T-RFLP) are commonly used for community structure analysis while Real Time Quantitative PCR (qPCR) methods are more frequently used for estimating community size by measuring gene copy number (Hirsch et al., 2011). Selesi et al. (2007) quantified the *cbbL* gene copy number by qPCR in agricultural soils and various other soil microhabitats. The study revealed the abundance and prevalence of RuBisCo coding *cbbL* gene in the various soil types. However, the authors were unable to ascribe a functional significance to the presence of the 'red-like' *cbbL* gene. Recently, the role of autotrophic microorganisms in soil under a continuously $^{14}CO_2$ labelled atmosphere was investigated by various techniques including qPCR of the *cbbL* gene (Yuan et al., 2012a). The authors reported a significant correlation between changes in *cbbL* gene diversity and soil organic carbon. In addition, a positive correlation between bacterial *cbbL* gene abundance and RuBisCo enzyme activity was also identified. It was concluded that the bacterial and chromophytic algal *cbbL* genes may offer a promising mechanism for the microbial assimilation of atmospheric CO_2 (Yuan et al., 2012a).

A concise understanding of soil microbial ecology is paramount to the assessment of terrestrial C cycle feedbacks and studies such as that by Yuan et al. (2012a) provides valuable information and possible mitigation options. However, a majority of investigations pertaining to climate change in biological ecosystems and the soil microbial community focus on single global change factors i.e. elevated atmospheric CO_2 concentrations, higher temperatures or drought. Considering that all these factors strongly impact various soil properties, it is reasonable to assume that combinations of these factors may have additive or antagonistic effects (Shaw et al., 2002). In addition, responses to single environmental changes are varied across systems and will also fluctuate annually (Dukes et al., 2005). Microbially mediated CO_2 fixation can aid in decreasing atmospheric CO_2 concentrations however, it is still unknown how changes in greenhouse gas concentrations and other environmental changes will affect the *cbbL* gene involved in this process. Thus, this study investigated the impact of single and/or multiple global change factors viz. CO_2, methane (CH_4), precipitation and temperature on 'red-like' soil bacterial *cbbL* gene copy numbers during spring and summer seasons in Durban, South Africa.

2. MATERIALS AND METHODS

2.1 Site description and experimental design

Sampling below the zone of short term agricultural influence with sampling depth of up to 60cm for perennial vegetation has been suggested (Carter and Gregorich, 2008), thus sandy loam soil from the top 0-50 cm was collected from an area adjacent to the University of KwaZulu Natal (Westville) which is situated within an environmental conservancy. The area is dominated by flowering plants such as *Tecomaria capensis, Strelitzia reginae, Plumbago auriculata and Aloe succotrina* ; while alien invasive species such as *Leucaena leucocephala, Lantana camara, Schinus terebinthifolius, Chromolaena odorata and Syringa vulgaris* are also frequently encountered (http://conservancy.ukzn.ac.za/IndigenousPlanting.aspx). The climate change experiments were started within 4 days of soil collection due to the complex experimental setup. Soil was stored at ambient environmental temperature (identical to the area from which soil was collected) in polyethylene bags that had holes to allow for aeration up until it was used for the experiments. The individual and/or interactive effects of elevated carbon dioxide (CO_2), methane (CH_4), precipitation and temperature were investigated using constructed modified Screen-Aided-Carbon-Dioxide-Control (Leadley et al. 1997) in two separate greenhouses (E and A) situated at the University of KwaZulu-Natal (Westville). Screen Aided Carbon Dioxide Control is recommended as the most suitable method for determining the effects of elevated CO_2 levels for short stature vegetation and is useful as it combines the advantages of Open Top Chambers and Free Air Carbon Dioxide Enrichment experiments (Leadley et al., 1997; Macháčová, 2010). Briefly, 1 m x 0.5 m polycarbonate screens were attached to stainless steel tables in the greenhouses and were arranged to achieve 1m x $1m^2$ plots within which the climate change simulations were conducted (Fig 1). The base of each plot was then lined with gas tubing (Afrox, South Africa) which had 10 mm holes drilled every 10 cm apart and a depth of 1.5cm.

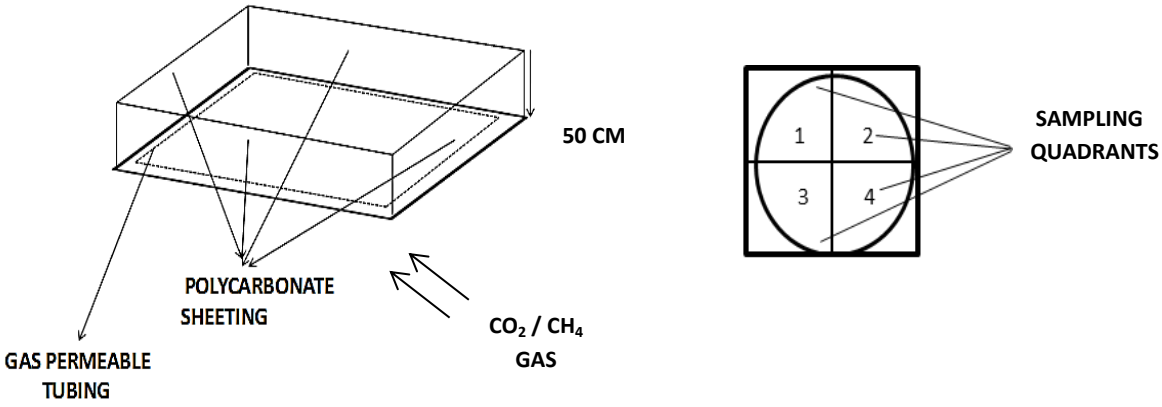

Fig 1: Experimental set-up used for investigating the effects of global change drivers. A – illustrates the 3D view of individual experimental plots covered at sides and base with polycarbonate sheeting, while B illustrates the top view with 4 sampling quadrants.

Sandy loam soil (25 kg) was then added to all the plots within the greenhouses. Greenhouse A corresponded to ambient environmental temperature, while Greenhouse E had temperatures approximately 5 – 10 ˚C higher than Greenhouse A (sunlight and humidity dependent). These experiments were conducted during spring and summer (September 2011- March 2012). Simulations of elevated greenhouse gas conditions was done once daily addition of 425 ppm CO_2 and 2.6 ppm CH_4 gas (Afrox, South Africa) to the soil at a flow rate of 10 litres per min for 5 min. The CO_2 and CH_4 concentrations used represent atmospheric concentrations plus a 10% increment (IPCC, 2001). Changes in rainfall were simulated weekly by addition of synthetic groundwater (Klier et al. 1999), based on the rainfall recorded for the relevant period in the previous year as per data from the South African Weather Service (72.6 and 28.4 mm for spring and summer, respectively) and increased by 10%. The effects of the following global change drivers were investigated: Carbon dioxide (CO_2); carbon dioxide and rainfall (CO_2R); carbon dioxide and methane (CO_2CH_4); carbon dioxide, methane and rainfall (CO_2CH_4R) and rainfall (R).Control plots that did not receive any treatment were also set up in both greenhouses (CTRL). At each sampling time, each experimental plot was divided into four quadrants and four soil samples were collected from each quadrant. Subsequently, these four samples from each quadrant were then homogenised. A single homogenised sample was then used from each quadrant (n = 4) to provide 4 replicates per plot.

2.2 DNA isolation, PCR amplification and cloning of *cbbL* gene

DNA was extracted from the soil samples collected from each experimental plot at days 0, 25 and 60 using the UltraClean Soil DNA Kit (MOBIO Laboratories Inc, California) according to the manufacturer's instructions. PCR targeting a 270 bp fragment was then performed using the primer set *cbbL* R1F and *cbbL* R1intR (Table 1, MetaBion Germany) specific for the 'red-like' RuBisCO gene according to a modified method of Selesi at al. (2007). Each 25 µl reaction contained 0.5 µM of each primer, 200 µM dNTPs, 1 mM $MgCl_2$, 10X Buffer , *Taq* polymerase (1.25 U), double distilled water and 2 µl template DNA (concentration ranged from 2 to 20 ng/ µl). The PCR reaction was then conducted in an Eppendorf MasterCycler according to the following conditions: 95 ℃ for 4 mins, 32 cycles of 95 °C for 1 min, 59 °C for 30 s, 72 °C for 1 min and a final elongation at 72 °C for 10 min.

Table 1: Primers and probe used for amplification and quantification of *cbbL* genes.

PRIMER	POSITION	SEQUENCE
cbbLR1F	634–651	AAG GAY GAC GAG AAC ATC
cbbLR1intR	892–908	TGC AGS ATC ATG TCR TT
cbbLRpro	666–683	CAT GCA YTG GCG CGA CCG

All PCR amplicons were then confirmed on a 2% agarose gel at 60 V for 90 min. The amplified 270 bp fragment was then ligated to the pJET plasmid (Thermo Scientific CloneJET PCR Cloning Kit #K1232) according to the manufacturer's instructions, followed by heat shock transformation and identification of positive clones from growth on LB agar plates containing ampicillin (25 $\mu g ml^{-1}$). Colony PCR was conducted on positive clones to verify presence of the correct insert according to manufacturers instructions described in the cloning kit mentioned above. All PCR reactions were then analysed on a 2% agarose gel at 60 V for 90 min. Clones that contained the correct insert size were chosen for further analysis.

2.3 DNA sequencing and analysis

Plasmids were isolated from overnight-grown LB broth cultures of positive transformants using the Invisorb Spin Plasmid Mini 2 Kit (InViTek, GmBH). Prior to sequencing, plasmid DNA samples were purified using ExoSAP-IT (USB Corporation, USA). Cycle sequencing was then conducted using the

Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to manufacturer's instructions. Subsequently, sequences obtained from the 3130 x1 Genetic Analyser (Applied Biosystems) were edited and analysed using Chromas Lite Version 2.01 and BioEdit Sequence Alignment Editor Version 7.0.9.0.

2.4 Restriction and copy number determination

Plasmid DNA to be used for the construction of a standard curve was restricted with *NCO* 1 Fast Digest (Fermentas) for 10 min at 37˚C. Linearised plasmid DNA was then measured spectrophotometrically using the Implemen Pearl BioPhotometer (Germany). Plasmid copy numbers were then calculated using the equation described by Whelan et al. (2003).

2.5 Real Time PCR (qPCR)

Primers and monocolour hydrolysis probes (Table 1) were synthesized (MetaBion, Germany) and used in the real time qPCR experiments conducted according to a modified method of Selesi et al. (2007). using the Roche LightCycler 480 System. Each PCR reaction was prepared using the LightCycler 480 Probes Master Kit (Germany) as per manufacturer's instructions. Amplification was performed for 10 min at 95 ˚C, 40 cycles at 95 ˚C for 15 s and 1 min at 56 ˚C for 1 min. A standard curve was first prepared by serially diluting linearized plasmid DNA (10^9 to 10^3 copies /µl). Subsequently, all results were compared against the standard curve and copy numbers were determined using the LightCycler Software Version 1.5.0.39.

2.6 Soil nutrient and organic carbon analysis

All soil samples were analysed by Soil Fertility and Analytical Services at the KwaZulu-Natal Department of Agriculture, Environmental Affairs and Rural Development. Phosphorus (P), Potassium (K), Zinc (Zn), copper (Cu) and Manganese (Mn) were determined using atomic absorption spectroscopy after extraction with Ambic-2 extracting solution (Murphy and Riley, 1962; Manson and Roberts, 2000). Calcium (Ca) and Magnesium (Mg) were determined by atomic absorption spectroscopy after incubation of soil with 1 M KCl and subsequent filtration with 0.0356 M $SrCl_2$

(Manson and Roberts, 2000). Soil pH was determined using 1 M KCl (Mason and Roberts, 2000). Nitrogen was determined using the Automated Dumas combustion method using a LECO CNS 2000 (LecoCorp, Michigan, USA, Matejovic, 1996). Total organic carbon was determined by the Walkley-Black method (Allison, 1965).

2.7 Statistical analysis

Data was analysed for normality and Pearson's correlation coefficients were calculated using IBM PASW 18 Statistics Processor.

3. RESULTS

3.1 Temperature and soil organic carbon profiles under various treatments

During spring, temperatures ranged from 26 to 38 ºC and 20 to 35 ºC in Greenhouse E (elevated) and Greenhouse A (ambient), respectively (Table 2). In summer, temperatures were approximately 5 to 10 ºC higher than those observed for spring for both greenhouses.

Table 2: Temperature profiles of greenhouses at elevated and ambient temperature during the spring and summer seasons

TEMPERATURE (˚C)				
SAMPLING TIME (Days)	**SPRING**		**SUMMER**	
	Elevated	**Ambient**	**Elevated**	**Ambient**
0	31	22	37	28
15	28	21	38	28
25	26	20	35	25
40	29	22	32	25
60	38	35	30	25

Overall, higher concentration of soil organic carbon (SOC) was observed for all plots under elevated temperature during spring (Table 3). From day 0 to day 25, all plots excluding the control and CO_2R plot under ambient temperature regimen showed a decrease in organic carbon content. At day 60, the CO_2R treated plot at elevated temperature conditions yielded a 41.18% increase in soil organic carbon compared to day 0. At ambient temperatures (Table 2), all plots had organic carbon values that ranged

between 2.2 to 2.5% SOC 100 g^{-1} by day 25, besides the plot that received a combination of elevated CO_2 and rainfall with 3.4% SOC 100 g^{-1}. From days 25 to day 60, there was no noticeable difference in organic carbon content between plots as a result of different treatments. Soil organic carbon concentrations measured at the beginning of summer season were much lower than those measured during spring (Table 3), thus overall lower SOC values were observed over the summer sampling periods. At elevated temperature, combination of CO_2 and rainfall resulted a substantial increase of 40.13% in SOC from day 0 to day 25 (Table 3), however, by day 60, this value decreased by 27.27%. At day 60, addition of elevated CO_2 lead to a 31.25% increase in SOC compared to day 25 and was the highest organic carbon measured during this sampling time. By day 25, at ambient temperatures (Table 3), all plots showed decreased SOC values compared to day 0, with little variation in SOC concentration observed in most plots from day 25 to day 60. However, by day 60, the addition of elevated CO_2 and the addition of increased rainfall lead to a 27.27% and 29.41% increase in SOC respectively when compared to day 25.

Table 3: Soil organic carbon (%) in soil exposed to global change drivers at elevated and ambient temperature during spring and summer

	SOIL ORGANIC CARBON IN SPRING [a]				SOIL ORGANIC CARBON IN SUMMER [b]			
	ELEVATED		AMBIENT		ELEVATED		AMBIENT	
TREATMENT	**Day 25**	**Day 60**	**Day 25**	**Day 60**	**Day 25**	**Day 60**	**Day 25**	**Day 60**
CO_2	2.2	2.9	2.2	2.5	1.6	2.1	1.1	1.4
CO_2 + R	2.4	3.6	3.4	2.5	2.2	1.6	1.4	1.3
CH_4	2.4	2.5	2.2	2.9	1.8	1.7	0.9	1
CH_4 + R	2.1	3	2.1	2.6	1.3	1.4	1.1	0.8
CO_2 + CH_4	2	2.3	2.2	2.4	1.6	1.5	1.2	1.4
CO_2 + CH_4 +R	2.3	2.3	2.5	2.4	1.2	1.9	1.4	1.4
R	2	2.4	2.3	2.5	1.6	1.8	1.2	1.7
CPG	2.7	2.9	2.5	2.4	1.2	1.8	1.3	1.2

[a] initial SOC = 2.55; [b] initial SOC = 1.57

3.2 Soil bacterial Rubisco gene (*cbbl*) gene copy number in response to various treatments

BLAST analysis of selected positive clones revealed 88- 91% similarity to sequences corresponding to to the 'red like' *cbbL* gene sequences in GenBank and were used for generation of the standard curve used in all qPCR experiments. Generally, an increase in *cbbL* gene copy number was observed in soil

from day 0 to day 60 in all plots (Fig 2a) with the exception of the plots that received CO_2CH_4 or rainfall during spring under elevated temperature conditions (Greenhouse E). Plots enriched with CO_2 and CH_4 showed a 76.60% and 73.34% increase in gene copy number, respectively, from day 0 to day 25. A high abundance of the *cbbL* gene was observed in plots exposed to multiple global change drivers with 2.20 x 10^5 copies g^{-1} soil in the CO_2R plot and 3.60 x 10^5 copies g^{-1} soil in the CO_2CH_4R plot obtained. At day 60, *cbbL* gene copy numbers in all plots showed an increase ranging from 90.9 – 93.09% compared to day 0.

An increase in *cbbL* gene copy number over time was also detected (Fig 2b) under ambient temperature (Greenhouse A) during spring with the exceptions of the plots that received CH_4 or CO_2CH_4R or rainfall. Addition of CO_2 led to the highest *cbbL* gene copy numbers of 2.85 x 10^5 copies g^{-1} soil quantified at day 25, reflecting a 86.31% difference compared to day 0. However, this plot also showed the lowest percentage increase of 32.46% from day 25 to day 60 while all other plots had increased copy numbers in the range of 71.01-96.20%. At day 60, the highest *cbbL* gene copy numbers were detected in the plot that received incremental rainfall with 9.22 x 10^5 copies g^{-1} soil observed which is 95.77% higher than that obtained at day 0. Overall, *cbbL* gene copy numbers were higher at ambient temperature compared to elevated temperature.

cbbL gene copy numbers were substantially lower during summer in Greenhouse E at elevated temperature (Fig 3a) compared to those measured in corresponding experimental plots during spring. For all plots, including the control a considerable reduction in the number of *cbbL* gene copies was evident from day 0 to day 25. Only the plots that received CO_2, CH_4R or a combination of CO_2CH_4 displayed significantly higher gene copy numbers from day 25 to day 60 with an increase of 92.88, 92.12 and 91.43% noted by day 60, respectively. Plots enriched with CO_2 in combination with rainfall (i.e. CO_2R and CO_2CH_4R) reflected a much lower abundance of *cbbL* gene copies compared to the other experimental plots.

Similarly, a decrease in *cbbL* gene copy numbers from day 0 to day 25 was noticed for all experimental plots during summer under the ambient temperature regimen (Fig 3b). All plots that received CO_2, either solely or in combination with other global change drivers, displayed noticeably higher gene copy numbers by day 60, compared to other plots. A high abundance of *cbbL* gene copies was observed in the CO_2R and control plots with 20.80 x 10^5 and 22. 48 x 10^5 copies g^{-1} soil, respectively.

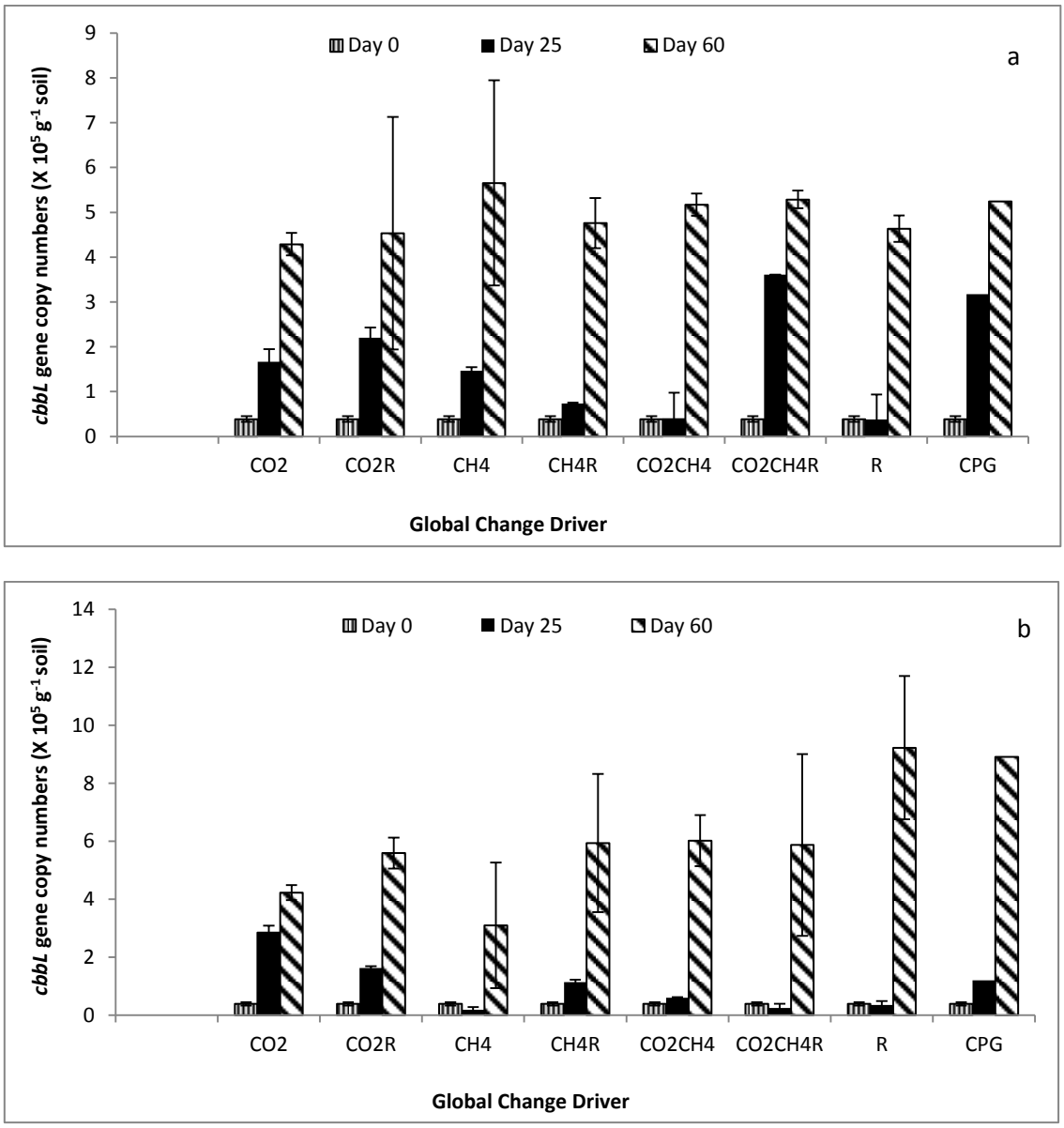

Fig 2: Effects of single and multiple global change drivers on bacterial *cbbL* gene copy numbers in soil under a) elevated temperature and b) ambient temperature during spring.

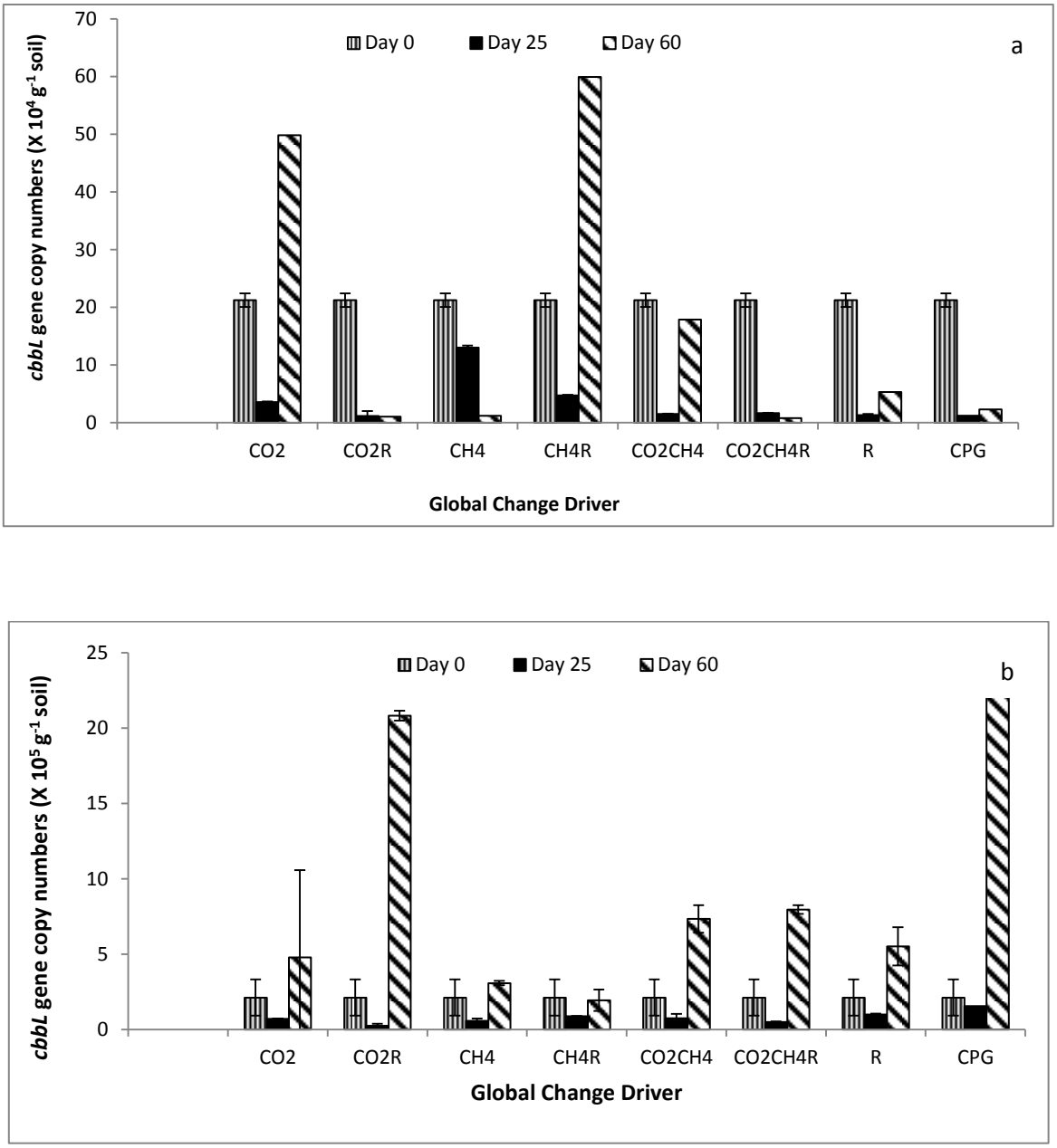

Fig 3: Effects of single and multiple global change drivers on bacterial *cbbL* gene copy numbers in soil under a) elevated temperature and b) ambient temperature during summer.

3.3 Correlation analysis between soil nutrients, organic carbon and *cbbL* gene copy number

By day 60 at elevated temperature during spring, *cbbL* gene copy number was negatively correlated with potassium, calcium and magnesium, while a positive correlation was observed with manganese (Table 4). In contrast, soil pH at day 60 showed a positive correlation with potassium, calcium, magnesium and phosphorus, and correlated negatively to manganese (r^2 = -0.542). A directly proportional relationship was also noted for the change in organic carbon vs. pH and nitrogen by the end of the sampling period. Similar to the elevated temperature conditions, organic carbon correlated with phosphorus, and strongly positively correlated with pH and nitrogen at ambient temperatures (Table 5). The ambient temperature incubations resulted in a highly significant ($p < 0.01$) negative correlation between organic carbon and *cbbL* gene copy number (r^2= -0.596). The *cbbL* gene copies also correlated negatively with phosphorus and nitrogen after exposure to the global change treatments and ambient temperatures.

In contrast to spring, *cbbL* gene copy number was positively correlated with phosphorus, potassium and calcium during the summer at elevated temperature incubation (Table 6). Zinc concentrations correlated negatively with copy number and soil pH ($p < 0.01$), while positive correlations between soil pH and phosphorus, calcium, magnesium or manganese were obtained. A significant positive relationship between organic carbon and nitrogen (r^2 = 0.829) was observed under the higher temperatures. This strong correlation between organic carbon and nitrogen (r^2 = 0.868) was also evident under ambient temperature conditions during the summer (Table 7). By day 60, *cbbL* gene copy number correlated with all nutrients evaluated except zinc, copper and organic carbon. Soil pH showed strong positive correlations to phosphorus, calcium, magnesium, manganese and nitrogen during summer at ambient temperatures.

Table 4: Pearsons correlation matrix for soil nutritional content and copy number during spring under elevated temperature

	Phosphorus	Potassium	Calcium	Magnesium	Zinc	Manganese	Copper	pH	Nitrogen	Organic Carbon	Copy Number
Phosphorus	1										
Potassium	.692**	1									
Calcium	.588**	.577**	1								
Magnesium	.753**	.904**	.821**	1							
Zinc	0.11	-0.33	-0.37	-0.25	1						
Manganese	-0.36	-.758**	-.772**	-.833**	.437*	1					
Copper	-0.07	-.545**	0.07	-0.40	0.05	0.27	1				
pH	.893**	.736**	.702**	.751**	-0.20	-.542**	0.12	1			
Nitrogen	0.10	0.04	0.25	0.02	-.557**	0.11	0.09	0.16	1		
Organic Carbon	.432*	0.17	0.31	0.17	-0.32	0.10	0.35	.546**	.420*	1	
Copy Number	-0.21	-.451*	-.421*	-.482*	0.39	.571**	0.16	-0.32	0.02	0.02	1

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 5: Pearsons correlation coeffecients for soil nutritional content and copy number during spring under ambient temperature

	Phosphorus	Potassium	Calcium	Magnesium	Zinc	Manganese	Copper	pH	Nitrogen	Organic Carbon	Copy Number
Phosphorus	1										
Potassium	.743**	1									
Calcium	-0.03	0.16	1								
Magnesium	0.04	-0.03	0.40	1							
Zinc	0.20	0.05	-0.08	-.526**	1						
Manganese	0.34	.406*	0.15	-0.02	-0.33	1					
Copper	-0.13	-0.01	-0.13	0.11	-.859**	.416*	1				
pH	.721**	.855**	0.38	0.19	-0.18	0.31	0.21	1			
Nitrogen	0.23	.439*	0.25	-0.34	.426*	0.31	-.511*	0.11	1		
Organic Carbon	.486*	.807**	0.38	-0.24	0.18	.475*	-0.18	.615**	.833**	1	
Copy Number	-.424*	-0.38	-0.17	0.08	-0.25	-0.14	0.38	-0.26	-.665**	-.596**	1

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 6: Pearsons correlation coeffecients for soil nutritional content and copy number during summer under elevated temperature

	Phosphorus	Potassium	Calcium	Magnesium	Zinc	Manganese	Copper	pH	Nitrogen	Organic Carbon	Copy Number
Phosphorus	1										
Potassium	-0.17	1									
Calcium	0.26	0.01	1								
Magnesium	0.05	0.36	.840**	1							
Zinc	0.02	.548**	-0.37	0.06	1						
Manganese	-0.38	-0.05	.696**	.644**	-.417*	1					
Copper	0.11	0.36	-.525**	-.587**	0.00	-.566**	1				
pH	.460*	-0.33	.835**	.521**	-.626**	.552**	-0.31	1			
Nitrogen	0.23	-0.04	0.12	0.25	-0.16	0.04	-0.09	0.24	1		
Organic Carbon	0.04	-0.30	0.31	0.24	-.532**	0.39	-0.29	0.40	.829**	1	
Copy Number	.570**	.462*	.411*	0.34	0.18	-0.06	0.24	0.29	-0.09	-0.20	1

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 7: Pearsons correlation coeffecients for soil nutritional content and copy number during summer under ambient temperature

	Phosphorus	Potassium	Calcium	Magnesium	Zinc	Manganese	Copper	pH	Nitrogen	Organic Carbon	Copy Number
Phosphorus	1										
Potassium	0.572**	1									
Calcium	0.716**	0.25	1								
Magnesium	0.737**	0.40	0.928**	1							
Zinc	0.405*	0.577**	-0.03	0.14	1						
Manganese	0.739**	0.595**	0.614**	.608**	.676**	1					
Copper	0.461*	0.426*	-0.02	0.03	0.22	0.12	1				
pH	0.868**	0.433*	.849**	.811**	.434*	.859**	0.20	1			
Nitrogen	0.770**	0.495*	.642**	.731**	0.36	.652**	-0.10	.652**	1		
Organic Carbon	0.511*	0.554**	0.39	.598**	.588**	.635**	-0.22	.462*	.868**	1	
Copy Number	0.754**	0.607**	.680**	.824**	0.22	.426*	.479*	.675**	.523**	0.39	1

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

4. DISCUSSION

This study focused on determining the variation in copies of the microbial *cbbL* genes that are necessary for carbon assimilation in response to global change factors, either singly or in combination and the related changes in SOC. Seasonal variation including changes in temperature, moisture, litterfall and soil microbial respiration accounts for the lower initial SOC values in summer than those for spring. If environmental conditions remain constant, it is expected that decomposition rates should increase as temperatures rise and soil carbon will be expected to decay more rapidly (Jones et al. 2005), and this was evident when comparing the elevated and ambient temperatures for both seasons. However, it is contradictory to the results obtained in this study when comparing the differences in SOC between spring and summer. In the present study, although temperature was higher during summer compared to spring, higher SOC values were not observed across seasons. Durban, KwaZulu-Natal is characterised by hot (temperatures range between 28 and 35˚C), dry and extremely humid summer conditions that also coincide with lower amounts of rainfall compared to spring. Lower soil moisture may have caused the lower soil organic carbon values observed in summer, as soil organic carbon is determined by soil moisture in different climatic zones (Reynaldo et al. 2012). Furthermore, it has been suggested that moisture limitation will inhibit microbial activity regardless of temperature (Davidson and Janssens, 2006), which will in turn affect decomposition rates and soil organic carbon stocks. The role of soil moisture on soil organic carbon is further emphasized in the present study as plots that received incremental rainfall during summer showed slightly higher SOC values than other plots on average.

SOC values did not change drastically between the two different temperature ranges investigated for both seasons. It is likely that drastic changes in SOC concentration were obscured by the short time-frame of the experiment. Kirschbaum (2000) suggests that massive changes in over a SOC time course cannot be anticipated because of the opposing effects of elevated temperature and elevated CO_2. The delayed change in SOC in other plots may be attributed to the close relationship between SOC and the soil nutrient cycles. Hungate et al. (1997) only reported significant changes in the soil organic pool

after four years of CO_2 fertilisation. It has been suggested that the effects of elevated CO_2 on SOC will be more pronounced in warmer regions due to the increased sensitivity of CO_2 to photosynthesis at higher temperatures (Kirschbaum, 1994) and changes in litter quality and quantity according to seasonal variation (Kirschbaum, 1995). In this regard, a combination of elevated temperature and elevated CO_2 resulted in the most substantial increase in SOC at day 60 compared to day 0 in summer.

Lower *cbbL* gene copy numbers were observed at the beginning of the spring season compared to the summer season and this is probably linked to the change in microbial communities with the accompanying seasonal change. Under fluctuating environmental conditions, microorganisms encounter stresses such as those related to environmental factors (e.g. temperature) and/or resource availability. This ultimately results in a community composition shift towards microorganisms that are better adapted to endure the new environment (Balser et al., 2001). Similarly, a change in the *cbbL* gene copy number in soil can indicate a change in the structure of the microbial community capable of carboxylation (Videmsek et al., 2009). During spring under elevated temperature, a simultaneous increase in the number of *cbbL* gene copies and total organic carbon was observed by day 60. Comparatively, the control plots exhibited higher copy numbers than most experimental treatment plots substantiating the observation that a combination of the global change drivers (either individually or interactively) and elevated temperature exerted a deleterious effect on *cbbL* gene copy number. *cbbL* gene copies were higher at ambient temperature compared to elevated temperatures during spring, further emphasising the negative effect of temperature.

