Symbiotic nitrogen fixation and soil carbon and nitrogen pools in a suburban native forest of subtropical Australia

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Symbiotic nitrogen fixation and soil carbon and nitrogen pools in a suburban native forest of subtropical Australia

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Abstract

Prescribed burning is a tool used in Toohey Forest management systems as a suburban forest fuel reduction burning to mitigate major bushfire risks and has played an important role in the development of plant communities in Australia. Fire influences terrestrial ecosystems and processes, which include vegetation distribution and structure, carbon (C) and nitrogen (N) cycling and climate. The current study examined the effects of prescribed burning (9-14 years after burning) on C and N cycling. We hypothesized that prescribed burning would be able to increase the availability of N, which may benefit regenerating plants shortly after the fire. The prescribed burning would influence C and N pools as well as BNF, water use efficiency, and plant growth of understory acacia species in short time, but its long-term impact might be minimal due to the ecosystem recovery and resilience in the suburban forest ecosystem of southeast Queensland, Australia. Forest litterfall and litter floor recovery would play important roles in the recovery process of soil C and N pools after 9-14 years. Soil C and N pools as well as forest litter floor would gradually be recovery after 9-14 years of prescribed burning in the suburban Toohey Forest ecosystem. Labile soil C and N pools could be affected 9-14 years after prescribed burning in the suburban Toohey Forest ecosystem of subtropical Australia. This study examined the effect of prescribed burning in the short- and medium-term on plant growth and eco-physiological responses of understory acacia species and symbiotic N fixation as well as soil C and N pools in the suburban forest ecosystem of subtropical Australia.

This project sought to determine the impacts of repeated prescribed burning on biological nitrogen fixation (BNF) by understorey acacia species, examine the N dynamics and plant N concentrations in Toohey Forest, a suburban forest in subtropical Australia, at different time periods following prescribed burning (9-14 years after prescribed burning). The study examined how prescribed burning could alter the soil biological, physical and chemical properties, particularly N cycling and availability, soil C pools and dynamics as well as soil

microbial composition, under specific fire regimes that are followed in a native forest ecosystem of southeast Queensland, Australia. However, the study explored also short-term responses of biochar amendment in typical Toohey Forest on BNF, water use efficiency, soil mineral N pools as well as tree growth of *Acacia leiocalyx* and *Acacia disparimma* 1, 2, 3, 6 and 9 months after three rates of biochar application (0, 5 and 10 t per ha) following about 12 months of prescribed burning at Site 7.

The studies were undertaken within Toohey Forest which is located 10 km south of the Brisbane, in South-East Queensland, Australia (27°30′S, 135°E). This forest has been subjected to fuel reduction prescribed burning over the last 30 years. The fuel reduction prescribed burning is usually done on a cycle of 7-10 year, depending on seasonal conditions and fuel loads. The studies were conducted at five sites within Toohey Forest. The first site, S7-B4 (B4-means 4 years after prescribed burning) was last burnt in August 2017. Site 4-B6 (B6-means 6 years after prescribed burning) was last burnt in May 2014. Site 1-B9 (9 years after prescribed burning) "27°32'26.79 S" & "153°02'39.81E", was last burned in August 2011. Site 2-B12 (12 years after prescribed burning) "27°32'34.02S" & "153°03'09.06E", was last burnt in April 2008. Finally, Site 3-B14 (14 years after prescribed burning) "27°32'28.61S"& "153°02'50.60E", was last burnt in May 2006. The main aims of this study were to assess the long-term impacts of prescribed burning on BNF, water use efficiency (WUE), and plant growth of understory acacia species as well as soil C and N pools in the Toohey Forest ecosystem.

The objectives of this PhD project, addressed in each of Chapters 3, 4 , 5, and 6 were: (1) to examine the long-term effect of prescribed burning on biological nitrogen fixation (BNF) and water use efficiency as well as tree growth of understory acacia species in a native forest ecosystem of subtropical Australia, as revealed by ^{15}N natural abundance method during the growing season (summer) and non-growing season (winter) (Chapter 3); (2) to evaluate the contribution of BNF by the understory acacia species to ecosystem N availability and cycling processes as well as ecosystem recovery in the Toohey Forest Ecosystem of subtropical Australia, in the context of fuel reduction prescribed burning and climate change (Chapter 3); (3) to examine whether biochar addition could reduce N loss and improve biological, physical and chemical properties of surface soils (Chapter 4); (4) to examine the short-term effect of biochar addition on BNF, WUE and tree growth of understory acacia species in the Toohey Forest of southeast Queensland, Australia (Chapter 4); (5) to quantify the recovery of forest litter floor and total C, total N, C and N isotope composition ($\delta^{13}C$ and $\delta^{15}N$) in the 0-5, 5-10, and 10-20 cm soil at Site 1 (S1, 9 years after burning), Site 2 (S2, 12 years after burning) and Site 3 (S3, 14 years after burning) in the suburban Toohey Forest ecosystem of subtropical Australia (Chapter 5); (6) to examine the role of forest litter floor in the nutrient cycling and forest litter floor recovery in the soil C and N pools and their dynamics in the periods of 9-14 years after prescribed burning in the suburban Toohey Forest ecosystem (Chapter 5); (7) to quantify the effect of prescribed burning on soil labile C and N pools 9-14 years after prescribed burning in the Toohey Forest ecosystems of subtropical Australia (Chapter 6); and (8) to examine the ability of soil labile C and N pools at the three burned sites to recover 9-14 years after the prescribed burning in the Toohey Forest ecosystems (Chapter 6).

In Chapter 3 (Study 1), there were no significant differences in foliar total C and $\delta^{13}C$ between *Acacia leiocalyx* and *Acacia disparimma* at all the four sites after long term prescribed burning (6-14 years after burning). However, our result showed that foliar total N concentrations of *A. leiocalyx* was higher at sites of S1, S2, S3, and S4 than those of *A. disparimma* after 6-14 years of prescribed burning. There were significant differences in foliar $\delta^{15}N$ between *Acacia leiocalyx* and *Acacia disparimma* at Site 1 (9 years after prescribed burning), and Site 3 (14 years after prescribed burning), while there were no significant differences in foliar $\delta^{15}N$ between *Acacia leiocalyx* and *Acacia disparimma* at Site 2 (12 years after prescribed burning), and Site 4 (6 years after prescribed burning). However, our result showed that $\delta^{15}N$ values of *A. leiocalyx* were significantly higher for August 2019 (winter-dry season) at sites of S1-B9 and S3- B14 after 9-14 years of prescribed burning respectively than those of *A. disparimma* in May 2019 (autumn) and August 2019 (winter) after 9 and 14 years of prescribed burning. Overall, at Site 4 (6 years after burning), BNF rates were in the range of 65-85% for *Acacia leiocalyx* and *Acacia disparimma*, while the corresponding BNF values were 40-55% at Site 1 (after 9 years of prescribed burning), 58-73% at Site 2 (after 12 years of prescribed burning), and 48-62% at Site 3 (after 14 years of prescribed burning). However, BNF of *Acacia leiocalyx* did not differ from that of *Acacia disparimma* at any of the study sites (S1, S2, S3, and S4). Meanwhile, we assessed the WUE of two acacia species growing at suburban Toohey Forest sites subjected to prescribed burns. We found that 6-14 years after prescribed burning, *A. disparimma* had higher WUE than that of *A. leiocalyx* for sites of S2, S3, and S4, but not at S1. In general, this study demonstrates that *A. disparimma* for all sites S1, S2, S3, and S4 had relatively higher WUE than *A. leiocalyx* in winter season sampling of August 2019.

In Chapter 4 (Study 2), the study explored short-term responses of biochar amendment in typical Toohey Forest on BNF, water use efficiency, soil mineral N pools as well as tree growth of *Acacia leiocalyx* and *Acacia disparimma* 1, 2, 3, 6 and 9 months after three rates of biochar application (0, 5 and 10 t per ha) following about 12 months of prescribed burning at Site 7. There were significant differences in foliar total C and total N as well as foliar $\delta^{15}N$, $\delta^{13}C$ and tree growth (tree height and diameter at ground level) of both *Acacia leiocalyx* and *Acacia disparimma* among the biochar application rates in the Toohey Forest, southeast Queensland, Australia, with the highest values in the biochar application at 5 t per ha compared with those of the control and the biochar rate at 10 t per ha, in the first 6 months. The present study indicated that the biochar treatment of 10 t ha^{-1} improved foliar total N, BNF, and $\delta^{15}N$ of understory acacia species for all sampling months, but not at month 2 (July 2019). However, our study showed that the biochar applied at 5 t ha^{-1} contributed to increases in total C, $\delta^{13}C$ and WUE, but did not differ from those of the 10 t ha^{-1} in the Toohey Forest ecosystem of subtropical Australia. Foliar total N concentrations of *A. leiocalyx* was higher in the autumn of March 2020 after 9 months of biochar application, also that foliar total N concentrations were significantly higher ($P < 0.05$) in the treatment of biochar applied at 10 t ha^{-1} in the autumn of March 2020 compared with those of the 5 t ha^{-1} and control. This study demonstrated that *A. disparimma* had relative higher BNF in the winter sampling than those of *A. leiocalyx*, and also the current study showed that BNF was increased after two months of biochar applied at 5 t ha^{-1}, which did not differ than those of biochar applied at 10 t ha^{-1}. The BNF ranged from 42.2 % to 80.7 % for *A. leiocalyx* and from 62.4 % to 93.4 % for *A. disparimma* for all foliar samples before and after biochar application during the autumn and winter of April and July 2019. There were positive relationships among $\delta^{13}C$, $\delta^{15}N$ and TN "R^2= 36.6% and R^2= 13.3%", $P < 0.05$ at month 0 and 6 for autumn and spring of April and November 2019 respectively, but there was no significant difference at month 9 for autumn of March 2020. Meanwhile, there were positive relationships among $\delta^{13}C$, $\delta^{15}N$ and TN "R^2=15.2%, R^2= 22.8% and R^2= 19.9%", $P <$ 0.05) at 1-2 months after biochar applied for winter of June and July 2019. There were no significant differences in plant growth for both tree height and diameter at ground level between of *A. leiocalyx* and *A. disparimma* at S7-B3. However, there were significant higher in plant growth for both tree height and diameter at ground level among biochar treatments at S7-B3 in the Toohey Forest. We conclude that different biochar application rates led to different effects on BNF and WUE due to the high variation in biochar properties, highlighting the role of BNF in improving plant WUE and subsequently tree growth after biochar was applied in the suburban Toohey Forest.

In Chapter 5 (Study 3), this study aimed to determine the long term (9-14 years) impact of prescribed burning on the dynamics of litter floor quantity and quality, and soil C and N pools as well as their dynamics in the Toohey Forest Ecosystems. The S1-B9 (9 years after prescribed burning), S2-B12 (12 years after prescribed burning), and S3-B14 (14 years after prescribed burning), were sampled for litter floors and 0-20 cm soil profile in August 2019. Soil samples were collected at 0-5, 5-10 and 10-20 cm depths from each of the four plots at each of the three sites (S1, S2, and S3) for total C, total N, $\delta^{13}C$ and $\delta^{15}N$ 9-14 years following the prescribed burning to examine the long term impact of prescribed burning on soil C and N pools (total C, total N, $\delta^{13}C$ and $\delta^{15}N$) and their dynamics . The litter floor samples were also collected from each plot of the three sites (S1, S2, and S3) for total C, total N, $\delta^{13}C$ and $\delta^{15}N$ 9-14 years after prescribed burning, which would be expected to be burnt in the next 1-2 years. The soil total C, total N, $\delta^{13}C$ and $\delta^{15}N$ at the 0-5, 5-10 and 10-20 cm soil depths at Site 1 after 9 years following the last prescribed burning were also compared with those of the corresponding properties shortly before the last prescribed burning in August 2011. Similarly, litter floor total C, total N, $\delta^{13}C$ and $\delta^{15}N$ as well as total C and N content at Site 1 after 9 years of prescribed burning would be compared with those of the corresponding properties in the Forest litter floor at Site 1 shortly before prescribed burning in August 2011 to quantify the recovery of C and N pools in the top 20 cm soil and litter floor 9 years after prescribed burning. Overall, after 9 years of prescribed burning at Site 1, soil total C and total N concentration at 0-5, 5-10 and 10-20 cm soil have recovered and indeed been higher than those of the corresponding soil depths shortly before the burning. Similarly, litter floor total C and N contents have also recovered after 9 years of the mild prescribed burning at Site 1, but the C:N ratio in the forest litter floor 9 years later is about 94, which is significantly higher than that of 68 sampled about 9 years ago, shortly before the prescribed burning. This highlights that the surface soil total C and total N concentrations have recovered and indeed been higher than those of the corresponding soil samples collected shortly before the prescribed burning about 9 years after prescribed burning. Similarly, the forest floor total C and N contents have also recovered about 9 years after prescribed burning at Site 1, but the C:N ratio of the forest litter floor 9 years later (96) is significantly higher than that of 68, highlighting that the forest litter floor would be more N limiting, with much less N losses or released into the soil via litter decomposition since litter floor $\delta^{15}N$ is much less 9 years after prescribed burning at Site 1 than that collected shortly before prescribed burning, while forest litter floor $\delta^{13}C$ is significantly higher 9 years later than that shortly before prescribed burning, suggesting that the vegetation / forest WUE would be higher, partly due to increasing water limitation under climate change and partly due to the suburban N deposition / N fertilization during the past 9 years. Soil total C and total N at Sites 2 and 3 have also been significantly higher 9 years later than those of soil samples collected about 9 years ago. Both soil $\delta^{13}C$ and $\delta^{15}N$ at Sites 1, 2 and 3 (9, 12 and 14 years after prescribed burning) have also been significantly lower 9 years later than those of soil samples collected 9 years ago, highlighting increasing N limitation and decreasing N availability as well as significant N deposition in the suburban forest ecosystems.

In Chapter 6 (Study 4), this study aimed to examine the availability of soil labile C and N pools such as hot water extractable organic C (HWEOC) and hot water extractable total N (HWETN), water soluble organic C (WSOC) and water soluble total N (WSTN), as well as microbial biomass C (MBC) and microbial biomass N (MBN) at the three burned sites to recover 9-14 years after prescribed burning; and to quantify the long term effect of prescribed burning on soil labile C and N pools 9-14 years after the prescribed burning in the Toohey Forest ecosystems of subtropical Australia. The S1-B9 (9 years after prescribed burning), S2-B12 (12 years after prescribed burning), and S3-B14 (14 years after prescribed burning), were sampled for 0-20 cm soil profile in August 2019. Soil samples were collected at 0-5, 5-10 and 10-20 cm depths from each of the four plots at each of the three sites (S1, S2, and S3) for determining the soil labile C and N pools. Labile C and N pools such as HWEOC, HWETN, WSOC, WSTN, MBC and MBN are commonly used as sensitive indicators of soil quality and fertility and soil organic matter stability. The repeated measures analysis revealed that labile C and N pools were recovered after 9-14 years of prescribed burning for HWEOC and HWETN, but in regards to the time since fire for 9-14 years, the present study indicated that WSOC and WSTN were decreased for all soil depth highlighting that both WSOC and WSTN were not yet recovered. Meanwhile, the results have also been indicated that fire had significantly increased MBC for both depths of 5-10 and 10-20 cm at sites of S2 and S3 after 12-14 years of prescribed burning compared with those of samples collected in August 2019. The current study also indicated that MBN was significantly decreased after 9-14 years of prescribed burning for all soil depths. In conclusion, all soil labile C and N pools were recovered 9-14 years after prescribed burning, except for WSOC and WSTN which were not recovered for S1 9 years after prescribed burning. At S1-B9, while the biomass of the litter floor was recovered, the C:N ratio of 96 was much higher than that of 68 for litter floor about 9 years ago before the last prescribed burning. This would limit the decomposition of litter floor, which would be closely linked to the soil WSOC and WSTN that were not recovered either. The lower soil $\delta^{15}N$ and higher $\delta^{13}C$ in the litter floor highlights that both water and N are becoming increasingly limiting due the climate change and prescribed burning. Overall, our study concluded that soil total C and total N were fully recovered after 9-14 years of prescribed burning, as well as litter floor leaf and twigs total C and total N were fully recovered 12-14 years after prescribed burning in the suburban Toohey forest ecosystem of subtropical Australia. Litter floor leaf and twigs total C and total N were not fully recovered 9 years after the prescribed burning at S1, compared with those of corresponding samples collected shortly before the last burn in June 2011. Litter floor leaf and twigs $\delta^{13}C$ 9, 12 and 14 years after prescribed burning at sites of S1, S2 and S3 were significantly higher compared with those of corresponding samples collected shortly before, 3 and 5 years after the prescribed burning.