A positive correlation between *cbbL* gene abundance and RuBisCo enzyme activity under different conditions has been observed (Yuan et al., 2012a; Xiao et al., 2014). Thus, it can be inferred that RuBisco enzyme activity would also increase under the experimental conditions investigated. Effectively, this should mean an increase in the amount of net CO_2 fixation. In bacteria, the number of *cbb* operons ordinarily differs between 1 and 2, thus gene copy number alone cannot directly be used as a proxy to estimate the *cbbL* bearing cell population size (Hugendieck and Meyer, 1991; Huseman et al., 1988). However, changes in the *cbbL* gene copy number can be linked to the presence of

different autotrophic bacteria, especially during the spring at elevated temperature. The RuBisCo gene is widely distributed amongst the aerobic, anaerobic, photoautotrophic and chemolithotrophic prokaryotic groups (Elsaied and Naganuma, 2001). Thus this gene is often used for the study of autotrophic microbial communities as the *cbbL* gene is characteristic amongst autotrophic microbial communities (Yousof et al., 2012). The chemo- and photolithotrophic bacteria are obligate autotrophs that are entirely reliant on CO_2 as a sole source of carbon for growth, whereas facultative autotrophic bacteria are more robust and are able to use a variety of organic substrates as alternative energy sources (Shively et al., 1998; Kusian and Bowien, 1997). Agricultural and forest soils have been shown to harbour large populations of previously unidentified autotrophic microorganisms including *Burkholderia*, *Bradyrhizobium* and *Nitrospira sp.* (Selesi et al., 2005; Tolli and King, 2005). Characterisation of microbial species in the present study that were dominant during the global change treatments by Denaturing Gradient Gel Electrophoresis (DGGE) revealed the presence of autotrophic *Burkholderia*, *Nitrospira* and 3 methanotrophic bacteria (Unpublished data). *Burkholderia sp.* in particular, are known to harbour red-like *cbbL* genes, while *Nitrospira* contain green-like *cbbL* genes (Selesi et al., 2007). Recently, Rasigraf et al. (2014) and Sharp et al. (2012) discovered two methanotrophic bacteria capable of autotrophic CO_2 fixation using the Calvin-Benson-Bassham cycle (CBB), while Hu et al. (2008) and Khadem et al. (2011) reported methanotrophic bacteria whose genomes contain RuBisCo genes and all other genes necessary to complete the CBB cycle.

In a clone library sequencing approach, facultative autotrophic bacteria and not obligate autrotrophic bacteria appeared more dominant (Yuan et al., 2012a). These researchers also proposed that lower *cbbL* gene copy numbers in soils with lower SOC was due to limited carbon resources available for the growth of the facultative autotrophic microbial population. This is in contrast to the observation in the present study where changes in SOC values did not necessarily reflect a corresponding change in *cbbL* gene copy number across each season. Soil microbial growth is strictly controlled by the quality and quantity of organic substrates that enter the soil ecosystem (Zak et al., 2004), and considering that *cbbL* gene copy numbers were not affected by SOC concentrations, it can be assumed, that the facultative autotrophs were dominant in the soil samples tested. This can be further confirmed by the fact that the probe used in this study targeted type 1C RuBisCo- containing organisms that generally

engage in a facultative autotrophic lifestyle. Additionally, facultative autotrophic microorganisms are able to switch their metabolism between the CBB cycle for CO_2 fixation and other external organic substrates as carbon sources (Badger and Bek, 2008). The nutritional versatility of the facultative autotrophic microbes allows them to also grow mixotrophically where they are able to exploit various inorganic and organic substrates for growth simultaneously (Bowien and Kusian, 2002). This would also explain the changes observed by day 25 in plots that received elevated CO_2 as it has been suggested that these organisms have evolved adaptation to environments with medium to high CO_2 levels (Bowien and Kusian, 2002). Furthermore, lower SOC values measured over time during the summer did not result in lower *cbbL* gene copy numbers as well, strengthening the hypothesis that facultative rather than obligate autotrophs were dominant in this study

Climate change studies investigating single factors or drivers can be extremely beneficial and are able to provide meaningful information regarding microbial responses to global change. However it has become crucial to determine microbial activities within the context of multiple interactive global change factors together with variations in annual weather conditions (Carmona-Moreno et al., 2005; IPCC, 2007, Gutknecht et al., 2010). Despite the fact that a combination of global change drivers can result in developments that could reduce, amplify or offset climate change effects, surprisingly little information is available regarding the effects of multiple factor experiments on the soil microbial community (Bardgett et al., 2008) or natural ecosystems (Zavaleta et al., 2003). Interestingly, plots that were supplied with a mixture of elevated CO_2, CH_4 and rainfall did not show much variation in *cbbL* gene copy numbers during spring and summer at elevated temperature compared to the control plot, suggesting that the individual effects of the global change drivers probably neutralized each other when applied in combination. A different trend was apparent at ambient temperature incubations for both seasons where substantially lower *cbbL* gene copy numbers were detected in the multifactor plots compared to control plots. This seasonal and temperature effect further emphasizes the need for more studies to better understand changes in microbial activity across different seasons in combination with various global change drivers.

Generally, lower *cbbL* gene copy numbers were discovered during the summer period at elevated temperature with a net decrease in values observed at day 25, which was dissimilar to the trend observed during the spring season. There can be several reasons for this observation: Firstly, change in substrates (i.e. SOC) and environmental conditions can drastically alter microbial physiology, especially if the autotrophic microorganisms are dominant, since an autotrophic lifestyle is considered to be energetically expensive and is under strict metabolic control (Adhikari and Kallmeyer, 2010). These species are also known to display a much greater flexibility in response to varied changes in substrates in fluctuating environments, thus ensuring their survival and enabling them to obtain a selective advantage (Kuenen, and Beudeker, 1982). Furthermore, there is clear evidence to support the fact that autotroph can be maintained even at reduced levels especially in the midst of fluctuating nutrient supplies (Gottschal et al., 1979; Smith and Kelly, 1979; Gottschal et al., 1981; Kuenen and Beudeker, 1982). This will possibly explain the lower *cbbL* gene copy values obtained at day 25 during summer and a substantial increase in copy number by day 60 even though the SOC contents were much lower. Secondly, adaptation mechanisms of the organisms harbouring the *cbbL* gene must also be taken into account. With regard to changing environmental conditions (especially increased temperatures) and photosynthesis, two different responses can be anticipated: a) steady and perhaps increased photosynthetic capacity in warmer growth conditions due to acclimation of photosynthesis apparatus after exposure to new thermal regimes, or b) natural selection and adaptation of selected populations due to long term exposure to higher temperatures (Sage et al., 2008). The red-type form I RubisCo genes such as those in *Ralstonia eutropha* are associated with a *cbbX* gene that is adjacent to the *cbbL* genes (Watson and Tabita, 1997). While the exact function of *cbbX* is unknown, it appears to be involved in the autotrophic growth of microorganisms since a loss of autotrophy was observed when *cbbX* was knocked out (Bowien and Kusian, 2002). Furthermore, it appears as if only photoautotrophic growth was inhibited in *cbbX*-deficient mutants. Thus, changes in gene copy numbers at the beginning of summer where sunlight conditions are optimal further supports bacterial autotrophy.

The transcriptional (Bradley and Gatenby, 1985) and translational (Gatenby et al., 1989) recognition sequences of many chloroplast genes present in plants including *rBCL* (analogous to bacterial *cbbL* genes) share several similarities with those found in prokaryotes, therefore chloroplast genes are easily expressed in *E. coli*. (Gatenby et al.,1981). With regards to *rBCL* genes in plants, Nie et al. (1995) observed a repression of genes involved in photosynthesis in wheat grown in the field exposed to elevated CO_2 concentrations, while similar results have been observed at high temperature and high CO_2 concentrations in cotton and tobacco leaves (Crafts-Brandner and Salvucci,2000). It is likely that similar mechanisms that regulate *rBCL* genes could regulate production of bacterial *cbbL* gene under various climatic conditions, but this is yet to be elucidated.

The *cbbL* genes were suggested to have a significant role in nutrient turnover and carbon sequestration in saline-alkaline soils (Keshri et al. 2013) and paddy soils (Xiao et al., 2014). Nutrient availability and enzyme activity have been shown to be closely linked to soil pH (Weber et al. 2010). Similarly several soil nutrients positively correlated with pH during both seasons in this study. CO_2 solubility and ionization in the soil solution is directly affected by pH, which can also affect autotrophic bacteria by interfering with the CO_2 concentrating mechanism (Hopkinson et al., 2011). Xiao et al. (2014) showed that soil properties such as pH, C/N ratio and CEC affected *cbbL* gene abundance and diversity, while Yuan et al. (2012 b) concluded that the *cbbL* – containing bacterial community were strongly influenced by both pH and SOC. In the present study, SOC correlated with soil pH for both seasons except during summer at elevated temperature, suggesting that the higher temperatures observed affected the SOC and pH values. SOC did not correlate with *cbbL* gene copy number for either season or temperature in contrast to several other studies (Yuan et al., 2012a; Yuan et al., 2012b; Ge et al., 2013; Xiao et al., 2014). However, soil pH correlated with *cbbL* gene copy number at ambient temperature conditions during summer, indicating that temperature and soil moisture can also be linked to changes in the *cbbL* gene bearing communities that are capable of carboxylation. Furthermore, several other soil nutrients also positively correlated with *cbbL* gene copy number during summer at ambient temperature which exhibited lower moisture conditions.

In conclusion, although a direct correlation between SOC and *cbbL* gene copy numbers was not observed in this study, seasonal changes in temperature and temperature increases as a result of global warming will have a profound effect on the number of bacterial cells that are able to participate in CO_2 fixation. In addition, the presence of certain global change drivers can also enhance or diminish the capacity of these cells to be involved in biogeochemical cycles. While elevated CO_2 is expected to enhance plant productivity in many regions in the world, higher temperatures and increasingly dry conditions will decrease vegetation cover. This effect is predicted to be significant in a variety of locations including the tropics (Jones et al. 2005) with the consequence that vegetation may not be able to fix CO_2 as effectively in the future. Thus it is pertinent to investigate soil microbial autotrophic processes together with their carbon assimilation properties and mechanisms. The activities of soil microorganisms involved in carbon cycling are affected by soil type, management practices and environmental conditions (Ge et al. 2013) and implementation of the proper agronomic measures will promote the growth of autotrophic microorganisms (Xiao et al.2014) thereby improving their carbon sequestration properties. Considering that the microbial community is such an integral component of soil, it is vital to understand their metabolic contributions especially if soil is to be considered as a viable mitigation option for the sequestration of CO_2 *in lieu* of rapid and on-going global climate change.

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CHAPTER FIVE

SEASONAL CHANGES IN SOIL BACTERIAL COMMUNITY AT ELEVATED GREENHOUSE GASES, RAINFALL AND TEMPERATURE

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SEASONAL CHANGES IN SOIL BACTERIAL COMMUNITY AT ELEVATED GREENHOUSE GASES, RAINFALL AND TEMPERATURE

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Abstract

The activity and diversity of soil microbial communities are imperative towards understanding terrestrial carbon cycle feedbacks. However, information regarding changes that may occur in microbial community structure and diversity owing to climate change is lacking due to various methodological constraints. Thus, the main objective of the study is to investigate possible changes to microbial community structure in soil exposed to either single or multiple climate change factors under elevated or ambient temperature incubations during spring and summer seasons. This was accomplished by the addition of carbon dioxide (CO_2), methane (CH_4) or rainfall to soil over a 60 day period using Screen Aided Carbon Dioxide Control experiments. DGGE profiles revealed seasonal variability changes in microbial diversity observed during spring. Soil moisture was a key factor in determining microbial responses during both seasons, with the elevated rainfall treatments able to counteract the adverse effects of elevated temperature during the spring season with communities in these plots appearing more robust. Increased temperatures and lower soil moisture during the summer period had a negative effect on microbial diversity; however sequence analysis of excised bands revealed the dominance of thermotolerant bacterial species. A combination of all the global change factors did not induce substantial change in community structure during spring at both temperature regimens. During summer at elevated temperature, growth of certain microbial species were inhibited by a combination of all the global change factors, highlighting the interactive effect between temperature, greenhouse gases and soil moisture. Furthermore, the loss of methanotrophic bacteria, (*Methylosinus* and *Methylocystis)* during both seasons can negatively impact greenhouse gas flux and consequently the carbon cycle at large. An in depth understanding of factors that can lead to changes in microbial community structure which influence nutrient and greenhouse gas cycling is essential towards developing carbon sequestration approaches which enhance current climate change mitigation strategies.

KEYWORDS: Climate change; Elevated greenhouse gases; Denaturing Gradient Gel Electrophoresis; Soil microbial community.

1. INTRODUCTION

Since the birth of the industrial revolution approximately 150 years ago, anthropogenic activities have drastically increased atmospheric carbon dioxide (CO_2) concentrations. This

has significantly contributed towards increased global temperatures and the related issue of climate change (IPCC, 2001), which is arguably one of the most urgent environmental threats. Global climate change will affect almost every facet of human society and economic, health, safety and food production are immediate concerns worldwide (Sutherst, 2004). The impact of climate-system feedbacks and environmental effects are increasing, with potentially irreversible phenomenon such as melting of the Greenland and west Antarctic ice sheets as well as accelerated global warming due to carbon cycle interactions observed (Haines and Patz, 2004, VijayaVenkataRamana et al., 2012).

Terrestrial ecosystems in particular, have a significant role in climate-system feedbacks and have been implicated as major global carbon sinks. Due to the massive quantities of organic carbon contained in Earths soils, a comprehensive understanding of the factors that mediate soil organic carbon transformations is imperative (Schimel et al., 1994; Billings and Ballantyne IV, 2013). Soil microorganisms are the living constituents of soil organic matter (Wang et al., 2001) and mediate a variety of ecosystem functions via their essential roles in soil nutrient cycling (Griffiths et al., 2003). The impacts of climate change on soil microbial communities could modify terrestrial ecosystem structure and biogeochemical cycles (Cruz-Martinez et al., 2009) since microbial community composition and diversity influence various soil processes (Griffiths et al., 2001).

Due to the quick generation times and rapid growth potential of microorganisms under favourable conditions, studies involving soil microbial community offer a useful and reliable opportunity to determine the effects of changing environmental conditions on terrestrial ecosystems (Prosser et al., 2007; Wolters et al., 2000). Despite this fact, investigations pertaining to soil biodiversity and their respective functions in terrestrial ecosystems are rare compared to similar studies for above ground organisms (Maron et al., 2011). Studies regarding the soil microbial community are frequently hampered by difficulties encountered

when attempting to culture and characterise these organisms (Kirk et al., 2004), mainly because only approximately 1% of the soil environmental bacterial population can be cultured using standard laboratory media. Furthermore, it has been speculated that this 1% may not be an accurate representation of the bacterial population present (Torsvik et al., 1998). Recently, however, microbial diversity studies have benefitted from a wide range of molecular biology tools that have enabled the examination of bacterial communities in environmental samples (Maron et al., 2011). These culture-independent techniques are based on extraction and analysis of nucleic acids directly from environmental samples (Zhang and Xu, 2008) and it has been suggested that more definitive information on natural communities can be obtained utilising such DNA based approaches (Yan et al., 2007).

In particular, the Denaturing Gradient Gel Electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments has proven to be effective in monitoring changes in microbial community structure (Zhao et al., 2008) and is presumably the most widely used method for the comparison and typing of microbial communities (Valášková and Baldrian, 2009) especially in environmental samples. The technique is based on the electrophoretic separation of double stranded DNA molecules in polyacrylamide gels that are composed of a linear denaturant of chemicals (formamide and urea). Thus, separation is achieved based on the melting behaviour of the DNA molecules (Zhang and Xu, 2008; Valášková and Baldrian, 2009). It is an easy, inexpensive and reliable method for studying microbial community diversity (Yan et al., 2007) and has been applied to characterise microbial communities from diverse environments including hydrothermal vents, hot springs, activated sludge, phyllosphere, biodegraded wall paintings, and soil (Heuer et al., 2001). Another inherent advantage of this technique is the ability to identify the bands within the gel by extraction and

sequence analysis or hybridisation with specific probes (Muyzer and Smalla, 1998; Zhao et al., 2008).

Temperature, soil moisture and nutrient availability are subject to intense variations as climate change progresses (IPCC, 2007). The combination of these factors will undoubtedly impact the soil ecosystem and hence soil microbial community structure, which can also have consequences for carbon cycle dynamics (Guenet et al., 2012). Despite the knowledge that ecosystem function can be impeded by microbial community composition (Carney et al., 2004; Hawkes *et al*., 2005), the response of soil microbial communities to anticipated global change is still poorly understood (Frey et al., 2008; Balser and Wixon, 2009; Allison et al., 2010). Recently, Guenet et al. (2012) found no significant change in soil bacterial DGGE profiles after a long term (10 years) experiment investigating the effects of CO_2 and moisture. These authors concluded that shifts in the soil microbial community do not always reflect corresponding changes in the soil carbon cycle. However, Singh et al. (2010) suggests that the interactive relationship between global change factors *viz*. changes in temperature, CO_2, and precipitation will affect the soil processes and will vary according to microbial community response. The interactive effects of these global change factors on soil microbial community dynamics is still debatable and it is not yet known if a combination of global change factors will exert an antagonistic or synergistic effect on microbial community composition. Therefore, the objective of this study was to investigate the effects of climate change factors viz. temperature, CO_2, methane (CH_4) and rainfall on soil microbial community diversity as well as identify the dominant surviving bacterial species.

2. MATERIALS AND METHODS

2.1 Site description and experimental design

Sampling below the zone of short term agricultural influence with sampling depth of up to 60cm for perennial vegetation has been suggested (Carter and Gregorich, 2008), thus sandy loam soil from the top 0-50 cm was collected from an area adjacent to the University of KwaZulu Natal (Westville) which is situated within an environmental conservancy. The area is dominated by flowering plants such as *Tecomaria capensis, Strelitzia reginae, Plumbago auriculata and Aloe succotrina* ; while alien invasive species such as *Leucaena leucocephala, Lantana camara, Schinus terebinthifolius, Chromolaena odorata and Syringa vulgaris* are also frequently encountered (http://conservancy.ukzn.ac.za/IndigenousPlanting.aspx). The climate change experiments were started within 4 days of soil collection due to the complex experimental setup. Soil was stored at ambient environmental temperature (identical to the area from which soil was collected) in polyethylene bags that had holes to allow for aeration up until it was used for the experiments. The individual and/or interactive effects of elevated carbon dioxide (CO_2), methane (CH_4), precipitation and temperature were investigated using constructed modified Screen-Aided-Carbon-Dioxide-Control (Leadley et al. 1997) in two separate greenhouses (E and A) situated at the University of KwaZulu-Natal (Westville). Screen Aided Carbon Dioxide Control is recommended as the most suitable method for determining the effects of elevated CO_2 levels for short stature vegetation and is useful as it combines the advantages of Open Top Chambers and Free Air Carbon Dioxide Enrichment experiments (Leadley et al., 1997; Macháčová, 2010). Briefly, 1 m x 0.5 m polycarbonate screens were attached to stainless steel tables in the greenhouses and were arranged to achieve 1m x $1m^2$ plots within which the climate change simulations were conducted (Fig 1). The base of each plot was then lined with gas tubing (Afrox, South Africa) which had 10 mm holes drilled every 10 cm apart and a depth of 1.5cm.

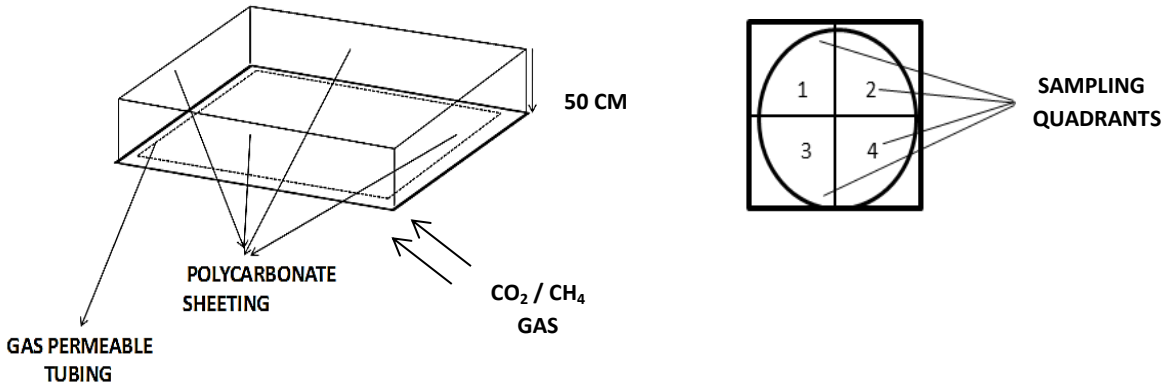

Fig 1: Experimental set-up used for investigating the effects of global change drivers. A – illustrates the 3D view of individual experimental plots covered at sides and base with polycarbonate sheeting, while B illustrates the top view with 4 sampling quadrants.

Sandy loam soil (25 kg) was then added to all the plots within the greenhouses. Greenhouse A corresponded to ambient environmental temperature, while Greenhouse E had temperatures approximately 5 – 10 ˚C higher than Greenhouse A (sunlight and humidity dependent). These experiments were conducted during spring and summer (September 2011-March 2012). Simulations of elevated greenhouse gas conditions was done once daily addition of 425 ppm CO_2 and 2.6 ppm CH_4 gas (Afrox, South Africa) to the soil at a flow rate of 10 litres per min for 5 min. The CO_2 and CH_4 concentrations used represent atmospheric concentrations plus a 10% increment (IPCC, 2001). Changes in rainfall were simulated weekly by addition of synthetic groundwater (Klier et al. 1999), based on the rainfall recorded for the relevant period in the previous year as per data from the South African Weather Service (72.6 and 28.4 mm for spring and summer, respectively) and increased by 10%. The effects of the following global change drivers were investigated: Carbon dioxide (CO_2); carbon dioxide and rainfall (CO_2R); carbon dioxide and methane (CO_2CH_4); carbon dioxide, methane and rainfall (CO_2CH_4R) and rainfall (R).Control plots that did not receive

any treatment were also set up in both greenhouses (CTRL). At each sampling time, each experimental plot was divided into four quadrants and four soil samples were collected from each quadrant. Subsequently, these four samples from each quadrant were then homogenised. A single homogenised sample was then used from each quadrant (n = 4) to provide 4 replicates per plot.

2.2 DNA Isolation

DNA was extracted from the soil samples collected from each experimental plot at days 0, 25 and 60 using the UltraClean Soil DNA Kit (MOBIO Laboratories Inc, California) according to the manufacturer's instructions. DNA purity and concentrations were determined spectrophotometrically using the NanoDrop 2000C Spectrophotometer (ThermoScientific).

2.3 Polymerase Chain Reaction (PCR)

16S rRNA gene was amplified from the soil DNA using the primer pair 341F-GC and 907R (Zhao et al., 2008). PCR reactions were conducted in a Bio-Rad T100 thermal cycler using a touchdown protocol and according to the following conditions: initial denaturation of 94 °C for 5 min, 10 cycles of 94 °C for 1 min, 65 °C for 1 min (decreasing by a degree each cycle) and 72 °C for 3 min, followed by 20 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min and a final elongation of 72 °C for 5 min. Each 50 µl reaction contained 0.5 µM of each primer, 200 µM dNTPs, 1 mM $MgCl_2$, 10 X Buffer, *Taq* polymerase (1.25 U), double distilled water and 2 µl of diluted template DNA. PCR amplicons were confirmed on a 2% agarose gel at 120 V for 60 min followed by ethidium bromide (1mg/ ml) staining.

2.4 Denaturing Gradient Gel Electrophoresis (DGGE)

PCR products were loaded onto 6% acrylamide gels composed of 40-70% denaturing urea-formamide gradient (100% denaturant = 7M urea and 40% formamide). Electrophoresis was performed using the D CODE Universal Mutation Detection System (Bio-Rad) at 50 V for 17 hr and maintained at 60 ºC in 1X TAE buffer. Subsequently, gels were stained in ethidium bromide (1mg/ml) for 30 min, destained in 1X TAE for 10 min and viewed with the GENE Genius Bio Imaging System (SYNGENE, UK). Bands of interest were excised with a sterile scalpel, suspended in 100 µl MilliQ water and allowed to elute overnight at 4 ºC. The samples were then centrifuged for 15 min at 13 000 rpm and the supernatant was used as a template for a further round of PCR as described above using the same primer set without the GC clamp. These PCR products were purified with the QIAquick PCR purification kit (Pretoria, USA) and sequenced by Inqaba Biotech (Gauteng, South Africa). Sequences were edited using Chromas Lite Version 2.01 and BioEdit Sequence Alignment Editor Version 7.0.9.0. BLAST search was then conducted to determine the identities of extracted bands.

2.5 DGGE Analysis

DGGE gel analysis was performed using BioNumerics Software Version 6.6 (Applied Maths, Belgium). All bands (strong and weak) were included in the band analysis and a background subtraction of 20% was applied. Cluster analysis was conducted by constructing a dendogram using Unweighted Pair Group for Mathematical Averages (UPGMA). Day 0 samples were run on each gel and used as a reference lane.

2.6 Statistical Analysis

Dice similarity coefficients were calculated using BioNumerics Software Version 6.6 (Applied Maths, Belgium).

3. RESULTS

3.1 Effects of global change factors on DGGE profiles during spring

All DGGE gel patterns were compared after normalization. Genetic fingerprints of soil samples during spring revealed the presence of 14 bands observed at day 0 (Fig 2). During spring, bands 1 and 2 were common to all treatments for both temperature incubations and across the sampling period. Under elevated temperature conditions (Fig. 2), plots with the addition of CO_2, CO_2R, CH_4, CO_2CH_4 and CO_2CH_4R (Fig 2a, b ,c ,e and f, respectively) were characterized by decreased intensity of most of the prominent bands (bands 1, 2, 3, 4, 5, 6 and 9) by day 25. Furthermore, a loss of band 2 was also evident either by day 25 or day 60 in all plots that received CO_2. A reduction in band intensity was observed for band 4 in the CH_4R treatment after 25 days, and after 60 days in the CO_2CH_4 treated plot. Bands that appeared in the samples which received elevated rainfall (Fig. 2 g) and the control soil sample (Fig. 2 h) were similar between days 25 and 60 with greater intensity observed for most bands, indicating the negative effect of the gas enrichments on certain microbial groups.

Overall, a reduction in band brightness accompanied by decreased diversity was observed for DGGE profiles of soils exposed to the climate change factors incubated under ambient temperature (Fig 3). Band 1 was present at all sampling times; however decreased intensity of this band was evident for most treatments. Band 1 was most negatively affected in CO_2 treated plots, with a decrease in intensity observed from day 0 to day 60 (Fig 3 a, b and e). Band 2 appeared prominently in the control, R and CO_2CH_4R treated soil samples (Fig 3 f, g and h) and did not change substantially from day 0 to day 60. Ambient temperature incubations also lead to the loss of band 4 in all treatments by day 60. Bands 10, 11, 12, 13 and 14 were not discernible in the CO_2CH_4R, R or control plots inferring that soils exposed to

CO_2, CH_4, CO_2R, CH_4R or CO_2CH_4 may have resulted in conditions that favoured the proliferation of certain microbial species.

Generally it was observed that Day 25 and Day 60 samples for most plots clustered together, under the respective temperature regimen investigated (Fig. 4). Several similarities were obtained between the various treatments, as illustrated by several small clusters; irrespective of the incubation temperature conditions. This is evident in plots that received a combination of CO_2CH_4 under elevated or ambient temperature incubation at day 25; which appeared in the same clade; however DGGE banding patterns of these plots were only 26.67% similar. By day 60, plots treated with CO_2CH_4R under elevated and ambient temperature showed 72.73% and 81.49% similarity, respectively to profiles of the control plots (Table 2) and were grouped together in an individual node corresponding to ambient temperature. The effects of temperature were further emphasized on comparison of similarity between the CO_2 and CO_2R treatments at elevated (63.64%) and ambient (37.04%) temperature regimens.

3.2 Effects of global change factors on DGGE profiles during summer

Bacterial diversity was negatively affected by elevated temperature during the summer (Fig 5) indicated by the reduction in the number (11) and intensity of bands present compared to day 0. Bands 15 and 17 were featured strongly in the DGGE profiles of the CO_2, CO_2R, CH_4 and CH_4R treated plots (Fig 5a, b, c and d) from day 0 to day 60. A significant loss of bands from day 25 to day 60 was noted in the CO_2CH_4, CO_2CH4_R and R treated plots (Fig 5e, f and g) with bands 15, 16, 17, 18, 20 and 21 almost completely indistinguishable. The considerable loss of bands and lowered band intensity apparent in the plots that received R highlights the effects of elevated moisture under higher temperatures on bacterial community structure. The species represented by band 23 was robust and appeared in all DGGE profiles

of soils incubated under elevated temperature. The control plot soil did not show much difference in banding patterns from day 25 to day 60, suggesting clearly showing that the higher temperature lead to changes in bacterial diversity.

Compared to elevated temperatures, bands produced from DGGE profiles of soils incubated under ambient temperatures were brighter and more prominent (Fig 6). However, CO_2 and CO_2R treated (Fig 6a and b) plots negatively impacted soil bacterial communities with a considerable reduction in the presence of bands and band intensity, with bands 15, 19, 22, 23, 24 and 29 only appearing by day 25 and removed by day 60. Band 2 was prominent at day 25 and day 60 under ambient temperature in the plots that were treated with CH_4 and CO_2CH_4. Band 19 also featured strongly in the all the profiles obtained from the various global change treatments. Under ambient temperature incubations, a combination of factors, CO_2CH_4R (Fig 6f) did not affect bacterial diversity profiles as strongly compared to each factor individually with 10 bands present by day 60, compared to day 0. Just as with the elevated temperature during summer, profiles from the control plot (Fig 6h) did not show much variation from day 25 to day 60 and had similar profile as the plots that received the rainfall treatment.

A slightly different trend for relationships amongst the treatments was observed during summer. Overall, 4 distinct branches are apparent; two in each temperature regimen were noted (Fig. 7). These clusters indicated strong associations with the same treatments for days 25 and 60. Under ambient temperatures, analogous to the spring season, the control and elevated rainfall plots (Table 3) shared a 68.75% similarity by day 60. However at elevated temperatures, the addition of rainfall was only 23.08% similar to the profile obtained under ambient temperature magnifying the negative interactive effect of elevated temperature and rainfall on microbial community structure. In contrast to the spring season, the CO_2CH_4R

treated plots and the control displayed lower similarity values of 31.58% and 32.43% for elevated and ambient temperature, respectively.

3.3 Sequence identity of extracted bands

A total of 27 bands were extracted for sequencing. While PCR reamplification of samples always yielded a single band, only 11 samples were sequenced successfully. These are represented in Table 1 and correspond to positions indicated in Figs 1, 2, 4 and 5. Bands 2 and 15; 4 and 16; 8 and 19 and 10 and 22 corresponded to the same microbial species. Two methanotrophic bacteria *Methylosinus sp*. and *Methylocystis sp.* were present during the spring season, while thermotolerant strains of *Geothermobacterium* and *Sediminbacterium* were present during the summer period.

4. DISCUSSION

Although many climate change studies focus on aboveground ecosystems, investigating belowground ecosystems are important for linking changes in soil microbial community structure with ecosystem functions and soil processes (Kirk et al., 2004; Rinnan et al., 2007). The observed variation in bacterial DGGE profiles across the spring and summer seasons in this study can be attributed to the seasonal change which was accompanied by changes in temperature, humidity and precipitation. DGGE profiles during summer were characterised by fewer bands than that observed during spring for the same treatment. Changes in temperature, pH, moisture and nutrient levels will allow for the proliferation of communities or species better suited for a particular set of environmental conditions, which substantiates the observation of certain bands/species across the sampling periods. The seasonal change from spring to summer is also often accompanied by changes in the plant litter quality and thus; the type and quality of substrates entering the carbon pool for use by the bacterial

populations present (Zogg et al., 1997). Substrate availability is also a key driver of changes in bacterial community structure and can have significant effects on microbial community structure which will have consequences for soil nutrient recycling as well (Budge et al., 2011). Furthermore, the degree of variation of spatial and temporal relationships, genetics and interactions between microorganisms can also profoundly affect community dynamics and structure in soil bacterial populations (Nannipieri et al., 2003) further substantiating the changes that occurred across the seasons.

In the present study, a shift in bacterial community composition is especially visible during the summer period at elevated temperature where a drastic reduction in the number and intensity of bands was observed under these experimental conditions. Consequently, this period was also characterised by decreased dehydrogenase enzyme activity that is directly linked to microbial activity (Chapter Three). Microbial processes are influenced by soil temperature and it is likely that majority of the soil microorganisms present were not capable of maintaining growth under the higher temperature regimens. Mineralization in soil increases when soil temperature increases resulting in a net decrease in the soil organic carbon pool (Lal, 2004). Thus, higher temperatures or warming can increase soil respiration rates initially, due to the loss of labile carbon (Kirschbaum, 2004). This will result in metabolic change for certain community members that are not able to adapt physiologically to changes in the available substrate pool. These adjustments can also lead to instability in vital soil processes such as extracellular enzyme production and respiration (Allison and Treseder, 2011). In the present study extracellular enzyme production was also linked to soil nutrient status (Chapter Three) which can also be related to the decreased diversity observed during the summer.

Soil is one of the most predominant microbial habitats exhibiting an astounding genetic diversity (Nannipieri et al., 2003), which is characterised by either genetic or functional diversity (Torsvik et al., 1990). In the present study, it was evident that genetic diversity was affected to a larger degree than functional diversity. Firstly, measurement of *cbbL* gene copy number, a functional gene required for autotrophic CO_2 fixation showed higher copy numbers in response to the global change treatments (Chapter Four). Secondly, sequence analysis of excised bands revealed a decrease in certain dominant species that harbour functional genes such as methane monoxygenase (functional diversity) however, the overall reduction in the number of bands (genetic diversity) was greater by comparison. Soil microbial populations are known to respond sensitively to fluctuating environmental conditions and may develop mechanisms to ensure their survival such as evolution of proteins with varying thermal optima to ensure survival (Hochachka and Somero, 2002; Bradford et al., 2008). Sequence analysis of DGGE bands recovered from the summer experimental samples reveal 88% and 90% homology of two of the bands, to uncultured *Geothermobacterium* sp. (Kashefi et al., 2002) and uncultured *Sediminibacterium* sp. (Clingenpeel et al., 2011) previously located in Yellowstone National Park. Their presence during the course of the experiment is related to their capabilities to flourish under conditions of thermal stress. As a community, such adaptations can be linked to variations in the activities of individual species and dissemination of new genetic traits via horizontal gene transfer (Winding et al., 2005). Furthermore, increased reproduction of species that have abilities and characteristics that could prove more beneficial to the community at large is also favoured (Winding et al., 2005).

During spring and summer; plots that received a 10% increment in rainfall, showed banding patterns similar to the control, as indicated by the dendogram analysis grouping them in the same cluster. The structure and function of soil bacterial communities may be fundamentally

altered due to selective pressures such as osmotic stress and resource competition especially during periods of moisture limitation (Griffiths et al., 2003). It is possible that the higher amount of rainfall was not sufficient to limit to microbial growth, thus little change in bacterial community structure was noted compared to the control. These results are consistent with Cruz-Martinez et al. (2009) who reported that microbial communities did not change significantly after a 7 year treatment of elevated rainfall simulations in Californian grasslands. These authors also reported a change in community structure across seasons under this treatment and ascribed the change in community structure to changes in the above ground biomass. The profiles obtained for the incremental rainfall plot during summer at elevated temperature showed reduced microbial diversity with a lower banding pattern similarity compared to the control. The coastal region of Durban, KwaZulu-Natal can be characterised as a temperate region that usually experiences moderate rainfall and humid conditions notably during the summer season. In addition, the rainfall patterns simulated during the summer season were quantitatively lower than that simulated for the spring season. It is therefore possible that the additional rainfall simulated during summer was still not sufficient to counteract the higher temperatures and humidity levels that characterised the summer season.

Soil moisture is also involved in regulating the flux of greenhouse gases and this has been documented for CO_2 (Craine et al., 2001; Frank et al., 2002), CH_4 (Gulledge and Schimel, 1998) and nitrous oxide (Hutchinson et al., 1993). The mechanisms underlying these interactions have been attributed to modifications and shifts in the activity and diversity of bacterial populations (Griffiths et al., 2003). This regulatory effect is evident in plots during the spring season for both temperature regimens and during the summer for ambient temperature conditions where plots with elevated rainfall displayed higher levels of similarity

to the control plot than to plots that received gas enrichment or a combination of gas and rainfall. Therefore, soil bacterial communities experienced a negative effect at elevated greenhouse gas concentrations, especially without the effects of additional soil moisture. Furthermore, the loss of microbial species such as *Methylocystis*, *Methylosinus* and *Nitropsira* due to the seasonal temperature and moisture differences could significantly influence global biogeochemical cycling. *Methylocystis* sp. and *Methylosinus* are aerobic methanotrophic microorganism that are usually found in diverse environments and are distinguished by their remarkable metabolic potential. These organisms possess methane monooxygenase that enables them to use CH_4 (second most abundant greenhouse gas on Earth) or other reduced C_1 compounds as growth substrates. *Hypomicrobium* sp. is a Gram negative, facultative methylotroph that is able to utilise methanol which results from the conversion of methane (Urakami et al., 1995). As a result, these methano- and methylotrophs are vitally important in the global carbon cycle (Gulledge et al., 2001, McDonald et al., 2008; Op den Camp et al., 2009). *Nitrospira* is central to the global nitrogen cycle as it is an important nitrite oxidising bacterium, responsible for the conversion of nitrite to nitrate (Haaijer et al,. 2013). The loss of either of these species from soil could have significant deleterious effects on soil ecosystems, soil nutrient and the biogeochemical cycling of carbon and nitrogen.

It has been acknowledged that soil bacterial communities will experience both direct and indirect effects resulting from climate change (Freeman et al., 2004; Bardgett et al., 2008; Singh et al., 2010). Factors such as changes in greenhouse gas (CO_2 and CH_4) concentrations, temperature, soil moisture and pH will control the way these microorganisms are able to mediate various soil processes and their abilities to maintain a healthy soil ecosystem. It has been suggested that the effects of elevated CO_2 on soil microbes may be predominantly

indirect and will occur as a result of changes in plant growth and productivity together with associated changes in soil physiochemical properties (Bardgett et al., 2008). The bacterial inhibitory effect of elevated CO_2 during summer at higher temperatures can be explained by these indirect changes and changes in the decomposition of available litter. Guenet et al. (2012) did not find any significant change in soil bacterial DGGE fingerprints after gradually elevating CO_2 concentrations (440 ppm) over a period of 10 years. The drastic change in microbial diversity after 60 days in the present study can be linked to the interactive effects of the global change factors, specifically temperature.

A change in microbial community structure can also infer a change in microbial activities which are related to environmental factors like temperature and soil moisture. Temperature and soil moisture content are two of the primary factors that can directly affect soil microorganisms (Singh et al., 2010). Furthermore, these factors can affect greenhouse gas concentrations in two ways. Firstly, the structure and/or physiology of soil microbial populations may be altered, and in the case of higher temperatures could mean fluctuations in some processes eg. respiration, while the main control mechanisms remain unchanged. Alternatively, climate change may produce a shift in microbial community structure, with a different control mechanism that will result in a microbial community with altered physiological abilities. The presence of organisms such as *Blastochloris sulfoviridis* and *Methylocystis* sp. provides an indication of a shift in soil metabolic diversities that enable them to survive despite the environmental perturbations. *Blastochloris sulfoviridi*s (sequenced from plots that were enriched with CO_2) is a purple non sulphur bacterium capable of photoautotrophic CO_2 fixation via the Calvin Benson Basham Cycle. These organisms have been reported to contain the ribulose-1,5 biphosphate carboxylase/ oxygenase

enzyme, utilising CO_2 as a sole source of carbon for growth (Selesi et al., 2007; Tabita et al., 2007).

Interestingly, a combination of all the climate change factors did not result in much variation in community structure compared to the control plot. In fact, across both seasons and under both temperature regimens, these conditions resulted in the least variation in microbial community structure by day 60 compared to the corresponding plots with individual climate change factors. Seasonal differences especially those related to soil moisture and temperature can have important consequences for soil microbial community structure and diversity. Climate change has been shown to affect both soil microbial activity and community structure (Zogg et al., 1997; Rey and Jarvis, 2006; Curiel Yuste et al., 2007; Castro et al., 2010; Curiel Yuste et al., 2011). In this study, changes in temperature and moisture affected soil microbial enzyme activities and soil nutrient status which in turn affected soil microbial community composition.

Common DNA fingerprinting techniques such as TRFLP, DGGE or SSCP enables researchers to screen the relative abundances of sequences which are grouped into phylotypes (Ramette et al., 2007). Next generation sequencing (Pyrosequencing) has emerged as a powerful tool to study microbial diversity and has been used to investigate microbial communities across various biomes, land uses, soil horizons and forest successional stages. It is a high resolution technique that provides detailed analysis on the structure, diversity and taxonomic composition of various microbial communities (Bastida et al., 2013; Swanson and Sliwinski, 2013; Curiel-Yaste et al., 2014). The application of pyrosequencing in climate change studies will provide a wealth of useful information about the successional changes within the soil microbial community. As climate change continues to advance, many factors

will undoubtedly determine the rate at which microbial processes will also advance. It is encouraging to observe that despite environmental disturbances and a loss of certain species; microorganisms with functional capabilities are still able to thrive and presumably contribute towards sustaining the soil ecosystem. Certainly, the direct effects of temperature and soil moisture on soil microorganisms warrants further investigation especially if soil management practices and the process of soil carbon sequestration to combat further climate changes is to be a successful and valuable alternative.

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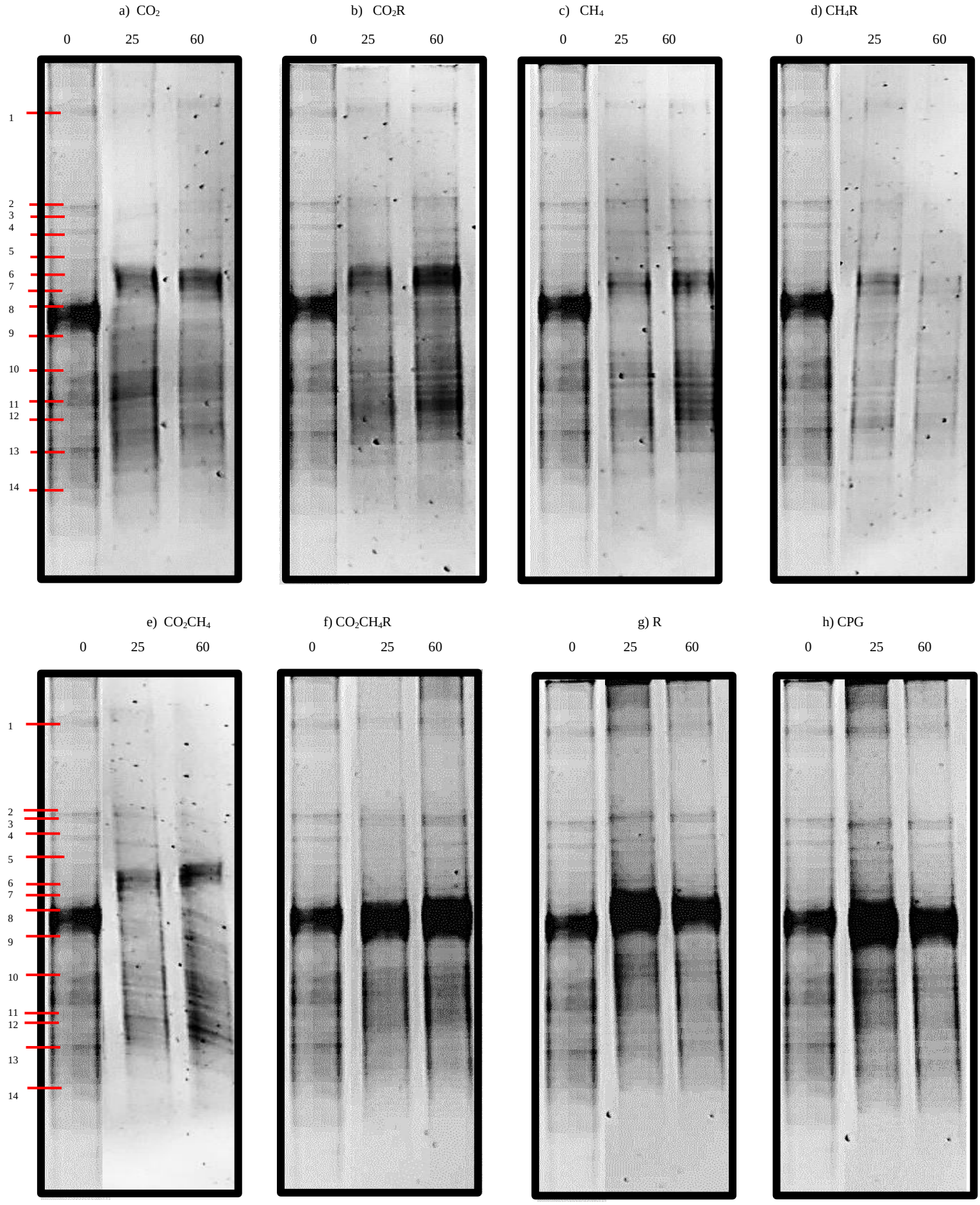

Fig 2: PCR-DGGE profiles of soil samples after exposure to various climate change simulations at day 0, day 25 and day 60 (L-R) during spring under elevated temperature conditions

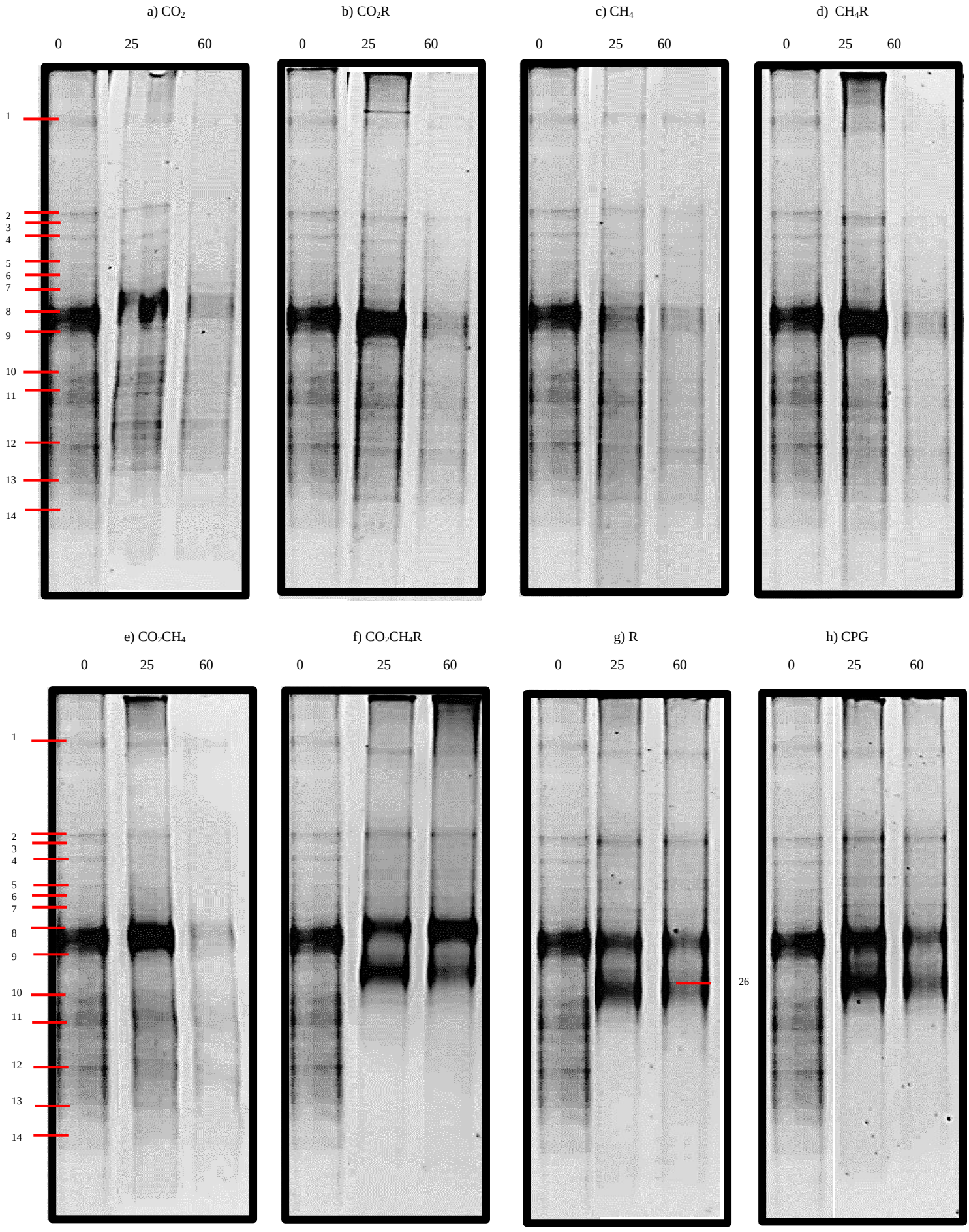

Fig 3: PCR-DGGE profiles of soil samples after exposure to various climate change simulations at day 0, day 25 and day 60 (L-R) during spring under ambient temperature conditions

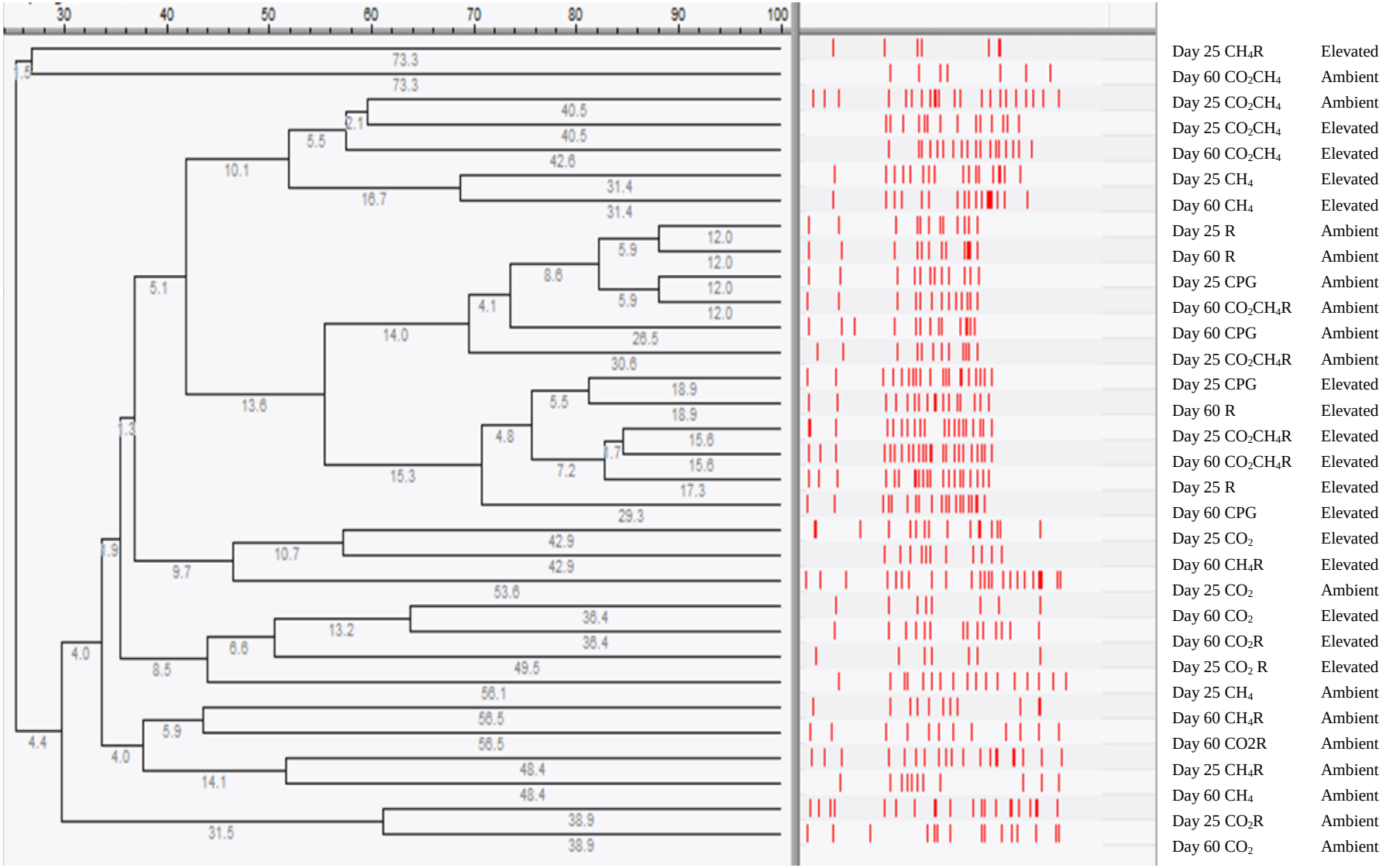

Fig 4: Cluster and band matching analysis of soil samples exposed to varying climate change simulations during spring

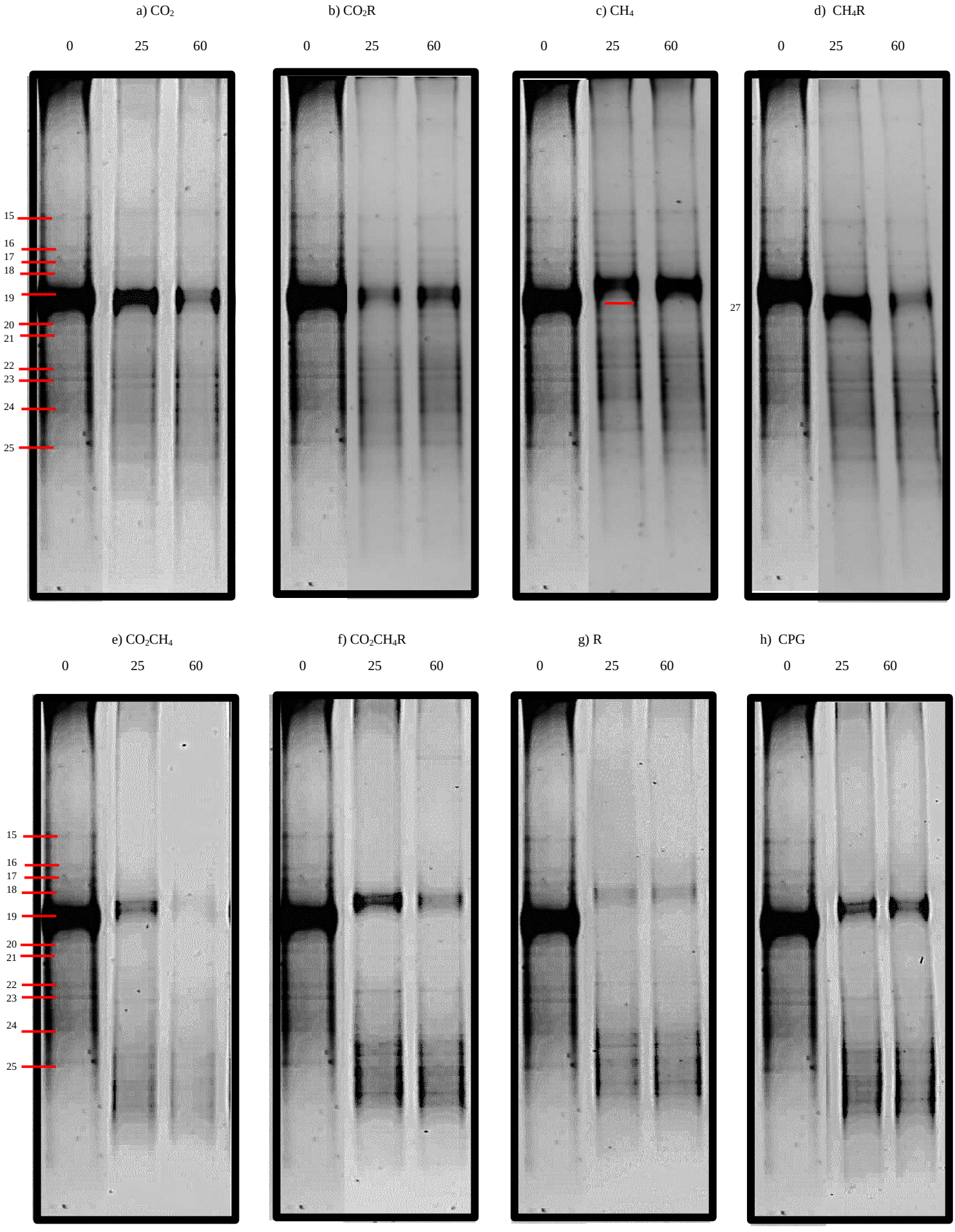

Fig 5: PCR-DGGE profiles of soil samples after exposure to various climate change simulations at day 0, day 25 and day 60 (L-R) during summer under elevated temperature conditions

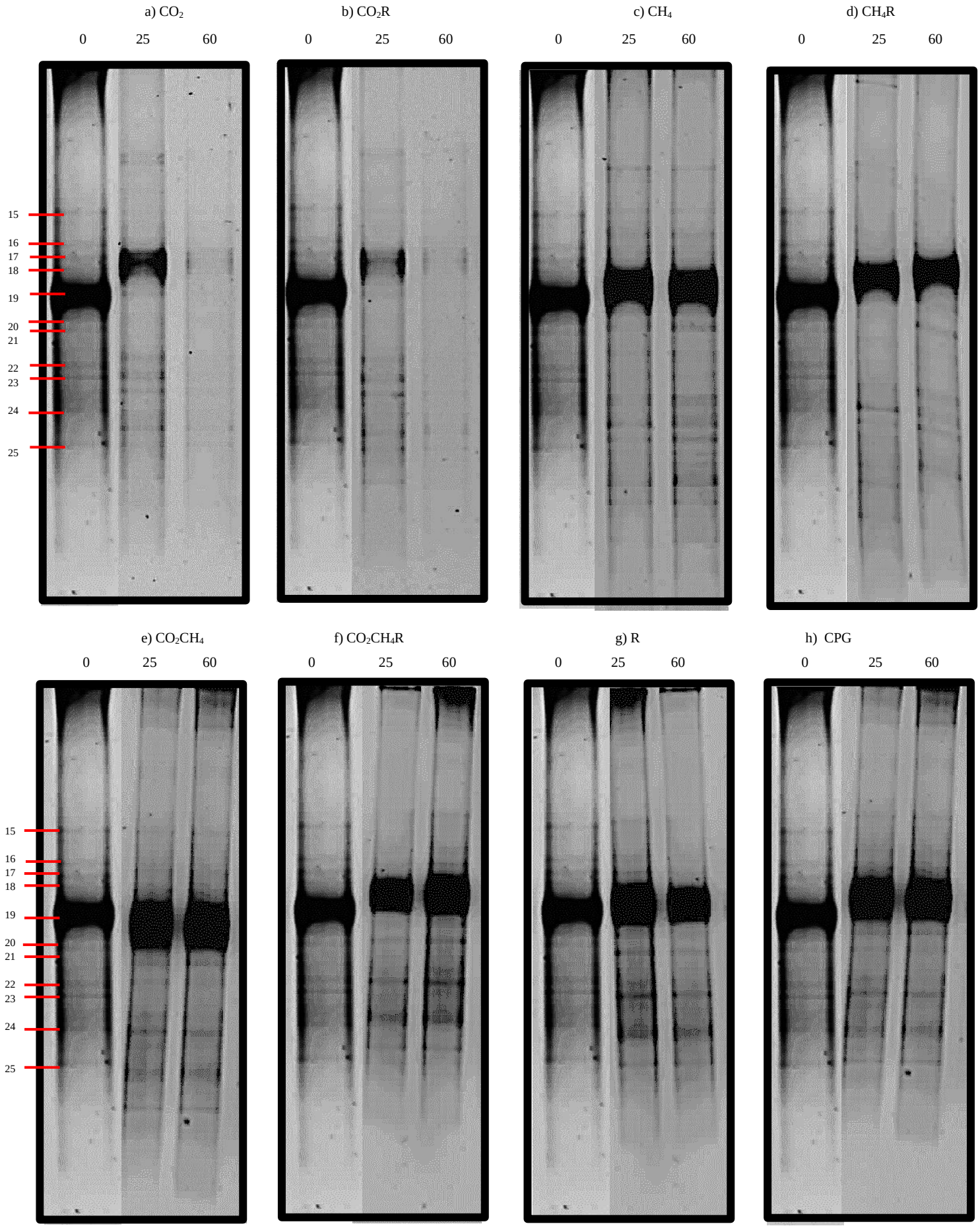

Fig 6: PCR-DGGE profiles of soil samples after exposure to various climate change simulations at day 0, day 25 and day 60 (L-R) during summer under ambient temperature conditions

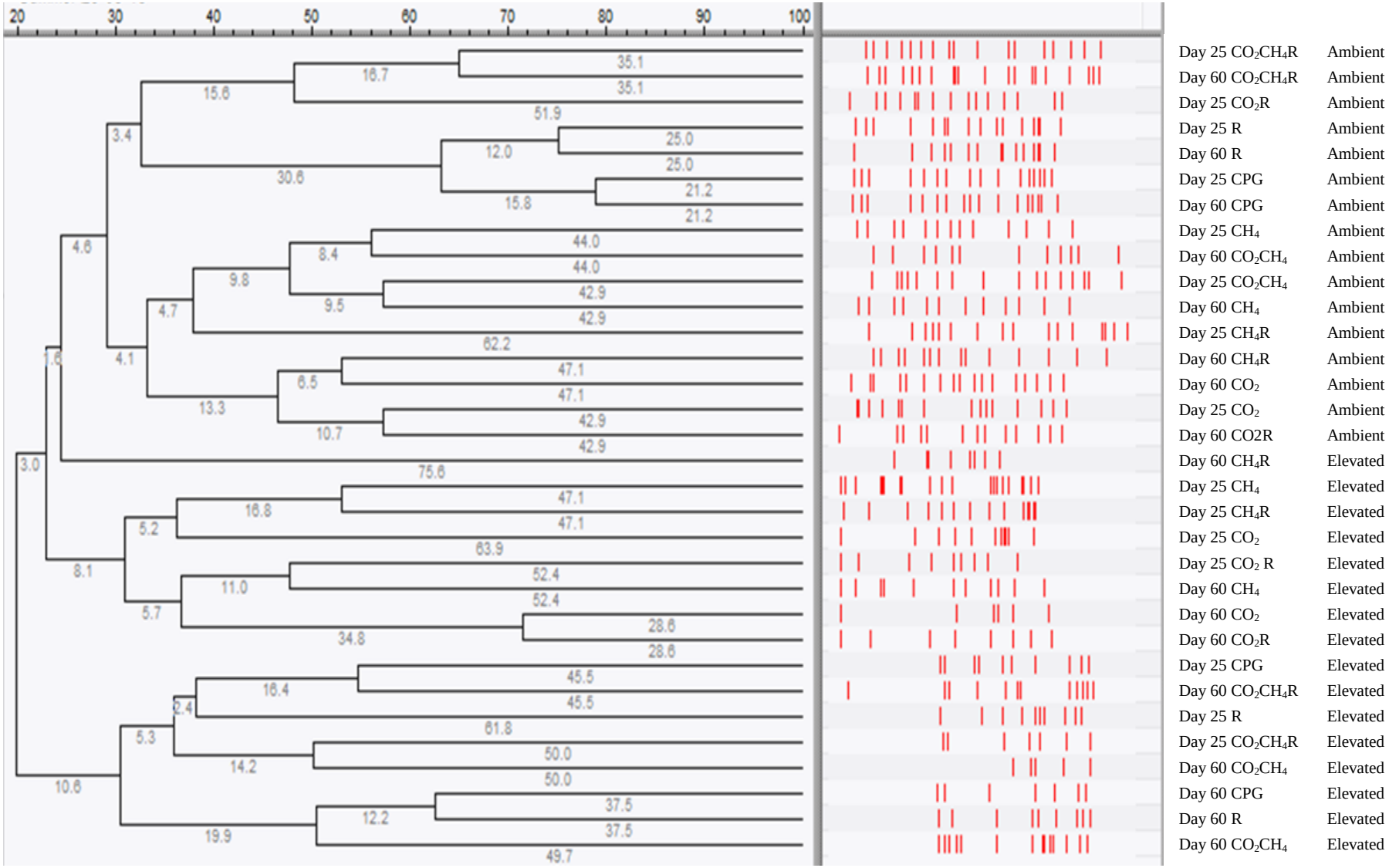

Fig 7 : Cluster and band matching analysis of soil samples exposed to varying climate change simulations during summer

Table 1: Dice coefficient similarity (%) analysis for comparison of climate change simulations effects in soil samples during spring under elevated (E) and ambient (A) conditions

	Day 25CH4R E	Day 60 CO2CH4 A	Day 25 CO2CH4 A	Day 25 CO2CH4 E	Day 60 C02CH4 E	Day 25 CH4 E	Day 60 CH4 E	Day 25 R A	Day 60 R A	Day 25 CPG A	Day 60 CO2CH4R A	Day 60 CPG A	Day 25 CO2CH4R A	Day 25 CPG E	Day 60 R E	Day 25 CO2CH4R E	Day 60 CO2CH4R E	Day 25 R E	Day 60 CPG E	Day 25 CO2 E	Day 60 CH4R E	Day 25 CO2 A	Day 60 CO2 E	Day 60 CO2R E	Day 25 CO2R E	Day 25 CH4 A	Day 60 CH4R A	Day 60 CO2R A	Day 25 CH4R A	Day 60 CH4 A	Day 25 CO2R A	Day 60 CO2 A
Day 25CH4R E	100																															
Day 60 CO2CH4 A	26.67	100.00																														
Day 25 CO2CH4 A	19.36	33.33	100.00																													
Day 25 CO2CH4 E	45.46	38.10	59.46	100.00																												
Day 60 C02CH4 E	30.77	32.01	58.54	56.25	100.00																											
Day 25 CH4 E	40.00	8.33	45.00	58.07	62.86	100.00																										
Day 60 CH4 E	46.15	24.00	39.03	56.25	50.00	68.57	100.00																									
Day 25 R A	20.00	21.05	28.57	38.47	53.33	41.38	46.67	100.00																								
Day 60 R A	19.05	30.00	16.67	29.63	45.17	40.00	38.71	88.00	100.00																							
Day 25 CPG A	20.00	31.58	28.57	30.77	46.67	41.38	33.33	83.33	80.01	100.0(																						
Day 60 CO2CH4R A	19.05	30.00	33.33	29.63	51.61	40.00	38.71	88.00	76.93	88.00	100.00																					
Day 60 CPG A	18.19	19.05	27.03	28.57	43.75	38.71	37.50	76.93	74.08	61.54	81.49	100.00																				
Day 25 CO2CH4R A	20.00	31.58	17.15	30.77	46.67	41.38	33.33	58.33	72.00	75.00	80.01	61.54	100.00																			
Day 25 CPG E	28.57	29.63	37.21	47.06	47.37	59.46	63.16	68.75	54.55	50.00	60.61	52.95	50.00	100.00																		
Day 60 R E	32.01	25.00	50.00	51.61	40.00	35.30	45.72	55.17	40.00	68.97	73.33	51.61	55.17	81.0	100.00																	
Day 25 CO2CH4R E	27.59	21.43	40.91	51.43	51.29	47.37	51.29	36.37	47.06	36.37	41.18	45.72	36.37	78.05	73.69	100.00																
Day 60 CO2CH4R E	31.25	25.81	42.55	57.90	47.62	58.54	61.91	50.00	54.06	55.56	59.46	52.63	50.00	81.82	73.17	84.45	100.00															
Day 25 R E	27.59	21.43	31.82	51.43	51.29	36.85	51.29	66.67	52.95	66.67	70.59	57.15	66.67	78.05	68.43	80.95	84.45	100.00														
Day 60 CPG E	28.57	29.63	37.21	35.30	42.11	43.25	42.11	62.50	60.61	56.25	66.67	64.71	56.25	80.01	59.46	73.17	72.73	68.29	100.00													
Day 25 CO2 E	32.01	25.00	40.00	32.26	40.00	58.83	34.29	27.59	33.33	34.49	33.33	19.36	34.49	43.25	29.41	36.85	48.78	36.85	32.43	100.00												
Day 60 CH4R E	42.11	33.33	35.30	48.00	34.49	42.86	48.28	26.09	33.33	26.09	25.00	24.00	17.39	51.61	50.00	50.00	57.15	43.75	38.71	57.15	100.00											
Day 25 CO2 A	18.19	25.00	50.00	35.90	32.56	47.62	41.87	32.43	31.58	27.03	26.32	15.39	27.03	35.56	42.86	39.13	44.90	39.13	35.56	42.86	50.00	100.00										
Day 60 CO2 E	25.00	40.00	25.81	36.37	38.47	48.00	30.77	30.00	28.57	30.00	28.57	18.19	30.00	28.57	32.01	34.49	37.50	27.59	35.72	56.01	31.58	24.25	100.00									
Day 60 CO2R E	27.27	19.05	27.03	42.86	50.00	64.52	50.00	38.47	37.04	38.47	37.04	35.72	38.47	47.06	25.81	45.72	47.37	34.29	52.95	51.61	32.01	35.90	63.64	100.00								
Day 25 CO2R E	13.33	0.00	13.33	19.05	32.01	33.33	24.00	31.58	40.00	42.11	40.00	28.57	63.16	22.23	8.33	7.15	19.36	35.72	22.23	50.00	33.33	25.00	53.33	47.62	100.00							
Day 25 CH4 A	16.00	33.33	45.00	32.26	51.43	35.30	45.72	34.49	40.00	27.59	46.67	38.71	34.49	54.06	23.53	42.11	43.91	36.85	37.84	35.30	14.29	38.10	32.01	58.07	41.67	100.00						
Day 60 CH4R A	10.53	33.33	29.41	48.00	41.38	21.43	13.79	34.79	25.00	34.79	41.67	24.00	26.09	45.17	42.86	37.50	34.29	31.25	45.17	50.00	27.27	38.89	42.11	32.01	33.33	42.86	100.00					
Day 60 CO2R A	20.00	10.53	40.00	38.47	33.33	34.49	33.33	33.33	24.00	25.00	40.00	30.77	8.33	25.00	41.38	30.31	33.33	30.31	31.25	20.69	26.09	32.43	20.00	30.77	21.05	34.49	43.48	100.00				
Day 25 CH4R A	21.43	29.63	55.81	41.18	52.63	37.84	31.58	37.50	30.31	37.50	24.25	29.41	31.25	35.00	43.25	48.78	45.46	29.27	30.00	54.06	25.81	40.00	42.86	41.18	14.82	43.25	38.71	31.25	100.00			
Day 60 CH4 A	21.05	33.33	47.06	40.00	27.59	21.43	13.79	34.79	33.33	26.09	33.33	32.01	26.09	38.71	28.57	37.50	40.00	25.00	25.81	35.72	27.27	33.33	31.58	32.01	22.23	42.86	45.46	34.79	51.61	100.00		
Day 25 CO2R A	20.69	7.15	27.27	28.57	35.90	36.85	41.03	18.19	23.53	36.37	23.53	22.86	30.31	34.15	47.37	38.10	44.45	38.10	39.03	26.32	18.75	43.48	34.49	34.29	14.29	31.58	25.00	48.49	34.15	12.50	100.00	
Day 60 CO2 A	17.39	9.09	47.37	27.59	36.37	37.50	24.25	22.23	14.29	37.04	21.43	20.69	14.82	34.29	37.50	33.33	30.77	22.23	28.57	18.75	23.08	45.00	26.09	41.38	9.09	18.75	15.39	37.04	34.29	7.69	61.11	100.00

Table 2: Dice coefficient similarity (%) analysis for comparison of climate change simulations effects in soil samples during summer under elevated (E) and ambient (A) conditions