Meanwhile, soil HWEOC and HWETN were recovered after 9-14 years of prescribed burning. MBN of 0-5 and 5-10 cm soil at S1 did not recover even 9 years after the prescribed burning. However, WEOC and WETN did not recover even 9 years after prescribed burning at S1. Similar trends of WEOC and WETN were also noted for sites of S2 and S3 12-14 years after prescribed burning in Toohey Forest ecosystem of subtropical Australia.

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Declaration of Originality

The experimentation, analyses, presentation and interpretation of results in this thesis represent original work that has not been previously submitted for a degree or diploma in any university. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due reference is made within the thesis itself.

(Signed)____________ _________________

Sabah Hasan Taresh

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Sabah Taresh, Shahla Hosseini Bai, Kadum Mohammed Abdullah, Jacinta Zalucki, Ashrafun Nessa, Negar Omidvar, Dianjie Wang, Juan Zhan, Fang Wang, Jiaping Yang, Edith Kichamu-Wachira, Zhihong Xu (2021). Long-term impact of prescribed burning on water use efficiency, biological nitrogen fixation and tree growth of understory acacia species in a suburban forest ecosystem of subtropical Australia. Journal of Soil and Sediments. (Published: 17 September 2021).

ALL PAPERS INCLUDED ARE CO-AUTHORED

Acknowledgment of Paper Included in this Thesis

Included in this thesis is paper in Chapter 3, which is co-authored with other researchers. My contribution to each co-authored paper is outlined at the front of the relevant chapter. The bibliographic details for the paper including all authors are:

Chapter 3: Sabah Taresh, Shahla Hosseini Bai, Kadum Mohammed Abdullah, Jacinta Zalucki, Ashrafun Nessa, Negar Omidvar, Dianjie Wang, Juan Zhan, Fang Wang, Jiaping Yang, Edith Kichamu-Wachira, Zhihong Xu (2021). Long-term impact of prescribed burning on water use efficiency, biological nitrogen fixation and tree growth of understory acacia species in a suburban forest ecosystem of subtropical Australia. Journal of Soil and Sediments. (Published: 17 September 2021).

Appropriate acknowledgments of those who contributed to the research but did not qualify as authors are included in each paper.

CHAPTER 1 General Introduction

1.1 Prescribed burning in forest management:

Fire is a major factor in the development of plant communities in Australia (Hamilton et al., 1991). The present study explores the possibility that prescribed burning has been widely used as an effective tool for protecting both native and plantation forests from the risk of damaging wildfires (Guinto et al., 1999a; Guinto et al., 1999b; Guinto, et al., 2000; Reverchon et al., 2012). Fire is able to increase the short-term availability of some nutrients (including nitrogen (N)), which may benefit regenerating plants shortly after the fire (Carter and Foster 2004).

However, plant growth and mineral nutrients, especially N, become limited by high- frequency burning (Durán et al., 2009). In the Mediterranean region, in particular, fires represent a major environmental problem, which could cause large-scale destruction of forests and a considerable increase of greenhouse gas (GHG) emissions (Fernandes et al., 2013; Moriondo et al., 2006). Prescribed burning is used in several countries to manage Mediterranean ecosystems mainly to reduce the risk of high-intensity wildfires (Battipaglia et al., 2014; Wang et al., 2014). Previous studies have noted the important effect of prescribed burning, in the short and medium-term, on tree productivity and plant eco-physiological responses which are considered of crucial importance for fuel management plans and burning prescriptions, especially in widespread flammable plantations (Ne'eman, 2000).

A study of forest ecosystem in China revealed that acacia species can fix atmospheric N_2 and be more tolerant to other stresses, which assists in the recovery after the fire (Yang et al., 2009). Previous studies have noted the importance of prescribed fire as for fire-fighting activities (Narayan et al., 2007). Prescribed burning has played an important role in changes in land use and the effectiveness achieved in suppressing wildfires, which leads to a general increase in biomass in many ecosystems (Kitzberger & Veblen, 1999; Morgan et al, 2003).

Another important finding is that fire is a natural force that has shaped many vegetation types worldwide, helping to maintain the function of ecological processes in many forests (Penman and York, 2010). Defossé et al. (2011) revealed that fire was one of the techniques used to reduce the risk of destructive wildfires, and prescribed burning has the potential of mitigating greenhouse gas (GHG) emissions. Other studies (Bowman et al., 2009; Harrison et al., 2010) found that prescribed burning can play an important role in forest maintenance and management worldwide. Penman and York (2010) observed that prescribed burning has been used to maintain ecological processes, such as maintaining the vegetation.

Wade and Lunsford (1989) revealed that prescribed burning can be described as the careful application to reduce the risk of wildfires, and has the objective to control, by fire, under specific fuel and weather conditions, silvicultural improvement, control of insects and diseases, habitat management biodiversity conservation and especially, the reduction of the risk of wildfires (Fernandes & Botelho, 2003). Fernandes et al. (2013) found that prescribed burning is increasingly being recognised and incorporated as a management tool for wildfire reduction in several European forests. Some studies showed that prescribed fires have the ability to reducing wildfire hazard, thus avoiding negative effects on different ecosystem components, such as soil, seeds, shrubs and trees (Catalanotti et al., 2010; Fernandes & Rigolot, 2007).

1.2 The importance of biological nitrogen fixation

Biological nitrogen fixation (BNF) is a natural process through which inert nitrogen (N) as N_2 gas is transformed into non-inert N, such as ammonium. It is an important biological process that moves (N) from the atmosphere into terrestrial ecosystems (Bohlool et al., 1992). Some studies have shown that BNF represents a primary source of new N input into terrestrial ecosystems (Sullivan et al., 2014; Vitousek et al. 2013). Another important finding is that the rate of BNF, at both regional and global scales, has decreased during recent decades, as a result of increasing atmospheric N deposition due to human activities(Sullivan et al., 2014).

BNF is the most important method among alternatives for N sources in the development of sustainable agricultural systems in the tropics (Dobereiner et al., 1995; Peoples et al., 1995). It has been estimated to contribute about 200 million tonnes N per year and about 80-90% of the N available to natural ecosystem (Rascio et al., 2008) and every tonne of shoot dry matter produced by crop legumes may be equivalent to 30-40 kg of N (Peoples et al., 2009), but that depends on many factors such as the type of rhizobia strain, crop variety, and interactions between specific strains and crop varieties (Hafeez et al., 2000; Shantharam & Mattoo, 1997).

BNF has a clear importance in the maintenance of N cycling and other nutrients after repeated fuel reduction burning, which is widely practiced in native forests around Australia (Raison, 1980; Guinto et al., 2000). Therefore, it is important to estimate N_2 fixation as a part of a comprehensive review of N cycling in a forest that has been subjected to prescribed burning regimes.

Investigations of forest ecosystems in a tropical plantation at Heshan National Field Research Station, Guangzhou, China, found that forest type is an important factor regulating the effects of external N input on BNF, and that elevated atmospheric N deposition in recent decades will suppress total N fixation in mature forests with legume trees (Zheng et al., 2016). Although, BNF differs in different ecosystem compartments, BNF appears to be sensitive to N input, at a whole of ecosystem scale (Reed et al., 2011).

In the review presented by Rees et al. (2005), BNF is mediated by the nitrogenase enzyme system that catalyses the adenosine triphosphate (ATP) dependent reduction of atmospheric dinitrogen (N_2) to ammonia (NH_3), whereby nitrogenase consists of two component metalloproteinase designated the molybdenum iron protein (MoFe-protein) with iron molybdenum protein (FeMo-protein), which provides the active site for substrate reduction, while Fe-protein couples ATP hydrolysis to electron transfer. The nitrogenase enzyme system is an important process in biochemical machinery, which is required for BNF (Lawson & Smith, 2001; Rees & Howard, 2000; Seefeldt et al, 2004). Both free-living soil microorganisms and symbiotic associations of microorganisms with higher plants bring about BNF. Previous studies have noted the importance of ATP and cellular metabolism in N-fixing organisms that require comparatively large amounts of P (Gutschick, 1981). The most interesting finding is that the relatively high P demand of N-fixers creates the potential for P limitation to BNF. Therefore, P makes a positive contribution to BNF (Boddey et al., 1997). The availability of P and water are important for increasing BNF under enriched CO_2 conditions.

Leguminous plants can fix atmospheric N by working symbiotically with special bacteria, called rhizobia, which live in root nodules. Rhizobia infect roots via the root hairs of some leguminous plants. The nodules that are produced by the plant as a response to the "infection" become the home for bacteria. Here they obtain energy from the host plant and take free N from the soil and air, and process it into plant-available N (Peoples et al., 1989). The result of studies by Dobereiner et al. (1995) and Peoples et al. (1995) revealed that BNF was an important alternative source of N in the development of sustainable agricultural systems in the tropics. Biological nitrogen fixation carried out by the legume-Rhizobium symbiosis contributes to agricultural sustainability by providing the required N input to agro-ecosystems. The essential mineral nutrients for symbiotic BNF by legume plants are those required for the normal establishment and functioning of the symbiosis. Macro-and-micro mineral nutrients are considered essential for several functions, and participate in various components in plants, such as chlorophyll, enzymatic activity and BNF (Marschner, 1995; Weisany et al., 2013). Biological N fixation can be affected by mineral nutrients, such as nodules and nitrogenase activity inhibition with the presence of mineral N in the soil (Sprent et al., 1988). Hedin et al. (2009) revealed that one of the major challenges for organisms is access to a sufficient supply of N, because N is not present in Earth's original crust. Previous studies have described how microorganisms use specific enzymes, called nitrogenase, to break the triple chemical bond of atmospheric N_2 and transform this molecule into bioavailable NH_3 (Reed et al., 2011).

Nitrogen is essential for all forms of life because N is required to biosynthesise basic building blocks of plant, animals and other life in the formation of, nucleotides for DNA and RNA and amino acid for the synthesis of proteins (Marschner, 1995). Another important finding is that growth of all organisms depends on the availability of mineral nutrients. Nitrogen is the most limiting nutrient and is required in large amounts. Also, N is considered an essential component of proteins, nucleic acids and other cellular constituents (Godfrey & Glass, 2011). Anand et al. (2007) found that N plays an important role in the accumulation of biomass and consequently the total production, as a result of its importance in photosynthesis through increasing leaf and mesophyll conductance and CO_2 exchange rate.

Therefore, as part of the N cycle, BNF is important for agriculture and natural forest ecosystems. Several reports have shown that N availability strongly regulates ecosystem structure and function (Elser et al., 2007), and that N is among the most abundant elements on Earth. However, it is the critical limiting element for the growth of most plants when unavailable (Graham & Vance, 2000). Various mechanisms, such as losses of C and N through volatilization and releasing inorganic C and N into soil through ashes, may influence the dynamics of C and N pools in the ecosystems after prescribed burning (Caon et al., 2014; Karhu et al., 2015; Prieto-Fernández et al., 2004). According to the results by Alcañiz et al. (2016) and Jones et al. (2015) that concentrations of total C (TC) and total N (TN) increase in soil immediately after prescribed burning, which in some cases the increased values can be observed up to one year post prescribed burning. The increased concentrations of TC and TN after prescribed burning were attributed to enhanced mineralisation of C and N and ash incorporation in soil (Alcañiz et al., 2016; Jones et al., 2015). Nutrient cycling, mainly N and carbon (C) dynamics in the soil and plant are important for ecosystem sustainability, which may be significantly affected by frequent burning used as a forest management practice (Bauhus et al., 1993; Bauhus et al., 2002; Kerkhoff et al., 2006; Alexis et al., 2007; Landsberg and Sands, 2011). Indeed, N and C dynamics can be affected by prescribed burning, whether directly or indirectly and the recovery after prescribed burning varies with fire type (Macedo et al., 2008; Rhoades et al., 2015), while the effect of time after fire will depend on soil depths (Fernández et al., 1998; Liu et al., 2007). The nutrients may be available for temporarily time after fire which benefits plants, although the leaching can contribute to nutrients losses (Yildiz et al., 2010). Fire regimes influence terrestrial ecosystems and processes, which include vegetation distribution and structure, thus the effect of fire on C and N cycling and climate (Hurteau and Brooks, 2011; Bai et al., 2012; Bai et al., 2014; Bai et al., 2015b).

1.3 The importance of biochar application for the soil and plant

Biochar is playing an important role as a soil additive to reduce N loss and improve physical and chemical soil fertility. Other promoted benefits of biochar application to soil include increased plant productivity and reduced nutrient leaching (Biederman & Harpole, 2013). Some reports have shown that biochar improves both chemical and physical soil properties, which will increase soil productivity (Glaser et al, 2002; Jeffery et al, 2011; Lehmann, 2007). Gaskin et al. (2008) observed that biochar also may alter nutrient cycles through the addition of nutrients (N, P, K, Ca and, Mg) to the soil and by influencing exchange of nutrients by addition of reactive surfaces (Gundale & DeLuca, 2006).

However, some have reported that specific combinations of biochar and soils led to decreases in crop productivity(Van Zwieten et al., 2010). One interesting finding is that the mixing of biochar into soils frequently stimulates the microbial population and activates dormant soil microorganisms (Hilscher et al., 2009; Smith et al., 2010), thus leading to significant increases in microbial respiration. Biochar, a carbon-rich product formed by the incomplete combustion of biomass, has been shown to improve soil quality and increase crop growth (Adams et al., 2013). Biochar is, produced through pyrolysis of a wide range of biomass sources, which may improve soil water storage, through altering the portion of pore size distribution related to improved aggregation (Lehmann et al. 2006).

In general, it seems that soil inorganic N adsorption by biochar is dependent on time and varies with the temperature and feedstock used to produce biochar (Hammes & Schmidt, 2009). Biochar, a carbon-rich product formed by the incomplete combustion of biomass, has been shown to improve soil quality and increase crop growth (Adams et al.2013). It has been suggested that Biochar application impacts on N_2-fixing bacteria (diazotrophs) including both free-living and symbiotic soil bacteria; thus, that biochar influences BNF, the conversion of atmospheric N_2 to ammonia (NH_3) (Giller, 2001). Increasing biochar application rate also led to increases nodulation of rhizobia due to increased availability of boron (B), molybdenum (Mo), K, P, and Ca (Rondon et al.,2007).