	Day 25 CO2CH4R A	Day 60 CO2CH4R A	Day 25 CO2R A	Day 25 R A	Day 60 R A	Day 25 CPG A	Day 60 CPG A	Day 25 CH4 A	Day 60 CO2CH4 A	Day 25 CO2CH4 A	Day 60 CH4 A	Day 25 CH4R A	Day 60 CH4R A	Day 60 CO2 A	Day 25 CO2 A	Day 60 CO2R A	Day 60 CH4R E	Day 25 CH4 E	Day 25 CH4R E	Day 25 CO2 E	Day 25 CO2R E	Day 60 CH4 E	Day 60 CO2 E	Day 60 CO2R E	Day 25 CPG E	Day 60 CO2CH4R E	Day 25 R E	Day 25 CO2CH4R E	Day 60 CO2CH4 E	Day 60 CPG E	Day 60 R E	Day 25 CO2CH4 E
Day 25 CO2CH4R A	100																														
Day 60 CO2CH4R A	64.87	100.00																													
Day 25 CO2R A	56.25	40.00	100.00																												
Day 25 R A	35.30	21.63	37.50	100.00																											
Day 60 R A	37.50	22.86	46.67	75.00	100.00																										
Day 25 CPG A	42.43	27.78	19.36	60.61	58.07	100.00																									
Day 60 CPG A	41.18	32.43	25.00	64.71	68.75	78.79	100.00																								
Day 25 CH4 A	33.33	30.31	7.15	20.00	14.29	41.38	26.67	100.00																							
Day 60 CO2CH4 A	34.49	25.00	22.23	13.79	14.82	28.57	27.59	56.01	100.00																						
Day 25 CO2CH4 A	42.43	44.45	32.26	24.25	12.91	31.25	24.25	34.49	50.00	100.00																					
Day 60 CH4 A	27.59	37.50	22.23	6.90	7.41	42.86	27.59	56.01	50.00	57.15	100.00																				
Day 25 CH4R A	42.43	33.33	32.26	30.31	45.17	37.50	36.37	41.38	42.86	31.25	35.72	100.00																			
Day 60 CH4R A	19.36	17.65	34.49	19.36	13.79	26.67	25.81	37.04	53.85	33.33	38.47	26.67	100.00																		
Day 60 CO2 A	37.84	35.00	35.30	21.63	28.57	28.57	27.78	42.43	31.25	38.89	25.00	38.89	52.95	100.00																	
Day 25 CO2 A	37.50	22.86	33.33	31.25	33.33	51.61	43.75	35.72	14.82	25.81	37.04	19.36	41.38	51.43	100.00																
Day 60 CO2R A	33.33	30.31	50.00	20.00	28.57	27.59	33.33	30.77	24.00	34.49	40.00	34.49	44.45	48.49	57.15	100.00															
Day 60 CH4R E	8.00	14.29	34.79	24.00	26.09	25.00	16.00	38.10	30.00	25.00	30.00	25.00	18.19	21.43	26.09	28.57	100.00														
Day 25 CH4 E	21.63	40.00	28.57	32.43	34.29	27.78	16.22	30.31	6.25	27.78	25.00	16.67	41.18	30.00	28.57	36.37	21.43	100.00													
Day 25 CH4R E	19.36	23.53	27.59	32.26	41.38	33.33	25.81	37.04	7.69	26.67	23.08	26.67	28.57	41.18	20.69	14.82	27.27	52.95	100.00												
Day 25 CO2 E	14.29	25.81	23.08	28.57	38.47	29.63	21.43	25.00	8.70	22.23	17.39	22.23	8.00	12.91	15.39	16.67	21.05	32.26	40.00	100.00											
Day 25 CO2R E	30.77	27.59	33.33	23.08	25.00	8.00	23.08	27.27	28.57	24.00	19.05	16.00	34.79	27.59	33.33	45.46	23.53	34.49	34.79	20.00	100.00										
Day 60 CH4 E	20.69	37.50	37.04	27.59	37.04	28.57	27.59	8.00	16.67	21.43	25.00	21.43	23.08	18.75	22.23	16.00	20.00	43.75	23.08	34.79	47.62	100.00									
Day 60 CO2 E	17.39	15.39	0.00	8.70	19.05	18.19	8.70	21.05	11.11	0.00	0.00	18.19	10.00	15.39	9.53	21.05	14.29	23.08	10.00	35.30	26.67	44.45	100.00								
Day 60 CO2R E	40.00	35.72	17.39	16.00	17.39	25.00	16.00	9.53	10.00	8.33	20.00	25.00	36.37	28.57	26.09	28.57	12.50	35.72	54.55	21.05	35.30	40.00	71.43	100.00							
Day 25 CPG E	22.23	26.67	16.00	29.63	32.01	30.77	29.63	34.79	9.09	38.47	18.19	30.77	8.33	20.00	24.00	26.09	22.23	26.67	25.00	47.62	10.53	0.00	12.50	11.11	100.00						
Day 60 CO2CH4R E	34.49	31.25	37.04	34.49	44.45	21.43	20.69	16.00	33.33	35.72	25.00	21.43	15.39	6.45	22.23	24.00	10.00	12.50	23.08	8.70	9.53	0.00	0.00	0.00	54.55	100.00					
Day 25 R E	14.82	20.00	16.00	37.04	32.01	46.15	29.63	17.39	18.19	53.85	36.37	23.08	25.00	26.67	24.00	26.09	11.11	26.67	33.33	28.57	0.00	9.09	0.00	0.00	40.00	36.37	100.00				
Day 25 CO2CH4R E	8.33	14.82	18.19	33.33	36.37	26.09	25.00	10.00	0.00	17.39	10.53	8.70	0.00	22.23	18.19	20.00	0.00	29.63	28.57	11.11	0.00	0.00	0.00	13.33	47.06	42.11	47.06	100.00			
Day 60 CO2CH4 E	9.09	32.01	10.00	18.19	20.00	19.05	9.09	0.00	11.77	28.57	0.00	9.53	0.00	24.00	10.00	22.23	0.00	16.00	21.05	12.50	0.00	11.77	18.19	30.77	40.00	11.77	26.67	50.00	100.00		
Day 60 CPG E	16.67	7.41	18.19	16.67	27.27	34.79	25.00	10.00	21.05	26.09	10.53	8.70	28.57	14.82	18.19	0.00	0.00	14.82	28.57	22.23	12.50	10.53	0.00	13.33	35.30	31.58	23.53	14.29	16.67	100.00	
Day 60 R E	15.39	27.59	16.67	23.08	33.33	32.01	38.47	18.19	28.57	40.00	9.53	24.00	17.39	20.69	0.00	9.09	11.77	34.49	17.39	40.00	11.11	19.05	13.33	23.53	42.11	28.57	42.11	37.50	28.57	62.50	100.00
Day 25 CO2CH4 E	32.26	23.53	13.79	32.26	34.49	40.00	38.71	29.63	38.47	26.67	15.39	20.00	21.43	29.41	20.69	14.82	9.09	17.65	21.43	32.01	17.39	23.08	30.00	27.27	33.33	30.77	33.33	38.10	21.05	57.15	43.48	100.00

Table 3: Identification of bacterial species represented by the DGGE bands based on closest neighbours in GenBank

BAND	SIMILARITY (%)	GENBANK MATCH	ACCESSION NO.
1	86	*Methylocystis sp.* M175	JN036514.1
2/15	92	Uncultured *Burkholderiaceae* bacterium clone Amb	EF018609.1
4/16	89	Uncultured *Methylosinus* sp. clone BG1-76	JX079158.1
8/19	88	*Blastochloris sulfoviridis* strain Wai3G1e	AY117150.1
26	91	Uncultured bacterium clone	GQ218679.1
12	91	Uncultured bacterium clone cs8A09	HM580790.1
27	88	Uncultured *Geothermobacterium sp*. clone SK520	DQ833881.1
10/22	88	Uncultured *Hyphomicrobium sp*. clone AUVE	EF651672.1
23	90	Uncultured *Sediminibacterium sp*. clone YL012	HM856387.1
24	97	*Nitrosospira sp*. APG3	KC477402.1
25	98	Uncultured bacterium clone 5B-14	DQ906815.1

CHAPTER SIX

CONCLUDING REMARKS

6.1 THE RESEARCH IN PERSPECTIVE

Charles Darwin, considered as the father of evolution once quoted: *"It is not the strongest of the species that survives, nor the most intelligent that survives. It is the one that is the most adaptable to change"* (http://timehuman.blogspot.com/2011/01/charles-darwin-quotes.html). Very few species on earth can validate this statement better than microorganisms that have persisted for millions of years, mainly through their remarkable ability to evolve and adapt to various factors. Decades of research can substantiate the intimate relationship between microbes, humans, animals and the environment and the foundations of these relationships still continue to provide new, insightful and useful information that is pertinent to all levels of organisation on this planet.

Despite their profound global significance, microorganisms are still considered the unseen majority. This is especially true regarding soil microorganisms that comprise one of the largest genetic diversities on Earth (van der Heijden *et al*., 2008). However, the importance of the soil microbial community in various soil processes remains poorly understood and underestimated. This is surprising considering that soil microbes are indispensable from an agronomic point of view and also essential for the degradation of nearly all organic compounds known to man, including some anthropogenic xenobiotics and polyphenolic compounds (Nannipieri *et al*., 2003; van der Heijden *et al*., 2008). Furthermore, the involvement of soil microbes in many significant biological processes such as photosynthesis, mineralisation, nitrification and decomposition (Mayr *et al*., 1999; Schimel, 2004) also

reinforces their importance in nutrient recycling processes in the soil ecosystems. Considerably less emphasis is placed on their ability to mediate and maintain biogeochemical cycles. Thus, this study focused on determining the effects of single and/or multiple global change drivers on soil microbial activity and diversity. Soil microorganisms can function either as sinks or sources of major greenhouse gases and their abundance coupled with high metabolic diversity make them key determinants of the fate of terrestrial ecosystems (King *et al*., 2001; Selesi *et al*., 2005, Mohanty *et al*., 2006). Considering the immense potential for soil microbial responses to climate change, now more than ever, it has become imperative to better understand the dynamics of the soil microbial community.

Chapter Three describes the effects of single or multiple global change factors on soil microbial enzyme activity across the spring and summer seasons. Soil microbial enzymes that mediate decomposition processes have become popular for determining microbial activities in various climate change experiments (Henry, 2012). However, measurements of these extracellular enzyme activities, particularly under temperature manipulation experiments at field scale are rare (McDaniel *et al*., 2013). This represents a major gap in knowledge considering that these enzymes can be used as bio-monitors of soil health while providing information on soil microbial activities (Kennedy and Papendick, 1995), especially as climate change progresses. Amongst the various enzymes in soil, activity of the dehydrogenase enzyme (belonging to the oxidoreductase enzyme family) is vital as it provides an indication of overall microbial activity. Dehydrogenase enzyme activity can be used to infer the metabolic potential of a soil and is also considered to be proportional to soil microbial biomass (Wolinska and Stepniewska, 2012). In this study, regardless of whether soils were incubated at elevated or ambient temperature conditions, soil microbial dehydrogenase activity decreased in response to the global change factors for both seasons by the end of the

sampling period resulting in an overall negative effect on soil microbial activity. However, for both seasons a greater loss in soil dehydrogenase activity was observed in the control at elevated temperature compared to ambient temperature incubation indicating that the global change drivers did not affect microbial activity as considerably when in combination with elevated temperature. Wolinska and Stepniewska (2012) state that the dehydrogenase enzyme is significantly affected by a variety of environmental factors including temperature, soil moisture, season of the year, organic matter content amongst others. In this study, seasonally related increases in temperature combined with lower soil moisture during summer were the main factors resulting in lower dehydrogenase activity compared to spring when soil was exposed to the global change drivers. In contrast, the activity of the hydrolytic enzymes viz. β-glucosidase, arylsulphatase and urease increased during summer at elevated temperatures. Hydrolytic enzymes are responsible for supporting primary metabolism by allowing microbes to directly acquire nutrients from labile organic matter (Sylvia *et al*., 2004). An increase in β–glucosidase, arylsulphatase and urease enzyme occurred in response to nutrient limitation under elevated temperature during summer. The cost: benefit ratio of enzyme production is a key determinant of extracellular enzyme synthesis. It is unlikely that microorganisms will direct effort towards the production of enzymes which generate resources that are not limiting (Burns *et al*, 2013). Although an increase in hydrolytic enzyme production was observed during summer, it was not sufficient to sustain the microbial biomass resulting in lower soil dehydrogenase and microbial activity.

Despite the lack of consistent results in many climate change studies, short-term studies will be useful to estimate temperature relationships which can be easily associated with the temperature adaptation abilities of a microbial community (Rinnan *et al*., 2011) and enhance the understanding of microbial responses towards projected climate change. Excess CO_2 may

be liberated to the atmosphere due to organic matter decomposition (by soil microorganisms), elevating atmospheric CO_2 concentrations even further (Hu *et al*., 1999; Bardgett *et al.,* 2008; Guenet *et al.,* 2012). Woodward (2007) suggested that while valuable information has been obtained from elevated CO_2 experiments, it is compulsory to investigate the effects of elevated temperature on various ecosystems. Lin and Zhang (2012) used stochastic modeling to simulate changes in soil organic carbon from 1998 to 2100 under higher temperatures and elevated CO_2 and found a counteracting effect of these two factors. In a similar study, Wang *et al*. (2007) reported that a 2.7 – 3.9 ºC temperature increase resulted in 5% - 6% higher soil organic carbon content and up to 8% decrease in soil organic carbon was observed when temperatures were 7.5- 7.8 ºC higher. These observations were consistent only under conditions of doubled CO_2 concentrations (Wang *et al*., 2007) and confirm the significant impact that temperature can exert on soil processes. The present study indicated a potential for interaction of elevated CO_2 and elevated temperature as reflected by the changes in soil microbial enzymes. This information is extremely relevant for a better understanding of climate change, and contextualises a key aspect of future global change, viz., interactions amongst multiple factors (Gutknecht *et al*., 2010). Single factor experiments have proven to be extremely valuable and have allowed the documentation and formulation of hypotheses regarding microbial response to climate change in a wide variety of ecosystems. However, it is possible that these single climate change factors may act in concert with each other, triggering unknown positive or negative effects. Therefore, this provided a motivation to investigate microbial responses when soils were exposed to a combination of global change factors (Carmona-Moreno *et al*., 2005; Bardgett *et al*., 2008; Singh *et al*., 2010), which is one of the objectives of this study. In this study, it was apparent that soil microbial enzymes were more sensitive towards changes in temperature and moisture related to seasonal differences, compared to changes in greenhouse gas concentrations viz. CO_2 or CH_4, either

individually or in combination with other global change factors. Freeman *et al*. (2004) reviewed the effect of elevated CO_2 concentrations on soil microbial extracellular enzymes and reported that many studies did not find any significant effect on soil enzyme activities (elevated vs. ambient CO_2 concentrations), which is in agreement with the results obtained in the present study. Some studies have revealed that higher temperatures lead to lower soil extracellular enzyme activities (Allison and Treseder, 2008; Cusack *et al*., 2010; Kardol *et al*., 2010). However, it is also clear that this trend does not apply to all soil extracellular enzymes or for all the experimental conditions investigated (McDaniel *et al*., 2013).

In this study, qPCR was used to evaluate the changes in the copy number of the *cbbL* gene coding for the highly conserved large subunit bacterial RuBisCO enzyme which is central to the removal and sequestration of environmental CO_2. Similar to the trend observed for the soil dehydrogenase activity, *cbbL* gene copy number was more negatively affected by the seasonal change rather than exposure to the global change drivers. In spring, the *cbbL* gene copy numbers increased by the end of the sampling period and were similar under both elevated and ambient temperatures. During summer, the interactive effect of CO_2 and temperature is again highlighted with higher *cbbL* gene copies observed at the end of the sampling period. Changes in *cbbL* gene copy number can be related to a change in the structure of the microbial community. Videmsek *et al.* (2009) observed a reduction in the number of *cbbL* gene copies in response to elevated CO_2 and proposed that this was due to either a change in the microbial community responsible for carboxylation or due to changes in various other parameters such as soil pH. The higher *cbbL* gene copy numbers observed at the end of the study is promising especially considering that changes in the *cbbL* genes are comparable to important genes that are involved in soil nutrient recycling and that the 'red-like' *cbbL* genes are closely associated with efficient functioning of soil bacterial

populations. Analysis of the functional genes harboured in soils could improve the understanding of the reactions that microorganisms mediate in biogeochemical elemental cycling (Selesi *et al*., 2005). Yuan *et al.* (2012) suggested that terrestrial ecosystems could fix approximately 4% of total CO_2 annually via soil microbially mediated autotrophic processes alone. Furthermore, the detection of *cbbL* genes in organisms not known to utilize the Calvin cycle (Eisen *et al*., 2002; Finn and Tabita, 2003) indicates the possibility of lateral *cbbL* gene transfer among bacteria, archaea and eukarya (Delwiche and Palmer, 1996), offering new insights into the often underestimated mechanisms of microbial CO_2 fixation potential and warranting further investigation of the *cbbL* gene especially in terrestrial ecosytems.

Interestingly, although *cbbL* gene copy number responded positively to the global change treatments under both elevated and ambient temperatures, results of DGGE profiles presented in Chapter Five revealed a substantial reduction in microbial diversity associated with some of the global change treatments. As with the soil dehydrogenase enzyme, this trend was most evident during summer under elevated temperature as the shift in bacterial community composition was most pronounced. While temperature and soil moisture due to the seasonal change greatly impacted soil microbial genetic diversity, the functional diversity of the soil microbial community was maintained. This is evidenced by the production of *cbbL* genes and increases in hydrolytic enzyme activity. Climate and ecosystem models including microbial processes use first order rate kinetics. These models are based on the assumption that the microbial population is sufficiently able to conduct various functions (e.g decomposition) however; the rate of the process may be altered by environmental factors such as temperature and moisture. Thus, climate change induces a microbial community structure shift where the process rate changes due to the emergence of a new microbial community with different physiologies (Singh *et al*., 2010). This new microbial community is presumably better adapted to the changes in environmental factors. Therefore, despite a loss of diversity

observed in the present study, the metabolic versatility and resilience of the soil microbial community was emphasised after identification of the prominent bands excised from the DGGE gels. Soil microorganisms that are commonly associated with biogeochemical cycling were identified reinforcing the significance of soil microbes towards the maintenance of the soil ecosystem. The detection of thermotolerant species, viz., *Geothermobacterium* and *Sediminibacterium* suggests that certain microbial species are able to persist amidst the harsh environmental conditions and possibly perform functions that are lacking by other community members. Furthermore, the presence of microbial species such as *Methylosinus*, *Methylocystis*, *Blastochloris*, *Burkholderia* and *Nitrospira*, known to harbour genes involved in the metabolism of either CO_2 or CH_4, is noted. This finding suggests that despite the drastic loss of microbial diversity observed in some experimental samples, it is apparent that microorganisms with the appropriate metabolic machinery will endure and still be involved in biogeochemical cycling. Recently, Yuste *et al*. (2014) used a pyrosequencing approach and concluded that seasonal shifts in both temperature and water availability were amongst the main factors controlling the selection of phenotypes adapted to semiarid conditions in Mediterranean ecosystems. Similar to the soil enzyme activities and qPCR experiments, it was encouraging to observe that a combination of all 3 global change factors investigated did not induce any significant change in microbial community structure relative to the individual factors. This information further emphasizes the need for more multifactor experiments, which can be used to provide a more valid representation of the trends in soil processes considering that climate change is also multifactorial.

Microbial activities are fundamental towards our understanding of regional and global ecology, due to their significant involvement in the carbon cycle (Selesi *et al*., 2005). Several factors regulate the dynamics of the soil microbial community, with a dominant role exerted

by temperature and moisture. This study supports the hypothesis that soil microbial activity and diversity were negatively affected by the global change treatments, most notably temperature. However, these results must be interpreted with caution as pseudoreplicate sampling was conducted in this study. While pseudoreplication is problematic, it is common practice in climate change studies due to financial and logistical issues. Nevertheless, soil microbial populations still offer the promise of autotrophic CO_2 fixation by increasing the numbers of their functional genes involved in this process. Climate change studies often disregard microbial contributions and if carbon sequestration strategies are to be successful, we must fully understand microbial responses under various environmental conditions. Despite the drastic increases in greenhouse gases, temperature and/or rainfall simulated in the present study, it was evident that certain species of soil microorganisms were still able to survive and mediate biochemical activities that are beneficial to the community as a whole. The response of the soil ecosystem towards the detrimental effects of the global change drivers is convincing evidence that nature has its very own immune system that will assist in its recovery in the future. Undoubtedly, this information can aid in curbing the overwhelming anxiety experienced by humans regarding climate change. Recently, Garcia-Pichel *et al.* (2013) reported that temperature change may even be responsible for the distribution of ecologically significant topsoil bacteria across continents. These new findings suggest that microbial responses towards climate change is not fully understood. It is clear that the ability of microorganisms to adapt to their surroundings will enhance their survival. Considering that microbes have been able to withstand a variety of environmental factors since the origin of life on Earth, it is reasonable to assume that they will continue to persist and drive important processes that sustain habitability on this planet. Certainly, since the time of Alexander Flemming and the discovery of penicillin, man has been intelligent enough to understand the importance of microorganisms. Through continued research and in depth studies of microbial

activity, man's intelligence will extend towards harnessing the amazing potential of microorganisms as bio-engineers of the climate system and administrators of our environment.

6.2 POTENTIAL FOR FUTURE DEVELOPMENT OF THE STUDY

Soil microbial communities are considered to be dynamic in response to environmental changes especially across seasons (Williams *et al*., 2013). In the present study, soil microbial community activity and structure were determined during the spring and summer seasons over a short term period. Thus, a proper understanding of microbial responses towards changes in temperature and/or rainfall and/or greenhouse gas concentrations will be achieved by extending the study to include the winter and autumn seasons. Rustad *et al*. (2001) suggests that there is an urgent need for numerous small-scale studies, rather than limited and costly large-scale studies incorporating few standardized methods that allow for simple comparisons and contrast of results across various ecosystem types (Aronson and McNulty, 2009). This will improve the understanding of microbial responses towards climate change and will assist the development of soil carbon sequestration strategies for possible mitigation of climate change effects in the context of the African climate system.

The incorporation of other sophisticated and current molecular and/or biochemical techniques will aid in enhancing our knowledge of the impacts of climate change on soil microorganisms and possible feedback mechanisms relating to greenhouse gas emissions (Singh *et al*., 2010). Phospholipid fatty acid analysis (PLFA) can reveal variations in microbial physiology and is a superb indicator of microbial community structural change (Williams *et al*., 2013). The use of this tool can also provide information regarding the changes in the fungal community and their relevance to soil processes in response to climate change. *In situ* culture-independent

molecular methods such as fluorescent *in situ* hybridization (FISH), *in situ* PCR (IS-PCR), *in situ* reverse transcription (ISRT) and *in situ* reverse transcription-PCR (ISRT-PCR) have been successful for visualizing and studying the expression of specific genes in whole cells (Sinigalliano *et al*., 2001). The use of these methods (including real-time PCR) to determine the expression of functional genes such as *cbbL*, *pmoA* and *amo* that code for enzymes important for methane and ammonia oxidation, respectively (Gilbert et al., 2008; Tourna *et al*., 2008), will certainly complement results obtained in the present study. Studies that are able to combine the emerging methods of metagenomics, metaproteomics and stable isotope probing (SIP) will enable investigation of the physiology and functions of individual taxa in specific environments providing the necessary information for determining whether particular soils are sources or sinks of carbon based on microbially mediated processes (Singh *et al*., 2010). Metagenomic studies are helpful in identifying the functional potential and composition of diverse microbial communities (Singh *et al*., 2009), while metaproetomics will allow for direct extraction of proteins from environmental samples followed by subsequent mass spectrometry analysis (Singh *et al*., 2010). The development of SIP techniques has increased the knowledge of the coupling between changes in ecosystem function and microbial diversity (Zak *et al*., 2006; Drigo *et al*., 2007; Neufeld *et al*., 2007; Bardgett *et al*., 2008). The combination of SIP and metagenomics make it possible to explore the roles of specific microbial populations within a community. Furthermore, this technique can also be used for studying certain functional genes that are central to biogeochemical processes (Bardgett *et al*., 2008; Singh *et al*., 2010). These techniques will also make it possible to study different microbial phylotypes like methanotrophs and methanogens that are central to biogeochemical cycles (Sun *et al*., 2012; Chowdhury and Dick, 2013)

Finally, it is important to develop models that can accurately predict microbial activities over a wide range of environments, time and scale. While modeling studies are fundamental

towards our understanding of future trends, such studies must be complemented by field studies which can provide information that can be incorporated into climate change models, and improve the reliability of the results that will be obtained. The incorporation of this information into existing soil carbon models will guarantee the success of soil carbon sequestration strategies and will greatly assist in mitigating the continuously increasing greenhouse gas concentrations and subsequently decreasing the effects of climate change.

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APPENDIX I – Additional data for Chapter 3

Reagents and Buffers

Tris Buffer (1M, pH 7.0)

Dissolve 30.28g of Tris in 200ml distilled water, adjust the pH to 7.0 with 3M HCl and bring up to 250ml with distilled water.

INT solution (9.88mM)

Dissolve 500mg of INT in 2ml N,N-dimethylformamide, then add 50ml distilled water, sonicate the solution (ultrasonic bath) and add distilled water to 100ml. Store in dark and use only freshly prepared solution.

Extractant

Mix 100ml N,N-dimethylformamide with 100ml ethanol (1:1 ratio).

INF standard solution (100ug/ml)

Dissolve 10mg INF in 80ml extractant and bring with the same extractant to 100ml.

Tris Buffer (0.1M, pH 12)

Dissolve 12.11g of Tris in 700ml distilled water, adjust the pH to 12 and bring up to 1000ml with distilled water.

Modified Universal Buffer (MUB) Stock

Dissolve 12.1g of Tris, 11.6g of maleic acid, 14g of citric acid and 6.3g of boric acid in approx. 500ml NaOH (1M) and dilute the solution to 1000ml with distilled water. Store at 4˚C.

Modified Universal Buffer (pH 6.0)

Titrate 200ml of MUB stock solution to pH 6.0 under continuous stirring with 0.1M HCl and dilute to 1000ml with distilled water.

***p*-Nitrophenyl-β-D-glucoside (25mM)**

Dissolve 0.377g of PNG in 40ml of MUB buffer and dilute to 50ml with the same buffer. Store at 4˚C.

Acetate buffer (0.5M, pH 5.8)

Dissolve 68g of sodium acetate trihydrate in 700ml distilled water, adjust the pH with the concentrated acetic acid to 5.8 and bring up to 1000ml with distilled water.

***p*-Nitrophenyl sulphate solution (25mM)**

Dissolve 0.312g of potassium-mitrophenyl sulphate in about 40ml acetate buffer and dilute the solution to 50ml with buffer. Store at 4˚C.

KCl solution

Dissolve 74.6g KCl in distilled water, add 10ml of 1M HCl and bring up with distilled water to 1000ml.

Sodium Salicylate solution

Dissolve 17g of sodium salicylate and 120 mg sodium nitroprusside in distilled water and bring to 100ml.

Sodium salicyclate/NaOH solution

Mix equal volumes of the NaOH and sodium salicyclate solutions, and distilled water (prepare fresh!).

Sodium dichloroisocyanide solution (0.1%)

Dissolve 0.1g sodium dichloroisocyanide in 100ml distilled water (prepare shortly before use).

Borate buffer (pH 10)

Dissolve 56.85g disodium tetraborate, or 30g disodium tetraborate (water free) in 1500ml warm distilled water. After cooling adjust pH to 10 with NaOH solution and bring up to 2000ml with distilled water.

Table a: Replicate optical density values for determination of dehydrogenase enzyme activity in spring at elevated temperature (Fig 1 a)

Day/Treatment	0	15	25	40	60
CO_2	1.531	2.062	0.836	1.071	1.1788
	1.448	2.047	1.004	1.043	1.09
	1.566	1.935	0.662	1.565	1.122
	1.431	2.066	1.158	1.197	1.291
Std Dev	13.50079	12.9395	44.48656	49.96243	18.37929
CO_2 + R	1.146	1.276	1.058	1.004	0.849
	1.144	1.401	1.45	0.905	1.016
	1.275	1.387	1.247	0.978	0.745
	1.336	1.257	1.086	0.91	0.888
Std Dev	19.95968	15.44255	37.48938	10.27678	23.29477
CO_2 + CH_4	1.417	1.353	0.716	1.066	0.77
	1.089	1.458	0.965	0.948	0.967
	1.112	1.488	0.935	0.827	0.831
	1.268	1.535	0.998	0.813	0.795
Std Dev	31.75768	16.04633	26.54613	24.62037	18.26559
CO_2 + CH_4 + R	1.147	1.251	0.716	0.86	0.689
	1.115	1.388	0.965	0.974	0.95
	1.072	1.368	0.935	0.817	0.861
	1.039	1.402	0.988	0.896	0.719
Std Dev	0.047458	0.068927	0.125228	0.06653	0.122492
R	1.36	1.202	0.937	0.832	0.777
	1.532	1.323	0.882	0.865	0.779
	1.316	1.151	0.878	0.773	0.74
	1.076	1.168	0.887	0.894	0.812
Std Dev	39.11663	16.14534	5.736908	10.80176	6.121243
CTRL	0.949	1.159	0.786	0.616	0.804
	1.254	1.291	0.791	0.741	0.72
	1.299	1.15	0.614	0.786	0.751
	1.068	1.067	0.823	0.848	0.766
Std Dev	33.94999	19.26324	19.64293	20.42184	7.258219

Table b: Replicate optical density values for determination of dehydrogenase enzyme activity in spring at ambient temperature (Fig 1 b)

Day/Treatment	0	15	25	40	60
CO_2	1.185	1.183	0.755	0.862	0.698
	0.845	1.233	0.795	1.074	0.856
	1.299	1.174	0.723	0.897	0.566
	1.068	1.117	0.726	1.013	0.626
Std Dev	40.34731	9.88915	6.955849	20.58091	26.04759
CO_2 + R	1.155	1.545	1.155	0.863	0.881
	1.294	1.623	1.061	0.966	1.03
	1.229	1.754	1.043	0.909	1.712
	1.033	1.668	1.265	0.687	0.698
Std Dev	23.29035	18.16116	21.20461	25.05292	92.04603
CO_2 + CH_4	0.924	1.27	0.793	0.829	0.836
	1.064	1.398	0.877	0.868	0.794
	1.167	1.119	0.773	0.877	1.131
	1.069	1.242	0.826	0.964	0.667
Std Dev	20.7932	23.81332	9.450654	11.84552	40.82937
CO_2 + CH_4 + R	1.168	1.301	0.863	0.797	0.833
	1.046	1.422	1.004	0.763	1.041
	1.246	1.416	0.901	0.631	0.877
	0.917	1.319	0.935	0.772	0.697
Std Dev	29.95434	13.189	12.45664	15.51057	29.49809
R	1.108	1.291	1.003	0.633	0.687
	0.996	1.214	0.859	0.633	0.69
	0.898	1.295	0.614	0.701	0.791
	0.97	1.402	0.849	0.723	0.692
Std Dev	18.12897	16.06968	33.49316	9.669591	10.54775
CTRL	1.047	0.909	0.963	0.914	1.061
	0.978	1.257	0.701	0.692	1.044
	1.044	1.143	0.884	0.802	0.772
	0.888	0.892	0.965	0.612	0.833
Std Dev	15.52452	37.27603	25.80163	27.36133	30.50119

Table c: Replicate optical density values for determination of dehydrogenase enzyme activity in summer at elevated temperature (Fig 2 a)

Day/Treatment	0	15	25	40	60
CO_2	1.374	0.713	0.708	1.092	0.572
	1.297	0.726	0.829	1.232	0.605
	1.401	0.71	0.671	1.067	0.711
	1.499	0.756	0.701	1.192	0.577
Std Dev	18.46599	4.651662	15.4302	17.46219	14.34742
CO_2 + R	0.995	0.566	0.487	0.704	0.347
	1.041	0.544	0.576	0.768	0.387
	1.093	0.565	0.577	0.638	0.429
	1.063	0.573	0.544	0.738	0.466
Std Dev	9.134119	2.770704	9.344492	12.35929	11.40584
CO_2 + CH_4	1.066	0.423	0.548	0.637	0.348
	1.157	0.538	0.543	0.583	0.347
	1.116	0.48	0.557	0.592	0.36
	1.172	0.523	0.604	0.601	0.368
Std Dev	10.51156	11.41532	6.184928	5.239535	2.231069
CO_2 + CH_4 + R	0.649	0.576	0.643	0.657	0.383
	1.126	0.491	0.631	0.712	0.415
	1.45	0.535	0.667	0.668	0.579
	0.743	0.574	0.716	0.689	0.413
Std Dev	8.157099	8.867358	8.32463	5.37533	19.67461
R	1.213	0.53	0.894	0.642	0.374
	0.99	0.533	0.592	0.69	0.402
	1.7	0.556	0.581	0.691	0.386
	1.295	0.574	0.594	0.694	0.403
Std Dev	8.533905	3.181897	3.649893	5.823697	2.537951
CTRL	1.156	0.609	0.638	0.686	0.338
	1.131	0.574	0.599	0.631	0.379
	1.112	0.551	0.662	0.783	0.387
	1.228	0.601	0.636	0.668	0.417
Std Dev	11.24576	5.860505	5.75646	14.35382	7.21012

Table d: Replicate optical density values for determination of dehydrogenase enzyme activity in summer at ambient temperature (Fig 2 b)

Day/Treatment	0	15	25	40	60
CO_2	1.037	0.697	0.758	0.84	0.594
	1.074	0.8	0.874	0.815	0.487
	1.056	0.738	0.847	0.82	0.48
	0.964	0.743	0.761	0.822	0.486
Std Dev	10.68265	9.375031	13.1395	2.413922	12.15725
CO_2 + R	1.201	0.623	0.832	0.96	0.52
	1.53	0.702	0.934	0.974	0.522
	1.187	0.743	0.819	0.801	0.464
	1.158	0.712	0.96	0.975	0.495
Std Dev	3.820316	11.30606	15.74871	18.72958	6.000962
CO_2 + CH_4	1.043	0.659	0.857	0.515	0.448
	1.012	0.742	0.965	0.487	0.513
	1.047	0.753	0.966	0.52	0.527
	1.057	0.719	1.019	0.372	0.429
Std Dev	4.297585	9.289049	15.05624	15.31985	10.63207
CO_2 + CH_4 + R	0.982	0.677	0.892	0.516	0.437
	1.076	0.556	1.105	0.524	0.439
	0.98	0.719	0.888	0.598	0.487
	1.026	0.608	0.897	0.532	0.454
Std Dev	10.02419	16.00858	23.5522	8.317023	5.116513
R	1.079	0.717	0.992	0.694	0.543
	1.032	0.768	0.948	0.613	0.504
	1.157	0.836	0.88	0.617	0.521
	1.073	0.839	0.98	0.652	0.435
Std Dev	11.55381	12.98947	11.11813	8.335659	10.32657
CTRL	1.155	1.005	1.473	0.727	0.556
	1.132	1.054	1.28	0.728	0.679
	1.292	1.003	1.214	0.763	0.55
	1.106	1.029	1.234	0.793	0.587
Std Dev	18.36221	5.301516	26.217	7.000936	13.18913

Table e: Replicate optical density values for determination of β- glucosidase enzyme activity in spring at elevated temperature (Fig 3 a)

Day/Treatment	0	15	25	40	60
CO_2	0.452	2.238	2.295	1.448	2.36
	2.307	2.215	2.29	1.593	2.368
	2.431	2.216	2.4	1.638	2.363
	2.381	2.348	2.384	1.342	2.35
Std Dev	3.735482	1.564391	1.427622	3.348878	0.187258
CO_2 + R	2.338	2.279	2.294	0.884	2.226
	2.21	1.826	2.309	1.276	2.226
	2.345	2.104	2.408	1.085	2.087
	2.361	1.826	2.373	1.227	1.888
Std Dev	1.719198	5.497707	1.324762	4.337571	3.945291
CO_2 + CH_4	2.234	1.91	2.35	1.424	2.189
	2.187	2.093	2.32	1.374	2.325
	2.174	2.038	2.321	1.514	2.258
	2.064	2.23	2.401	1.401	2.314
Std Dev	1.775295	3.271677	0.937073	1.498111	1.538238
CO_2 + CH_4 + R	2.192	1.878	2.269	1.36	2.121
	2.049	1.82	2.325	1.24	2.295
	2.111	2.107	2.304	1.44	2.23
	2.054	2.261	2.215	0.455	2.258
Std Dev	1.642637	5.052544	1.18644	11.18719	1.848079
R	2.125	1.465	2.205	0.201	2.097
	2.278	1.79	2.12	0.972	2.045
	1.913	1.615	2.173	1.431	2.222
	2.19	1.937	2.135	1.197	1.859
Std Dev	3.838007	5.071426	0.945778	13.16504	3.719774
CTRL	2.069	1.509	2.12	1.08	2.243
	2.197	1.876	2.134	1.484	2.107
	2.305	1.967	2.245	1.228	2.226
	2.173	2.097	2.166	1.221	2.008
Std Dev	2.389969	6.227434	1.379904	4.152247	2.717226

Table f: Replicate optical density values for determination of β- glucosidase enzyme activity in spring at ambient temperature (Fig 3 b)

Day/Treatment	0	15	25	40	60
CO_2	2.4	2.09	2.341	1.529	2.381
	2.41	1.918	2.317	1.101	2.396
	2.457	2.076	2.44	1.677	2.358
	2.479	1.986	2.356	1.706	2.4
Std Dev	0.930006	1.993135	1.319465	6.888422	0.46924
CO_2 + R	2.377	2.47	2.45	2.085	2.364
	2.41	2.32	2.429	2.263	2.37
	2.424	2.476	2.451	2.134	2.365
	2.469	2.461	2.384	2.17	2.343
Std Dev	0.941395	1.844713	0.773691	1.856189	0.295093
CO_2 + CH_4	2.503	2.266	2.454	1.934	2.351
	2.42	2.147	2.487	2.014	2.39
	2.309	2.381	2.302	1.875	2.357
	2.433	2.104	2.402	1.088	2.377
Std Dev	1.980454	3.080202	1.993937	1.061816	0.444356
CO_2 + CH_4 + R	2.339	2.429	2.435	0.47	2.365
	2.509	2.344	2.481	2.068	2.435
	2.444	2.366	2.412	2.015	2.38
	2.427	2.373	2.198	2.073	2.387
Std Dev	1.728955	0.8927	3.100694	1.953018	0.746689
R	2.477	2.19	2.428	1.78	2.372
	2.466	2.21	2.429	1.674	2.417
	2.352	2.081	2.451	1.783	2.36
	2.423	2.329	2.37	1.753	2.422
Std Dev	1.398625	2.507552	0.855427	1.25416	0.773396
CTRL	2.196	2.322	2.356	1.814	2.351
	2.369	1.5	2.322	1.668	2.43
	2.424	2.323	2.425	0.986	2.36
	2.442	1.889	2.417	1.699	2.35
Std Dev	2.769125	9.767871	1.220115	9.273232	0.94835

Table g: Replicate optical density values for determination of β- glucosidase enzyme activity in summer at elevated temperature (Fig 4 a)

Day/Treatment	0	15	25	40	60
CO_2	1.54	2.327	1.304	2.242	1.886
	1.59	2.377	1.453	2.23	2.266
	1.474	2.452	1.212	2.349	2.15
	1.596	2.382	1.606	2.25	2.282
Std Dev	1.48493	1.349743	4.539661	1.438756	4.806847
CO_2 + R	1.33	2.059	1.073	2.089	1.729
	1.53	2.341	1.162	2.263	1.971
	1.289	2.054	0.871	2.127	1.786
	1.319	2.233	0.891	2.117	1.836
Std Dev	2.889749	3.682005	3.716742	2.04004	2.714474
CO_2 + CH_4	1.675	2.173	1.223	1.896	1.825
	1.585	2.265	1.213	2.117	2.163
	1.476	2.117	1.015	1.922	1.817
	1.448	2.316	1.088	1.781	2
Std Dev	2.740308	2.350754	2.645151	3.66358	4.319835
CO_2 + CH_4 + R	1.602	2.333	1.336	1.986	2.163
	1.463	2.39	1.549	2.23	2.293
	1.803	2.31	1.329	1.953	2.222
	1.851	2.451	1.358	2.012	2.191
Std Dev	4.735315	1.655765	2.750356	3.29604	1.469426
R	1.439	2.306	1.286	1.715	2.147
	1.437	2.44	1.222	2.011	2.364
	1.536	2.309	1.119	1.689	1.975
	1.462	2.391	1.303	1.964	2.255
Std Dev	1.218712	1.719981	2.187982	4.366834	4.354499
CTRL	1.745	2.512	1.712	2.061	2.383
	2.032	2.468	1.491	1.888	2.255
	2.011	2.44	1.325	1.881	2.321
	1.9	2.507	1.34	2.021	2.394
Std Dev	3.451904	0.895029	4.719541	2.412433	1.684174

Table h: Table 6: Replicate optical density values for determination of β- glucosidase enzyme activity in summer at ambient temperature (Fig 4 b)

Day/Treatment	0	15	25	40	60
CO_2	1.913	2.474	1.85	1.709	2.128
	1.58	2.48	1.866	1.944	2.105
	1.681	2.496	1.734	1.743	2.15
	1.494	2.502	1.687	1.67	2.282
Std Dev	4.751483	0.345743	2.298841	3.204744	2.083123
CO_2 + R	1.625	2.429	2.068	2.077	2.305
	1.677	2.495	2.118	2.288	2.439
	1.87	2.441	1.904	2.009	2.315
	1.985	2.501	1.875	2.18	2.391
Std Dev	4.4052	0.965964	3.147046	3.202878	1.67653
CO_2 + CH_4	2.02	2.3	1.426	2.217	2.266
	1.728	2.46	1.541	2.283	2.43
	2.06	2.26	1.427	2.158	2.186
	1.795	2.357	1.569	2.39	2.326
Std Dev	4.304642	2.280166	1.971314	2.611445	2.699996
CO_2 + CH_4 + R	1.95	2.196	1.457	2.288	2.247
	1.581	2.497	1.836	2.384	2.454
	1.676	2.503	1.663	2.259	2.31
	1.803	2.45	1.786	2.29	2.386
Std Dev	4.202341	3.823816	4.43257	1.428011	2.364405
R	1.787	2.46	1.568	2.377	2.44
	1.932	2.514	1.867	2.438	2.447
	1.851	2.43	1.614	2.388	2.453
	1.905	2.492	1.807	2.47	2.47
Std Dev	1.682398	0.965607	3.816866	1.14316	0.336647
CTRL	2.172	2.51	2.05	2.456	2.432
	2.062	2.503	1.813	2.471	2.401
	2.047	2.524	1.838	2.33	2.477
	2.011	2.45	2.046	2.423	2.451
Std Dev	1.82207	0.849975	3.384407	1.661309	0.840729

Table i: Replicate optical density values for determination of arylsulphatase enzyme activity in spring at elevated temperature (Fig 5 a)

Day/Treatment	0	15	25	40	60
CO_2	0.803	1.569	2.142	1.5	1.058
	0.87	1.541	1.694	1.693	1.055
	0.933	1.763	1.808	1.601	1.415
	0.922	1.565	1.949	1.443	1.196
Std Dev	1.649106	2.861593	5.360509	3.068737	4.699986
CO_2 + R	0.908	1.138	2.04	1.14	0.858
	0.917	1.32	2.117	0.404	1.09
	0.84	1.058	2.422	1.044	0.662
	0.926	1.191	1.921	1.254	0.833
Std Dev	1.088114	3.054893	5.93504	10.5716	4.883577
CO_2 + CH_4	1.107	1.23	1.939	1.043	0.838
	1.065	0.982	2.042	1.183	1.038
	1.129	1.313	2.019	1.15	0.921
	1.057	1.08	2.061	1.194	0.888
Std Dev	0.951347	4.120405	1.48956	1.913264	2.359281
CO_2 + CH_4 + R	0.955	1.187	2.039	0.973	0.727
	1.235	1.125	2.017	1.246	0.803
	1.212	1.206	1.791	0.468	0.795
	1.073	0.952	1.73	1.348	0.77
Std Dev	3.623801	3.20999	4.350056	10.93051	0.949151
R	1.003	0.97	1.807	0.987	0.659
	1.166	0.821	1.878	1.139	0.851
	1.273	0.936	2.253	1.122	0.61
	1.153	0.737	2.152	1.018	0.739
Std Dev	3.082936	2.971996	5.937355	2.090365	2.921903
CTRL	1.314	0.816	1.816	1.294	1.211
	1.171	1.269	1.894	1.136	0.818
	1.172	1.141	1.919	1.078	0.775
	0.964	0.952	1.941	1.317	1.175
Std Dev	4.000832	5.563422	1.512855	3.259222	6.386856

Table j: Replicate optical density values for determination of arylsulphatase enzyme activity in spring at ambient temperature (Fig 5 b)

Day/Treatment	0	15	25	40	60
CO_2	1.425	1.323	1.935	1.734	1.583
	1.305	1.186	2.02	2.093	1.148
	1.317	1.5	2.018	1.675	1.517
	1.403	1.157	2.024	1.699	1.314
Std Dev	1.67515	4.350832	1.191133	5.459706	5.499235
CO_2 + R	1.132	1.218	2.008	1.549	1.417
	1.431	1.545	2.123	1.81	1.246
	1.283	0.858	2.017	1.489	1.133
	1.563	1.525	2.02	1.855	1.579
Std Dev	5.166995	8.952953	1.505791	5.096207	5.427805
CO_2 + CH_4	1.185	2.005	2.419	1.63	1.163
	1.29	1.476		2.16	1.495
	1.085	1.796	2.327	1.599	1.186
	1.237	1.407	2.381	2.005	1.275
Std Dev	2.426382	7.768302	3.992714	7.712127	4.203366
CO_2 + CH_4 + R	1.474	1.854	2.117	1.464	1.021
	1.297	1.448	1.823	1.978	1.296
	1.141	1.698	2.409	1.684	0.922
	1.24	1.276	2.135	1.605	1.291
Std Dev	3.879667	7.138474	6.646553	6.020065	5.281918
R	1.353	1.076	2.105	1.464	1.107
	1.466	1.346	2.299	1.978	1.341
	1.36	1.161	2.123	1.684	1.187
	1.134	1.287	2.024	1.605	1.28
Std Dev	3.871008	3.383301	3.214858	6.020065	2.857898
CTRL	1.282	1.261	2.43	1.64	1.338
	1.279	1.357	2.077	1.961	1.477
	1.397	1.183	2.006	1.575	1.526
	1.246	1.354	2.193	2.025	1.308
Std Dev	1.833522	2.315426	5.156599	6.264737	2.934187

Table k: Replicate optical density values for determination of arylsulphatase enzyme activity in summer at elevated temperature (Fig 6 a)

Day/Treatment	0	15	25	40	60
CO_2	1.85	1.263	1.647	1.25	1.654
	1.831	1.173	1.865	1.595	2.112
	1.662	1.311	1.76	1.249	1.757
	1.858	1.232	1.782	1.155	2.017
Std Dev	2.743415	1.708496	2.655043	5.722398	6.352416
CO_2 + R	1.783	1.133	1.592	1.457	1.786
	1.671	1.165	1.947	1.367	1.971
	1.562	1.223	1.577	1.234	1.587
	1.552	1.006	1.861	0.998	1.937
Std Dev	3.201186	2.710233	5.55051	5.896757	5.171824
CO_2 + CH_4	1.63	0.941	1.698	1.368	1.67
	1.613	1.215	2.055	1.443	2.074
	1.606	0.958	1.561	1.152	1.716
	1.83	1.109	1.776	1.271	2.067
Std Dev	3.17026	3.848954	6.152394	3.718632	6.463445
CO_2 + CH_4 + R	1.648	1.241	1.667	1.344	1.793
	1.491	0.961	1.917	1.38	1.824
	1.474	1.171	1.759	1.334	1.573
	1.773	1.145	1.624	1.233	2.05
Std Dev	4.176052	3.528253	3.831681	1.861544	5.765534
R	1.873	1.35	1.814	1.363	1.551
	1.584	1.193	2.179	1.711	2.016
	1.779	1.358	1.604	1.43	1.832
	1.705	1.098	1.921	1.52	2.058
Std Dev	3.605488	3.737664	7.066086	4.462497	6.817118
CTRL	1.579	1.444	1.958	1.317	1.754
	1.939	1.09	1.654	1.498	2.106
	1.95	1.368	1.688	1.337	1.931
	1.81	1.1	1.876	1.454	2.299
Std Dev	5.095491	5.384708	4.330475	2.607498	6.90524

Table l: Replicate optical density values for determination of arylsulphatase enzyme activity in summer at ambient temperature (Fig 6 b)

Day/Treatment	0	15	25	40	60
CO_2	1.491	1.225	1.475	1.281	1.702
	1.757	1.333	1.718	1.368	2.277
	1.294	1.304	1.677	1.663	1.813
	1.742	1.466	1.635	1.654	2.238
Std Dev	6.539014	2.963708	3.1427	5.7939	8.64577
CO_2 + R	1.591	1.555	1.983	1.494	1.977
	1.9	1.891	2.12	1.594	2.45
	1.923	1.64	1.883	1.445	2.37
	1.441	1.781	2.233	1.689	2.151
Std Dev	6.989951	4.400082	4.534633	3.204592	6.337651
CO_2 + CH_4	1.567	1.094	1.642	1.288	1.768
	1.59	1.465	1.384	1.508	2.21
	1.871	1.487	1.787	1.128	1.842
	1.96	1.193	1.941	1.514	2.157
Std Dev	5.854172	5.801873	6.998401	5.517289	6.548768
CO_2 + CH_4 + R	1.35	1.382	1.797	0.989	2.275
	1.728	1.656	2.087	1.562	1.905
	1.71	1.67	1.82	1.42	1.554
	1.841	1.4	1.897	1.6	2.26
Std Dev	6.289282	4.647662	3.88911	8.274949	10.10775
R	1.771	1.514	2.096	1.252	1.805
	1.833	1.277	2.011	1.61	2.358
	1.757	1.417	1.786	1.087	1.725
	1.826	1.141	1.976	1.51	2.097
Std Dev	1.133075	4.816759	3.867805	7.055574	8.548402
CTRL	1.729	1.293	1.981	1.321	2.228
	1.686	1.271	1.706	1.595	2.307
	1.763	1.549	1.987	1.347	2.059
	1.684	1.351	1.963	1.507	2.171
Std Dev	1.118635	3.739658	4.014489	3.863299	3.082032

Table m: Replicate optical density values for determination of urease enzyme activity in spring at elevated temperature (Fig 7 a)

Day/Treatment	0	15	25	40	60
CO_2	0.628	0.406	0.243	0.415	0.471
	0.545	0.27	0.228	0.404	0.429
	0.26	0.267	0.206	0.461	0.499
	0.497	0.351	0.313	0.416	0.509
Std Dev	4.573709	19.51546	13.39272	7.316595	10.37422
CO_2 + R	0.52	0.28	0.335	0.507	0.409
	0.425	0.192	0.329	0.483	0.445
	0.405	0.445	0.284	0.531	0.435
	0.251	0.32	0.311	0.56	0.525
Std Dev	3.227792	30.44798	6.632357	9.546926	14.4901
CO_2 + CH_4	0.476	0.465	0.303	0.494	0.535
	0.45	0.275	0.344	0.603	0.572
	0.784	0.498	0.235	0.497	0.37
	0.526	0.406	0.295	0.484	0.411
Std Dev	4.440003	28.48275	13.02186	16.20495	28.0314
CO_2 + CH_4 + R	0.452	0.442	0.308	0.422	0.406
	0.485	0.195	0.261	0.484	0.382
	0.571	0.469	0.348	0.517	0.497
	0.453	0.537	0.27	0.492	0.476
Std Dev	16.72148	4.324249	11.52222	11.6909	15.92267
R	0.461	0.377	0.368	0.431	0.434
	0.631	0.323	0.316	0.469	0.475
	0.625	0.367	0.299	0.466	0.324
	0.5	0.287	0.231	0.464	0.469
Std Dev	0.086669	0.041581	0.056548	0.017786	0.07004
CTRL	0.411	0.086	0.357	0.497	0.432
	0.384	0.333	0.334	0.435	0.558
	0.439	0.516	0.382	0.512	0.367
	0.568	0.334	0.348	0.405	0.494
Std Dev	23.60443	5.113986	5.847993	14.6778	23.74723

Table n: Replicate optical density values for determination of urease enzyme activity in spring at ambient temperature (Fig 7 b)

Day/Treatment	0	15	25	40	60
CO_2	0.686	0.821	0.274	0.557	0.368
	0.683	0.293	0.343	0.543	0.395
	0.569	0.16	0.351	0.54	0.348
	0.619	0.495	0.321	0.575	0.448
Std Dev	16.25299	83.31378	10.01587	4.630895	12.55603
CO_2 + R	0.526	0.299	0.446	0.685	0.469
	0.646	0.421	0.499	0.651	0.573
	0.687	0.582	0.444	0.694	0.248
	0.696	0.469	0.458	0.494	0.486
Std Dev	22.66739	33.95275	7.41296	26.9945	40.08371
CO_2 + CH_4	0.366	0.545	0.431	0.6	0.4
	0.592	0.408	0.428	0.578	0.533
	0.322	0.26	0.465	0.631	0.482
	0.674	0.276	0.459	0.512	0.503
Std Dev	49.56914	38.49581	5.492859	14.60712	16.50592
CO_2 + CH_4 + R	0.703	0.326	0.428	0.608	0.391
	0.585	0.337	0.423	0.625	0.427
	0.419	0.714	0.398	0.682	0.388
	0.542	0.519	0.381	0.483	0.528
Std Dev	33.97004	52.96441	6.374863	24.29347	18.95713
R	0.509	0.243	0.362	0.659	0.365
	0.577	0.461	0.357	0.534	0.394
	0.595	0.419	0.327	0.42	0.438
	0.551	0.431	0.402	0.563	0.471
Std Dev	10.8125	28.55973	8.928129	28.51023	13.57628
CTRL	0.398	0.197	0.349	0.529	0.321
	0.395	0.377	0.402	0.628	0.409
	0.639	0.217	0.43	0.53	0.376
	0.628	0.368	0.362	0.539	0.414
Std Dev	39.65861	27.79933	10.75642	13.86876	12.39602

Table o: Replicate optical density values for determination of urease enzyme activity in summer at elevated temperature (Fig 8 a)

Day/Treatment	0	15	25	40	60
CO_2	0.219	0.246	0.168	0.303	0.193
	0.221	0.307	0.169	0.221	0.242
	0.169	0.257	0.191	0.224	0.301
	0.2	0.293	0.17	0.18	0.234
Std Dev	7.429793	8.917865	3.400209	15.84878	1.372256
CO_2 + R	0.162	0.241	0.169	0.249	0.135
	0.18	0.257	0.175	0.259	0.218
	0.161	0.216	0.151	0.192	0.287
	0.13	0.249	0.161	0.201	0.147
Std Dev	6.399064	5.470382	3.203551	10.37268	2.171351
CO_2 + CH_4	0.188	0.259	0.189	0.197	0.264
	0.166	0.241	0.179	0.192	0.2
	0.158	0.244	0.125	0.217	0.185
	0.21	0.199	0.265	0.2	0.231
Std Dev	7.21403	7.932878	1.777168	3.343848	10.79944
CO_2 + CH_4 + R	0.164	0.162	0.273	0.105	0.251
	0.169	0.206	0.217	0.116	0.211
	0.205	0.273	0.206	0.169	0.235
	0.208	0.281	0.194	0.22	0.182
Std Dev	7.156722	1.748237	10.77448	1.632818	9.266248
R	0.135	0.28	0.123	0.116	0.128
	0.181	0.266	0.168	0.146	0.243
	0.21	0.266	0.135	0.127	0.187
	0.192	0.264	0.178	0.17	0.184
Std Dev	9.859562	2.279193	8.073872	7.296435	1.447737
CTRL	0.2	0.34	0.147	0.159	0.21
	0.222	0.303	0.205	0.219	0.37
	0.25	0.288	0.108	0.18	0.236
	0.196	0.34	0.189	0.206	0.263
Std Dev	7.642573	8.141751	13.4589	8.260037	2.165508

Table p: Replicate optical density values for determination of urease enzyme activity in summer at ambient temperature (Fig 8 b)

Day/Treatment	0	15	25	40	60
CO_2	0.2	0.277	0.107	0.192	0.375
	0.181	0.278	0.108	0.167	0.19
	0.156	0.269	0.104	0.237	0.226
	0.181	0.273	0.118	0.289	0.24
Std Dev	5.562967	1.267877	1.872971	1.653996	2.495509
CO_2 + R	0.144	0.306	0.136	0.243	0.198
	0.181	0.245	0.131	0.273	0.221
	0.171	0.237	0.123	0.217	0.196
	0.129	0.32	0.193	0.217	0.343
Std Dev	7.387037	1.297388	9.849517	8.21582	2.155557
CO_2 + CH_4	0.183	0.12	0.1	0.171	0.34
	0.195	0.23	0.161	0.132	0.308
	0.299	0.14	0.141	0.18	0.212
	0.159	0.151	0.129	0.173	0.225
Std Dev	9.062451	1.486997	7.864706	6.682958	1.927294
CO_2 + CH_4 + R	0.157	0.312	0.254	0.213	0.323
	0.205	0.262	0.268	0.167	0.254
	0.23	0.211	0.268	0.227	0.24
	0.199	0.121	0.181	0.153	0.257
Std Dev	9.341148	2.513232	1.285215	10.96534	1.143066
R	0.186	0.136	0.101	0.2	0.338
	0.205	0.145	0.168	0.109	0.258
	0.22	0.227	0.132	0.103	0.129
	0.242	0.171	0.227	0.129	0.213
Std Dev	7.300775	1.262338	1.667846	13.74072	2.690722
CTRL	0.184	0.229	0.28	0.213	0.147
	0.189	0.208	0.263	0.147	0.192
	0.161	0.296	0.234	0.149	0.189
	0.218	0.279	0.152	0.202	0.216
Std Dev	7.220613	1.274232	1.750025	10.68258	8.838035

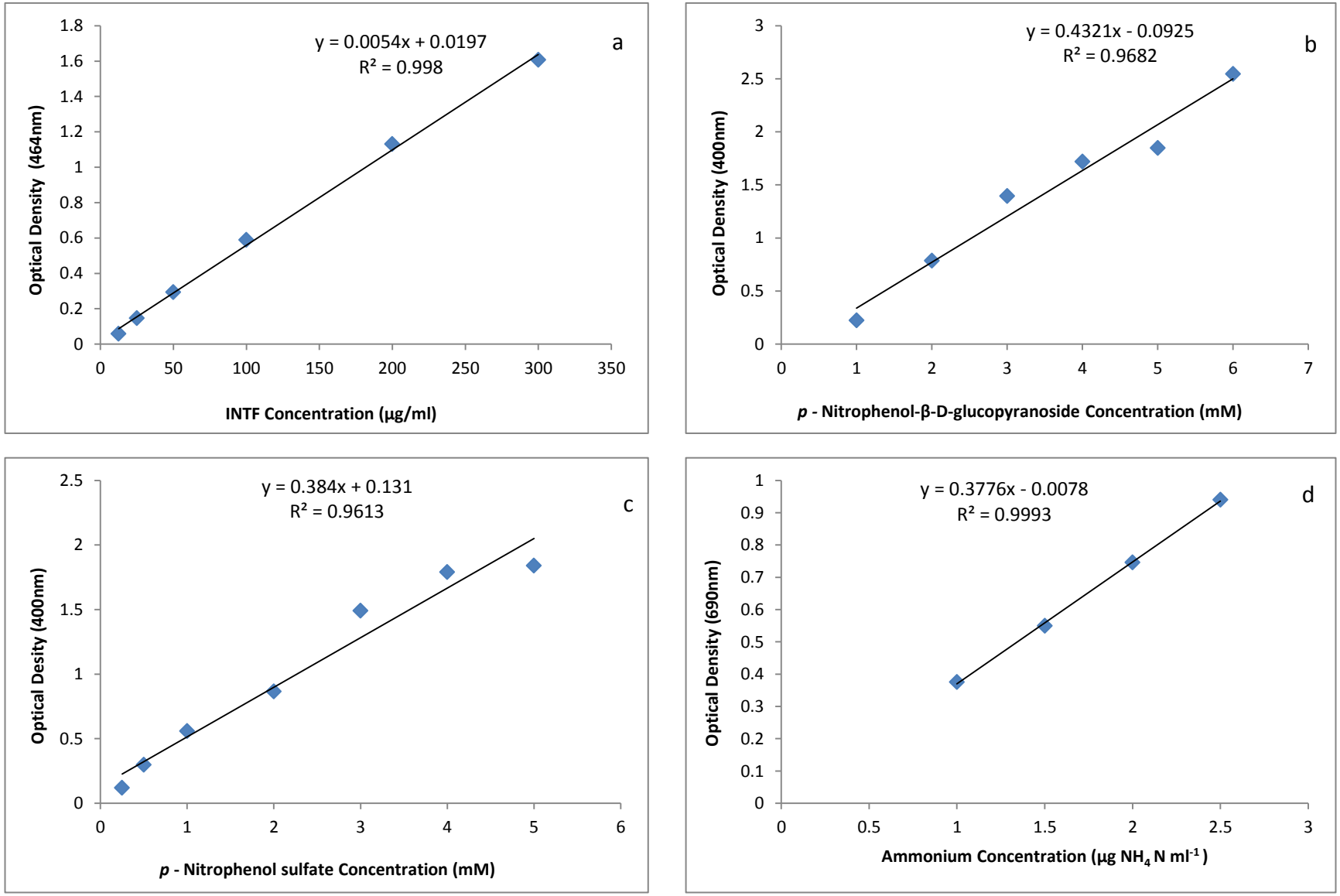

Fig 1: Standard curves for determination of a) dehydrogenase, b) β- glucosidase, c) arylsulphatase and d) urease enzyme values

STATISTICAL DATA

Descriptive Statistics and Pairwise Comparison Analysis among global change factors during spring at elevated temperature for dehydrogenase enzyme activity

Descriptive Statistics

	TIME	Mean	Std. Deviation	N
CO2	0	263.3905	13.50079	4
	1	374.363	12.9395	4
	2	142.9537	44.48656	4
	3	206.1882	49.96243	4
	4	196.0894	18.37929	4
	Total	236.597	85.7218	20
CO2R	0	207.4883	19.95968	4
	1	229.3292	15.44255	4
	2	204.3682	37.48938	4
	3	150.078	10.27678	4
	4	134.5294	23.29477	4
	Total	185.1586	42.62581	20
CH4	0	201.9761	34.65961	4
	1	186.2715	1.52263	4
	2	165.6266	39.2462	4
	3	138.3775	13.17423	4
	4	94.6438	7.84456	4
	Total	157.3791	44.53901	20
CH4R	0	207.1763	32.65151	4
	1	237.0255	11.95807	4
	2	140.6136	33.25418	4
	3	151.5861	14.00572	4
	4	116.9527	38.88847	4
	Total	170.6708	52.12629	20
CO2CH4	0	206.7083	31.75768	4
	1	256.0062	16.04633	4
	2	140.5616	26.54613	4
	3	142.6417	24.62037	4
	4	127.5091	18.26559	4
	Total	174.6854	54.74971	20
CO2CH4R	0	180.0312	9.87163	4
	1	233.9054	14.33737	4
	2	140.0416	26.04842	4
	3	137.0775	13.83881	4
	4	120.0208	25.47932	4
	Total	162.2153	45.25369	20
R	0	227.4051	39.11663	4
	1	204.5242	16.14534	4
	2	139.0016	5.73691	4
	3	127.5611	10.80176	4
	4	114.2486	6.12124	4
	Total	162.5481	49.33916	20
CPG	0	190.2756	33.94999	4
	1	195.3198	19.26324	4
	2	109.3604	19.64293	4
	3	108.1643	20.42184	4
	4	110.7644	7.25822	4
	Total	142.7769	46.20507	20

Pairwise Comparisons

Measure: MEASURE_1

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for	
					Lower Bound	Upper Bound
1	2	51.438*	9.031	0.001	17.228	85.649
	3	79.218*	7.532	0	50.683	107.752
	4	65.926*	7.54	0	37.363	94.489
	5	61.912*	9.573	0	25.649	98.174
	6	74.382*	8.829	0	40.937	107.826
	7	74.049*	8.64	0	41.317	106.781
	8	93.820*	6.972	0	67.409	120.232
2	1	-51.438*	9.031	0.001	-85.649	-17.228
	3	27.78	9.403	0.276	-7.839	63.398
	4	14.488	7.019	1	-12.1	41.076
	5	10.473	6.267	1	-13.266	34.213
	6	22.943*	5.955	0.044	0.383	45.503
	7	22.611	7.792	0.307	-6.907	52.128
	8	42.382*	7.193	0.001	15.132	69.632
3	1	-79.218*	7.532	0	-107.75	-50.683
	2	-27.78	9.403	0.276	-63.398	7.839
	4	-13.292	8.176	1	-44.266	17.682
	5	-17.306	6.3	0.419	-41.173	6.561
	6	-4.836	6.042	1	-27.725	18.053
	7	-5.169	6.501	1	-29.797	19.459
	8	14.602	7.908	1	-15.356	44.56
4	1	-65.926*	7.54	0	-94.489	-37.363
	2	-14.488	7.019	1	-41.076	12.1
	3	13.292	8.176	1	-17.682	44.266
	5	-4.015	7.108	1	-30.942	22.913
	6	8.456	6.658	1	-16.766	33.677
	7	8.123	8.209	1	-22.973	39.219
	8	27.894	8.066	0.098	-2.662	58.45
5	1	-61.912*	9.573	0	-98.174	-25.649
	2	-10.473	6.267	1	-34.213	13.266
	3	17.306	6.3	0.419	-6.561	41.173
	4	4.015	7.108	1	-22.913	30.942
	6	12.47	4.155	0.251	-3.271	28.211
	7	12.137	7.889	1	-17.748	42.022
	8	31.908	9.42	0.114	-3.777	67.594
6	1	-74.382*	8.829	0	-107.83	-40.937
	2	-22.943*	5.955	0.044	-45.503	-0.383
	3	4.836	6.042	1	-18.053	27.725
	4	-8.456	6.658	1	-33.677	16.766
	5	-12.47	4.155	0.251	-28.211	3.271
	7	-0.333	5.808	1	-22.333	21.668
	8	19.438	6.919	0.37	-6.772	45.648
7	1	-74.049*	8.64	0	-106.78	-41.317
	2	-22.611	7.792	0.307	-52.128	6.907
	3	5.169	6.501	1	-19.459	29.797
	4	-8.123	8.209	1	-39.219	22.973
	5	-12.137	7.889	1	-42.022	17.748
	6	0.333	5.808	1	-21.668	22.333
	8	19.771*	5.135	0.044	0.317	39.225
8	1	-93.820*	6.972	0	-120.23	-67.409
	2	-42.382*	7.193	0.001	-69.632	-15.132
	3	-14.602	7.908	1	-44.56	15.356
	4	-27.894	8.066	0.098	-58.45	2.662
	5	-31.908	9.42	0.114	-67.594	3.777
	6	-19.438	6.919	0.37	-45.648	6.772
	7	-19.771*	5.135	0.044	-39.225	-0.317

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Descriptive Statistics and Pairwise Comparison Analysis among global change factors during spring at ambient temperature for dehydrogenase enzyme activity

Descriptive Statistics

	TIME	Mean	Std. Deviation	N
CO2	0	181.279	40.3473	4
	1	197.4	9.88915	4
	2	108.58	6.95585	4
	3	152.626	20.5809	4
	4	95.4238	26.0476	4
	Total	147.062	45.9751	20
CO2R	0	197.608	23.2904	4
	1	295.32	18.1612	4
	2	187.884	21.2046	4
	3	130.733	25.0529	4
	4	177.327	92.046	4
	Total	197.774	68.5828	20
CH4	0	220.801	48.9895	4
	1	220.697	17.8898	4
	2	114.561	17.6709	4
	3	119.241	27.3908	4
	4	133.749	36.5165	4
	Total	161.81	57.3386	20
CH4R	0	195.372	13.3396	4
	1	222.569	15.7706	4
	2	126.521	14.4602	4
	3	109.984	6.8999	4
	4	114.561	15.7844	4
	Total	153.801	48.9047	20
CO2CH4	0	172.283	20.7932	4
	1	214.145	23.8133	4
	2	122.621	9.45065	4
	3	136.61	11.8455	4
	4	130.889	40.8294	4
	Total	155.309	40.8755	20
CO2CH4R	0	180.239	29.9543	4
	1	236.454	13.189	4
	2	145.19	12.4566	4
	3	106.708	15.5106	4
	4	131.929	29.4981	4
	Total	160.104	49.9575	20
R	0	159.178	18.129	4
	1	223.141	16.0697	4
	2	125.533	33.4932	4
	3	92.5117	9.66959	4
	4	101.352	10.5478	4
	Total	140.343	51.6523	20
CPG	0	158.398	15.5245	4
	1	171.087	37.276	4
	2	135.309	25.8016	4
	3	109.672	27.3613	4
	4	145.554	30.5012	4
	Total	144.004	33.0191	20

Pairwise Comparisons

Measure: MEASURE_1

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for	
					Lower Bound	Upper Bound
1	2	-50.712*	12.589	0.031	-98.402	-3.023
	3	-14.748	9.061	1	-49.072	19.576
	4	-6.739	6.492	1	-31.332	17.853
	5	-8.248	7.653	1	-37.24	20.745
	6	-13.042	4.603	0.352	-30.478	4.394
	7	6.719	7.417	1	-21.378	34.815
	8	3.058	7.462	1	-25.209	31.324
2	1	50.712*	12.589	0.031	3.023	98.402
	3	35.965	14.704	0.763	-19.738	91.667
	4	43.973*	11.594	0.05	0.053	87.893
	5	42.465*	8.258	0.003	11.181	73.748
	6	37.67	10.034	0.054	-0.34	75.68
	7	57.431*	10.065	0.001	19.303	95.559
	8	53.770*	12.205	0.014	7.536	100.005
3	1	14.748	9.061	1	-19.576	49.072
	2	-35.965	14.704	0.763	-91.667	19.738
	4	8.008	6.959	1	-18.355	34.372
	5	6.5	9.271	1	-28.622	41.622
	6	1.706	10.368	1	-37.57	40.982
	7	21.466	8.006	0.479	-8.863	51.796
	8	17.806	10.046	1	-20.251	55.862
4	1	6.739	6.492	1	-17.853	31.332
	2	-43.973*	11.594	0.05	-87.893	-0.053
	3	-8.008	6.959	1	-34.372	18.355
	5	-1.508	6.715	1	-26.946	23.93
	6	-6.303	6.519	1	-30.997	18.392
	7	13.458	6.393	1	-10.761	37.677
	8	9.797	7.44	1	-18.386	37.981
5	1	8.248	7.653	1	-20.745	37.24
	2	-42.465*	8.258	0.003	-73.748	-11.181
	3	-6.5	9.271	1	-41.622	28.622
	4	1.508	6.715	1	-23.93	26.946
	6	-4.795	6.449	1	-29.223	19.634
	7	14.966	6.89	1	-11.133	41.066
	8	11.305	9.157	1	-23.385	45.996
6	1	13.042	4.603	0.352	-4.394	30.478
	2	-37.67	10.034	0.054	-75.68	0.34
	3	-1.706	10.368	1	-40.982	37.57
	4	6.303	6.519	1	-18.392	30.997
	5	4.795	6.449	1	-19.634	29.223
	7	19.761	6.9	0.331	-6.38	45.901
	8	16.1	6.474	0.704	-8.426	40.626
7	1	-6.719	7.417	1	-34.815	21.378
	2	-57.431*	10.065	0.001	-95.559	-19.303
	3	-21.466	8.006	0.479	-51.796	8.863
	4	-13.458	6.393	1	-37.677	10.761
	5	-14.966	6.89	1	-41.066	11.133
	6	-19.761	6.9	0.331	-45.901	6.38
	8	-3.661	8.485	1	-35.802	28.481
8	1	-3.058	7.462	1	-31.324	25.209
	2	-53.770*	12.205	0.014	-100.01	-7.536
	3	-17.806	10.046	1	-55.862	20.251
	4	-9.797	7.44	1	-37.981	18.386
	5	-11.305	9.157	1	-45.996	23.385
	6	-16.1	6.474	0.704	-40.626	8.426
	7	3.661	8.485	1	-28.481	35.802