Biochar improves soil moisture and pH, and so it stimulates N mineralisation and nitrification, leading to improved plant uptake (Saarnio et al., 2013). Thies et al., (2015) showed that free-living diazotrophs (e.g. Azotobacter sp. and Azospirillum) are stimulated in biochar treatments due to an enlarged habitat with limited O_2 inside biochar pores. As mentioned in the literature review, the overall reduction of approximately 10% of soil inorganic N (SIN) after biochar addition regardless of experimental conditions (Nguyen et al., 2017).

Another important finding in the literature review was that the main implications of this reduction are decreases of SIN leaching, and reduced SIN availability for crops. And also, this review indicated that biochar decreased SIN when it stayed in soil for a short term (Nguyen et al., 2017). The study in macadamia orchards by Bai et al. (2016) concluded that the application of biochar on soil soluble organic C and N, led to increased soil nitrate concentration after months 12, 18 and 24. In another study, Bai et al. (2015a) observed after five years of biochar application that NH_4^+-N and NO_3^--N did not differ between control and biochar plots. Biochar can increase, decrease or have no effect on the conversion of organic N to inorganic N (soil N mineralisation) (Bai et al., 2015a; Kuzyakov et al., 2009).

1.4 The importance of litter floor and soil C and N pools recovered in the forest ecosystem

Litter floor plays an important role in nutrient cycling within forest ecosystems and litter production constitutes an important process controlling nutrient cycling within forest ecosystems and is considered to be the main mechanism of transfer of dead organic matter and nutrients from the living biomass to soils (Bansal et al., 2014; Tutua et al., 2014). The study explored that the possibility of prescribed burning may affect the quantity and quality, as result to combustion of the litter layer, which lead to loss of an important source of nutrients (Guinto et al., 1999a; Guinto et al., 1999b; Guinto et al., 2001; Tuininga et al., 2002; Penman and York, 2010). The nutrients in the soil for plant may alter after prescribed burning, which will affect nutrients cycling, thus consequently affecting litter quality and quantity (Tuininga et al., 2002; Dezzeo et al., 2004; Dezzeo and Chacón, 2006). There is a need to study litter floor and nutrient return at different periods 9-14 years following the fire to gain a full understanding about the contribution of litter floor to nutrient cycling and the effect of this disturbance on the nutritional status of the system during fire cycle for Toohey Forest ecosystems. The nutrients may be available temporarily shortly after fire, which can be beneficial for plants, although the nutrient leaching can contribute to nutrients losses too (Yildiz et al., 2010). Fire regimes influence terrestrial ecosystems and processes, which include vegetation distribution and structure, thus the effect of fire on carbon (C) and nitrogen (N) cycling and climate (Hurteau and Brooks, 2011; Bai et al., 2012; Bai et al., 2014; Bai et al., 2015b). Several reports have shown that the stable C and N isotope compositions ($\delta^{13}C$ and $\delta^{15}N$) can be a useful tool to acquire a better understanding of C and N cycling processes in forest ecosystems (Guinto et al., 2000; Xu et al., 2009; Bai et al., 2015b), and soil organic matter composition can also be affected by prescribed burning in different ecosystems (Martí-Roura et al., 2013; Ando et al., 2014; Roaldson et al., 2014; Grande et al., 2015; Rhoades et al., 2015). Other studies by Rumpel et al (2006); Rumpel et al (2007); Saito et al (2007) have found that the fire may lead to a selective loss of light isotopes in soil organic matter compared with the heavy isotopes, but this selective loss is not substantial. Previous studies have noted that the effect of fire intensity has played an important role in the change of soil organic matter composition after fire (Guinto et al., 1999; Knicker et al., 2006; Cécillon et al., 2009; Guénon et al., 2011). However, several reports have shown that the low intensity fire led to increased soil C content, as a result of partly charred material or litter from decaying trees (Knicker et al., 2006; Guénon et al., 2011).

Richards et al. (2012a, 2012b) reported that water extractable total N (WETN) increased in the soil of burnt plots compared with that of the unburnt plots in Australian tropical savannas, but the time since fire did not have a significant impact on N pools. On the other hand, some studies have also reported that soluble organic C and N decreased after the fire (Rodríguez et al., 2009; Tokareva et al., 2011; Vergnoux et al., 2011). Other studies indicated that soil type, vegetation cover, climate, soil microbes, and analytical methods used played an important role in the variations of soluble organic C and N after the fire (Chen et al., 2005; Rodríguez et al., 2009; Lu et al., 2011; Guigue et al., 2015). The effect of the time since fire on hot water extractable organic C (HWEOC) showed variation from 12% to 42% over the sampling time at three sites. Meanwhile, the 18%-27% variation of hot water extractable total N (HWETN) was explained by the time since fire at the B3 and B5 sites (Abdullah., 2016). Another study demonstrated that HWEOC and HWETN had no significant difference among the winters shortly before burning, and 5 year after prescribed burning (Wang et al. 2020). The results of the studies conducted by Boerner (1982); Raison et al (1985a); Raison et al., (1985b); and Mills and Fey (2004) indicated that prescribed burning may affect microbial activities, the particulate ash which movement by wind or erosion from burned area, leaching of ions by precipitation which led to dissolve the ash and movement on the surface and leaching beyond the root zone (Yildiz et al., 2010).

This thesis is intended to investigate the long-term effect of prescribed burning (9-14 years) on biological nitrogen fixation (BNF) of understory acacia species using the ^{15}N natural abundance method, water use efficiency, and plant growth in a suburban forest of south-east Queensland with a well-known burning history. The experiments in this project were focused on examining the ability of soil hot water extractable organic C (HWEOC) and hot water extractable total N (HWETN), water extractable organic C (WSOC) and water extractable organic N (WSON), as well as microbial biomass C (MBC) and microbial biomass N (MBN) at the three burned sites to recover 9-14 years after prescribed burning; and to quantify the long term effect of prescribed burning on soil labile C and N pools 9-14 years after the prescribed burning, as well as focused on changes in soil fertility in relation to C and N cycling and the factors that contribute to their variations after different times following repeated low intensity prescribed burning. These factors include: litterfall quantity and quality; soil labile C and N pools, soil microbial community and function; and local climate variables (rainfall and temperature) in the Toohey Forest ecosystems of subtropical Australia. The suitable interactions among these variables were also investigated in this thesis.

1.5 Research Approach and Objectives of this thesis

This project was conducted to investigate a series of research questions about the BNF, water use efficiency, and plant growth, in response to 6-14 years of prescribed burning and biochar application as well as labile soil organic C and N pools, soil microbial biomass C and N after 9-14 years of prescribed burning to assess the recovery processes over a full fire cycle in a suburban native forest of south-east Queensland. The main research questions include:

Research question 1: How does long-term prescribed burning influence biological N fixation (BNF), water use efficiency and plant growth of understory acacia species, and their BNF's relationship with ecosystem N availability and fuel reduction prescribed burning in suburban forest ecosystem? (Chapter 3)

Research question 2: What would be the short-term effect of biochar application on BNF, water use efficiency (WUE), and tree growth of understorey *Acacia leiocalyx* and *Acacia disparimma* in the Toohey Forest of subtropical Australia? (Chapter 4)

Research question 3: How would forest litter floor and soil carbon (C) and nitrogen pools (0-20 cm) recover after 9-14 years of prescribed burning in the Toohey Forest ecosystems of subtropical Australia? (Chapter 5)

Research question 4: How important would the forest litter floor recovery be considered in view of soil C and N pools and their recovery after 9-14 years of prescribed burning in the Toohey Forest ecosystem of subtropical Australia? (Chapter 5)

Research question 5: How would prescribed burning influence soil labile C and N pools 9-14 years after prescribed burning in the Toohey Forest ecosystems of subtropical Australia? (Chapter 6)

The objectives of this research were therefore to:

1. Examine the long-term effect of prescribed burning on biological nitrogen fixation (BNF) and water use efficiency as well as tree growth of understory acacia species in a native forest ecosystem of subtropical Australia, as revealed by ^{15}N natural abundance method during the growing season (summer) and non-growing season (winter); (Chapter 3)
2. Evaluate the contribution of BNF by the understory acacia species to ecosystem N availability and cycling processes as well as ecosystem recovery in the Toohey Forest Ecosystem of subtropical Australia, in the context of fuel reduction prescribed burning and climate change. (Chapter 3)
3. Assess whether biochar addition could reduce N loss and improve biological, physical and chemical properties of surface soils; and (Chapter 4)
4. Examine the short-term effect of biochar addition on BNF, WUE and tree growth of understory acacia species in the Toohey Forest of southeast Queensland, Australia. (Chapter 4)
5. Quantify the recovery of forest litter floor and total C, total N, C and N isotope composition ($\delta^{13}C$ and $\delta^{15}N$) in the 0-5, 5-10, and 10-20 cm soil at Site 1 (S1, 9 years after burning), Site 2 (S2, 12 years after burning) and Site 3 (S3, 14 years after burning) in the suburban Toohey Forest ecosystem of subtropical Australia. (Chapter 5)
6. Examine the role of forest litter floor in the nutrient cycling and forest litter floor recovery in the soil C and N pools and their dynamics in the periods of 9-14 years after prescribed burning in the suburban Toohey Forest ecosystem. (Chapter 5)
7. Quantify the effect of prescribed burning on soil labile C and N pools 9-14 years after prescribed burning in the Toohey Forest ecosystems of subtropical Australia? (Chapter 6)

8. Examine the ability of soil labile C and N pools at the three burned sites to recover 9-14 years after the prescribed burning in the Toohey Forest ecosystems. (Chapter 6)

1.6 Overview of this thesis

In this research, a series of field and laboratory investigations (Fig. 1.2) was conducted using plots established at four different sites in Toohey Forest according to the fire history (6-14 years) provided by Brisbane City Council. These investigations were carried out in order to achieve the above objectives.

Paper 1- Chapter 3 reports changes in the biological nitrogen fixation (BNF) by understory legumes; and the variation in the water use efficiency, and plant growth by understory legumes in winter, spring and autumn after 6-14 years of prescribed burning.

Paper 2- Chapter 4 reports the changes in the BNF, water use efficiency, and plant growth by understory legumes after short term biochar application.

Paper 3- Chapter 5 reports the variation in quality and quantity of litter floor, changes in litter floor C and N pools recovery, as well as changes in soil total C, total N, C and N isotope composition after 9-14 years of prescribed burning.

Paper 4- Chapter 6 reports the changes in soil labile C and N pools, and microbial biomass after 9-14 years of prescribed burning.

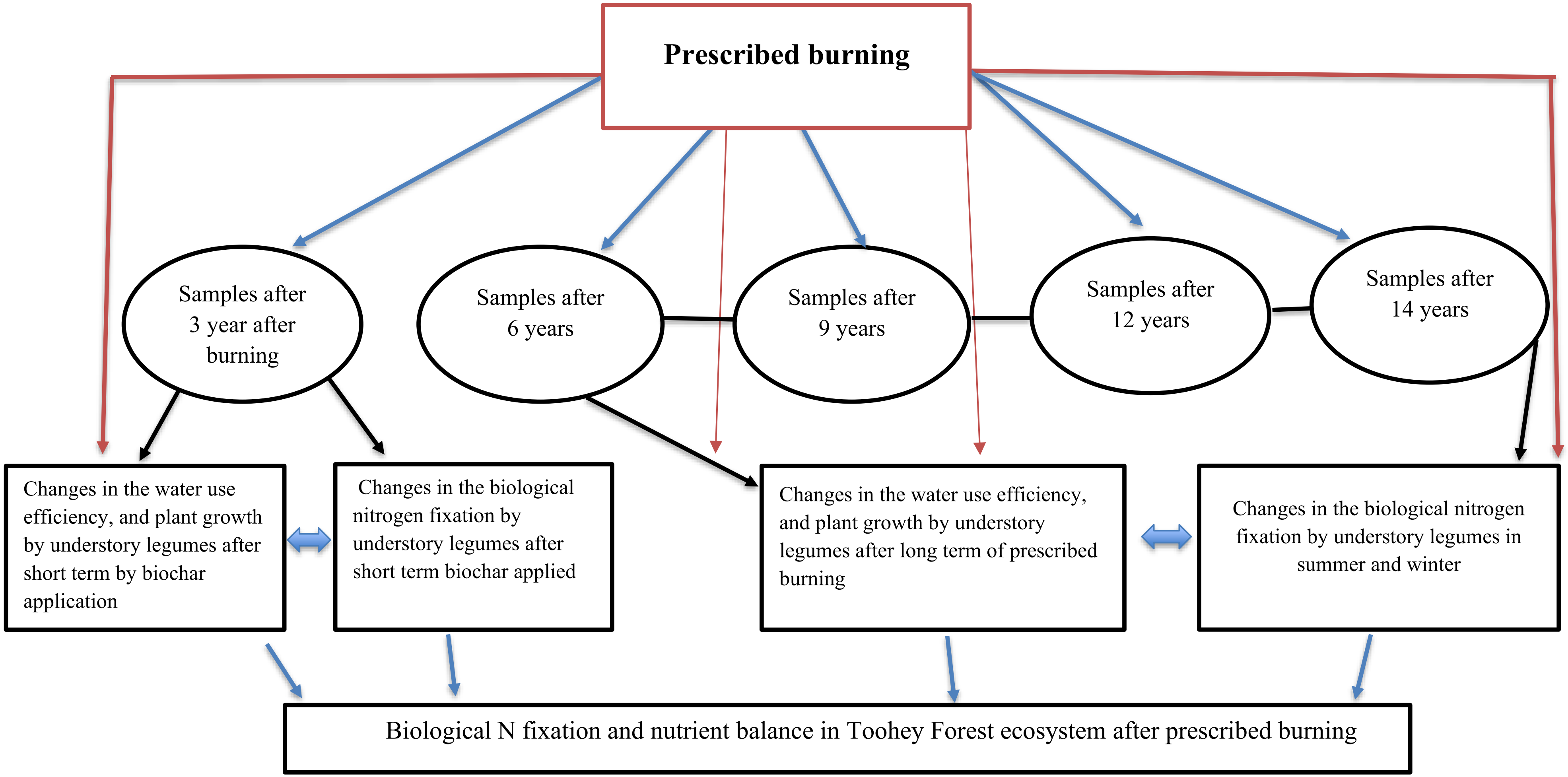

Figure 1.1 A simplified flowchart encompassing biological nitrogen fixation, nutrient balance, and eco-physiological status of understory acacia species in Toohey Forest after burning.