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Descriptive Statistics and Pairwise Comparison Analysis among global change factors during summer at elevated temperature for dehydrogenase enzyme activity

Descriptive Statistics

	TIME	Mean	Std. Deviation	N
CO2	0	257.886	18.466	4
	1	110.349	4.65166	4
	2	110.57	15.4302	4
	3	203.21	17.4622	4
	4	85.9989	14.3474	4
	Total	153.603	68.7673	20
CO2R	0	181.572	9.13412	4
	1	73.99	2.7707	4
	2	70.4483	9.34449	4
	3	107.194	12.3593	4
	4	39.7344	11.4058	4
	Total	94.5877	50.4443	20
CH4	0	183.841	16.9293	4
	1	74.3774	9.58502	4
	2	78.0299	8.74938	4
	3	104.261	8.05945	4
	4	34.5877	3.3327	4
	Total	95.0194	51.7884	20
CH4R	0	202.656	11.0834	4
	1	78.1959	9.06411	4
	2	83.2872	10.8233	4
	3	101.273	10.5689	4
	4	40.9519	11.5863	4
	Total	101.273	56.5673	20
CO2CH4	0	199.225	10.5116	4
	1	58.2734	11.4153	4
	2	74.2114	6.18493	4
	3	83.1212	5.23954	4
	4	28.3343	2.23107	4
	Total	88.6331	60.2805	20
CO2CH4R	0	169.175	81.571	4
	1	70.0055	8.86736	4
	2	96.6242	8.32463	4
	3	100.443	5.37533	4
	4	48.6442	19.6746	4
	Total	96.9784	53.7079	20
R	0	237.244	65.6282	4
	1	70.9463	4.58802	4
	2	96.8456	33.7813	4
	3	99.9447	5.51	4
	4	36.1926	3.07459	4
	Total	108.235	76.1635	20
CPG	0	205.645	11.2458	4
	1	78.8046	5.8605	4
	2	89.8727	5.75646	4
	3	102.767	14.3538	4
	4	33.7576	7.21012	4
	Total	102.169	58.8017	20

Pairwise Comparisons

Measure: MEASURE_1

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for	
					Lower Bound	Upper Bound
1	2	59.015*	2.962	0	47.796	70.234
	3	58.583*	4.828	0	40.292	76.874
	4	52.330*	3.562	0	38.836	65.823
	5	64.970*	3.769	0	50.693	79.246
	6	56.624*	9.313	0.001	21.343	91.905
	7	45.368*	7.465	0.001	17.09	73.646
	8	51.433*	4.234	0	35.396	67.471
2	1	-59.015*	2.962	0	-70.234	-47.796
	3	-0.432	3.477	1	-13.605	12.741
	4	-6.685	2.251	0.267	-15.213	1.843
	5	5.955	2.582	1	-3.827	15.736
	6	-2.391	7.896	1	-32.302	27.521
	7	-13.647	7.537	1	-42.199	14.905
	8	-7.582	3.361	1	-20.315	5.151
3	1	-58.583*	4.828	0	-76.874	-40.292
	2	0.432	3.477	1	-12.741	13.605
	4	-6.253	3.59	1	-19.855	7.348
	5	6.386	3.273	1	-6.012	18.785
	6	-1.959	9.008	1	-36.082	32.164
	7	-13.215	8.464	1	-45.28	18.85
	8	-7.15	3.087	0.983	-18.845	4.545
4	1	-52.330*	3.562	0	-65.823	-38.836
	2	6.685	2.251	0.267	-1.843	15.213
	3	6.253	3.59	1	-7.348	19.855
	5	12.640*	2.539	0.005	3.023	22.257
	6	4.294	8.634	1	-28.413	37.001
	7	-6.962	7.496	1	-35.359	21.435
	8	-0.897	2.911	1	-11.925	10.132
5	1	-64.970*	3.769	0	-79.246	-50.693
	2	-5.955	2.582	1	-15.736	3.827
	3	-6.386	3.273	1	-18.785	6.012
	4	-12.640*	2.539	0.005	-22.257	-3.023
	6	-8.345	8.529	1	-40.656	23.965
	7	-19.602	7.907	0.715	-49.556	10.352
	8	-13.536*	2.571	0.003	-23.274	-3.798
6	1	-56.624*	9.313	0.001	-91.905	-21.343
	2	2.391	7.896	1	-27.521	32.302
	3	1.959	9.008	1	-32.164	36.082
	4	-4.294	8.634	1	-37.001	28.413
	5	8.345	8.529	1	-23.965	40.656
	7	-11.256	8.504	1	-43.473	20.961
	8	-5.191	9.453	1	-41.002	30.62
7	1	-45.368*	7.465	0.001	-73.646	-17.09
	2	13.647	7.537	1	-14.905	42.199
	3	13.215	8.464	1	-18.85	45.28
	4	6.962	7.496	1	-21.435	35.359
	5	19.602	7.907	0.715	-10.352	49.556
	6	11.256	8.504	1	-20.961	43.473
	8	6.065	7.887	1	-23.814	35.944
8	1	-51.433*	4.234	0	-67.471	-35.396
	2	7.582	3.361	1	-5.151	20.315
	3	7.15	3.087	0.983	-4.545	18.845
	4	0.897	2.911	1	-10.132	11.925
	5	13.536*	2.571	0.003	3.798	23.274
	6	5.191	9.453	1	-30.62	41.002
	7	-6.065	7.887	1	-35.944	23.814

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Descriptive Statistics and Pairwise Comparison Analysis among global change factors during summer at ambient temperature for dehydrogenase enzyme activity

Descriptive Statistics

	TIME	Mean	Std. Deviation	N
CO2	0	181.738	10.6827	4
	1	117.93	9.37503	4
	2	132.429	13.1395	4
	3	135.584	2.41392	4
	4	66.4084	12.1573	4
	Total	126.818	39.0655	20
CO2R	0	234.034	38.7203	4
	1	106.973	11.3061	4
	2	149.308	15.7487	4
	3	158.439	18.7296	4
	4	63.8628	6.00096	4
	Total	142.524	61.2525	20
CH4	0	199.391	43.1742	4
	1	58.8821	7.07289	4
	2	81.793	9.10793	4
	3	84.9474	4.35844	4
	4	38.8489	4.42861	4
	Total	92.7726	60.0567	20
CH4R	0	93.4698	4.02123	4
	1	35.2518	9.35497	4
	2	57.554	6.2254	4
	3	14.3885	2.7788	4
	4	11.9535	3.81005	4
	Total	42.5235	31.5359	20
CO2CH4	0	183.287	4.29759	4
	1	112.12	9.28905	4
	2	163.807	15.0562	4
	3	57.9413	15.3199	4
	4	59.2142	10.6321	4
	Total	115.274	54.1545	20
CO2CH4R	0	178.03	10.0242	4
	1	94.798	16.0086	4
	2	162.424	23.5522	4
	3	73.2153	8.31702	4
	4	53.6801	5.11651	4
	Total	112.429	52.0285	20
R	0	193.359	11.5538	4
	1	128.002	12.9895	4
	2	163.42	11.1181	4
	3	95.6835	8.33566	4
	4	63.9734	10.3266	4
	Total	128.888	48.3794	20
CPG	0	212.396	18.3622	4
	1	179.524	5.30152	4
	2	240.952	26.217	4
	3	119.757	7.00094	4
	4	84.394	13.1891	4
	Total	167.405	61.0403	20

Pairwise Comparisons

Measure: MEASURE_1

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for	
					Lower Bound	Upper Bound
1	2	-15.706	4.539	0.098	-32.902	1.491
	3	34.045*	5.131	0	14.61	53.481
	4	84.294*	2.004	0	76.701	91.888
	5	11.544	3.394	0.111	-1.314	24.402
	6	14.388*	3.72	0.043	0.296	28.481
	7	-2.07	3.293	1	-14.544	10.405
	8	-40.587*	4.468	0	-57.512	-23.662
2	1	15.706	4.539	0.098	-1.491	32.902
	3	49.751*	5.054	0	30.606	68.896
	4	100.000*	4.867	0	81.564	118.436
	5	27.250*	5.547	0.005	6.236	48.263
	6	30.094*	5.129	0.001	10.663	49.525
	7	13.636	5.376	0.639	-6.731	34.002
	8	-24.881*	6.454	0.044	-49.329	-0.433
3	1	-34.045*	5.131	0	-53.481	-14.61
	2	-49.751*	5.054	0	-68.896	-30.606
	4	50.249*	4.595	0	32.844	67.655
	5	-22.501*	5.186	0.016	-42.148	-2.855
	6	-19.657*	4.487	0.015	-36.655	-2.659
	7	-36.115*	5.947	0.001	-58.644	-13.587
	8	-74.632*	6.894	0	-100.75	-48.516
4	1	-84.294*	2.004	0	-91.888	-76.701
	2	-100.000*	4.867	0	-118.44	-81.564
	3	-50.249*	4.595	0	-67.655	-32.844
	5	-72.750*	2.348	0	-81.644	-63.857
	6	-69.906*	3.44	0	-82.939	-56.873
	7	-86.364*	2.826	0	-97.071	-75.658
	8	-124.881*	3.733	0	-139.02	-110.74
5	1	-11.544	3.394	0.111	-24.402	1.314
	2	-27.250*	5.547	0.005	-48.263	-6.236
	3	22.501*	5.186	0.016	2.855	42.148
	4	72.750*	2.348	0	63.857	81.644
	6	2.844	3.957	1	-12.147	17.836
	7	-13.614*	3.251	0.022	-25.929	-1.299
	8	-52.131*	5.206	0	-71.852	-32.409
6	1	-14.388*	3.72	0.043	-28.481	-0.296
	2	-30.094*	5.129	0.001	-49.525	-10.663
	3	19.657*	4.487	0.015	2.659	36.655
	4	69.906*	3.44	0	56.873	82.939
	5	-2.844	3.957	1	-17.836	12.147
	7	-16.458*	4.283	0.045	-32.683	-0.234
	8	-54.975*	5.325	0	-75.148	-34.802
7	1	2.07	3.293	1	-10.405	14.544
	2	-13.636	5.376	0.639	-34.002	6.731
	3	36.115*	5.947	0.001	13.587	58.644
	4	86.364*	2.826	0	75.658	97.071
	5	13.614*	3.251	0.022	1.299	25.929
	6	16.458*	4.283	0.045	0.234	32.683
	8	-38.517*	3.463	0	-51.634	-25.4
8	1	40.587*	4.468	0	23.662	57.512
	2	24.881*	6.454	0.044	0.433	49.329
	3	74.632*	6.894	0	48.516	100.748
	4	124.881*	3.733	0	110.739	139.023
	5	52.131*	5.206	0	32.409	71.852
	6	54.975*	5.325	0	34.802	75.148
	7	38.517*	3.463	0	25.4	51.634

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Descriptive Statistics and Pairwise Comparison Analysis among global change factors during spring at elevated temperature for β- glucosidase enzyme activity

Descriptive Statistics

	TIME	Mean	Std. Deviation	N
CO2	0	48.9775	23.7355	4
	1	57.8982	1.56439	4
	2	60.0698	1.42762	4
	3	39.4152	3.34888	4
	4	60.514	0.18726	4
	Total	53.3749	12.6889	20
CO2R	0	59.3603	1.7192	4
	1	51.84	5.49771	4
	2	60.1623	1.32476	4
	3	29.8591	4.33757	4
	4	54.2584	3.94529	4
	Total	51.096	11.8237	20
CH4	0	57.9537	1.94625	4
	1	49.1749	6.07782	4
	2	57.0962	2.48158	4
	3	31.8456	3.88928	4
	4	55.301	2.00061	4
	Total	50.2743	10.4742	20
CH4R	0	55.0172	4.05506	4
	1	45.3562	4.86333	4
	2	58.4843	1.05756	4
	3	27.5394	9.07679	4
	4	49.9091	6.73213	4
	Total	47.2612	12.2465	20
CO2CH4	0	55.6896	1.77529	4
	1	53.296	3.27168	4
	2	60.2117	0.93707	4
	3	37.5151	1.49811	4
	4	58.3239	1.53824	4
	Total	53.0072	8.48484	20
CO2CH4R	0	54.1288	1.64264	4
	1	52.0313	5.05254	4
	2	58.4905	1.18644	4
	3	30.001	11.1872	4
	4	57.2011	1.84808	4
	Total	50.3705	11.8147	20
R	0	54.7457	3.83801	4
	1	44.2642	5.07143	4
	2	55.5292	0.94578	4
	3	25.7195	13.165	4
	4	52.9998	3.71977	4
	Total	46.6517	12.9765	20
CPG	0	56.214	2.38997	4
	1	48.2249	6.22743	4
	2	55.7266	1.3799	4
	3	33.1966	4.15225	4
	4	55.2269	2.71723	4
	Total	49.7178	9.59278	20

Pairwise Comparisons

Measure: MEASURE_1

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for	
					Lower Bound	Upper Bound
1	2	2.279	2.577	1	-7.484	12.042
	3	3.101	2.56	1	-6.596	12.798
	4	6.114	2.471	0.721	-3.245	15.473
	5	0.368	2.531	1	-9.22	9.955
	6	3.004	2.712	1	-7.27	13.279
	7	6.723	2.786	0.814	-3.831	17.278
	8	3.657	2.302	1	-5.064	12.379
2	1	-2.279	2.577	1	-12.042	7.484
	3	0.822	1.314	1	-4.158	5.801
	4	3.835	1.849	1	-3.171	10.84
	5	-1.911	1.155	1	-6.285	2.463
	6	0.726	1.716	1	-5.775	7.226
	7	4.444	1.627	0.432	-1.719	10.607
	8	1.378	1.207	1	-3.196	5.952
3	1	-3.101	2.56	1	-12.798	6.596
	2	-0.822	1.314	1	-5.801	4.158
	4	3.013	1.339	1	-2.061	8.087
	5	-2.733	0.756	0.072	-5.599	0.133
	6	-0.096	1.045	1	-4.055	3.862
	7	3.623	1.854	1	-3.402	10.647
	8	0.556	1.242	1	-4.148	5.261
4	1	-6.114	2.471	0.721	-15.473	3.245
	2	-3.835	1.849	1	-10.84	3.171
	3	-3.013	1.339	1	-8.087	2.061
	5	-5.746*	1.155	0.005	-10.123	-1.369
	6	-3.109	1.718	1	-9.619	3.4
	7	0.61	2.066	1	-7.216	8.435
	8	-2.457	1.606	1	-8.54	3.626
5	1	-0.368	2.531	1	-9.955	9.22
	2	1.911	1.155	1	-2.463	6.285
	3	2.733	0.756	0.072	-0.133	5.599
	4	5.746*	1.155	0.005	1.369	10.123
	6	2.637	1.153	1	-1.729	7.003
	7	6.356*	1.444	0.014	0.884	11.827
	8	3.289*	0.827	0.034	0.157	6.422
6	1	-3.004	2.712	1	-13.279	7.27
	2	-0.726	1.716	1	-7.226	5.775
	3	0.096	1.045	1	-3.862	4.055
	4	3.109	1.718	1	-3.4	9.619
	5	-2.637	1.153	1	-7.003	1.729
	7	3.719	2.112	1	-4.283	11.721
	8	0.653	1.392	1	-4.622	5.927
7	1	-6.723	2.786	0.814	-17.278	3.831
	2	-4.444	1.627	0.432	-10.607	1.719
	3	-3.623	1.854	1	-10.647	3.402
	4	-0.61	2.066	1	-8.435	7.216
	5	-6.356*	1.444	0.014	-11.827	-0.884
	6	-3.719	2.112	1	-11.721	4.283
	8	-3.066	1.383	1	-8.306	2.174
8	1	-3.657	2.302	1	-12.379	5.064
	2	-1.378	1.207	1	-5.952	3.196
	3	-0.556	1.242	1	-5.261	4.148
	4	2.457	1.606	1	-3.626	8.54
	5	-3.289*	0.827	0.034	-6.422	-0.157
	6	-0.653	1.392	1	-5.927	4.622
	7	3.066	1.383	1	-2.174	8.306

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Descriptive Statistics and Pairwise Comparison Analysis among global change factors during spring at ambient temperature for β-glucosidase enzyme activity

Descriptive Statistics

	TIME	Mean	Std. Deviation	N
CO2	0	62.3956	0.93001	4
	1	52.0559	1.99313	4
	2	60.5942	1.31947	4
	3	39.3658	6.88842	4
	4	61.0939	0.46924	4
	Total	55.1011	9.36797	20
CO2R	0	61.9884	0.9414	4
	1	62.2784	1.84471	4
	2	62.1982	0.77369	4
	3	55.6464	1.85619	4
	4	60.5201	0.29509	4
	Total	60.5263	2.83329	20
CH4	0	58.984	5.46983	4
	1	57.3862	3.61107	4
	2	61.2296	0.95326	4
	3	44.9182	14.3901	4
	4	60.7669	0.37829	4
	Total	56.657	8.82414	20
CH4R	0	62.6177	1.32514	4
	1	54.7025	3.93019	4
	2	61.6799	0.2853	4
	3	50.3532	3.8482	4
	4	60.5325	0.45278	4
	Total	57.9772	5.32808	20
CO2CH4	0	61.8959	1.98045	4
	1	57.1641	3.0802	4
	2	61.7725	1.99394	4
	3	44.9058	10.6182	4
	4	60.7237	0.44436	4
	Total	57.2924	8.00299	20
CO2CH4R	0	62.229	1.72895	4
	1	60.952	0.8927	4
	2	61.0383	3.10069	4
	3	43.1476	19.5302	4
	4	61.2913	0.74669	4
	Total	57.7316	10.8912	20
R	0	62.2228	1.39862	4
	1	56.6212	2.50755	4
	2	61.9761	0.85543	4
	3	45.3932	1.25416	4
	4	61.316	0.7734	4
	Total	57.5058	6.69028	20
CPG	0	60.4523	2.76912	4
	1	51.8339	9.76787	4
	2	61.0013	1.22011	4
	3	40.3159	9.27323	4
	4	60.8224	0.94835	4
	Total	54.8852	9.93474	20

Pairwise Comparisons

Measure: MEASURE_1

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for	
					Lower Bound	Upper Bound
1	2	-5.425*	0.853	0	-8.658	-2.192
	3	-1.556	2.028	1	-9.238	6.126
	4	-2.876	1.102	0.553	-7.052	1.3
	5	-2.191	1.639	1	-8.4	4.018
	6	-2.631	2.161	1	-10.817	5.556
	7	-2.405	0.745	0.158	-5.229	0.419
	8	0.216	1.561	1	-5.699	6.131
2	1	5.425*	0.853	0	2.192	8.658
	3	3.869	1.534	0.657	-1.942	9.681
	4	2.549*	0.457	0.001	0.819	4.279
	5	3.234	1.155	0.378	-1.143	7.611
	6	2.795	1.858	1	-4.242	9.832
	7	3.020*	0.504	0.001	1.112	4.929
	8	5.641*	1.264	0.013	0.852	10.43
3	1	1.556	2.028	1	-6.126	9.238
	2	-3.869	1.534	0.657	-9.681	1.942
	4	-1.32	1.515	1	-7.06	4.42
	5	-0.635	0.814	1	-3.72	2.45
	6	-1.075	2.857	1	-11.897	9.748
	7	-0.849	1.713	1	-7.337	5.64
	8	1.772	1.997	1	-5.794	9.338
4	1	2.876	1.102	0.553	-1.3	7.052
	2	-2.549*	0.457	0.001	-4.279	-0.819
	3	1.32	1.515	1	-4.42	7.06
	5	0.685	1.193	1	-3.835	5.205
	6	0.246	2	1	-7.33	7.821
	7	0.471	0.733	1	-2.305	3.248
	8	3.092	1.134	0.437	-1.205	7.389
5	1	2.191	1.639	1	-4.018	8.4
	2	-3.234	1.155	0.378	-7.611	1.143
	3	0.635	0.814	1	-2.45	3.72
	4	-0.685	1.193	1	-5.205	3.835
	6	-0.439	2.574	1	-10.189	9.31
	7	-0.213	1.249	1	-4.946	4.519
	8	2.407	1.797	1	-4.401	9.215
6	1	2.631	2.161	1	-5.556	10.817
	2	-2.795	1.858	1	-9.832	4.242
	3	1.075	2.857	1	-9.748	11.897
	4	-0.246	2	1	-7.821	7.33
	5	0.439	2.574	1	-9.31	10.189
	7	0.226	2.059	1	-7.575	8.026
	8	2.846	2.713	1	-7.431	13.124
7	1	2.405	0.745	0.158	-0.419	5.229
	2	-3.020*	0.504	0.001	-4.929	-1.112
	3	0.849	1.713	1	-5.64	7.337
	4	-0.471	0.733	1	-3.248	2.305
	5	0.213	1.249	1	-4.519	4.946
	6	-0.226	2.059	1	-8.026	7.575
	8	2.621	1.55	1	-3.252	8.493
8	1	-0.216	1.561	1	-6.131	5.699
	2	-5.641*	1.264	0.013	-10.43	-0.852
	3	-1.772	1.997	1	-9.338	5.794
	4	-3.092	1.134	0.437	-7.389	1.205
	5	-2.407	1.797	1	-9.215	4.401
	6	-2.846	2.713	1	-13.124	7.431
	7	-2.621	1.55	1	-8.493	3.252

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Descriptive Statistics and Pairwise Comparison Analysis among global change factors during summer at elevated temperature for β- glucosidase enzyme activity

Descriptive Statistics

	TIME	Mean	Std. Deviation	N
CO2	0	43.1206	1.48493	4
	1	65.0355	1.34974	4
	2	39.0173	4.53966	4
	3	61.9695	1.43876	4
	4	58.7722	4.80685	4
	Total	53.583	11.1204	20
CO2R	0	38.3149	2.88975	4
	1	59.4484	3.68201	4
	2	28.6574	3.71674	4
	3	58.851	2.04004	4
	4	50.4869	2.71447	4
	Total	47.1517	12.6056	20
CH4	0	47.8607	1.65208	4
	1	66.2763	2.04605	4
	2	33.9424	1.32186	4
	3	63.3745	2.0625	4
	4	55.3977	2.88684	4
	Total	53.3703	12.0917	20
CH4R	0	39.6936	7.64524	4
	1	63.6633	2.2295	4
	2	30.7123	1.09889	4
	3	51.7736	1.64497	4
	4	56.7042	4.54811	4
	Total	48.5094	12.7149	20
CO2CH4	0	43.0156	2.74031	4
	1	60.6564	2.35075	4
	2	32.2157	2.64515	4
	3	53.0736	3.66358	4
	4	53.6579	4.31984	4
	Total	48.5238	10.5595	20
CO2CH4R	0	46.528	4.73531	4
	1	64.6809	1.65577	4
	2	38.9976	2.75036	4
	3	56.1264	3.29604	4
	4	60.6433	1.46943	4
	Total	53.3953	10.0118	20
R	0	40.9804	1.21871	4
	1	64.4315	1.71998	4
	2	34.7828	2.18798	4
	3	50.8611	4.36683	4
	4	59.803	4.3545	4
	Total	50.1717	11.7278	20
CPG	0	52.8897	3.4519	4
	1	67.5894	0.89503	4
	2	40.941	4.71954	4
	3	53.9599	2.41243	4
	4	63.8209	1.68417	4
	Total	55.8402	9.93344	20

Pairwise Comparisons

Measure: MEASURE_1

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for	
					Lower Bound	Upper Bound
1	2	6.431*	0.858	0	3.18	9.682
	3	0.213	0.949	1	-3.384	3.809
	4	5.074*	1.019	0.005	1.213	8.935
	5	5.059*	0.849	0.001	1.843	8.275
	6	0.188	0.944	1	-3.387	3.762
	7	3.411*	0.874	0.039	0.101	6.721
	8	-2.257	1.078	1	-6.34	1.825
2	1	-6.431*	0.858	0	-9.682	-3.18
	3	-6.219*	0.714	0	-8.924	-3.513
	4	-1.358	0.819	1	-4.46	1.745
	5	-1.372	0.511	0.472	-3.306	0.562
	6	-6.244*	0.848	0	-9.455	-3.032
	7	-3.020*	0.72	0.022	-5.748	-0.292
	8	-8.688*	0.842	0	-11.877	-5.5
3	1	-0.213	0.949	1	-3.809	3.384
	2	6.219*	0.714	0	3.513	8.924
	4	4.861*	0.991	0.005	1.109	8.613
	5	4.846*	0.632	0	2.451	7.242
	6	-0.025	0.622	1	-2.381	2.331
	7	3.199*	0.577	0.002	1.014	5.384
	8	-2.47	0.811	0.229	-5.541	0.602
4	1	-5.074*	1.019	0.005	-8.935	-1.213
	2	1.358	0.819	1	-1.745	4.46
	3	-4.861*	0.991	0.005	-8.613	-1.109
	5	-0.014	0.735	1	-2.797	2.768
	6	-4.886	1.36	0.075	-10.038	0.267
	7	-1.662	0.972	1	-5.345	2.02
	8	-7.331*	1.24	0.001	-12.028	-2.633
5	1	-5.059*	0.849	0.001	-8.275	-1.843
	2	1.372	0.511	0.472	-0.562	3.306
	3	-4.846*	0.632	0	-7.242	-2.451
	4	0.014	0.735	1	-2.768	2.797
	6	-4.871*	0.859	0.001	-8.125	-1.618
	7	-1.648	0.69	0.852	-4.26	0.964
	8	-7.316*	1.013	0	-11.155	-3.477
6	1	-0.188	0.944	1	-3.762	3.387
	2	6.244*	0.848	0	3.032	9.455
	3	0.025	0.622	1	-2.331	2.381
	4	4.886	1.36	0.075	-0.267	10.038
	5	4.871*	0.859	0.001	1.618	8.125
	7	3.224*	0.728	0.014	0.468	5.98
	8	-2.445	0.995	0.745	-6.213	1.323
7	1	-3.411*	0.874	0.039	-6.721	-0.101
	2	3.020*	0.72	0.022	0.292	5.748
	3	-3.199*	0.577	0.002	-5.384	-1.014
	4	1.662	0.972	1	-2.02	5.345
	5	1.648	0.69	0.852	-0.964	4.26
	6	-3.224*	0.728	0.014	-5.98	-0.468
	8	-5.668*	0.92	0.001	-9.155	-2.182
8	1	2.257	1.078	1	-1.825	6.34
	2	8.688*	0.842	0	5.5	11.877
	3	2.47	0.811	0.229	-0.602	5.541
	4	7.331*	1.24	0.001	2.633	12.028
	5	7.316*	1.013	0	3.477	11.155
	6	2.445	0.995	0.745	-1.323	6.213
	7	5.668*	0.92	0.001	2.182	9.155

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Descriptive Statistics and Pairwise Comparison Analysis among global change factors during summer at ambient temperature for β-glucosidase enzyme activity

Descriptive Statistics

	TIME	Mean	Std. Deviation	N
CO2	0	46.1932	4.75148	4
	1	67.7535	0.34574	4
	2	49.2723	2.29884	4
	3	48.8061	3.20474	4
	4	59.304	2.08312	4
	Total	54.2658	8.69549	20
CO2R	0	49.4036	4.4052	4
	1	67.1889	0.96596	4
	2	54.7083	3.14705	4
	3	58.5753	3.20288	4
	4	64.4577	1.67653	4
	Total	58.8668	7.10715	20
CH4	0	36.1943	1.11279	4
	1	53.2508	7.62889	4
	2	37.6452	1.7677	4
	3	35.9973	5.41242	4
	4	52.115	3.47298	4
	Total	43.0405	9.06263	20
CH4R	0	25.8343	1.77521	4
	1	32.7803	1.15357	4
	2	36.0302	1.62578	4
	3	36.1089	1.72085	4
	4	34.7237	1.0562	4
	Total	33.0955	4.14398	20
CO2CH4	0	52.3317	4.30464	4
	1	63.9785	2.28017	4
	2	41.5647	1.97131	4
	3	61.8185	2.61144	4
	4	62.8689	2.7	4
	Total	56.5125	9.1365	20
CO2CH4R	0	48.4385	4.20234	4
	1	65.7445	3.82382	4
	2	46.679	4.43257	4
	3	62.9543	1.42801	4
	4	64.1098	2.36441	4
	Total	57.5852	9.00582	20
R	0	51.4913	1.6824	4
	1	67.3858	0.96561	4
	2	47.4274	3.81687	4
	3	65.9218	1.14316	4
	4	66.8212	0.33665	4
	Total	59.8095	8.95892	20
CPG	0	56.8552	1.82207	4
	1	67.9833	0.84998	4
	2	53.2771	3.38441	4
	3	65.9677	1.66131	4
	4	66.4995	0.84073	4
	Total	62.1166	6.29956	20

Pairwise Comparisons

Measure: MEASURE_1

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference	
					Lower Bound	Upper Bound
1	2	-4.601*	0.929	0.005	-8.121	-1.08
	3	11.225*	1.146	0	6.883	15.568
	4	21.170*	0.794	0	18.162	24.178
	5	-2.247	0.771	0.299	-5.167	0.674
	6	-3.319*	0.848	0.039	-6.531	-0.108
	7	-5.544*	0.87	0	-8.84	-2.248
	8	-7.851*	0.661	0	-10.355	-5.347
2	1	4.601*	0.929	0.005	1.08	8.121
	3	15.826*	1.069	0	11.776	19.877
	4	25.771*	0.737	0	22.979	28.564
	5	2.354	0.816	0.317	-0.737	5.446
	6	1.282	0.951	1	-2.323	4.886
	7	-0.943	0.689	1	-3.554	1.668
	8	-3.25	0.871	0.056	-6.551	0.052
3	1	-11.225*	1.146	0	-15.568	-6.883
	2	-15.826*	1.069	0	-19.877	-11.776
	4	9.945*	0.99	0	6.196	13.694
	5	-13.472*	1.175	0	-17.924	-9.02
	6	-14.545*	1.194	0	-19.066	-10.023
	7	-16.769*	1.088	0	-20.891	-12.647
	8	-19.076*	1.034	0	-22.992	-15.16
4	1	-21.170*	0.794	0	-24.178	-18.162
	2	-25.771*	0.737	0	-28.564	-22.979
	3	-9.945*	0.99	0	-13.694	-6.196
	5	-23.417*	0.729	0	-26.177	-20.657
	6	-24.490*	0.821	0	-27.601	-21.378
	7	-26.714*	0.537	0	-28.748	-24.68
	8	-29.021*	0.438	0	-30.679	-27.363
5	1	2.247	0.771	0.299	-0.674	5.167
	2	-2.354	0.816	0.317	-5.446	0.737
	3	13.472*	1.175	0	9.02	17.924
	4	23.417*	0.729	0	20.657	26.177
	6	-1.073	0.721	1	-3.803	1.657
	7	-3.297*	0.708	0.009	-5.978	-0.616
	8	-5.604*	0.733	0	-8.381	-2.827
6	1	3.319*	0.848	0.039	0.108	6.531
	2	-1.282	0.951	1	-4.886	2.323
	3	14.545*	1.194	0	10.023	19.066
	4	24.490*	0.821	0	21.378	27.601
	5	1.073	0.721	1	-1.657	3.803
	7	-2.224	0.746	0.261	-5.05	0.601
	8	-4.531*	0.91	0.005	-7.98	-1.083
7	1	5.544*	0.87	0	2.248	8.84
	2	0.943	0.689	1	-1.668	3.554
	3	16.769*	1.088	0	12.647	20.891
	4	26.714*	0.537	0	24.68	28.748
	5	3.297*	0.708	0.009	0.616	5.978
	6	2.224	0.746	0.261	-0.601	5.05
	8	-2.307	0.709	0.149	-4.992	0.378
8	1	7.851*	0.661	0	5.347	10.355
	2	3.25	0.871	0.056	-0.052	6.551
	3	19.076*	1.034	0	15.16	22.992
	4	29.021*	0.438	0	27.363	30.679
	5	5.604*	0.733	0	2.827	8.381
	6	4.531*	0.91	0.005	1.083	7.98
	7	2.307	0.709	0.149	-0.378	4.992

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Descriptive Statistics and Pairwise Comparison Analysis among global change factors during spring at elevated temperature for arylsulphatase enzyme activity

Descriptive Statistics

	TIME	Mean	Std. Deviation	N
CO2	0	20.8489	1.64911	4
	1	41.0454	2.86159	4
	2	49.0616	5.36051	4
	3	39.6504	3.06874	4
	4	29.1496	4.69999	4
	Total	35.9512	10.6546	20
CO2R	0	21.2862	1.08811	4
	1	29.0316	3.05489	4
	2	55.3565	5.93504	4
	3	23.0282	10.5716	4
	4	20.259	4.88358	4
	Total	29.7923	14.5031	20
CH4	0	22.5632	5.22115	4
	1	29.7187	3.96582	4
	2	53.4409	3.43802	4
	3	27.7476	10.7053	4
	4	18.7599	2.67419	4
	Total	30.4461	13.5157	20
CH4R	0	23.0074	1.60511	4
	1	27.5464	1.07409	4
	2	45.3832	3.13212	4
	3	29.1635	4.10986	4
	4	18.2046	3.62096	4
	Total	28.661	9.78809	20
CO2CH4	0	26.6094	0.95135	4
	1	28.3237	4.12041	4
	2	52.3097	1.48956	4
	3	28.0808	1.91326	4
	4	21.9385	2.35928	4
	Total	31.4524	11.1652	20
CO2CH4R	0	27.4214	3.6238	4
	1	27.3867	3.20999	4
	2	48.9505	4.35006	4
	3	24.3677	10.9305	4
	4	17.8437	0.94915	4
	Total	29.194	11.8846	20
R	0	28.2543	3.08294	4
	1	20.4047	2.972	4
	2	52.5109	5.93735	4
	3	25.9709	2.09036	4
	4	16.2058	2.9219	4
	Total	28.6693	13.3723	20
CPG	0	28.4347	4.00083	4
	1	25.3602	5.56342	4
	2	48.9019	1.51285	4
	3	29.8506	3.25922	4
	4	23.979	6.38686	4
	Total	31.3053	10.0996	20

Pairwise Comparisons

Measure: MEASURE_1

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for	
					Lower Bound	Upper Bound
1	2	6.159	1.987	0.205	-1.37	13.688
	3	5.505	1.494	0.062	-0.154	11.164
	4	7.290*	1.142	0	2.964	11.616
	5	4.499*	0.964	0.009	0.846	8.152
	6	6.757*	1.492	0.011	1.106	12.408
	7	7.282*	1.208	0.001	2.705	11.858
	8	4.646	1.514	0.218	-1.088	10.38
2	1	-6.159	1.987	0.205	-13.688	1.37
	3	-0.654	2.22	1	-9.064	7.756
	4	1.131	1.241	1	-3.568	5.831
	5	-1.66	1.537	1	-7.482	4.162
	6	0.598	2.005	1	-6.995	8.192
	7	1.123	1.541	1	-4.716	6.962
	8	-1.513	1.514	1	-7.25	4.224
3	1	-5.505	1.494	0.062	-11.164	0.154
	2	0.654	2.22	1	-7.756	9.064
	4	1.785	1.196	1	-2.747	6.318
	5	-1.006	1.216	1	-5.611	3.599
	6	1.252	1.294	1	-3.651	6.155
	7	1.777	1.477	1	-3.819	7.373
	8	-0.859	1.675	1	-7.206	5.487
4	1	-7.290*	1.142	0	-11.616	-2.964
	2	-1.131	1.241	1	-5.831	3.568
	3	-1.785	1.196	1	-6.318	2.747
	5	-2.791	0.784	0.08	-5.762	0.179
	6	-0.533	1.389	1	-5.793	4.727
	7	-0.008	0.945	1	-3.588	3.571
	8	-2.644	1.116	0.887	-6.872	1.584
5	1	-4.499*	0.964	0.009	-8.152	-0.846
	2	1.66	1.537	1	-4.162	7.482
	3	1.006	1.216	1	-3.599	5.611
	4	2.791	0.784	0.08	-0.179	5.762
	6	2.258	1.294	1	-2.642	7.159
	7	2.783*	0.719	0.042	0.061	5.505
	8	0.147	1.284	1	-4.716	5.01
6	1	-6.757*	1.492	0.011	-12.408	-1.106
	2	-0.598	2.005	1	-8.192	6.995
	3	-1.252	1.294	1	-6.155	3.651
	4	0.533	1.389	1	-4.727	5.793
	5	-2.258	1.294	1	-7.159	2.642
	7	0.525	1.588	1	-5.49	6.539
	8	-2.111	1.557	1	-8.01	3.788
7	1	-7.282*	1.208	0.001	-11.858	-2.705
	2	-1.123	1.541	1	-6.962	4.716
	3	-1.777	1.477	1	-7.373	3.819
	4	0.008	0.945	1	-3.571	3.588
	5	-2.783*	0.719	0.042	-5.505	-0.061
	6	-0.525	1.588	1	-6.539	5.49
	8	-2.636	1.373	1	-7.836	2.564
8	1	-4.646	1.514	0.218	-10.38	1.088
	2	1.513	1.514	1	-4.224	7.25
	3	0.859	1.675	1	-5.487	7.206
	4	2.644	1.116	0.887	-1.584	6.872
	5	-0.147	1.284	1	-5.01	4.716
	6	2.111	1.557	1	-3.788	8.01
	7	2.636	1.373	1	-2.564	7.836

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Descriptive Statistics and Pairwise Comparison Analysis among global change factors during spring at ambient temperature for arylsulphatase enzyme activity

Descriptive Statistics

	TIME	Mean	Std. Deviation	N
CO2	0	34.1883	1.67515	4
	1	32.2173	4.35083	4
	2	51.8655	1.19113	4
	3	46.3409	5.45971	4
	4	34.9656	5.49924	4
	Total	39.9155	8.74259	20
CO2R	0	33.9038	5.16699	4
	1	32.0784	8.95295	4
	2	53.0523	1.50579	4
	3	42.8846	5.09621	4
	4	33.6678	5.4278	4
	Total	39.1174	9.60498	20
CH4	0	34.6811	7.35041	4
	1	38.1929	2.53542	4
	2	55.6757	7.55622	4
	3	45.3138	2.64609	4
	4	39.8031	4.58871	4
	Total	42.7333	8.91378	20
CH4R	0	27.2757	4.91212	4
	1	44.7516	3.45903	4
	2	60.2148	4.83949	4
	3	41.8574	4.39003	4
	4	29.1843	1.91794	4
	Total	40.6568	12.7588	20
CO2CH4	0	29.6563	2.42638	4
	1	42.7528	7.7683	4
	2	62.6457	1.23882	4
	3	47.6804	7.71213	4
	4	31.8911	4.20337	4
	Total	42.9252	13.1233	20
CO2CH4R	0	32.1201	3.87967	4
	1	39.9211	7.13847	4
	2	55.2454	6.64655	4
	3	43.0789	6.02007	4
	4	27.8032	5.28192	4
	Total	39.6337	11.0771	20
R	0	33.2375	3.87101	4
	1	30.1629	3.3833	4
	2	55.7104	3.21486	4
	3	43.0789	6.02007	4
	4	30.4752	2.8579	4
	Total	38.533	10.6587	20
CPG	0	32.481	1.83352	4
	1	32.1409	2.31543	4
	2	56.7862	5.1566	4
	3	46.3409	6.26474	4
	4	35.5695	2.93419	4
	Total	40.6637	10.4639	20

Pairwise Comparisons

Measure: MEASURE_1

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for	
					Lower Bound	Upper Bound
1	2	0.798	1.742	1	-5.802	7.398
	3	-2.818	1.65	1	-9.07	3.434
	4	-0.741	1.078	1	-4.826	3.344
	5	-3.01	1.279	0.915	-7.855	1.835
	6	0.282	1.413	1	-5.072	5.636
	7	1.383	1.289	1	-3.502	6.267
	8	-0.748	1.312	1	-5.717	4.221
2	1	-0.798	1.742	1	-7.398	5.802
	3	-3.616	1.636	1	-9.815	2.583
	4	-1.539	1.609	1	-7.633	4.554
	5	-3.808	1.798	1	-10.62	3.004
	6	-0.516	2.023	1	-8.18	7.147
	7	0.584	1.362	1	-4.576	5.744
	8	-1.546	1.372	1	-6.743	3.651
3	1	2.818	1.65	1	-3.434	9.07
	2	3.616	1.636	1	-2.583	9.815
	4	2.077	1.594	1	-3.962	8.115
	5	-0.192	1.557	1	-6.09	5.706
	6	3.1	1.564	1	-2.827	9.026
	7	4.2	1.459	0.321	-1.325	9.726
	8	2.07	1.429	1	-3.344	7.483
4	1	0.741	1.078	1	-3.344	4.826
	2	1.539	1.609	1	-4.554	7.633
	3	-2.077	1.594	1	-8.115	3.962
	5	-2.268	0.868	0.549	-5.558	1.021
	6	1.023	1.367	1	-4.154	6.2
	7	2.124	1.14	1	-2.195	6.443
	8	-0.007	1.129	1	-4.282	4.268
5	1	3.01	1.279	0.915	-1.835	7.855
	2	3.808	1.798	1	-3.004	10.62
	3	0.192	1.557	1	-5.706	6.09
	4	2.268	0.868	0.549	-1.021	5.558
	6	3.291	1.086	0.237	-0.824	7.407
	7	4.392	1.362	0.158	-0.766	9.551
	8	2.262	1.258	1	-2.505	7.028
6	1	-0.282	1.413	1	-5.636	5.072
	2	0.516	2.023	1	-7.147	8.18
	3	-3.1	1.564	1	-9.026	2.827
	4	-1.023	1.367	1	-6.2	4.154
	5	-3.291	1.086	0.237	-7.407	0.824
	7	1.101	1.494	1	-4.557	6.759
	8	-1.03	1.669	1	-7.354	5.294
7	1	-1.383	1.289	1	-6.267	3.502
	2	-0.584	1.362	1	-5.744	4.576
	3	-4.2	1.459	0.321	-9.726	1.325
	4	-2.124	1.14	1	-6.443	2.195
	5	-4.392	1.362	0.158	-9.551	0.766
	6	-1.101	1.494	1	-6.759	4.557
	8	-2.131	1.109	1	-6.33	2.069
8	1	0.748	1.312	1	-4.221	5.717
	2	1.546	1.372	1	-3.651	6.743
	3	-2.07	1.429	1	-7.483	3.344
	4	0.007	1.129	1	-4.268	4.282
	5	-2.262	1.258	1	-7.028	2.505
	6	1.03	1.669	1	-5.294	7.354
	7	2.131	1.109	1	-2.069	6.33

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

Descriptive Statistics and Pairwise Comparison Analysis among global change factors during summer at elevated temperature for arylsulphatase enzyme activity

Descriptive Statistics

	TIME	Mean	Std. Deviation	N
CO2	0	49.3158	2.74342	4
	1	32.9043	1.7085	4
	2	48.23	2.65504	4
	3	34.8985	5.7224	4
	4	51.8196	6.35242	4
	Total	43.4336	8.93946	20
CO2R	0	44.6405	3.20119	4
	1	29.5658	2.71023	4
	2	47.6613	5.55051	4
	3	33.473	5.89676	4
	4	49.9066	5.17182	4
	Total	41.0495	9.25407	20
CH4	0	51.2287	3.11028	4
	1	27.158	1.23764	4
	2	51.8122	6.28257	4
	3	38.0227	5.69332	4
	4	53.5996	4.70332	4
	Total	44.3643	11.2677	20
CH4R	0	51.6571	3.7676	4
	1	29.6175	5.9979	4
	2	49.7663	6.86721	4
	3	39.9652	4.55442	4
	4	54.1683	4.72493	4
	Total	45.0349	10.4464	20
CO2CH4	0	45.4603	3.17026	4
	1	27.3205	3.84895	4
	2	48.4959	6.15239	4
	3	34.7877	3.71863	4
	4	51.7236	6.46345	4
	Total	41.5576	10.3006	20
CO2CH4R	0	43.2963	4.17605	4
	1	29.4994	3.52825	4
	2	47.5875	3.83168	4
	3	35.2087	1.86154	4
	4	49.6038	5.76553	4
	Total	41.0391	8.57717	20
R	0	47.3954	3.60549	4
	1	33.052	3.73766	4
	2	51.6571	7.06609	4
	3	40.6226	4.4625	4
	4	51.2066	6.81712	4
	Total	44.7867	8.67824	20
CPG	0	49.8845	5.09549	4
	1	33.0741	5.38471	4
	2	49.1311	4.33047	4
	3	37.5352	2.6075	4
	4	55.8819	6.90524	4
	Total	45.1014	9.76979	20

Pairwise Comparisons

Measure: MEASURE_1

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for	
					Lower Bound	Upper Bound
1	2	2.384	0.89	0.482	-0.989	5.757
	3	-0.931	0.837	1	-4.102	2.241
	4	-1.601	1.247	1	-6.324	3.122
	5	1.876	0.899	1	-1.529	5.281
	6	2.395	0.872	0.42	-0.908	5.697
	7	-1.353	0.924	1	-4.854	2.148
	8	-1.668	1.211	1	-6.255	2.92
2	1	-2.384	0.89	0.482	-5.757	0.989
	3	-3.315*	0.769	0.017	-6.229	-0.4
	4	-3.985	1.093	0.067	-8.127	0.156
	5	-0.508	1.019	1	-4.367	3.351
	6	0.01	1.036	1	-3.912	3.933
	7	-3.737	1.122	0.128	-7.987	0.512
	8	-4.052	1.514	0.483	-9.786	1.682
3	1	0.931	0.837	1	-2.241	4.102
	2	3.315*	0.769	0.017	0.4	6.229
	4	-0.671	0.933	1	-4.206	2.864
	5	2.807	0.948	0.272	-0.784	6.397
	6	3.325	1.054	0.183	-0.669	7.319
	7	-0.422	1.187	1	-4.92	4.075
	8	-0.737	1.306	1	-5.685	4.21
4	1	1.601	1.247	1	-3.122	6.324
	2	3.985	1.093	0.067	-0.156	8.127
	3	0.671	0.933	1	-2.864	4.206
	5	3.477	0.936	0.058	-0.069	7.023
	6	3.996	1.462	0.432	-1.543	9.535
	7	0.248	1.388	1	-5.009	5.505
	8	-0.066	1.577	1	-6.039	5.906
5	1	-1.876	0.899	1	-5.281	1.529
	2	0.508	1.019	1	-3.351	4.367
	3	-2.807	0.948	0.272	-6.397	0.784
	4	-3.477	0.936	0.058	-7.023	0.069
	6	0.518	1.063	1	-3.509	4.546
	7	-3.229	1.018	0.177	-7.087	0.629
	8	-3.544	1.471	0.819	-9.115	2.027
6	1	-2.395	0.872	0.42	-5.697	0.908
	2	-0.01	1.036	1	-3.933	3.912
	3	-3.325	1.054	0.183	-7.319	0.669
	4	-3.996	1.462	0.432	-9.535	1.543
	5	-0.518	1.063	1	-4.546	3.509
	7	-3.748	1.169	0.165	-8.175	0.68
	8	-4.062	1.338	0.233	-9.131	1.007
7	1	1.353	0.924	1	-2.148	4.854
	2	3.737	1.122	0.128	-0.512	7.987
	3	0.422	1.187	1	-4.075	4.92
	4	-0.248	1.388	1	-5.505	5.009
	5	3.229	1.018	0.177	-0.629	7.087
	6	3.748	1.169	0.165	-0.68	8.175
	8	-0.315	1.269	1	-5.124	4.494
8	1	1.668	1.211	1	-2.92	6.255
	2	4.052	1.514	0.483	-1.682	9.786
	3	0.737	1.306	1	-4.21	5.685
	4	0.066	1.577	1	-5.906	6.039
	5	3.544	1.471	0.819	-2.027	9.115
	6	4.062	1.338	0.233	-1.007	9.131
	7	0.315	1.269	1	-4.494	5.124

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Descriptive Statistics and Pairwise Comparison Analysis among global change factors during summer at ambient temperature for arylsulphatase enzyme activity

Descriptive Statistics

	TIME	Mean	Std. Deviation	N
CO2	0	42.5429	6.53901	4
	1	35.482	2.96371	4
	2	44.1752	3.1427	4
	3	40.1942	5.7939	4
	4	55.4387	8.64577	4
	Total	43.5666	8.53973	20
COR	0	46.7602	6.98995	4
	1	46.8489	4.40008	4
	2	56.8346	4.53463	4
	3	42.085	3.20459	4
	4	62.219	6.33765	4
	Total	50.9495	8.93243	20
CH4	0	48.0528	5.31904	4
	1	40.1203	3.06306	4
	2	55.4904	3.68617	4
	3	38.163	4.05803	4
	4	59.9515	6.8184	4
	Total	48.3556	9.65549	20
CH4R	0	42.3139	3.38051	4
	1	32.0327	4.60461	4
	2	44.7439	5.50314	4
	3	54.0649	6.09951	4
	4	49.2715	6.2828	4
	Total	44.4854	8.93508	20
CO2CH4	0	47.7426	5.85417	4
	1	34.8246	5.80187	4
	2	46.0143	6.9984	4
	3	36.2944	5.51729	4
	4	55.0472	6.54877	4
	Total	43.9846	9.47403	20
CO2CH4R	0	45.091	6.28928	4
	1	41.243	4.64766	4
	2	52.2701	3.88911	4
	3	37.2767	8.27495	4
	4	55.1728	10.1078	4
	Total	46.2107	9.26767	20
R	0	49.2124	1.13307	4
	1	35.6371	4.81676	4
	2	54.2496	3.86781	4
	3	36.4495	7.05557	4
	4	55.1063	8.5484	4
	Total	46.131	10.0686	20
CPG	0	46.8119	1.11864	4
	1	36.4864	3.73966	4
	2	52.536	4.01449	4
	3	38.7465	3.8633	4
	4	60.8674	3.08203	4
	Total	47.0897	9.66201	20

Pairwise Comparisons

Measure: MEASURE_1

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for	
					Lower Bound	Upper Bound
1	2	-7.383*	1.62	0.011	-13.52	-1.245
	3	-4.789*	1.194	0.032	-9.312	-0.266
	4	-0.919	1.282	1	-5.775	3.938
	5	-0.418	1.647	1	-6.656	5.819
	6	-2.644	1.704	1	-9.098	3.809
	7	-2.564	1.557	1	-8.462	3.334
	8	-3.523	1.517	0.97	-9.268	2.222
2	1	7.383*	1.62	0.011	1.245	13.52
	3	2.594	1.201	1	-1.956	7.144
	4	6.464*	1.245	0.003	1.749	11.179
	5	6.965*	1.575	0.014	0.999	12.931
	6	4.739	2.032	0.953	-2.959	12.437
	7	4.819	1.54	0.193	-1.015	10.652
	8	3.86	1.394	0.401	-1.421	9.14
3	1	4.789*	1.194	0.032	0.266	9.312
	2	-2.594	1.201	1	-7.144	1.956
	4	3.870*	0.877	0.014	0.547	7.193
	5	4.371	1.532	0.339	-1.433	10.175
	6	2.145	1.876	1	-4.961	9.251
	7	2.225	1.244	1	-2.487	6.936
	8	1.266	1.187	1	-3.232	5.764
4	1	0.919	1.282	1	-3.938	5.775
	2	-6.464*	1.245	0.003	-11.179	-1.749
	3	-3.870*	0.877	0.014	-7.193	-0.547
	5	0.501	1.355	1	-4.631	5.632
	6	-1.725	1.841	1	-8.7	5.25
	7	-1.646	1.491	1	-7.294	4.002
	8	-2.604	1.16	1	-6.997	1.789
5	1	0.418	1.647	1	-5.819	6.656
	2	-6.965*	1.575	0.014	-12.931	-0.999
	3	-4.371	1.532	0.339	-10.175	1.433
	4	-0.501	1.355	1	-5.632	4.631
	6	-2.226	1.745	1	-8.838	4.386
	7	-2.146	1.408	1	-7.481	3.188
	8	-3.105	1.104	0.368	-7.288	1.078
6	1	2.644	1.704	1	-3.809	9.098
	2	-4.739	2.032	0.953	-12.437	2.959
	3	-2.145	1.876	1	-9.251	4.961
	4	1.725	1.841	1	-5.25	8.7
	5	2.226	1.745	1	-4.386	8.838
	7	0.08	1.718	1	-6.43	6.589
	8	-0.879	1.557	1	-6.777	5.019
7	1	2.564	1.557	1	-3.334	8.462
	2	-4.819	1.54	0.193	-10.652	1.015
	3	-2.225	1.244	1	-6.936	2.487
	4	1.646	1.491	1	-4.002	7.294
	5	2.146	1.408	1	-3.188	7.481
	6	-0.08	1.718	1	-6.589	6.43
	8	-0.959	1.147	1	-5.305	3.388
8	1	3.523	1.517	0.97	-2.222	9.268
	2	-3.86	1.394	0.401	-9.14	1.421
	3	-1.266	1.187	1	-5.764	3.232
	4	2.604	1.16	1	-1.789	6.997
	5	3.105	1.104	0.368	-1.078	7.288
	6	0.879	1.557	1	-5.019	6.777
	7	0.959	1.147	1	-3.388	5.305

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Descriptive Statistics and Pairwise Comparison Analysis among global change factors during spring at elevated temperature for urease enzyme activity

Descriptive Statistics

	TIME	Mean	Std. Deviation	N
CO2	0	137.737	45.7371	4
	1	91.6795	19.5155	4
	2	69.6649	13.3927	4
	3	120.791	7.31659	4
	4	136.143	10.3742	4
	Total	111.203	34.4414	20
CO2R	0	113.912	32.2779	4
	1	87.5518	30.448	4
	2	89.1449	6.63236	4
	3	148.672	9.54693	4
	4	129.336	14.4901	4
	Total	113.723	30.7443	20
CH4	0	144.182	26.613	4
	1	100.659	28.2802	4
	2	78.8618	13.482	4
	3	142.733	7.94826	4
	4	141.213	26.3215	4
	Total	121.53	33.8435	20
CH4R	0	138.244	41.2002	4
	1	99.2109	51.744	4
	2	105.149	9.86002	4
	3	172.062	11.3403	4
	4	149.106	45.8726	4
	Total	132.754	42.9408	20
CO2CH4	0	159.896	44.4	4
	1	117.025	28.4828	4
	2	83.2068	13.0219	4
	3	148.454	16.205	4
	4	134.695	28.0314	4
	Total	128.656	37.2918	20
CO2CH4R	0	107.177	74.2388	4
	1	116.953	43.2425	4
	2	83.9309	11.5222	4
	3	136.65	11.6909	4
	4	125.498	15.9227	4
	Total	114.042	39.8229	20
R	0	158.52	25.1052	4
	1	96.0245	12.0447	4
	2	85.8862	16.3801	4
	3	130.495	5.15194	4
	4	121.226	20.2884	4
	Total	118.43	30.5496	20
CPG	0	128.467	23.6044	4
	1	89.8691	51.1399	4
	2	100.876	5.84799	4
	3	131.871	14.6778	4
	4	132.016	23.7472	4
	Total	116.62	30.9939	20

Pairwise Comparisons

Measure: MEASURE_1

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.[a]	95% Confidence Interval for	
					Lower Bound	Upper Bound
1	2	-2.52	6.489	1	-27.1	22.06
	3	-10.327	6.848	1	-36.267	15.614
	4	-21.551	9.934	1	-59.182	16.08
	5	-17.452	10.429	1	-56.959	22.054
	6	-2.839	10.693	1	-43.344	37.667
	7	-7.227	7.816	1	-36.834	22.38
	8	-5.417	9.52	1	-41.481	30.647
2	1	2.52	6.489	1	-22.06	27.1
	3	-7.807	6.672	1	-33.083	17.47
	4	-19.031	9.608	1	-55.43	17.368
	5	-14.932	7.526	1	-43.442	13.578
	6	-0.319	6.56	1	-25.168	24.53
	7	-4.707	5.712	1	-26.346	16.932
	8	-2.897	7.561	1	-31.539	25.746
3	1	10.327	6.848	1	-15.614	36.267
	2	7.807	6.672	1	-17.47	33.083
	4	-11.225	7.521	1	-39.716	17.267
	5	-7.126	9.017	1	-41.286	27.034
	6	7.488	9.545	1	-28.672	43.648
	7	3.099	4.753	1	-14.906	21.104
	8	4.91	8.748	1	-28.229	38.048
4	1	21.551	9.934	1	-16.08	59.182
	2	19.031	9.608	1	-17.368	55.43
	3	11.225	7.521	1	-17.267	39.716
	5	4.099	11.317	1	-38.772	46.97
	6	18.712	9.949	1	-18.977	56.402
	7	14.324	8.696	1	-18.619	47.267
	8	16.134	12.815	1	-32.411	64.68
5	1	17.452	10.429	1	-22.054	56.959
	2	14.932	7.526	1	-13.578	43.442
	3	7.126	9.017	1	-27.034	41.286
	4	-4.099	11.317	1	-46.97	38.772
	6	14.614	9.443	1	-21.157	50.384
	7	10.225	5.706	1	-11.39	31.841
	8	12.036	8.381	1	-19.713	43.785
6	1	2.839	10.693	1	-37.667	43.344
	2	0.319	6.56	1	-24.53	25.168
	3	-7.488	9.545	1	-43.648	28.672
	4	-18.712	9.949	1	-56.402	18.977
	5	-14.614	9.443	1	-50.384	21.157
	7	-4.388	8.723	1	-37.434	28.657
	8	-2.578	12.371	1	-49.443	44.287
7	1	7.227	7.816	1	-22.38	36.834
	2	4.707	5.712	1	-16.932	26.346
	3	-3.099	4.753	1	-21.104	14.906
	4	-14.324	8.696	1	-47.267	18.619
	5	-10.225	5.706	1	-31.841	11.39
	6	4.388	8.723	1	-28.657	37.434
	8	1.81	7.332	1	-25.965	29.585
8	1	5.417	9.52	1	-30.647	41.481
	2	2.897	7.561	1	-25.746	31.539
	3	-4.91	8.748	1	-38.048	28.229
	4	-16.134	12.815	1	-64.68	32.411
	5	-12.036	8.381	1	-43.785	19.713
	6	2.578	12.371	1	-44.287	49.443
	7	-1.81	7.332	1	-29.585	25.965

Based on estimated marginal means

a. Adjustment for multiple comparisons: Bonferroni.

Descriptive Statistics and Pairwise Comparison Analysis among global change factors during spring at ambient temperature for urease enzyme activity

Descriptive Statistics

	TIME	Mean	Std. Deviation	N
CO2	0	183.142	16.253	4
	1	126.078	83.3138	4
	2	91.3174	10.0159	4
	3	158.375	4.63089	4
	4	110.87	12.556	4
	Total	133.956	48.2149	20
CO2R	0	182.997	22.6674	4
	1	126.222	33.9528	4
	2	131.726	7.41296	4
	3	180.752	26.9945	4
	4	126.584	40.0837	4
	Total	149.656	37.0611	20
CH4	0	171.99	36.2129	4
	1	104.932	43.6763	4
	2	111.123	9.19768	4
	3	168.659	8.62661	4
	4	96.7487	20.0296	4
	Total	130.69	41.4983	20
CH4R	0	151.061	28.678	4
	1	116.374	29.9245	4
	2	92.3313	19.5797	4
	3	133.464	17.2059	4
	4	94.0693	3.63625	4
	Total	117.46	30.3413	20
CO2CH4	0	139.475	49.5691	4
	1	105.801	38.4958	4
	2	127.091	5.49286	4
	3	166.052	14.6071	4
	4	136.868	16.5059	4
	Total	135.057	33.2268	20
CO2CH4R	0	160.838	33.97	4
	1	135.274	52.9644	4
	2	116.012	6.37486	4
	3	171.628	24.2935	4
	4	123.543	18.9571	4
	Total	141.459	35.5543	20
R	0	159.606	10.8125	4
	1	110.508	28.5597	4
	2	102.832	8.92813	4
	3	155.551	28.5102	4
	4	118.763	13.5763	4
	Total	129.452	30.015	20
CPG	0	147.151	39.6586	4
	1	81.9033	27.7993	4
	2	109.711	10.7564	4
	3	159.172	13.8688	4
	4	108.046	12.396	4
	Total	121.197	35.7478	20

Pairwise Comparisons

Measure: MEASURE_1

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for	
					Lower Bound	Upper Bound
1	2	-15.7	12.889	1	-64.526	33.126
	3	3.266	13.542	1	-48.033	54.565
	4	16.497	10.833	1	-24.542	57.535
	5	-1.101	8.121	1	-31.866	29.665
	6	-7.502	12.991	1	-56.713	41.709
	7	4.504	11.616	1	-39.501	48.51
	8	12.76	11.171	1	-29.557	55.076
2	1	15.7	12.889	1	-33.126	64.526
	3	18.966	5.619	0.117	-2.322	40.254
	4	32.196	8.688	0.059	-0.715	65.108
	5	14.599	9.479	1	-21.309	50.508
	6	8.198	7.185	1	-19.021	35.416
	7	20.204	6.973	0.31	-6.213	46.621
	8	28.460*	7.053	0.03	1.74	55.18
3	1	-3.266	13.542	1	-54.565	48.033
	2	-18.966	5.619	0.117	-40.254	2.322
	4	13.231	7.796	1	-16.303	42.764
	5	-4.367	10.296	1	-43.37	34.637
	6	-10.768	8.12	1	-41.529	19.992
	7	1.238	6.381	1	-22.934	25.411
	8	9.494	4.988	1	-9.403	28.391
4	1	-16.497	10.833	1	-57.535	24.542
	2	-32.196	8.688	0.059	-65.108	0.715
	3	-13.231	7.796	1	-42.764	16.303
	5	-17.597	8.529	1	-49.907	14.713
	6	-23.999	9.074	0.515	-58.372	10.374
	7	-11.992	6.368	1	-36.116	12.131
	8	-3.737	7.358	1	-31.609	24.136
5	1	1.101	8.121	1	-29.665	31.866
	2	-14.599	9.479	1	-50.508	21.309
	3	4.367	10.296	1	-34.637	43.37
	4	17.597	8.529	1	-14.713	49.907
	6	-6.402	10.796	1	-47.301	34.497
	7	5.605	9.044	1	-28.657	39.868
	8	13.861	8.622	1	-18.802	46.523
6	1	7.502	12.991	1	-41.709	56.713
	2	-8.198	7.185	1	-35.416	19.021
	3	10.768	8.12	1	-19.992	41.529
	4	23.999	9.074	0.515	-10.374	58.372
	5	6.402	10.796	1	-34.497	47.301
	7	12.007	8.234	1	-19.186	43.2
	8	20.262	10.124	1	-18.088	58.613
7	1	-4.504	11.616	1	-48.51	39.501
	2	-20.204	6.973	0.31	-46.621	6.213
	3	-1.238	6.381	1	-25.411	22.934
	4	11.992	6.368	1	-12.131	36.116
	5	-5.605	9.044	1	-39.868	28.657
	6	-12.007	8.234	1	-43.2	19.186
	8	8.256	5.661	1	-13.188	29.699
8	1	-12.76	11.171	1	-55.076	29.557
	2	-28.460*	7.053	0.03	-55.18	-1.74
	3	-9.494	4.988	1	-28.391	9.403
	4	3.737	7.358	1	-24.136	31.609
	5	-13.861	8.622	1	-46.523	18.802
	6	-20.262	10.124	1	-58.613	18.088
	7	-8.256	5.661	1	-29.699	13.188

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Descriptive Statistics and Pairwise Comparison Analysis among global change factors during summer at elevated temperature for urease enzyme activity

Descriptive Statistics

	TIME	Mean	Std. Deviation	N
CO2	0	60.1881	7.42979	4
	1	82.8454	8.91787	4
	2	51.6339	3.40021	4
	3	69.3589	15.8488	4
	4	72.5957	13.7226	4
	Total	67.3244	14.5767	20
CO2R	0	46.6246	6.39906	4
	1	72.0562	5.47038	4
	2	48.3971	3.20355	4
	3	67.2782	10.3727	4
	4	58.4927	21.7135	4
	Total	58.5698	14.5026	20
CH4	0	55.9495	15.4531	4
	1	73.5205	5.01182	4
	2	58.6468	12.2205	4
	3	67.047	22.762	4
	4	56.9514	16.3841	4
	Total	62.423	15.4272	20
CH4R	0	58.1074	9.20494	4
	1	48.0118	10.8707	4
	2	59.2633	9.45909	4
	3	67.047	22.5031	4
	4	67.5093	18.2092	4
	Total	59.9878	15.2291	20
CO2CH4	0	53.4834	7.21403	4
	1	70.5149	7.93288	4
	2	56.2578	17.7717	4
	3	59.9569	3.34385	4
	4	65.6598	10.7994	4
	Total	61.1746	11.3428	20
CO2CH4R	0	55.333	7.15672	4
	1	68.8965	17.4824	4
	2	66.4304	10.7745	4
	3	44.8521	16.3282	4
	4	65.5827	9.26625	4
	Total	60.219	14.6663	20
R	0	53.1752	9.85956	4
	1	80.7646	2.27919	4
	2	44.3897	8.07387	4
	3	40.9218	7.29644	4
	4	55.0247	14.4774	4
	Total	54.8552	16.5474	20
CPG	0	64.735	7.64257	4
	1	95.7924	8.14175	4
	2	47.8577	13.4589	4
	3	56.7202	8.26004	4
	4	80.9958	21.6551	4
	Total	69.2202	21.075	20

Pairwise Comparisons

Measure: MEASURE_1

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for	
					Lower Bound	Upper Bound
1	2	8.755*	2.079	0.021	0.879	16.63
	3	4.901	4.175	1	-10.914	20.717
	4	7.337	3.544	1	-6.087	20.76
	5	6.15	3.96	1	-8.853	21.153
	6	7.105	4.336	1	-9.322	23.533
	7	12.469	3.456	0.072	-0.624	25.562
	8	-1.896	4.167	1	-17.681	13.89
2	1	-8.755*	2.079	0.021	-16.63	-0.879
	3	-3.853	4.485	1	-20.842	13.135
	4	-1.418	3.686	1	-15.38	12.544
	5	-2.605	4.219	1	-18.588	13.378
	6	-1.649	4.244	1	-17.728	14.43
	7	3.715	2.885	1	-7.215	14.644
	8	-10.65	3.377	0.183	-23.442	2.141
3	1	-4.901	4.175	1	-20.717	10.914
	2	3.853	4.485	1	-13.135	20.842
	4	2.435	3.752	1	-11.778	16.648
	5	1.248	3.81	1	-13.183	15.68
	6	2.204	4.449	1	-14.65	19.058
	7	7.568	4.437	1	-9.241	24.377
	8	-6.797	4.701	1	-24.604	11.009
4	1	-7.337	3.544	1	-20.76	6.087
	2	1.418	3.686	1	-12.544	15.38
	3	-2.435	3.752	1	-16.648	11.778
	5	-1.187	3.801	1	-15.585	13.211
	6	-0.231	4.756	1	-18.249	17.787
	7	5.133	4.282	1	-11.087	21.352
	8	-9.232	4.795	1	-27.398	8.933
5	1	-6.15	3.96	1	-21.153	8.853
	2	2.605	4.219	1	-13.378	18.588
	3	-1.248	3.81	1	-15.68	13.183
	4	1.187	3.801	1	-13.211	15.585
	6	0.956	3.94	1	-13.972	15.883
	7	6.319	3.274	1	-6.085	18.724
	8	-8.046	3.823	1	-22.529	6.438
6	1	-7.105	4.336	1	-23.533	9.322
	2	1.649	4.244	1	-14.43	17.728
	3	-2.204	4.449	1	-19.058	14.65
	4	0.231	4.756	1	-17.787	18.249
	5	-0.956	3.94	1	-15.883	13.972
	7	5.364	3.611	1	-8.314	19.041
	8	-9.001	4.34	1	-25.442	7.44
7	1	-12.469	3.456	0.072	-25.562	0.624
	2	-3.715	2.885	1	-14.644	7.215
	3	-7.568	4.437	1	-24.377	9.241
	4	-5.133	4.282	1	-21.352	11.087
	5	-6.319	3.274	1	-18.724	6.085
	6	-5.364	3.611	1	-19.041	8.314
	8	-14.365*	1.761	0	-21.035	-7.695
8	1	1.896	4.167	1	-13.89	17.681
	2	10.65	3.377	0.183	-2.141	23.442
	3	6.797	4.701	1	-11.009	24.604
	4	9.232	4.795	1	-8.933	27.398
	5	8.046	3.823	1	-6.438	22.529
	6	9.001	4.34	1	-7.44	25.442
	7	14.365*	1.761	0	7.695	21.035

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

Descriptive Statistics and Pairwise Comparison Analysis among global change factors during summer at ambient temperature for urease enzyme activity

Descriptive Statistics

	TIME	Mean	Std. Deviation	N
CO2	0	53.1752	5.56297	4
	1	82.383	1.26788	4
	2	31.5198	1.87297	4
	3	66.0451	16.54	4
	4	77.2967	24.9551	4
	Total	62.0839	22.3465	20
CO2R	0	46.0081	7.38704	4
	1	83.2307	12.9739	4
	2	42.7713	9.84952	4
	3	71.0544	8.21582	4
	4	71.6709	21.5556	4
	Total	62.9471	19.9277	20
CH4	0	36.529	6.27977	4
	1	64.5809	2.3409	4
	2	44.9292	16.3473	4
	3	58.7239	29.2849	4
	4	78.7609	13.2577	4
	Total	56.7048	21.0636	20
CH4R	0	38.9181	6.96779	4
	1	58.4927	1.85598	4
	2	44.0044	11.5104	4
	3	45.3916	5.72502	4
	4	65.6598	18.0713	4
	Total	50.4933	13.7955	20
CO2CH4	0	62.2689	19.0625	4
	1	47.2411	14.87	4
	2	38.7639	7.86471	4
	3	48.3971	6.68296	4
	4	81.4582	19.2729	4
	Total	55.6259	20.0797	20
CO2CH4R	0	58.8009	9.34115	4
	1	67.6635	25.1323	4
	2	72.6727	12.8522	4
	3	56.4119	10.9653	4
	4	80.6105	11.4307	4
	Total	67.2319	16.2102	20
R	0	63.579	7.30077	4
	1	50.1696	12.6234	4
	2	46.2393	16.6785	4
	3	39.5346	13.7407	4
	4	70.1296	26.9072	4
	Total	53.9304	18.8555	20
CPG	0	55.7954	7.22061	4
	1	75.8324	12.7423	4
	2	69.436	17.5003	4
	3	52.6357	10.6826	4
	4	55.1789	8.83803	4
	Total	61.7757	14.1707	20

Pairwise Comparisons

Measure: MEASURE_1

(I) TREATMENT	(J) TREATMENT	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for	
					Lower Bound	Upper Bound
1	2	-0.863	4.772	1	-18.941	17.214
	3	5.379	4.615	1	-12.102	22.86
	4	11.591	4.304	0.467	-4.712	27.894
	5	6.458	3.845	1	-8.107	21.023
	6	-5.148	4.153	1	-20.882	10.586
	7	8.154	3.925	1	-6.716	23.023
	8	0.308	4.396	1	-16.345	16.961
2	1	0.863	4.772	1	-17.214	18.941
	3	6.242	4.619	1	-11.257	23.742
	4	12.454	3.672	0.113	-1.457	26.364
	5	7.321	4.93	1	-11.353	25.996
	6	-4.285	4.998	1	-23.217	14.647
	7	9.017	4.807	1	-9.194	27.227
	8	1.171	4.119	1	-14.431	16.774
3	1	-5.379	4.615	1	-22.86	12.102
	2	-6.242	4.619	1	-23.742	11.257
	4	6.211	4.214	1	-9.75	22.173
	5	1.079	4.712	1	-16.771	18.929
	6	-10.527	4.991	1	-29.433	8.379
	7	2.774	4.027	1	-12.481	18.03
	8	-5.071	4.679	1	-22.797	12.655
4	1	-11.591	4.304	0.467	-27.894	4.712
	2	-12.454	3.672	0.113	-26.364	1.457
	3	-6.211	4.214	1	-22.173	9.75
	5	-5.133	3.886	1	-19.855	9.59
	6	-16.739*	4.133	0.029	-32.394	-1.083
	7	-3.437	3.573	1	-16.973	10.099
	8	-11.282	3.216	0.089	-23.467	0.902
5	1	-6.458	3.845	1	-21.023	8.107
	2	-7.321	4.93	1	-25.996	11.353
	3	-1.079	4.712	1	-18.929	16.771
	4	5.133	3.886	1	-9.59	19.855
	6	-11.606	3.833	0.237	-26.126	2.913
	7	1.695	3.805	1	-12.718	16.109
	8	-6.15	4.943	1	-24.875	12.575
6	1	5.148	4.153	1	-10.586	20.882
	2	4.285	4.998	1	-14.647	23.217
	3	10.527	4.991	1	-8.379	29.433
	4	16.739*	4.133	0.029	1.083	32.394
	5	11.606	3.833	0.237	-2.913	26.126
	7	13.301	4.982	0.489	-5.57	32.173
	8	5.456	4.618	1	-12.038	22.951
7	1	-8.154	3.925	1	-23.023	6.716
	2	-9.017	4.807	1	-27.227	9.194
	3	-2.774	4.027	1	-18.03	12.481
	4	3.437	3.573	1	-10.099	16.973
	5	-1.695	3.805	1	-16.109	12.718
	6	-13.301	4.982	0.489	-32.173	5.57
	8	-7.845	4.855	1	-26.236	10.545
8	1	-0.308	4.396	1	-16.961	16.345
	2	-1.171	4.119	1	-16.774	14.431
	3	5.071	4.679	1	-12.655	22.797
	4	11.282	3.216	0.089	-0.902	23.467
	5	6.15	4.943	1	-12.575	24.875
	6	-5.456	4.618	1	-22.951	12.038
	7	7.845	4.855	1	-10.545	26.236

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Bonferroni.