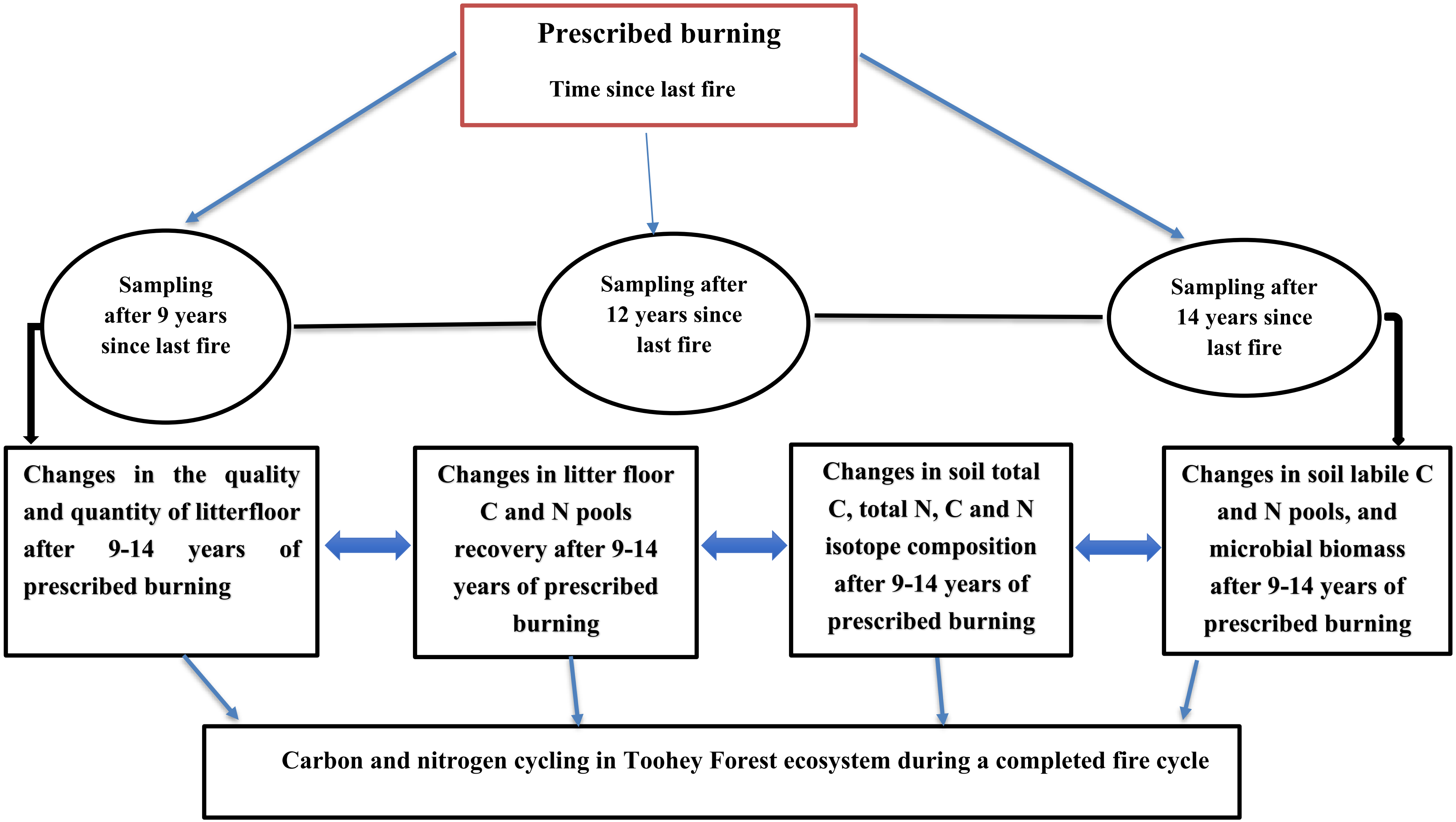

Figure 1.2 A simplified flowchart encompassing the recovery stages of C and N cycling after burning.

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CHAPTER 2 Material and Methods

2.1 General site description

The studies were undertaken within Toohey Forest which is located 10 km south of the Brisbane, in South-East, Queensland, Australia “27°30′S & 135°E”. This dry sclerophyll forest is approximately 400 ha and is dominated by different species of eucalypt and acacia species. About half of this ecosystem is a local government conservations reserve and it is surrounded by suburbans areas (Catterall et al., 2001; Farmer et al., 2004).

The climate of the region is subtropical with annual rainfall of about 1332.2 mm. Minimum temperatures were 17°C in winter, and maximum temperatures were 27°C in summer in Brisbane. In general, summer is hot and wet, whereas winter is cool and dry (Stock et al., 1987).

Toohey Forest consists of four soil types, which are different from the other regions in the same forest, whereby these soil types consist of lithosols; red yellow podzolic, red earth, and alluvial. The lithosols soil, which contains both stony components and loam to silty loam, may be rich with humus. Whereas, the podzols are mainly less than 60 cm deep in soil, and are low in nutrients, and horizon-B is sandy clay, while horizon-A is sandy loam with coarser particles (Catterall & Wallace, 1987; Stock et al., 1987).

The history of burning in Toohey Forest before 1950s is unknown. During the period between 1950s and the early 1970s, individual fires may have burned across a large proportion of the area. Whereas, individual fires were confined to more localised areas, and also created a spatially heterogeneous regime in the early 1970s. In general, fires usually occurred at the end of the dry season in September-October. From 1993, prescribed burning has been applied in the forests, including Toohey Forest, whereby there is an establishment of 27 blocks within the forest. Prescribed burning has been applied in frequencies between 4-5 years and 8-10 years (Catterall & Wallace, 1987).

Figure 2.1 Toohey Forest satellite view. The labelled zones show the sites studied in the current studies.

2.2 Study areas:

This study encompasses fieldwork at 4 sites within the Toohey Forest:

2.2.1 Site 1 (B9) (Chapters 3, 5 and 6)

This study Site 1 (S1) (27°32'26.79"S, 153°02'39.81E), is in the Block 17B of Toohey Forest, and was last burned in August 2011. This site is dominated by more than 10 species of eucalypt as well as, *Acacia leiocalyx* and *Acacia disparimma*, and contains some species of vascular plants (Catterall & Wallace, 1987). The site had also previously been burnt in March 2003. The site is located on Neranleigh-Fernvale beds and the soil type is a combination of Lithosols, Podzolic and Alluvial (Catterall & Wallace, 1987). The soil is podzolic with thickness between 60 cm and rarely 1 m. Horizon-A (10-20 cm) is sandy silt loam to sandy loam, while horizon-B (20-40 cm) ranges from sandy clay loam to sandy clay (Catterall & Wallace, 1987).

Figure 2.2 Map of Toohey Forest showing the position of Site S1 (B9), designated as Block 17b.

2.2.2 Site 2 (B12) (Chapters 3, 5 and 6)

The study site (S2, B12) (27°32'34.02"S, 153°03'09.06E) is in the Block 24 of Toohey Forest and was last burnt in April 2008. The site had also previously been burnt in September 1999. The ecological units are the Neranleigh-Fernvale beds, which is the oldest, most elevated and most common rocks in the Toohey Forest (Catterall & Wallace, 1987). The soil is lithosol which is stony and has a little profile development. Non stony components are ranging from loam to silty loam with abundant humus (Catterall & Wallace, 1987).

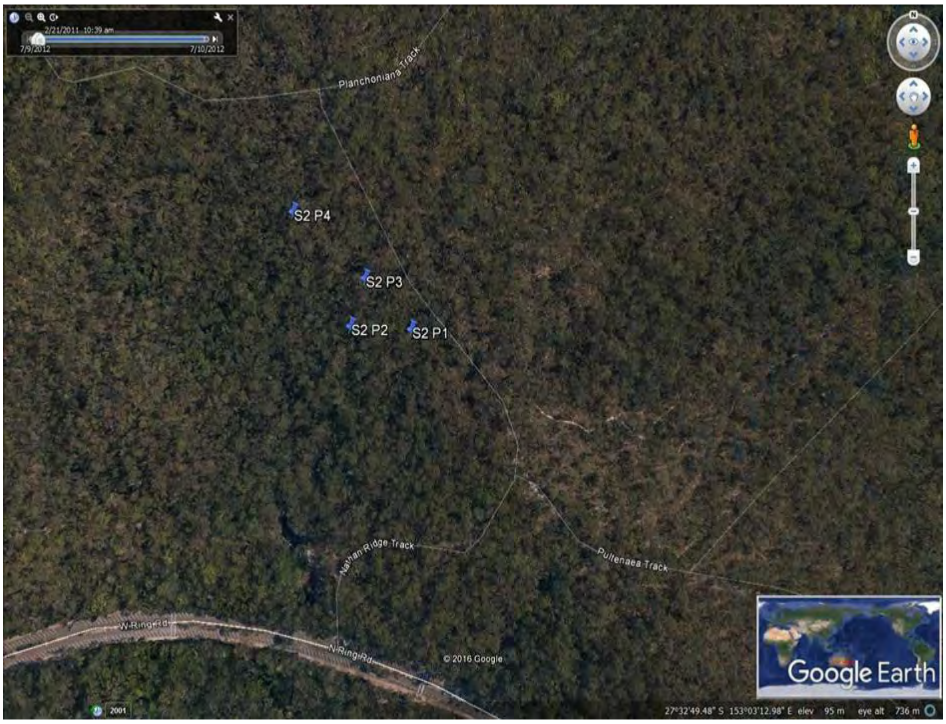

Figure 2.3 Map of Toohey Forest showing the position of Site S2 (B12), designated as Block 24.

2.2.3 Site 3 (B14) (Chapters 3, 5 and 6)

The study site (S3, B14) (27°32'28.61"S, 153°02'50.60E) is in the Block 17A of Toohey Forest and was last burnt in May 2006. The site had also previously been burnt in August 1995. The ecological units are the Neranleigh-Fernvale bed, which is the oldest, most elevated and most common rocks in the Toohey Forest (Catterall & Wallace, 1987). The soil is podzolic with thickness between 60 cm and rarely 1 m. Horizon-A (10-20 cm) is sandy silt loam to sandy loam, while B-horizon (20-40 cm) ranges from sandy clay loam to sandy clay (Catterall & Wallace, 1987).

Figure 2.4 Map of Toohey Forest showing the position of site S3 (B14), designated as Block 17A.

2.2.4 Site 4 (B6) (Chapter 3)

The study site (S4, B6) is in the Block 22 of Toohey Forest and was last burnt in May 2014. The site had also been previously been burnt in June 1999. The site (B6) is located on Neranleigh-Fernvale beds, Woogaroo subgroup and Tingalpa formation and the soil type is a combination of lithosols, podzolic and alluvial (Catterall & Wallace, 1987).

Figure 2.5 Map of Toohey Forest showing the position of site S4 (B6), designated as Block 22.

2.2.5 Site 7 (B3) (Chapter 4)

The study site (B3) is in the Block 12B of Toohey Forest and was last burnt in August 2017. The site had also been previously been burnt in March 2003. The B3 site is located on Neranleigh-Fernvale beds and the soil type is a combination of lithosols, podzolic and alluvial (Catterall & Wallace, 1987).

2.3 Study design: - (Chapters 3, 4, 5 and 6)

The trial plots were randomly located within four sites (Study 1- Chapter 3) and contained a mixture of understorey legumes such as acacia species and overstory such as eucalyptus that is typical of the area. Canopy samples were taken from the upper, and lower part of the tall plant canopy of understory acacia species; as for plant upper part, plants were collected for three seasons (spring, autumn and winter). Samples were taken from two acacia species and eucalyptus in each plot at each site. There were four sites, and four plots each site. For each plot, there were two plants of *Acacia leiocalyx*, two plants of *Acacia disparimma*, and one reference plant (*Eucalyptus psamitica*). These four circular plots with a radius of 12.62 m, giving an internal area of 500 m^2, were randomly located within each site, giving a total sampling area of one-fifth (1/5) of a hectare.

As for Study 2 (Chapter 4), The trial plots were randomly located within one site (S7-B3) and contained a mixture of understorey legumes such as *Acacia leiocalyx*, *Acacia disparimma* and overstory such as *Eucalyptus psammitica*, which is typical of the area. Each plant area was defined by four steel pegs providing an area of two square metre. Biochar treatment was applied in May 2019 with the pyrolysis temperatures of 600 °C. In this experiment, pine wood (*Pinus radiata*) were used to produce the biochar material through slow pyrolysis at temperature 600°C. The biochar produced was released via a jacketed cooling screw. The biochar was applied at three rates (0 as control, 5 t ha^{-1} (2 kg), and 10 t ha^{-1} (4 kg)) in each of the two square meters subplots. Soil samples were collected before biochar application was made in April

2019, and immediately after biochar application by 1, 2, 3, 6 and 9 months. Canopy samples were taken from only the upper part of the plants in April, June, July, August, and November of 2019, and March 2020. Samples was taken from two species of acacia (*Acacia leiocalyx*, *Acacia disparimma*) and *Eucalyptus psammitica* from each plot within the study site. In each plot, there are 6 plants of *Acacia leiocalyx* (2 plants for each of the three biochar rates of 0, 5 and 10 t ha^{-1}), 6 plants of *Acacia disparimma* (like *Acacia leiocalyx*) and one plant as *Eucalyptus psammitica* as the reference plant (no biochar added), making a total of 13 plants for each plot. These four circular plots with a radius of 12.62 m, giving an internal area of 500 m^2, were randomly located within the site, giving a total sampling area of one-fifth of a hectare. A randomised complete block design was used in this study.

For Chapters 5 and 6, there were four circular plots with a radius of 12.62 m, giving an internal area of 500 m^2, were randomly located within each site giving a total sampling area of 1/5 of hectare. Locations of the plots were randomised within the trial area but contained a mixture of understory (*Acacia leiocalyx* and *Acacia disparimma*) and overstory (*Eucalyptus psamittica*) that are typical of the area. Each plot was divided into four sections and each section had one litterfall and soil collection area. The experimental design was a randomised block design with 4 replications.

2.4 The treatments

2.4.1 Chapter 3

For the purpose of this study, treatments were taken to be the time that has elapsed since the last burn (B means year after prescribed burning). The foliar samples were taken from five different sites after the last burn. The soil samples at different depths were used in this study, which would be the horizon 0-20 cm.

- T1 Site 4-B6 (sampling 6 years after the burning).

- T2 Site 1-B9 (sampling 9 years after the burning).
- T3 Site 2-B12 (sampling 12 years after the burning).
- T4 Site 3-B14 (sampling 14 year after the burning).
- D1 depth of 0-5 cm.
- D2 depth of 5-10 cm.
- D3 depth of 10-20 cm.
- *Acacia leiocalyx*
- *Acacia disparimma*
- *Eucalyptus psammitica*

2.4.2 Chapter 4

For the purpose of this study, treatments were taken to be the time that has elapsed since the last burn. The samples were taken from the site before and after biochar application. The soil samples at different depths were used in this study, which would be the depth of 0-20 cm.

- T0 Control (No biochar)
- T1 Biochar application of 5 t / ha (2 kg for each plant)
- T3 Biochar application of 10 t / ha (4 kg for each plant)
- D1 depth of 0-5 cm.
- D2 depth of 5-10 cm.
- D3 depth of 10-20 cm.
- *Acacia leiocalyx*
- *Acacia disparimma*
- *Eucalyptus psammitica*

- Sampling time (before and 1, 2, 3, 6, and 9 months immediately after biochar application)

2.4.3 Chapters 5 and 6

For the purpose of this study, treatments were taken to be the time that has elapsed since the last burn with one treatment (T1) being the samples that were taken 9 years prior to the burn and for the T2 and T3, the samples were taken 12 and 14 years after the last burning. The soil samples at different depths were used in this study, which would be the depth of 0-20 cm.

- T1 Site 1-B9 (sampling 9 years after the burning).
- T2 Site 2-B12 (sampling 12 years after the burning).
- T3 Site 3-B14 (sampling 14 year after the burning).
- D1 depth of 0-5 cm.
- D2 depth of 5-10 cm.
- D3 depth of 10-20 cm.

2.5 Plant sample collection

In Chapter 3, the trial plots were randomly located within four sites (S1, S2, S3 and S4) and will contain a mixture of understorey legumes such as acacia species and overstory such as eucalyptus that is typical of the area. At each site, there are four plots. Within each plot, there are two plants for each of *Acacia leiocalyx* and *Acacia disparimma*, and one reference plant (*Eucalyptus psammitica*). These four circular plots with a radius of 12.62 m, giving an internal area of 500 m^2, were randomly located within each site, giving a total sampling area of one-fifth (1/5) of a hectare. There were four replicated plots at each study site. The foliar samples were collected from the upper part from two individuals of *A. leiocalyx* and *A. disparimma*, and one reference *E. psammitica*. These foliar samples were collected in three seasons (growing seasons of Spring October 2018 and Autumn May 2019, and non-growing season of Winter August 2019). In summary, the sampling regime consisted of five foliar samples (two *A. leiocalyx*, two *A. disparimma* and one *E. psammitica*) from each plot at each of the four sites over three seasons. Overall, 80 foliar samples were collected for each sampling season (*A. leiocalyx* = 32, *A. disparimma* = 32), as well as 16 samples of *E. psammitica* and 240 foliar samples were collected over three sampling seasons (80 × 3 sampling seasons = 240).

Five leaves per plant were collected and kept in separate paper bags and transferred to the laboratory. These samples were then oven-dried at 60°C to a constant weight and ground to a fine powder by a Rocklabs™ ring grinder (Guinto et al. 2000). The homogenised powder was weighed into 8 mm × 5 mm tin capsules for analysis using an isotope ratio mass spectrometer (GV Isoprime Manchester, UK). In this study, total N (TN), N isotope composition ($\delta^{15}N$), and C isotope composition ($\delta^{13}C$) were assessed using an isotope ratio mass spectrometer as described by Prasolova et al. (2000) and Xu et al. (2003).

Whilst for foliar samples (Chapter 4), the leaf samples (canopy position) were collected from two species of acacia and eucalyptus as reference plants within one site (S7). There were four replicated plots at the study site, with foliar samples collected from two individuals of *A. leiocalyx*, and *A. disparimma* for each of the 3 biochar rates (0, 5 and 10 t ha^{-1}) and one reference plant of *E. psammitica* (with no biochar added). These foliar samples were collected in six sampling months (April, June, July, August and November of 2019 and March of 2019). In summary, the sampling regime consisted of six *A. leiocalyx,* six *A. disparimma* and one *E. psammitica* from each plot of the one site over 9 months. Overall, 52 foliar samples were collected for each sampling month (*A. leiocalyx* = 24, *A. disparimma* = 24), as well as 4 samples of *E. psammitica* and 312 foliar samples were collected over 6 sampling months (52 × 6 sampling month = 312).

Three leaves per plant were collected and kept in separate paper bags and transferred to the laboratory, oven-dried at 60°C to a constant weight and ground to a fine powder by a Rocklabs™ ring grinder (Guinto et al. 2000). As described by Prasolova et al. (2000) and Xu et al. (2003), 6-7 mg plant material was packed into tin capsule to determine foliar total C and N as well as $\delta^{13}C$ and $\delta^{15}N$ by isotope ratio mass spectrometer with Eurovector Elemental Analyser (Isoprime-EuroEA 3000, Milan, Italy) (Sollins et al., 1999; Molero et al., 2011).

As for the Chapters 5 and 6, the trial plots were randomly located within three sites (S1, S2 and S3). There were four replicated plots at each study site, and within each plot there were four quadrants. The litter floor samples were collected from each quadrant in each plot at each study site of S1-B9 (9 years after prescribed burning), S2-B12 (12 years after prescribed burning), and S3-B14 (14 years after prescribed burning). Each litter floor sampling area was defined by four pegs driven into the soil marking out an area of 1 square meter. In August 2019, litter floor collection entailed using a metal square that was placed over the marker pegs and defined the edges of the sampling area. All litter deposited within the square was collected in paper bags for subsequent drying and sorting. The ground was swept clear at the start of the experiment. The four collection areas were distributed randomly, one within each quadrant of the circular plot. Sample collection was undertaken just at Site 1 (9 years after burning), Site 2 (12 years after burning), and at Site 3 (14 years after burning), while the litter floor collection was just once in 23 August 2019 at S1-B9 S2-B12 and S3-B14 following the last burning. In summary, the sampling regime consisted of one litter floor samples (leaves and twigs) in August 2019 from each quadrant within each plot at each of the three sites. Overall, 16 litter floor samples (leaves and twigs) were collected for each study site (16 × 3 sites = 48).

The litter floor were kept in separate paper bags and transferred to the laboratory, and then these samples were oven-dried at 60°C for 7 days to a constant weight and ground to a fine powder by a Rocklabs™ ring grinder (Guinto et al., 2000). The homogenised powder was weighed into 8 mm × 5 mm tin capsules for analysis using an isotope ratio mass spectrometer (GV Isoprime Manchester, UK). In this study, total N, total C, $\delta^{13}C$ and $\delta^{15}N$ were assessed using an isotope ratio mass spectrometer. As described by Prasolova et al. (2000) and Xu et al. (2003), 6-7 mg plant material was packed into tin capsule to determine foliar total C and N as well as $\delta^{13}C$ and $\delta^{15}N$ by isotope ratio mass spectrometer with a Eurovector Elemental Analyser (Isoprime-EuroEA 3000, Milan, Italy) (Sollins et al., 1999; Molero et al., 2011).

2.6 Soil sample collection

In Chapter 4, soil samples were taken after identifying each area. Each plant area was defined by four steel pegs providing an area of two square metres. Soil sampling was carried out before and 1, 2, 3, 6, and 9 months immediately after biochar application within the two square metres for each plant. The biochar was applied on 13 May 2019 within the three biochar treatments (control 0, 5 t ha^{-1}, and 10 t ha^{-1}) in each of the two square meters for 6 plants of *A. leiocalyx* and 6 plants of *A. disparimma*. Two soil cores were randomly collected within 2 m^2 for each plant in each plot within the study site, at three depths (0-5, 5-10, 10-20 cm) before, 3, 6 and 9 months, while 8 soil cores were collected randomly from each plant after 1 and 2 months of biochar application at the one depth (0-5 cm), using a 2.5 cm diameter auger. The soil samples were kept in separate plastic bags and transferred to the laboratory to measurement of soil properties. Total C, total N, $\delta^{13}C$ and $\delta^{15}N$ were determined using mass spectrometer (GV Isoprime Manchester, UK), as described by Prasolova et al. (2000) and Farquhar et al. (1982).

While, for Chapters 5, three soil cores were randomly collected from each quarter in each plot within each site. Three depths were sampled using a 2.5 cm diameter auger: 0-5, 5-10, and 10-20 cm. The soil samples were kept in separate plastic bags and transferred to the laboratory. The air-dried samples were ground (< 150 μm) and stored at room temperature. In the laboratory, soil samples were passed through 2 mm sieve. Total C and N were analysed by isotope ratio mass spectrometer with a Eurovector Elemental Analyser (Isoprime-EuroEA 3000, Milan, Italy). The 40-50 mg soil material was packed into tin capsule for both 0-5 and 5-10cm depths, and 50-60 mg soil material was packed into tin capsule for 10-20cm depth which were loaded into the auto-sampler that dropped the capsule and sample into the combustion column. A sub-sample of the soil was air-dried, and the rest was refrigerated at 4°C and processed shortly after sampling. Total C, total N, $\delta^{13}C$ and $\delta^{15}N$ were assessed using mass spectrometer (GV Isoprime Manchester, UK), as described by Prasolova et al. (2000); Xu et al., 2000, 2003 and Farquhar et al. (1982).

As for Chapter 6, Three soil samples were randomly collected from each quarter in each plot at each study site. Three depths were sampled using a 2.5 cm diameter auger: 0-5, 5-10, and 10-20 cm. The soil samples were kept in separate plastic bags and transferred to the laboratory. The air-dried samples were ground (< 150 µm) and stored at room temperature. All samples were bulked from each quarter to make one sample at each depth per plot. In the laboratory, soil samples were passed through a 2-mm sieve. A sub-sample of the soil was air-dried, and the rest was refrigerated at 4°C and processed shortly after sampling. The concentrations of organic C and total N in the filtered solutions for hot and cold water, as well as MBC and MBN were determined using Shimadzu TOC-VCSH/CSN TOC/N analyser (Chen and Xu, 2005).

2.7 Foliar and forest floor litter analysis

2.7.1 Foliar total N, total C, $\delta^{15}N$ and $\delta^{13}C$ (Chapters 3, 4 and 5)

Total C, total N, $\delta^{13}C$ and $\delta^{15}N$ were analysed by isotope ratio mass spectrometer with a Eurovector Elemental Analyser (Isoprime-EuroEA 3000, Milan, Italy). The 7-10 mg plant material was packed into tin capsule, which was loaded into the auto-sampler that dropped the capsule and sample into the combustion column. Samples were determined for total C and N, $^{13}C/^{12}C$ ratio, and $^{15}N/^{14}N$ ratio which were used to calculate $\delta^{13}C$ or $\delta^{15}N$ (‰) as:

$$\delta^{13}C \text{ or } \delta^{15}N\ (‰) = (R_{sample} / R_{std} - 1) \times 1000$$

where R_{sample} is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio of the samples and R_{std} is the $^{13}C/^{12}C$ ratio of the international PeeDee Belemnite (PDB) standard or $^{15}N/^{14}N$ ratio of N_2 gas (Xu et al., 2000; Xu et al., 2003; Molero et al., 2011).

2.7.2 Assessment of biological nitrogen fixation

Several techniques are available to assess the biological N_2 fixation (BNF) potential of plants because the best management of BNF needs the better understanding of the suitable method to assess this activity accurately (Peoples et al., 2002). The methods include:

a) The acetylene reduction assay (ARA) connects the measurement of nitrogenase activity with BNF. It is not useful to quantify BNF but is an ideal qualitative measurement (Brockwell et al., 2005) because this enzyme has the ability to reduce acetylene to ethylene and both gases are readily measured at very low concentration by gas chromatographic technique. However, the major problem with this technique to estimate absolute rates of BNF is the calibration and extrapolation in the field (Bergensen, 1980; Peoples and Research, 1989).

b) The N-difference method (NDM) measures the difference in N uptake between an N fixing legume and a non-fixing control plant (Brockwell et al., 2005; Cadisch et al., 2000; Carranca et al., 1999). It is considered as the simplest method, but it is less accurate and reliable in field applications because of the difficulty of the selection of the control plants (Brockwell et al., 2005; Cadisch et al., 2000). It may not be suitable for plants with different root traits (Yu et al., 2010). The reliability of NDM in legume-based intercropping systems was tested by two field experiments in Northwest China and the results suggested that, instead of traditional N difference method (TNDM), improved N difference method (INDM) could be more accurate to quantify the %Ndfa of N_2-fixing plants in intercropping systems with a positive correlation between INDM and natural abundance method (NA) (Yu et al., 2010).

c) The ^{15}N isotope dilution techniques separate N source in the legume as a soil N and atmospheric N. These methods can be divided to: ^{15}N enrichment and ^{15}N natural abundance (Brockwell et al., 2005; Peoples et al., 2009). The ^{15}N natural abundance method is based on the difference of ^{15}N analysis, which is enriched naturally in this method, between legumes and non-legumes plants over a period (Chalk, 1985; Peoples et al., 2009). The calculation of % Ndfa needs to calculate ^{15}N natural abundance in the non-fixing plant (references plant), which refers to ^{15}N derived from the soil, and ^{15}N natural abundance in the fixing plant, the ^{15}N natural abundance of N in the plant derived from BNF that is equivalent to the ^{15}N natural abundance in air = 0.3663 (Boddey et al., 2000; Boddey et al., 2001; Schwartz, 2009).

In Chapter 3, BNF was estimated by using the ^{15}N natural abundance method. The foliar samples were collected from two species of acacia and reference plants within each study site (S1, S2, S3, S4). The foliar samples were taken from the upper and lower parts of the tall plants; as for the short plants only the upper parts of plants were collected for three seasons (spring, winter, and summer) from two species of acacia and eucalyptus species in each plot within each site. As for Chapter 4, foliar samples were taken from the upper part from *Acacia leiocalyx* and *Acacia disparimma* and reference plants within the study site (S7, B3). The leaves were kept in separate paper bags and transferred to the laboratory. These samples were oven-dried at 60°C to a constant weight and ground to a fine powder by a Rocklabs™ ring grinder (Guinto et al., 2000). The homogenised powder was weighed into 8×5 mm tin capsules for analysis using an isotope ratio mass spectrometer (GV Isoprime Manchester, UK) to assess value of $\delta^{15}N$.

The BNF was estimated by using the ^{15}N natural abundance of two acacia species and reference plant. Also, two reference plants were identified for all estimations which could minimize the potential errors. The $\delta^{15}N$ was determined by using the following equation:

$$\delta^{15}N = 1000 \times [(R_{sample} - R_{std}) / (R_{std}]$$

Where

R_{sample} is the isotopic ratio of $^{15}N/^{14}N$ of sample and R_{std} was the $^{15}N/^{14}N$ of the atmospheric N_2.

The percentage of N derived from atmospheric N_2 (%Ndfa) was calculated using the following equation (Shearer & Kohl 1986):

$$\%Ndfa = [\delta^{15}N_{ref} - \delta^{15}N_{acacia}) / (\delta^{15}N_{ref} - B_{value})] \times 100$$

Where:

$\delta^{15}N_{ref}$: The $\delta^{15}N$ value of the reference plant

$\delta^{15}N_{acacia}$: The $\delta^{15}N$ value of the Acacia spp.

B_{value}: The relative isotopic abundance of acacia spp. growing under N-free nutrient condition.

2.7.3 Water use efficiency: (Chapters 3 and 4)

Water use efficiency (WUE) was calculated based on expression of C isotope composition ($\delta^{13}C$) (McCarroll and Loader, 2004), carbon fixed ratio to water lost during vapor through stomatal conductance, like carbon isotope discrimination, controlled by stomatal conductance and photosynthetic rate (Farquhar et al., 1982; Ehleringer et al., 1993; Leavitt et al., 2003).

Water use efficiency was defined on the concept of work by Saurer et al. (2004); and Penuelas et al. (2008), it is the ratio of CO_2 assimilation (A) to stomatal conductance for water vapor (gw):

$$A/gw = (C_a - C_i) / 1.6$$

Francey and Farquhar (1982), showing relation between the isotope ratios in plants ($\delta^{13}Cp$) and the source of CO_2 for photosynthesis, atmospheric CO_2 ($\delta^{13}Ca$) by the equation:

$$\delta^{13}C_p = \delta^{13}C_a - a - (b-a)\, C_i / C_a$$

Xu et al. (2014) calculated of WUE by using the equation:

$$A/gw = C_a\, (\delta^{13}C_p - \delta^{13}C_a + b) / (b-a) / 1.6$$

Where

C_a and C_i are the CO_2 concentration in the atmosphere and the leaf intercellular space, respectively.

a = (4.4‰) which refers to the enrichment during CO_2 diffusion, and b = (27‰) is the fractionation by Rubisco against $^{13}CO_2$. Historical annual atmospheric CO_2 concentrations were obtained by using the direct measurements at the South Pole. As for the trend in $\delta^{13}Ca$, the exponential functions adjusted for the compiled data by Feng (1998) were used.

2.7.4 Plant growth measurement (Chapters 3 and 4)

Plant height (H) and diameter at ground (D) were measured in October 2018 and October 2019 at four sites (S1, S2, S3, and S4). All understory plants such as acacia species, as well as eucalyptus species for all plots were included for growth measurements, in total, 16 plots containing an average of 80 plants, each species of acacia 32 plants (*A. leiocalyx* = 32, *A. disparimma* = 32), as well as 16 plants of *E. psammitica*.

Diameter (D) at the ground level was measured at the four sites (S1, S2, S3, and S4). The D and height (H) measurement were determined in August 2019 and November 2019 as well as the BNF was measured in October 2018 and October 2019 at four sites by using diameter tape and height measurements hypsometer (Vertex IV).

As for Chapter 4, plant height (H) and diameter at ground (D) were measured in August 2019 and November 2019 at S7. All understory plants such as acacia species, as well as eucalyptus species for all plots were included for growth measurements, in total, 4 plots containing an average of 52 plants, each species of acacia 24 plants (*A. leiocalyx* = 24, *A. disparimma* = 24), as well as 4 plants of *E. psammitica*.