APPENDIX II - Additional data for Chapter Four

Reagents and Buffers

0.5 M Disodium ethylenediaminetetraacetate (EDTA)

Dissolve 186.12g of EDTA and a few NaOH pellets in approximately 800 ml double distilled water with vigorous stirring. Adjust the pH of the solution to 8 with NaOH pellets and bring up to 1000 ml with double distilled water.

50 X Tris-acetate EDTA buffer (TAE)

Add 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5M EDTA (pH 8) in approximately 600 ml of double distilled. Adjust the pH to 8 and bring up to 1000ml.

Ethidium Bromide solution

Add 50 µl of a 100mg/ml solution to 500 µl of distilled water. Store in a dark.

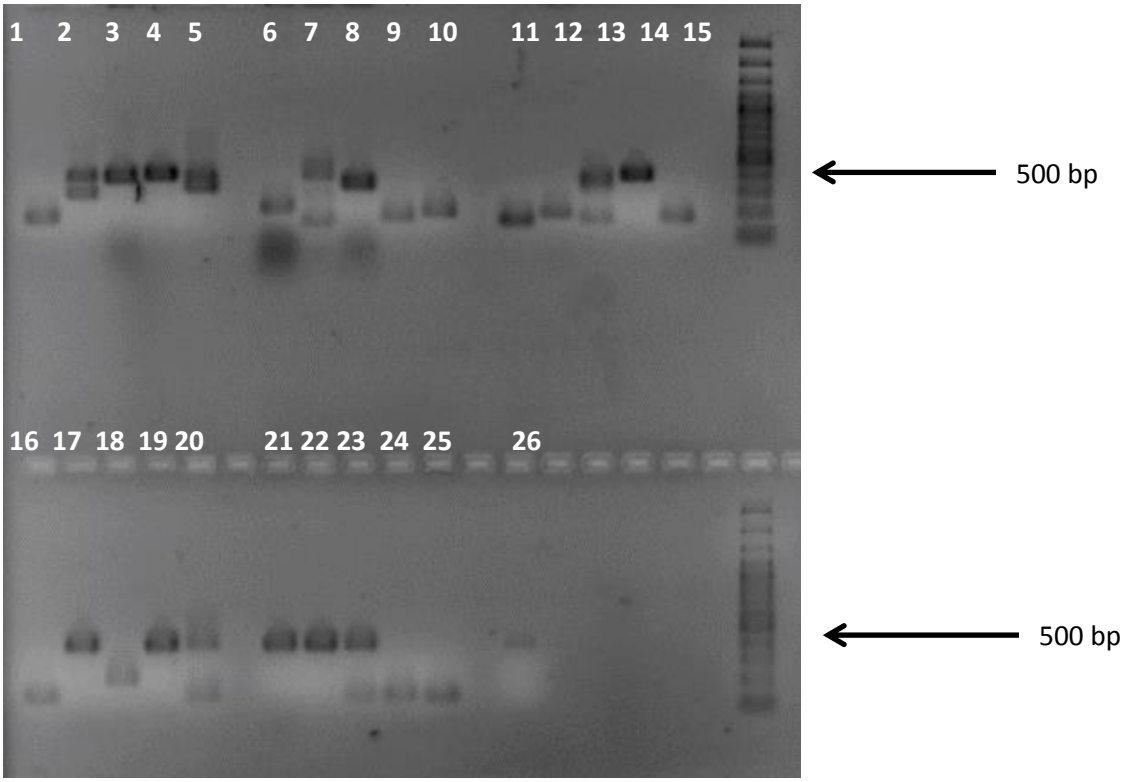

Fig. i: Agarose gel (2%) showing colony PCR of positive clones (Lanes 1-15 and 16- 26 positive tranformants; Lane 16 and 27 Fermentas 100 bp plus marker)

Nucleotide Sequence of clone 4 (88% identity to the *cbbL* gene, GenBank Accession number JN162638.1

aaggacgacg agaacatcaa ttcccagccc ttcatgcact ggcgcgaccg Gtacctctac tgcatggagg
gcgtcaacaa ggcgatggcc gaaaccggcg agatcaaggg cacctacctg aacgtcaccg ccgcgactat
ggaagacatg tacgagcggg cggaattcgc caagcagctc ggcagcgtca tcatcatgat cgacctggtg
atcggctata ccgcgatcca gtcgatggcg aagtgggcgc gcaaaaacga catgatcccg cacctgcacc
gcgccggcca ttcgacatat acccggcaga agtcgcacgg cgtgtcgttc cgggtcatcg ccaagtggat
gcgcatggcc ggcgtcgatc acatccatgc cggcaccgtc gtcggcaagc tggaaggcga tcccaacgtc
atccgcggca tctacgacac ctgccgcgag gtcaacgtcc cgcagaaact cgagcacggc atcctgttcg
accagccctg ggccggtctg aagaagatga tgccggtggc gtcgggcggc atccatgccg gccagatgca
ccagttgctg acatatctgg gcgaggatgt cgtgcttcag ttcggcggcg gcaccatcgg ccatccgggc
ggcatccagg ccggagccac cgccaaccgg gtggcgctgg aatgcatggt caaggcccgc aacgagggca
tcgatatctg gaacgaggga cctgacgtcc tggtcaaggc ggccaagtgg tgccagccgc tgcgcgcggc
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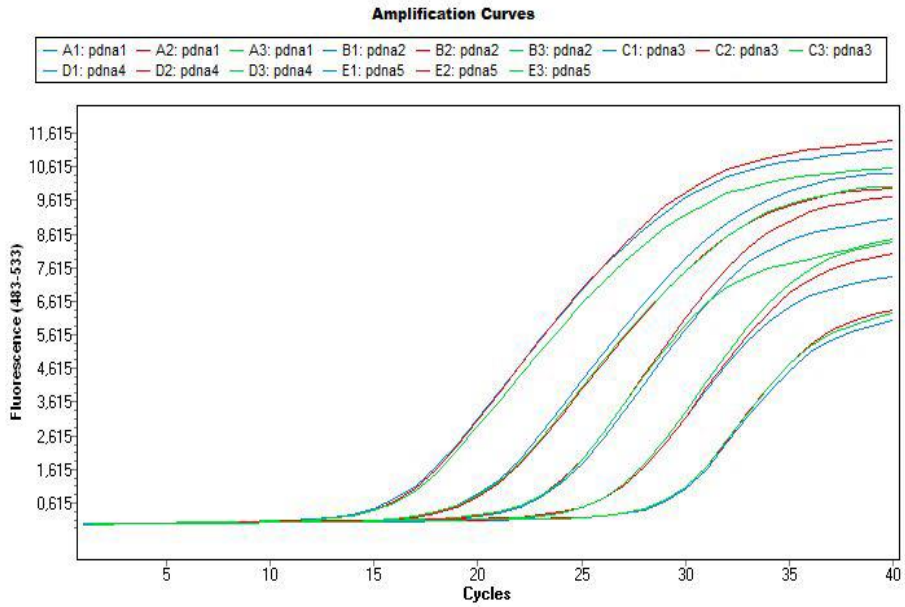

Fig. ii: Amplification curves produced from dilutions of plasmid DNA used for standard curve generation

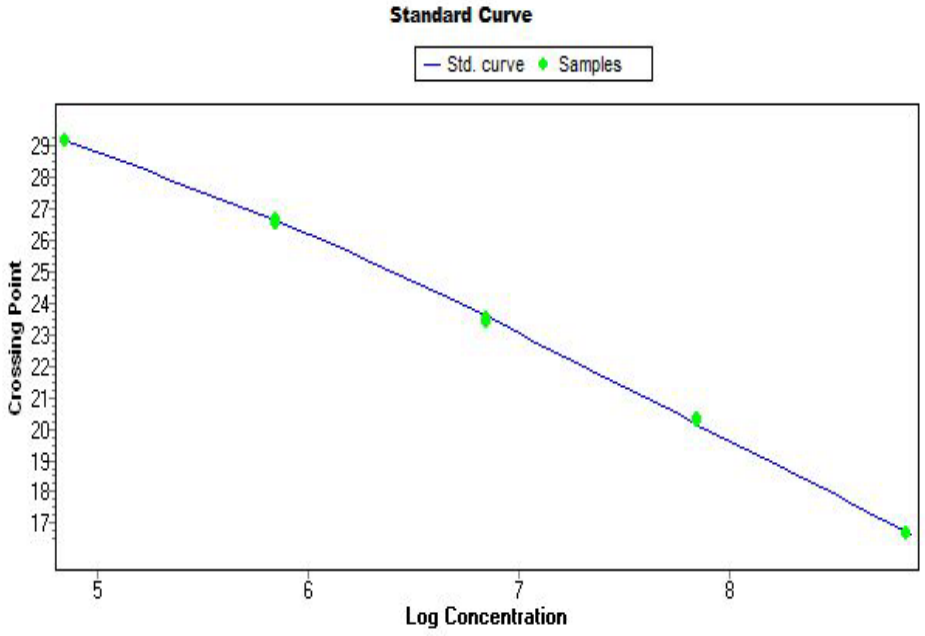

Fig. iii: Standard curve used for calculation of *cbbL* gene copy numbers

Table a: Replicate plasmid DNA copy numbers used for generation of standard curve

Copy Number	700000000	70000000	7000000	700000	70000
Cp Value	16.65	20.21	23.54	26.52	29.15
	16.68	20.34	23.51	26.70	29.20
	16.70	20.28	23.35	26.64	29.16

Table b: Replicate Cp values obtained from qPCR experiments with soil samples exposed to global change treatments during spring and summer

	SPRING				SUMMER			
	ELEVATED		AMBIENT		ELEVATED		AMBIENT	
	Day 25	**Day 60**	**Day 25**	**Day 60**	**Day 25**	**Day 60**	**Day 25**	**Day 60**
	28.81	27.25	27.88	26.98	27.67	27.86	27.10	27.79
CO_2	28.82	26.79	27.32	27.11	27.93	27.94	27.08	27.99
	28.81	27.21	27.95	27.17	27.87	27.78	26.95	27.95
	27.81	26.98	28.14	26.80	28.88	31.52	28.31	26.44
CO_2R	28.02	27.08	28.44	26.69	29.01	27.20	28.24	26.32
	28.06	26.95	28.35	26.77	29.13	31.71	28.13	25.85
	28.45	26.23	30.88	27.36	26.22	31.30	26.98	28.27
CH_4	28.23	27.09	30.92	27.30	26.37	31.61	27.47	28.50
	28.55	27.11	30.18	27.68	26.34	31.95	27.48	28.39
	29.24	26.85	28.61	26.88	27.46	27.83	26.56	28.83
CH_4R	29.11	26.51	28.79	27.04	27.45	27.89	26.92	28.82
	29.02	25.15	28.64	26.26	27.60	27.33	26.92	28.97
	29.79	26.87	29.47	26.36	28.75	29.08	26.90	27.45
CO_2CH_4	29.66	26.72	29.32	24.45	28.67	28.86	26.95	27.31
	29.70	26.96	29.22	26.48	28.77	28.94	27.01	27.53
	27.50	26.83	30.18	26.51	28.66	31.97	27.98	27.25
CO_2CH_4R	27.21	26.87	30.29	26.75	28.63	31.82	27.78	27.27
	27.53	26.76	30.13	26.85	28.62	32.00	26.87	27.49
	29.77	27.01	29.51	26.64	28.91	30.63	26.60	27.81
R	29.78	27.00	29.92	26.16	28.86	30.82	26.60	27.74
	29.76	26.92	30.36	25.78	28.87	29.56	26.71	27.69
	27.77	26.76	28.67	26.09	29.04	30.96	26.05	26.06
CTRL	27.55	26.84	28.50	26.64	28.79	31.07	26.05	26.06
	27.37	26.90	28.72	25.94	29.11	30.88	26.15	26.18

APPENDIX III - Additional data for Chapter Five

Reagents and Buffers

0.5 M Disodium ethylenediaminetetraacetate (EDTA)

Dissolve 186.12g of EDTA and a few NaOH pellets in approximately 800 ml double distilled water with vigorous stirring. Adjust the pH of the solution to 8 with NaOH pellets and bring up to 1000 ml with double distilled water.

50 X Tris-acetate EDTA buffer (TAE)

Add 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5M EDTA (pH 8) in approximately 600 ml of double distilled. Adjust the pH to 8 and bring up to 1000ml.

Ethidium Bromide solution

Add 50 µl of a 100mg/ml solution to 500 µl of distilled water. Store in a dark.

Denaturing solution (0%)

For a 6 % gel, add 15 ml of 40% acrylamide/bis acrylamide and 2 ml of 50 X TAE. Bring up to 100 ml by adding 83 ml of distilled water. Store at 4 ˚C in a brown bottle.

Denaturing solution (100%)

For a 6 % gel, dissolve 42 g of 7 M urea in 15 ml of 40% acrylamide/bis acrylamide, 2 ml of 50 X TAE buffer and 40 ml of 40 % deionized formamide. Bring up to 100 ml by adding distilled water. Store at 4 ˚C in a brown bottle.

Ammonium persulphate (APS)

Dissolve 1 g of APS in 10 ml distilled water. Aliquot and store at - 20 ˚C. Thaw shortly before use.

16S rRNA Gene sequences of bacteria represented on the DGGE bands

Nucleotide sequence of Methylocystis sp. M175 (Table 3)

ttggacaatg ggcgcaagcc tgatccagcc atgccgcgtg agtgatgaag gccytagggt
tgtaaagctc tttcgccagg gacgataatg acggtacctg gataagaagc cccggctaac
ttcgtgccag cagccgcggt aatacgaagg gggctagcgt tgttcggatt tactgggcgt
aaagcgcacg taggcggatc tttaagtcag gggtgaaatc ccggggctca acctcggaac
wgcctttgat actggaggtc tcgagtccgg gagaggtgag tggaactgcg agtgtagagg
tgaaattcgt agatattcgc aagaacacca gtggcgaagg cggctcactg gcccggaact
gacgctgagg tgcgaaagcg tggggagcaa acaggattag ataccctggt agtccacgcc
gtaaacgatg gatgctagcc gttggggagc ttgctcttca gtggcgcagc taacgcttta
agcatcccgc ctggggagta cggtcgcaag attaaaactc aaaggaattg acgggggccc
gcacaagcgg tggagcatgt ggtttaattc gaagcaacgc gcagaacctt accagctttt
gacatgcccg gtatggtcga cagagatgtc ttccttcccg caaggggccg gagcacaggt
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gccgcgagga aggtggggat gacgtcaagt cctcatggcc cttacaggct gggctacaca
cgtgctacaa tggcggtgac agagggatgc gaaggggcga cctggagcaa atcctttaaa
agccgtctca gttcggattg cactctgcaa ctcgggtgca tgaaggtgga atcgctagta
atcgcagatc agcacgctgc ggtgaatacg ttcccgggcc ttgtacacac cgcccgtcac
accatgggag ttggttttac c

Nucleotide sequence of Uncultured Burkholderiaceae bacterium clone Amb (Table 3)

gagtttgatc ctggctcaga ttgaacgctg gcggcatgcc ttacacatgc aagtcgaacg
gcagcacggg agcaatcctg gtggcgagtg gcgaacgggt gagtaataca tcggaacgtg
cccagtcgtg ggggataacg tagcgaaagc tacgctaata ccgcatacga accctggttg
aaagcagggg acccgcaagg gccttgcgcg agaggagcgg ccgatgccgg attagctagt
tggtggggta aaagcccacc aaggcgacga tccgtagctg gtctgagagg acgaccagcc
acactgggac tgggacacgg cccagactcc tacgggaggc agcagtgggg aattttggac
aatgggggca accctgatcc agccatgccg cgtgtgtgaa gaaggccttc gggttgtaaa
gcactttcgg ccggaacgaa atcgcgcggg cgaatatccc gcgtggatga cggtaccgga
agaagaagca ccggctaact acgtgccagc agccgcggta atacgtaggg tgcgagcgtt
aatcggaatt actgggcgta aagtgtgcgc aggcggctct gcaagtcgag tgtgaaatcc
ccgagcttaa cttgggaatt gcgctcgaaa ctatggagct ggagtgtggc agaggaaggt
ggaattccac gtgtagcggt gaaatgcgta gagatgtgga ggaacaccga tggcgaaggc
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taccctggta gtccacgccc taaacgatga tgactagttg ttggaggagt taaatccttt
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cgaaaaacct tacctaccct tgacatgcca ggaacgctcc agaaatgggg cggtgcccga
aagggaacct ggacacaggt gctgcatggc tgtcgtcagc tcgtgtcgtg agatgttggg
ttaagtcccg caacgagcgc aacccttgtc attagttgct acattcagtt gagcactcta
atgagactgc cggtgtaaac cggaggaagg cggggatgac gtcaagtcct catggccttt
atgcccaggg ctgcacacgt gctacaatgg cgtgaacaaa gggaagcaaa cccgcgaggg
ggagccaatc ccaaaaaaca cgccccagtt cagatcgaag gctgcaactc gccttcgtga
agtcggaatc gctagtaatc gcaggtcagc aacactgcgg tgaatgtgtt cctgagcctt
gcacacac

Nucleotide sequence of Uncultured Methylosinus sp. clone BG1-76 (Table 3)

gtcgaacgca tccttcgggg tgagtggcag acgggtgagt aacacgtggg aacgtgcctt
ctggttcgga ataacccagg gaaacttggg ctaataccgg atacgccctt acggggaaag
attcatcgcc ggaagatcgg cccgcgtctg attagcttgt tggtggggta atggcccacc
aaggcgacca tcagtagctg gtctgagagg atgatcagcc acactgggac tgagacacgg
cccagactcc tacgggaggc agcagtgggg aatactggac aatgggggca accctgatcc
agccatgccg cgtgagtgat gaaggcctta gggttgtaaa gctctttcgc cggtgaagat
aatgacggta accggagaag aagccccggc taacttcgtg ccagcagccg cggtaatacg
aaggggcta gcgttgttcg gaattactgg gcgtaaagcg cacgtaggcg gattgttaag
tcgggggtga aatcccaggg ctcaaccctg gaactgcctc cgatactggc aatctcgagt
ccgggagagg tgagtggaat tccgagtgta gaggtgaaat tcgtagatat tcggaagaac
accagtggcg aaggcggctc actggcccgg tactgacgct gaggtgcgaa agcgtgggga
gcaaacagga ttagataccc tggtagtcca cgccgtaaac gatggatgct agccgttggt
cagcttgctg atcagtggcg ccgctaacgc tttaagcatt ccgcctgggg ggtacggtcg
caagattaaa actcaaagga attgacgggg gcccgcacaa gcggtggagc atgtggttta

Nucleotide sequence of Blastochloris sulfoviridis strain Wai3G1e (Table 3)

ctggcggcag gcttaacaca tgcaagtcga gcgccgtagc aatacggagc ggcagacggg
tgagtaacgc gtgggaacgt gccctaaggt acggaacaac caagggaaac tttggctaat
accgtatgtg cccgagaggg gaaagattta tcgccttagg agcggcccgc gtctgattag
ctagttggtg aggtaacggc tcaccaaggc gacgatcagt agctggtctg agaggatgat
cagccacact gggactgaga cacggcccag actcctacgg gaggcagcag tggggaatat
tggacaatgg gcgcaagcct gatccagcca tgccgcgtga gtgatgaagg ccttagggtt
gtaaagctct ttcgacgggg aagataatga cggtacccgt agaagaagcc ccggctaact
tcgtgccagc agccgcggta atacgaaggg ggctagcgtt gctcggattt actgggcgta
aagcgcacgt aggcgggtcg ttaagtcggg ggtgaaatcc cggagctcaa ctccggaact
gccctcgata ctggcgatct cgagtccggg agaggtgagt ggaattccga gtgtagaggt
gaaattcgta gatattcgga agaacaccag tggcgaaggc ggctcactgg cccggtactg
acgctgaggt gcgaaagcgt ggggagcaaa caggattaga taccctggta gtccacgccg
taaacgatgg aggctagccg ttggtgagca tgctcatcag tggcgcagct aacgctttaa
gcctcccgcc tggggagtac ggtcgcaaga ttaaaactca aaggaattga cgggggcccg
cacaagcggt ggagcatgtg gtttaattcg aagcaacgcg cagaacctta ccagctcttg
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ccctcgccct tagttgccat cattcagttg ggcactctaa ggggactgcc ggtgataagc
cgagaggaag gtggggatga cgtcaagtcc tcatgggcct tacgggctgg gctacacacg
tgctacaatg gcggtgacag tgggcagcga aggggtgacc ctaagctaat ctccaaaagc
cgtctcagtt cggattgcac tctgcaactc gagtgcatga agtcggaatc gctagtaatc
gcagatcagc atgctgcggt gaatacgttc ccgggccttg tacacaccgc ccgtcacacc
atgggagttg gctttacccg aaggcgctgc gccaacccgc aaggggggca ggcgaccacg
gtagggtcag cgactggggt gaagtcgtaa caaggtagcc gtaggggaac c

Nucleotide sequence of Uncultured bacterium clone (Table 3)

cacgcaggtg gctgagtcag tcgattgtga aagccctggg cttaacctgg gaattgcagt
cgatactact cagctagagt atgggagagg gtagtggaat tcccggtgtg gcggtgaaat
gcgtagatat cgggaggaac atcagtggcg aaggcggcta cctggcccaa tactgacact
caggtgcgac agcgtgggag caaaca

Nucleotide sequence of Uncultured bacterium clone cs8A09 (Table 3)

gctggcggcg tgcctaacac atgcaagtcg aacgagaaag tggagcaatc catgagtaaa
gtggcgaccg ggtgagtaac acgtgactaa cctacctccg agtggggaat aactccggga
aaccggggct aataccgcat aacatcgcaa gatcaaagca gcaatgcgct tggagagggg
gtcgcggctg attagctcgt tggcggggta acggcccacc aaggcgaaga tcggtatccg
gcctgagagg gcgcacggac acactggaac tgaaacacgg tccagactcc tacgggaggc
agcagtgggg aattttgcgc aatgggggaa accctgacgc agcaacgccg cgtggaggat
gaagcccttt ggggcgtaaa ctcctttcga ctcggacgat aatgacggta cgagtggaag
aagcaccggc taactctgtg ccagcagccg cggtaataca gagggtgcga gcgttgttcg
gaattattgg gcgtaaaggg cgcgtangcg gtgcggtaag tcacctgtga aatctcccgg
cttaactggg agtctgcagg cgaaactacc gtgctggagt gtgggaaagg tgcgtggaat
tcccggtgtg gcggtgaaat gcg

Nucleotide sequence of Uncultured Geothermobacterium sp. clone SK520 (Table 3)

ggcgaacgct agcggcgcgc ctaacacatg caagtcgtgc gggaaagggc ttcggcccca
gtaccgcggc agacgggtga gtaacacgtg agtaacctgt cctcgggtct gggataacca
cccgaaaggg tggctaatac cggataaagt caccgggcgc aagctcggtg atgaaagggg
gcctctgcat agcaagctcc tgcctgagga ggggctcgcg gcccatcagc tagttggtgg
ggtaacggcc caccaaggct atgacgggta gccggcctga gagggtggtc ggccacacgg
gcactgagac acgggcccga ctcctacggg aggcagcagt ggggaatctt gggcaatggg
cgaaagcctg acccagcgac gccgcgtggg ggaagaaggc cttcgggtcg taaacccctg
ttctgggggа agaaccctgg ctgggttaag agcccagtca ggctgacggt accccaggag
aaagccccgg ctaactacgt gccagcagcc gcggtaagac gtaggggcgc agcgttgtcc
ggagtcactg ggcgtaaagg gcgcgtaggc ggtttagcaa gtcaggtgta aaaggccacg
gctcaaccgt ggaggtgcgc ctgaaactgc taggctagag agcaggagag gggagtggaa
ttcccggtgt aggggtgaaa tccgtagata tcgggaggaa caccggaggg gaagccggcc
agctggacag ctcctgacgc tgaggcacga aagcgtgggg agcaaaccgg attagatacc
cgggtagtcc acgccgtaaa cgatgggtgc taggtctggg gaggtaatct ctctgggccg
aagctaacgc gttaagcacc ccgcctgggg agtacggccg caaggctgaa actcaaagga
attgacgggg gcccgcacaa gcggtggagc acgtggttta attcgatgca aagcgaagaa
ccttacccgg gcttgacatg ccagggttgt accccggtgg aaacactggg gagcgtgggg
ttttctcacg cgctctggca caggtgctgc atggctgtcg tcagctcgtg tcgtgagatg
ttgggttaag tcccgcaacg agcgcaaccc ctgcccttag ttgctaccgg gtaaagccgg
gcactctaag gggactgccg gggataaccc ggaggaagga ggggatgacg tcaagtcctc
atggcccttа tgcccggggc tacacacgtg ctacaatggg gggtacagag ggttgcgaac
ccgcaagggg gagctaatcc cagaaagccc tcctcagttc ggatcggggt ctgcaactcg
accccgtgaa gccggaatcg ctagtaatgg cggatcagca tgccgccgtg aatacgttcc
cgggcctt

Nucleotide sequence of Uncultured Hyphomicrobium sp. clone AUVE (Table 3)

caatagagag tggcagacgg gtgagtaacg cgtgggaatc ttcctatcgg tacggaatag
ctcagggaaa cttggggtaa taccgcatac gcccttcggg ggaaagattt atcgccgata
gatgagcccg cgtctgatta gctagttggt gaggtaatgg ctcaccaagg cgacgatcag
tagctggtct gagaggatga ccagccacac tgggactgag acacggccca gactcctacg
ggaggcagca gtggggaata ttggacagtg ggcgcaagcc tgatccagcc atgccgcgtg
agtgacgaag gtcttcggat tgtaaaactc ttttggcggg gacgataatg acggtacccg
cagaataagc cccggctaac ttcgtgccag cagccgcggt aatacgaagg gggctagcgt
tgttcggaat tactgggcgt aaagcgcacg taggcggatt tgtaagtcag gggtgaaatc
ccggggctca acctcggaac tgcctttgat actgcaagtc ttgagtccgg aagaggtgag
tggaattcct agtgtagagg tgaaattcgt agatattagg aagaacacca gtggcgaagg
cggctcactg gtccggtact gacgctgagg tgcgaaagcg tggggagcaa acaggattag
ataccctggt agtccacgcc gtaaactatg gatgctagcc gtcggcaagc ttgcttgtcg
gtggcgcagc taacgcttta agcatcccgc ctggggagta cggccgcaag gttaaaactc
aaaggaattg ac

Nucleotide sequence of Uncultured Sediminibacterium sp. clone YL012 (Table 3)

gatgaacgct agcggcaagc ttaatacatg caagtcgagg ggcagcatga agtagcaata
ctttgatggc gaccggcaaa cgggtgcgga acacgtacac aaccttcctt ttagtgggga
atagcccaga gaaatttgga ttaatacccc gtaacataac gatgtggcat cacattgtta
ttatagcttc ggcgctagat gatgggtgtg cgtatgatta gatagttggc gaggtaacgg
ctcaccaagt ctacgatcat tagctgatgt gagagcatga tcagccacac gggcactgag
acacgggccc gactcctacg ggaggcagca gtaaggaata ttggtcaatg gacgcaagtc
tgaaccagct atgccgcgtg aaggattaag gtcctctgga ttgtaaactt cttttatctg
ggacgaaaaa aggtctttct agatcacttg acggtaccag atgaataagc accggctaac
tccgtgccag cagccgcggt aatacggagg gtgcaagcgt tatccggatt cactgggttt
aaagggtgcg taggcgggca ggtaagtcag tggtgaaatc ctggagctca actccagaac
tgccattgat actatctgtc ttgaatattg tggaggtaag cggaatatgt catgtagcgg
tgaaatgctt agatatgaca tagaacaccc attgcgaagg cagcttacta cgcatatatt
gacgctgagg cacgaaagcg tggggatcaa acaggattag ataccctggt agtccacgcc
ctaaacgatg gatactcgac atacgcgata cactgtgtgt gtctgagcga aagcattaag
tatcccacct gggaagtacg ttcgcaagaa tgaaactcaa aggaattggc gggggtccgc
acaagcggag gagtatgtgg tttaattcga tgatacgcga ggaaccttac ctgggctaga
atgctgggag accgtgggtg aaagctcact ttgtagcaat acactgccag taaggtgctg
catggctgtc gtcagctcgt gccgtgaggt gttgggttaa gtcccgcaac gagcgcaacc
cctatcatta gttgccaaca ggttaagctg ggaactctaa tgaaactgcc gccgtaaggt
gtgaggaagg aggggatgat gtcaagtcat catggccttt atgcccaggg ctacacacgt
actacaatgg gggagacaaa gggctgcaac atagcgatat gaagccaatc ccaaaaactc
cctctcagtt cagattgcag gctgcaactc gcctgcatga agctggattc gctagtaatc
gtatatcagc aatgatacgg tgaatacgtt cccggacctt gcacacaccg cccgtcaagc
catgggagtc gggtgtacct aaagtcggta accgtaagga gctgcctagg gtaaaatcga
tgactggggc t

Nucleotide sequence of Nitrosospira sp. APG3 (Table 3)

attgaacgct ggcggcatgc tttacacatg caagtcgaac ggcagcacgg gggcaaccct
ggtggcgagt ggcgaacggg tgagtaatgc atcggaacgt atccttaagt gggggataac
gcatcgaaag atgcgctaat accgcataat ctctgaggag aaaagcaggg gatcgcaaga
ccttgcgctt ttggagcggc cgatgtctga ttagctagtt ggtgaggtaa aggcttacca
aggcttcgat cagtagctgg tctgagagga cgaccagcca cactgggact gagacacggc
ccagactcct acgggaggca gcagtgggga attttggaca atgggggaaa ccctgatcca
gccatgccgc gtgagtgaag aaggccttcg ggttgtaaag ctctttcagc cggaacgaaa
aggttacggt taatacccgt gactaatgac ggtaccggaa gaagaagcac cggctaacta
cgtgccagca gccgcggtaa tacgtagggt gcgagcgtta atcggaatta ctgggcgtaa
agcgtgcgca ggcggttttg taagtcagat gtgaaatccc cgggctcaac ctgggaactg
cgtttgaaac tacaaggcta gagtgtggca gagggggggtg gaattccacg tgtagcagtg
aaatgcgtag agatgtggag gaacaccgat ggcgaaggca gcccccctggg ttaacactga
cgctcaggca cgaaagcgtg gggagcaaac aggattagat accctggtag tccacgccct
aaacgatgtc aactagttgt cgggtcttaa cggacttggt aacgtagcta acgcgtgaag
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acaagcggtg gattatgtgg attaattcga tgcaacgcga aaaaccttac ctacccttga
catgtaccga agcttgccga gaggtgagcg tgcccgaaag ggaacggtaa cacaggtgct
gcatggctgt cgtcagctcg tgtcgtgaga tgttgggtta agtcccgcaa cgagcgcaac
ccttgtcatt aattgccatc attcagttgg gcactttaat gaaactgccg gtgacaaacc
ggaggaaggt ggggatgacg tcaagtcctc atggcccttа tgggtagggc ttcacacgta
atacaatggc gcgtacagag ggttgccaac ccgcgagggg gagctaatct cagaaagcgc
gtcgtagtcc ggatcggagt ctgcaactcg actccgtgaa gtcggaatcg ctagtaatcg
cggatcagca tgtcgcggtg aatacgttcc cgggtcttgt acacaccgcc cgtcacacca
tgggagtggg tttcaccaga agcaggtagt ctaaccgcaa ggagggcgct tgccacggtg
agattcatga ctggggtg

Nucleotide sequence of Uncultured bacterium clone 5B-14 (Table 3)

attgaacgct ggcggcatgc cttacacatg caagtcgagc ggcagcgcgg gggcaaccct
ggcggcgagc ggcgaacggg tgagtaatgc atcggaacgt gtccttttgt gggggataac
cagtcgaaag actggctaat accgcatgag ctcgagagag gaaagcaggg gacccgcaag
ggccttgcgc gagaggagcg gccgatgccc gattagctag ttggtggggt aatagcccac
caaggcgacg atcggtagct ggtctgagag gacgaccagc cacactggga ctgagacacg
gcccagactc ctacgggagg cagcagtggg gaattttgga caatgggggc aaccctgatc
cagccatgcc gcgtgtgtga agaaggcctt cgggttgtaa agcactttcg gccggaacga
aatcgcgcgg attaatactc cgcgtggatg acggtaccgg aagaagaagc accggctaac
tacgtgccag cagccgcggt aatacgtagg gtgcgagcgt taatcggaat tactgggcgt
aaagtgtgcg caggtggccg cgcaagtcga gtgtgaaatc cccgggctta acttgggaat
tgcgctcgaa actacgtggc tggagtgtgg cagaggaagg tggaattcca cgtgtagcgg
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caccatggga gtggggttca ccagaagcag attgcctaac cgcaaggagg gcgtctacca
cggtgagctt catgatctgg ggtg