Diameter (D) at the ground level was measured at S7. The D and height (H) measurement were determined in August 2019 and November 2019 using diameter tape and height measurements hypsometer (Vertex IV).

2.8 Soil chemical analysis

2.8.1 Labile C and N pools

Labile C and N fractions were measured for hot water extractable organic C (HWEOC) and total N (HWETN), water soluble organic C (WSOC) and water-soluble total N (WSTN). HWEOC and HWETN were extracted by adding 25 ml water to 5.0 g of air-dried soil and the suspensions were incubated in a capped test tube at 70°C for 18 h. After the incubation, the samples were shaken by an end-to-end shaker for 1 hour followed by centrifuging at 10,000 rpm for 10 min. The suspensions were filtered through a Whatman 42 paper, then were filtered by a 33-mm Millex syringe driven 0.45-µm filter membrane. WSOC and WSTN were extracted using only cold water for soil: water ratio (1:5) and followed the same steps used for hot water extraction. The concentrations of organic C and total N in the filtered solution were determined using Shimadzu TOC-VCSH/CSN TOC/N analyser (Chen and Xu, 2005).

2.8.2 Microbial biomass carbon (MBC) and nitrogen (MBN)

The fumigation-extraction method was used on fresh soil and it is now widely used in heated soil because of its simplicity and accuracy (Brookes et al., 1985; Vance et al., 1987; Diaz-Raviña et al., 1992). Briefly, 10 g of fresh soil fumigated with purified chloroform $CHCl_3$. Both fumigated and non-fumigated soils were extracted with 40 ml of 0.5 M K_2SO_4. After adding K_2SO_4 to soil samples, all were shaken for 30 min. The samples were then filtered through a Whatman 42 paper, then were filtered by a 33-mm Millex syringe driven 0.45-µm filter membrane. A SHIMADZU TOC-VCPH/CPN analyser was used to determine total organic C (TOC) and total N (TSN) in the fumigated and non- fumigated samples (Chen and Xu, 2005). The MBC and MBN were calculated as described by Vance et al. (1987), Brookes et al. (1985), and Jenkinson (1988).

2.9 Statistical analysis

Two-way analysis of variance (ANOVA) was conducted to detect the differences in BNF, WUE and plant growth with respect to the seasonal variation and species (study 1); to evaluate the differences in BNF, WUE and plant growth with respect to the biochar application rates and understory acacia species (study 2); to detect the differences in total N, total C, $\delta^{13}C$ and $\delta^{15}N$ with respect to the seasonal sampling times and soil depths (study 3); and to detect the differences in HWEOC, HWETN, WEOC, WEON, MBC and MBN with respect to the sampling time and soil depths (study 4). Statistical analyses were conducted with Statistix software (version 8). Differences among means of all variables over time were determined with post-hoc pairwise comparison using the Tukey HSD test at $P < 0.05$ level (Idol et al. 2002;

Schafer and Mack 2010; Köster et al. 2014). Multiple regressions were carried out to quantify the relationships among plant properties such as $\delta^{13}C$, $\delta^{15}N$, and total TN across the four sites for studies 1, 3 and 4, and as $\delta^{13}C$, $\delta^{15}N$, and total TN across any given site for study 2.

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CHAPTER 3 Long-term impact of prescribed burning on water use efficiency, biological nitrogen fixation and tree growth of understory acacia species in a suburban forest ecosystem of subtropical Australia

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My contribution to the paper involved:

Experimental design; data collection; categorisation of the data; statistical analysis, data analysis, and writing the manuscript.

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3.1 Abstract

Purpose Prescribed burning is a useful tool in a suburban forest of subtropical Australia for bushfire risk management. Toohey Forest has been managed by controlled burns and was used in this study. This study examined the long-term impacts of prescribed burning on biological nitrogen fixation (BNF) of understory acacia species estimated by ^{15}N natural abundance method, water use efficiency (WUE) as reflected in foliar carbon isotope composition ($\delta^{13}C$), and plant growth 6-14 years after prescribed burning in a suburban Toohey forest ecosystem of subtropical Australia.

Materials and methods Four sites (S1, S2, S3, and S4) were established in Toohey Forest, Queensland, Australia. Foliar samples were collected from two understory species of acacia (*A. leiocalyx* and *A. disparimma*) and reference plants (*E. psammitica*) at each study site for three seasons (growing seasons of Spring October 2018 and Autumn May 2019, and non-growing season of Winter August 2019).

Results and discussion Foliar total nitrogen (N) concentrations of *A. leiocalyx* was higher at sites of S1, S3, and S4 than those of *A. disparimma* after 6-14 years of prescribed burning. Both species still depended upon BNF for their N supply, with a higher dependence maybe in winter than in summer at S3. The highest BNF rates for *A. leiocalyx* at all sites were also found in the winter of August 2019, while the lowest BNF rates were detected during the autumn of May 2019. The BNF was estimated as 56.5% for *A. leiocalyx* and 52.8% for *A. disparimma* at S1, with the corresponding values of 74.6% at S2, 63.4% at S3, and 85.0% at S4, respectively. This study demonstrates that *A. disparimma* for S1, S2, S3, and S4 had relative higher WUE than *A. leiocalyx* in the winter of August 2019 after 6-14 years of prescribed burning. There was a significant higher plant growth rate (tree height) of *A. leiocalyx* at sites of S1, S2 and S4, compared with that of *A. disparimma*.

Conclusions Both young acacia plants were more active in BNF than older plants, with the BNF peaked between 6 and 8 years after prescribed burning. Conversely, BNF was lower after 9-14 years of prescribed burning. There were significant and positive relationship between foliar $\delta^{13}C$ and $\delta^{15}N$, highlighting the role of BNF in improving plant WUE and subsequently tree growth in the suburban native forest

Keywords: Biological nitrogen fixation. Understorey *Acacia* spp. Water use efficiency. Plant growth

3.2 Introduction

Fire plays an important role in the development of plant communities in Australia (Hamilton et al. 1991). Prescribed burning can be used as an effective tool for protecting both native and plantation forests from the risk of damaging wildfires (Guinto et al. 1999 a, b; 2000; Reverchon et al. 2012). Fire is able to increase the short-term availability of some nutrients (including nitrogen (N)), which may benefit regenerating plants shortly after the fire (Carter and Foster 2004). Fire regimes influence terrestrial ecosystems and processes, which include vegetation distribution and structure, thus the effect of fire on carbon (C) and N cycling and climate (Hurteau and Brooks 2011; Bai et al. 2012; Bai et al. 2015). Plant growth and mineral nutrients, especially N, can become limited by high-frequency burning (Durán et al. 2009). Therefore, it is important to understand the effects of high frequency burning on N cycling and plant growth.

Moving N from the atmosphere into terrestrial ecosystems is an important biological process (Bohlool et al. 1992; Reverchon et al. 2012, 2020). Some studies have shown that biological N fixation (BNF) represents a primary source of new N inputs into terrestrial ecosystems (Vitousek et al. 2013; Sullivan et al. 2014). Several reports have found that the rate of BNF, at both regional and global scales, has decreased during recent decades. This could be a result of increasing atmospheric N deposition due to human activities (Sullivan et al. 2014), and of increasing water limitation due to climate change (Sun et al. 2010; Xu et al. 2014; Fu et al. 2020; Succarie et al. 2020).

The BNF is a natural process through which inert N_2 gas is transformed into non-inert N, such as ammonium. Leguminous plants can fix atmospheric N_2 by working symbiotically with special bacteria, called rhizobia, which live in root nodules (Peoples et al. 1989). Rhizobia obtain energy from the host plant and take N from the soil and air as well and process it into plant-available N (Peoples et al. 1989; Reverchon et al. 2012, 2020). However, BNF can be affected by environmental factors and management practices such as prescribed burning. In another study (Bai et al. 2012), high BNF was reported at a site 8 years after fire, suggesting a high reliance of acacia species on BNF to compensate N limitation in the forest imposed by frequent burning. It has been reported that putative N fixer abundance increases after fires tenfold but associated symbiotic BNF do not increase to those levels (Wong et al. 2020). A study of the mixtures of *Eucalyptus globulus* and *Acacia mearnsii*, has found that planting N-fixing species increases the rates of N and P cycling in leaves of the N-fixing species (Forrester et al. 2005, 2007). Additionally, nitrate and ammonium are usually considered the most important forms of N for plant nutrition, which are both highly soluble in water and can be lost via leaching (Peoples et al. 1995; Wang et al. 2015; Wang et al. 2020). Nitrate and ammonium loss can further exacerbate N limitation and may lead to increase plant reliance on BNF.

The BNF also plays an important role in reducing fertiliser usage designed for the maintenance and sustainability of production and compensation of nutrients losses in many ecosystems (Adams and Attiwill 1984; Bai et al. 2017). *Acacia* spp. due to their BNF properties may be important for the conservation, replacement and redistribution of nutrients (particularly N). Organic N forms in soil are difficult for plants to be used directly, and firstly need to be converted to inorganic N before the absorption by plants. Meanwhile, in agricultural soils, different organic matter inputs may lead to differences in rates of soil N transformations, competition for ammonium (NH_4) by immobilisers, nitrification, and loss of nitrate (Nguyen et al. 2017; Farrar et al. 2021; Hannet et al. 2021). The N mineralisation, nitrification, and denitrification are major processes in the soil N cycle (Wang et al. 2020). However, high soil N mineralisation rates are also one of the main drivers of denitrification or leaching of ^{15}N depleted N_2O and N_2 or NO_3 respectively (Pardo et al. 2006; Hilscher et al. 2009; Wang et al. 2015; Wang et al. 2020).

Water use efficiency (WUE) is considered a useful index in providing information about plant photosynthesis and plant response to environmental factors including water availability (Huang et al. 2008; Otieno et al. 2005). Water use efficiency can be reflected in foliar C isotope composition ($\delta^{13}C$) (Huang et al. 2008). No significant difference in WUE, as shown by $\delta^{13}C$, between the two species of *A. leiocalyx* and *A. disparimma*, have been reported one year since last burnt, although *A. leiocalyx* had higher WUE than *A. disparrima* (Bai et al. 2013). Frequently burnt forest may become N limited (Reverchon et al. 2020) and hence it is important to understand species response to frequent burning in long term.

Eucalyptus dominated forests with understory acacia species in suburban areas are subject to frequent burning. Toohey Forest in subtropical Australia has been managed by controlled burns for approximately the last 25 years. The current study was designed to determine the effect of N_2-fixing species of leguminous plants (acacia species) mixed with non-N fixing trees, (eucalypt species) in response to changes in macro and micronutrient contents and cycling following prescribed burning. We hypothesised that the prescribed burning would influence C and N pools as well as BNF, WUE, and plant growth of understory acacia species in short time, but its long-term impact might be minimal due to the ecosystem recovery and resilience in the suburban forest ecosystem of southeast Queensland, Australia. In addition, BNF by understory legumes such as acacia species, which plays an important role in replenishing the N lost by volatilisation and the N losses following fuel reduction burning, could be affected by prescribed burning.

3.3 Material and methods

3.3.1 General site description

A series of studies has been conducted within Toohey Forest which is located 10 km south of Brisbane, in South-East Queensland, Australia (27°30′S, 135°E). This dry sclerophyll forest is approximately 400 ha and is dominated by different species of eucalypt and acacia species. About half of this ecosystem is a local government conservations reserve and is surrounded by suburbans areas (Catterall et al. 2001; Farmer et al. 2004). The climate of the region is subtropical, with annual rainfall 859.2 mm and 613.4 mm for 2018 and 2019, respectively (Fig. 1). Annual minimum and maximum temperatures ranged between 16.5 and 27.0 °C in 2018, and between 17.1 and 27.8 °C in 2019 (Fig. 1). In general, summer is hot and wet, whereas winter is cool and dry (Catterall and Wallace 1987).

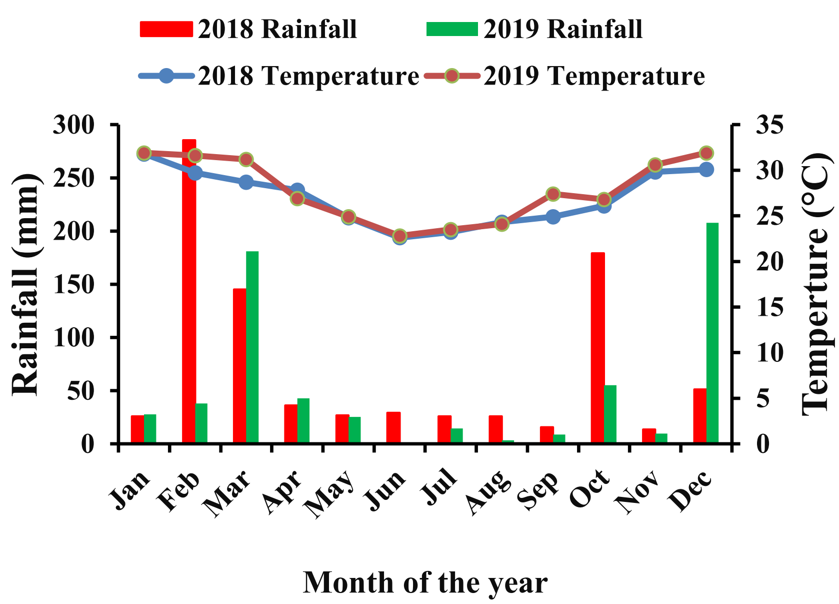

Figure. 3.1 Monthly air temperatures and rainfall during the study period of 2018-2019 in Toohey Forest.

Toohey Forest consists of four soil types, whereby these soil types consist of lithosols, red yellow podzolic, red earth, and alluvial. The lithosols soil, which contains both stony components and loam to silty loam, may be rich with humus. Whereas, the podzols are mainly less than 60 cm deep in soil, and are low in nutrients, and horizon-B is sandy clay, while horizon-A is sandy loam with coarser particles (Catterall and Wallace 1987; Stock et al. 1987). Soil properties are presented in Tables 3.1 and 3.2.

Before 1950s, the history of burning in Toohey Forest is unknown. During the period between 1950s and the early 1970s, individual fires might have burned across a large proportion of the area. In the early 1970's individual fires were confined to more localised areas, and also created a spatially heterogeneous landscape. In general, fires usually occurred at the end of the dry season in September-October. From 1993, prescribed burning has been applied in the forests, including Toohey Forest, whereby there is an establishment of 27 blocks within the forest. Prescribed burning has been applied in frequencies between 4-5 years and 8-10 years (Catterall and Wallace 1987).

Table 3.1 Soil pH, total carbon (TC), total nitrogen (TN), C isotope composition ($\delta^{13}C$) and N isotope composition ($\delta^{15}N$) at two sites: S1-B9 9 years after burning, and S3-B14 - 14 years after burning under *A. leiocalyx* and *A. disparimma* in Toohey Forest collected at three soil depths (cm).

		A. leiocalyx					*A. disparimma*					*E. psammitica*				
Site	soil depth (cm)	pH	TC (%)	TN (%)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	pH	TC (%)	TN (%)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	pH	TC (%)	TN (%)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
	0-5	5.16	5.35 (1.09)	0.23 (0.02)	-26.70 (0.27)	-0.28 (0.23)	5.14	6.15 (0.58)	0.25 (0.03)	-26.85 (0.18)	0.03(0.35)	5.43	5.10 (0.86)	0.23 (0.02)	-26.25 (0.18)	0.03(0.29)
S1-B9	5-10	5.29	4.05 (0.92)	0.18 (0.02)	-25.80 (0.29)	0.48 (0.26)	5.24	3.97 (0.49)	0.17 (0.02)	-25.90 (0.17)	0.68 (0.42)	5.37	4.32 (0.55)	0.17 (0.02)	-25.68 (0.13)	0.85 (0.16)
	10-20	5.17	3.20 (0.78)	0.13 (0.02)	-25.30 (0.47)	1.08 (0.27)	5.17	3.70 (0.66)	0.12 (0.05)	-25.58 (0.40)	1.23 (0.19)	5.25	3.65 (0.33)	0.15 (0.03)	-25.60 (0.19)	1.15 (0.39)
	0-5	4.92	2.90 (0.90)	0.13 (0.02)	-26.53 (0.27)	-2.08 (0.71)	5.11	2.85 (0.48)	0.13 (0.02)	-26.18 (0.08)	-1.60 (0.49	5.04	2.12 (0.42)	0.10 (0.01)	-26.60 (0.16)	-2.50 (0.26)
S3-B14	5-10	4.99	2.67 (1.09)	0.13 (0.02)	-25.65 (0.25)	-1.15 (0.92)	5.10	2.33 (0.42)	0.10 (0.01)	-25.50 (0.10)	-1.40 (0.69)	5.13	1.98 (0.32)	0.05 (0.03)	-25.90 (0.10)	-1.90 (0.34)
	10-20	5.10	2.32 (0.77)	0.13 (0.02)	-25.30 (0.19)	-0.58 (0.93)	5.15	1.80 (0.43)	0.08 (0.02)	-25.20 (0.08)	0.35 (0.36)	5.15	1.42 (0.36)	0.05 (0.03)	-25.50 (0.14)	-0.83 (0.47)

Numbers in the brackets denote standard errors; TC: total carbon (C); TN: total nitrogen (N); $\delta^{15}N$: stable N isotope composition; $\delta^{13}C$: stable C isotope composition.

Table 3.2 Soil pH, total carbon (TC), total nitrogen (TN), C isotope composition ($\delta^{13}C$) and N isotope composition ($\delta^{15}N$) at two sites: S2-B12 after 12 years prescribed burning (combination species), and S4-B6 - 6 years after prescribed burning (combination species) at located in Toohey Forest.

Site	soil depth (cm)	pH	TC (%)	TN (%)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
	0-5	4.80	5.97 (0.29)	0.25 (0.03)	-27.10 (0.14)	-0.30 (0.25)
S2-B12	5-10	4.84	2.92 (0.36)	0.12 (0.02)	-26.40 (0.07)	0.25 (0.24)
	10-20	4.93	1.80 (0.12)	0.10 (0.01)	-25.98 (0.20)	1.70 (0.38)
	0-5	5.41	4.72 (0.63)	0.15 (0.03)	-27.15 (0.20)	0.95 (0.35)
S4-B6	5-10	5.51	3.47 (0.42)	0.13 (0.02)	-26.20 (0.18)	1.13 (0.36)
	10-20	5.50	2.72 (0.25)	0.10 (0.01)	-25.65 (0.27)	1.55 (0.73)

Numbers in the brackets denote standard errors; TC: total carbon (C); TN: total nitrogen (N); $\delta^{15}N$: stable N isotope composition; $\delta^{13}C$: stable C isotope composition.

Four sites within Toohey Forest were used in this study. Each site had a different history of prescribed burns. Site 1 in Block 17B was last burned in August 2011 (9 years after prescribed burning), and earlier in March 2003. Site 2 in Block 24 of Toohey Forest was last burnt in April 2008 (12 years after prescribed burning), and earlier in September 1999. Site 3 in Block 17 was last burnt in May 2006 (14 years after prescribed burning), and earlier in August 1995. Site 4 in Block 22 was last burnt in May 2014 (6 years after prescribed burning) and burnt earlier in June 1999.

3.3.2 Plant sample collection and analysis

The trial plots were randomly located within four sites (S1, S2, S3 and S4) and will contain a mixture of understorey legumes such as acacia species and overstory such as eucalyptus that is typical of the area. Each plot, there are two *Acacia leiocalyx*, two *Acacia disparimma*, and one reference plants (*Eucalyptus psammitica*). These four circular plots with a radius of 12.62 m, giving an internal area of 500 m^2, were randomly located within each site, giving a total sampling area of one-fifth (1/5) of a hectare. There were four replicated plots at each study site. The foliar samples were collected from the upper part from two individuals of *A. leiocalyx* and *A. disparimma*, and one reference *E. psammitica*. These foliar samples were collected in three seasons (growing seasons of Spring October 2018 and Autumn May 2019, and non-growing season of Winter August 2019). In summary, the sampling regime consisted of five foliar samples (two *A. leiocalyx,* two *A. disparimma* and one *E. psammitica*) from each plot at each of the four sites over three seasons. Overall, 80 foliar samples were collected for each sampling season (*A. leiocalyx* = 32, *A. disparimma* = 32), as well as 16 samples of *E. psammitica* and 240 foliar samples were collected over three sampling seasons (80 × 3 sampling seasons = 240).

Five leaves per plant were collected and kept in separate paper bags and transferred to the laboratory. These samples were then oven-dried at 60°C to a constant weight and ground to a fine powder by a Rocklabs™ ring grinder (Guinto et al. 2000). The homogenised powder was weighed into 8 mm × 5 mm tin capsules for analysis using an isotope ratio mass spectrometer (GV Isoprime Manchester, UK). In this study, total N (TN), N isotope composition ($\delta^{15}N$), and C isotope composition ($\delta^{13}C$) were assessed using an isotope ratio mass spectrometer as described by Prasolova et al. (2000) and Xu et al. (2003). The BNF by the ^{15}N natural abundance for two acacia species was estimated. Also, two reference plants were identified for all estimations which could minimize the potential error. The values of $\delta^{15}N$ were determined using the following equation:

$\delta^{15}N = 1000 \times [(R_{sample} - R_{std}) / R_{std}]$ ………………………………. (1)

Where, R sample is the isotopic ratio $^{15}N/^{14}N$ of sample and R std refers to the atmospheric N_2.

The percentage of N derived from atmospheric N_2 (% Ndfa) was calculated using the following equation (Shearer and Kohl 1986):

$\%Ndfa = [\delta^{15}N_{ref} - \delta^{15}N_{acacia}) / (\delta^{15}N_{ref} - B_{value})] \times 100$ ………. (2)

Where:

$\delta^{15}N_{ref}$: The $\delta^{15}N$ value of the reference plants

$\delta^{15}N_{acacia}$: The $\delta^{15}N$ value of the *Acacia* spp.

B_{value}: The relative isotopic abundance of acacia species growing under N-free nutrient condition.

The present study used different *B* values varying from -0.3‰ to 1.0‰ in this study to determine an applicable *B* value for acacia species to estimate BNF at the four sites. In our study, using *B* value of -0.3‰ resulted in an estimation of %Ndfa that was a better and more reasonable than that of 1.0 ‰ for all the study sites.

WUE was defined as the ratio of CO_2 assimilation (A) to stomatal conductance for water vapor (gw):

$A/gw = (C_a - C_i) / 1.6$ ………………………………. (3)

The relation between the isotope ratios in plants ($\delta^{13}Cp$) and the source of CO_2 for photosynthesis and the atmospheric CO_2 ($\delta^{13}Ca$) is described by the equation:

$\delta^{13}Cp = \delta^{13}C_a - a - (b-a)\, C_i / C_a$ …………………… (4)

Xu et al. (2014) calculated of WUE by using the equation:

$A/gw = C_a (\delta^{13}C_p - \delta^{13}C_a + b) / (b-a) /1.6$

Where

C_a and C_i are the CO_2 concentration in the atmosphere and the leaf intercellular space, respectively.

a = (4.4‰) refers to the enrichment during CO_2 diffusion, and b = (27‰) is the fractionation by Rubisco against $^{13}CO_2$. Historical annual atmospheric CO_2 concentrations were obtained by using the direct measurements at the South Pole. As for the trend in $\delta^{13}C_a$, the exponential functions adjusted for the compiled data by Feng (1998) were used.

3.3.3 Plant growth measurement

Plant height (H) and diameter at ground level (D) were measured in October 2018 and October 2019 at four sites (S1, S2, S3, and S4). All understory plants such as acacia species, as well as eucalyptus species for all plots were included for growth measurements, in total, 16 plots containing an average of 80 plants, 32 plants for each *Acacia* sp. (*A. leiocalyx* = 32, *A. disparimma* = 32) and 16 plants of *E. psammitica*.

The D and height (H) measurement were determined in October 2018 and October 2019 at four sites by using diameter tape and height measurements hypsometer (Vertex IV).

3.3.4 Statistical analysis

Two-way repeated analysis of variance (ANOVA) was conducted to detect the differences in BNF, WUE and plant growth with respect to the seasonal variation and species. Statistical analyses were conducted with Statistix software (version 8). Direct comparison among experimental sites was not possible due to the fact that they had different properties and plant cover, so each site has been independently assessed. Differences among means of all variables over time were determined with post-hoc pairwise comparison using the Tukey HSD test at $p < 0.05$ level (Idol et al. 2002; Schafer and Mack 2010; Köster et al. 2014). Multiple regressions were carried out to quantify the relationships among plant properties such as $\delta^{13}C$, $\delta^{15}N$, and total TN across the four sites.

3.4 Results

3.4.1 Foliar total N, BNF and $\delta^{15}N$

Foliar total N concentrations of *A. leiocalyx* and *A. disparimma* were significantly different ($P < 0.05$) between the two species 9, 14, and 6 years after prescribed burning at sites of S1, S3, and S4, respectively, while there was no significant difference in foliar total N concentrations between them at S2 (12 years after prescribed burning). Our result showed that foliar total N concentrations of *A. leiocalyx* was higher (2.5%, 2.4%, 2.4%, and 2.3%) at sites of S1, S2, S3, and S4 than those of *A. disparimma* (2.3%, 2.0%, 2.0%, and 1.9%) after 6-14 years of prescribed burning (Fig. 3.2).

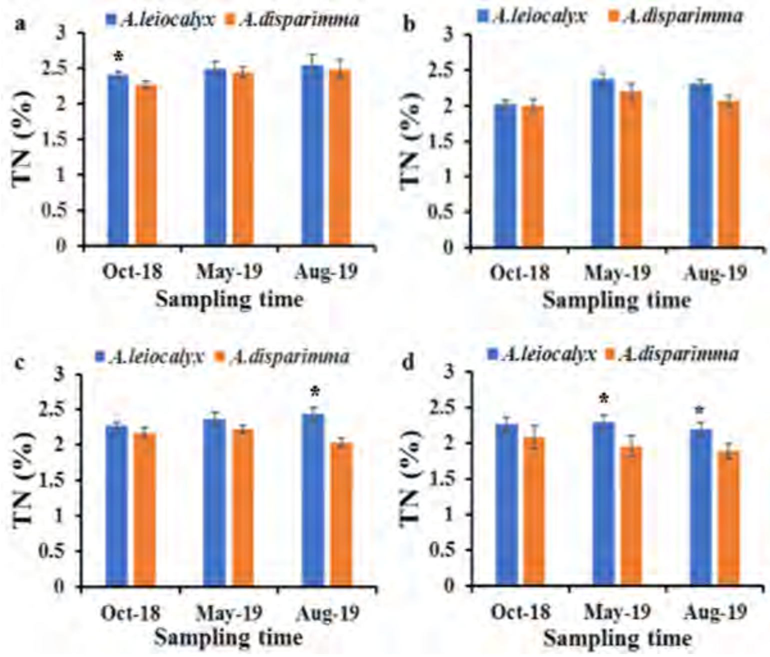

Figure. 3.2 Total nitrogen (TN) of *Acacia leiocalyx* and *Acacia disparimma* of all study sites: (a) S1-B9 (9 years after burning), (b) S2-B12 (12 years after burning); (c) S3-B14 (14 years after burning); and (d) S4-B6 (6 years after burning). Asterisk indicates significant differences between *Acacia leiocalyx* and *Acacia disparimma* at the level $P<0.05$. Bars are means ± standard errors.

The present study indicated that % Ndfa of *A. leiocalyx* was significantly higher at sites of S1 and S3 than those of *A. disparimma* after 9 and 14 years of prescribed burning on only one occasion at each site (Fig. 3.3). The % Ndfa ranged from 56.5% to 76.6% for *A. leiocalyx* and from 49.0% to 78.1% for *A. disparimma* at four sites for the spring of October 2018. The estimated %Ndfa varied from 47.6% to 85.1% for *A. leiocalyx* during the autumn and winter seasons of May and August 2019 compared with the 40.1% to 83.6% for *A. disparimma* (Fig. 3.3).

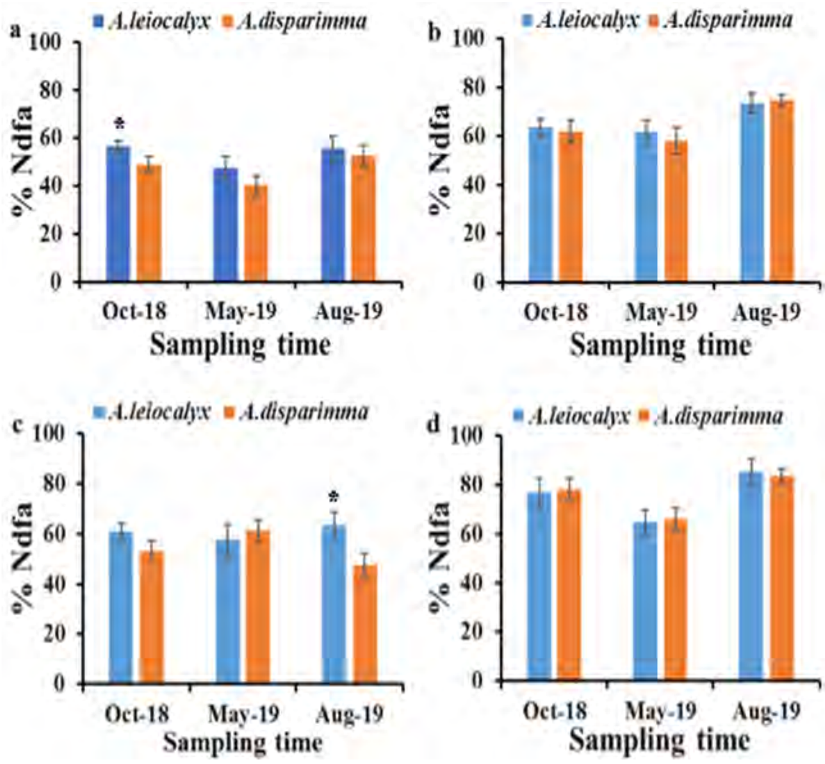

Figure. 3.3 Percentage of N derived from atmosphere (%Ndfa) of understorey *Acacia leiocalyx* and *Acacia disparimma* at (a) S1-B9 (9 years after burning), (b) S2-B12 (12 years after burning); (c) S3-B14 (14 years after burning); and (d) S4-B6 (6 years after burning). Asterisk indicates significant differences between %Ndfa of *Acacia leiocalyx* and that of *Acacia disparimma* at $P < 0.05$. Bars are means ± standard errors.

Our result showed that $\delta^{15}N$ values of *A. leiocalyx* were significantly higher (-1.8‰ and -1.6‰) for August 2019 (winter-dry season) at sites of S1-B9 and S3- B14 after 9-14 years of prescribed burning respectively than those of *A. disparimma* (-2.3‰ and -2.2‰) in May 2019 (autumn) and August 2019 (winter) after 9 and 14 years of prescribed burning, but not at S2 and S4 after 12 and 6 years of prescribed burning (Fig. 3.4).

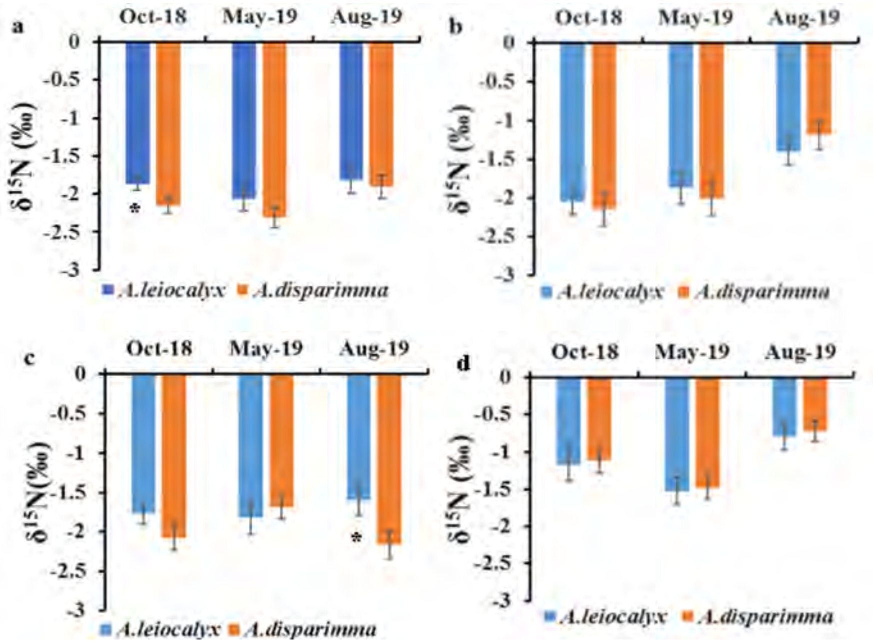

Figure. 3.4 Foliar $\delta^{15}N$ of *Acacia leiocalyx* and *Acacia disparimma* for (a) S1-B9 (9 years after burning), (b) S2-B12 (12 years after burning), (c) S3-B14 (14 years after burning), and (d) S4-B6 (6 years after burning). Asterisk indicates significant differences between $\delta^{15}N$ of *Acacia leiocalyx* and *Acacia disparimma* at the level $P < 0.05$. Bars are means ± standard errors.

3.4.2 $\delta^{13}C$ and WUE

Our result showed that foliar $\delta^{13}C$ values of *A. disparimma* were higher (-29.5‰, -30.9‰, and -29.5‰) in August 2019 (winter dry season) at sites of S2, S3, and S4 than those of *A. leiocalyx* (-31.2‰, -31.6‰, and -31.4‰) in October 2018 (spring) and May 2019 (autumn) after 12, 14, and 6 years of prescribed burning (Table 3.3), but not at S1 after 9 years of prescribed burning. The WUE of *A. disparimma* was higher (62.8, 45.8, and 62.8 μmol mmol^{-1}) in August 2019 (winter-dry season) at sites of S2, S3 and S4, respectively than those of *A. leiocalyx* (44.3, 35.0, and 39.1 μmol mmol^{-1}) after 12, 14 and 6 years of prescribed burning (Table 3.3), but not at S1.

Table 3.3 Foliar carbon (C) isotope composition ($\delta^{13}C$) and water use efficiency (WUE) of *A. leiocalyx* and *A. disparimma* at (a) S1-B9 (9 years after burning), (b) S2-B12 (12 years after burning), (c) S3-B14 (14 years after burning), and (d) S4-B4 (4 years after burning). Different letters indicate significant differences at $P < 0.05$.

		Site 1		Site 2		Site 3		Site 4	
Sampling time	Species	$\delta^{13}C$ (‰)	WUE ($\mu mol\ mmol^{-1}$)	$\delta^{13}C$ (‰)	WUE ($\mu mol\ mmol^{-1}$)	$\delta^{13}C$ (‰)	WUE ($\mu mol\ mmol^{-1}$)	$\delta^{13}C$ (‰)	WUE ($\mu mol\ mmol^{-1}$)
October 2018	*A.leiocalyx*	-30.8 a	46.3 a	-30.7 a	47.3 a	-31.5 a	38.2 a	-31.4 a	39.1 a
	A.disparimma	-31.2 a	41.8 a	-30.7 a	46.9 a	-31.7 a	35.0 a	-31.4 a	39.4 a
May 2019	*A.leiocalyx*	-30.6 a	49.9 a	-31.2 a	44.3 a	-31.6 a	37.9 a	-31.3 a	40.8 a
	A.disparimma	-30.9 a	45.9 a	-30.8 a	47.3 a	-31.3 a	41.1 a	-30.6 a	49.5 a
August 2019	*A.leiocalyx*	-30.4 a	52.0 a	-30.8 b	50.0 a	-31.1 a	43.4 a	-30.2 a	54.2 a
	A.disparimma	-30.4 a	51.8 a	-29.5 a	62.8 a	-30.9 a	45.8 a	-29.5 a	62.5 a

3.4.3 Relationship between plant physiological variables

For the spring sampling in October 2018, 22.3% of the variation in foliar $\delta^{13}C$ (Table 3.4) of both acacia species was explained by foliar $\delta^{15}N$ (12.6%) and total N (9.7%) at S1-B9, while 37.3% of the foliar $\delta^{13}C$ was explained by foliar $\delta^{15}N$ (26.5%) and total N (10.8%) at S2-B12 (Table 3.4). There were 37.8% and 22.6% of foliar total N at S2-B12 and S3-B14, which were explained by the corresponding foliar $\delta^{15}N$ values respectively (Fig. 3.5).

For the autumn sampling of May 2019, there were 21.6%, 61.5% and 44.0% of the variation in foliar total N of both acacia species at sites of S1-B9, S2-B12, and S3-14 respectively explained by the corresponding foliar $\delta^{15}N$ (Fig. 3.5). About 40.6% of the variation in foliar $\delta^{13}C$ of acacia species at site of S4-B6 was explained by $\delta^{15}N$ and total N (Table 3.4).

For the winter sampling of August 2019, 28.7% of the variation in foliar $\delta^{13}C$ of both acacia species at site of S4-B6 was explained by the corresponding foliar $\delta^{15}N$, while 62.1% of the variation in foliar $\delta^{13}C$ of both acacia species at site of S1-B9 was explained by the corresponding foliar $\delta^{15}N$ and total N (Table 3.4).

Table 3.4 Relationship among foliar $\delta^{13}C$, total N (TN) and $\delta^{15}N$; and between $\delta^{13}C$ and $\delta^{15}N$ of both acacia species for the spring sampling in spring (October 2018), autumn (May 2019), and winter (August 2019) at sites of S1-B9, S2-B12, and S3-B14 and S4-B6 in Toohey Forest south-east Queensland.

Sampling time	S1-B8	S2-B11	S3-B13	S4-B4
Spring October 2018	$\delta^{13}C$ (‰) = -27.42 + 0.595 ($\delta^{15}N$) -1.044(TN) ($R^2 = 0.223$, n = 32, P< 0.05)	$\delta^{13}C$ (‰) = - 25.76 + 0.953 ($\delta^{15}N$) – 1.495 (TN) ($R^2 = 0.373$, n = 24, P < 0.05)	ns	ns
	$\delta^{13}C$ (‰) = -29.87+0.592 ($\delta^{15}N$) ($R^2 = 0.126$, n = 32, P < 0.05)	$\delta^{13}C$ (‰) = - 29.46 + 0.599 ($\delta^{15}N$) ($R^2 = 0.265$, n = 16, P < 0.05)	ns	ns
Autumn May 2019	ns	ns	ns	$\delta^{13}C$ (‰) = -26.18 + 0.667 ($\delta^{15}N$) - 1.805(TN) ($R^2 = 0.406$, n = 16, P < 0.05)
Winter August 2019	$\delta^{13}C$ (‰) = -37.70 + 0.450 ($\delta^{15}N$) + 3.434 (TN) ($R^2 = 0.621$, n = 32, P < 0.05)	ns	ns	ns
	ns	ns	ns	$\delta^{13}C$ (‰) = -29.00 + 1.239 ($\delta^{15}N$) (R^2= 0.287%, n = 16, P < 0.05)

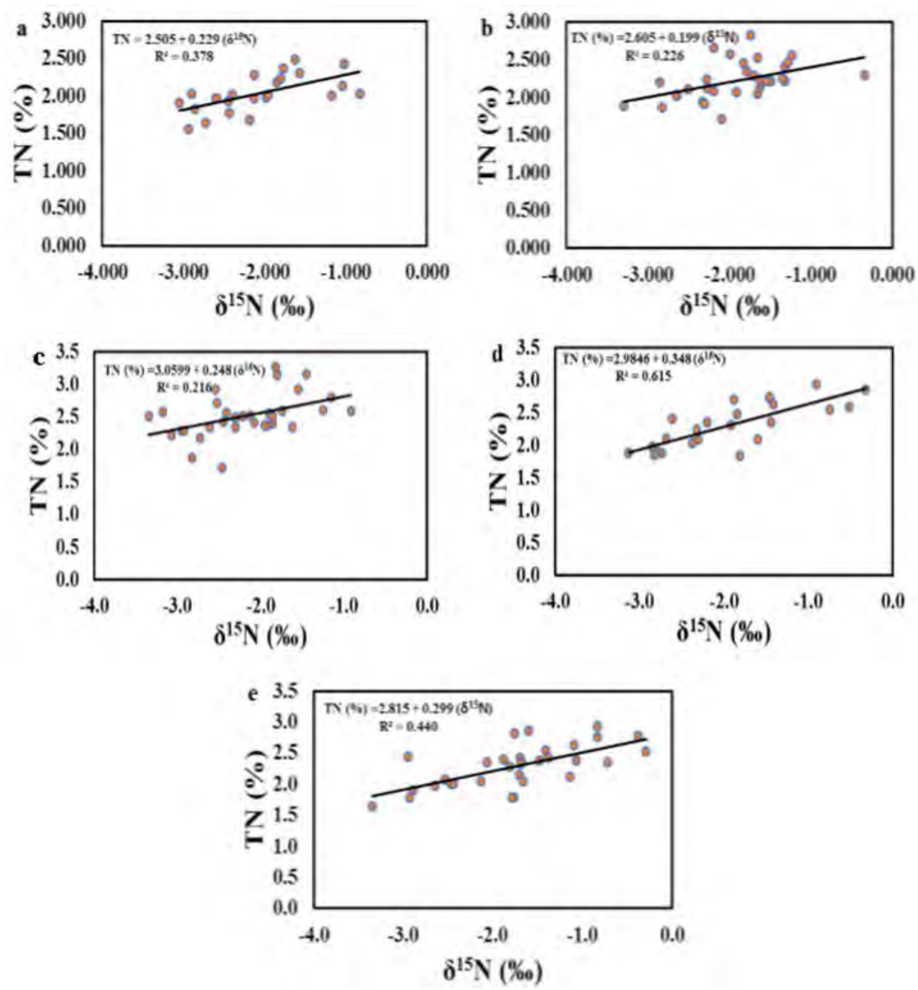

Figure. 3.5 Relationships between foliar TN and $\delta^{15}N$ of both acacia species for the spring sampling in October 2018 at (a) S2-B12; (b) S3-B14; and for the autumn sampling (May 2019) at (a) S1-B9; (b) S2-B12; and (c) S3-B14 in Toohey Forest south-east Queensland.

3.4.4 Plant height and diameter at ground level

The heights of *A. leiocalyx* were significantly higher than those of *A. disparimma* at all sites of S1-B9, S2-B12, and S3-B14, and S4-B6 ($P < 0.05$; Table 3.5). Similarly, diameters at ground level (DGL) of *A. leiocalyx* were also higher than those of *A. disparimma*, but only statically significant ($P < 0.05$) at S1-B9, S2-B12 and S4-B6 (Table 3.5).

Table 3.5 Heights (m) and diameter at ground level (DGL (mm)) of *A. leiocalyx* and *A. disparimma* at all study sites, (a) S1-B9 (9 years after burning), (b) S2-B12 (12 years after burning), (c) S3-B14 (14 years after burning), and (d) S4-B6 (6 years after burning). Different letters indicate significant differences in Heights and DGL between the acacia species at $P < 0.05$.

	Site 1		Site 2		Site 3		Site 4	
Species	Height (m)	DGL (mm)	Height (m)	DGL (mm)	Height (m)	DGL (mm)	Height (m)	DGL (mm)
A.leiocalyx	2.03 (0.16) a	26.6 (2.73) a	1.1 (0.08) a	20.2 (2.25) a	1.2 (0.13) a	19.5 (2.77) a	1.1(0.09) a	8.4 (0.95) a
A.disparimma	1.01 (0.13) b	18.3 (1.37) a	0.9 (0.08) b	10.0 (0.73) b	0.8 (0.10) a	14.3 (2.66) b	0.7(0.06) b	6.0 (0.36) b

3.5 Discussion

3.5.1 Foliar total N, BNF, and $\delta^{15}N$

This study demonstrates that *A. leiocalyx* for all sites of S1, S2, S3, and S4 had relative higher total N than *A. disparimma*, which is similar to the findings from Bai et al. (2012, 2013), Ma et al. (2015) and Reverchon et al. (2020). The present study showed that *A. leiocalyx* may recover more quickly than *A. disparimma* after 6-14 years of prescribed burning. However, the prescribed burning may also make a greater contribution to the N recovery, considering that N is one of the main components in Rubisco, the enzyme facilitating C fixation at the carboxylation site (Wilson et al. 2000).

In general, there were significant differences in foliar total N concentrations between both acacia species at S1, S3, and S4 after 9, 14, and 6 years of prescribed burning, while there was no significant difference in foliar total N concentrations at S2 (Fig. 2). This is similar to the findings of Bai et al. (2012) and Ma et al. (2015) who found that foliar total N at S1 was significantly greater in *A. leiocalyx* than that of *A. disparrima*, but not at S2, but this contradicts the findings from Bai et al. (2013) at Site 2. This might be somewhat due to the fact that this study was undertaken quite a few years later compared with that of Bai et al. (2013). The temporal and spatial variations in foliar total N concentrations might account for these differences in response to the prescribed burning events.

We assessed the BNF of two acacia species growing at suburban Toohey Forest sites subjected to prescribed burns. We found that even 6-14 years after the prescribed burning, both acacia species still depended upon BNF for their N supply. This study demonstrates that *A. leiocalyx* at all sites of S1, S2 S3, and S4 had relatively higher % Ndfa than those of *A. disparimma*. The highest BNF rate for *A. leiocalyx* at all sites were also found in the winter season of August 2019, while the lowest BNF rates were detected during the second subsequent samplings in the autumn season of May 2019. There were significant differences in BNF between *A. leiocalyx* and *A. disparimma* and among the sampling seasons for both acacia species at S1-B9 and S3-B14, but not at sites of S2-B12 and S4-B6. This contradicts the findings from Ma et al. (2015) and Reverchon et al. (2020). The seasonal differences in BNF were found for both *A. disparrima* and *A. leiocalyx* (Reverchon et al. 2020). However, both Ma et al. (2015) and Reverchon et al. (2020) reported the BNF for the short and medium- term impact of prescribed burning at the relevant sites.

The estimated % Ndfa based on ^{15}N natural abundance technique for *A. leiocalyx* and *A. disparimma* at S1 agreed with those for other acacia species reported in Australia: 59.6% to 82.5% for *A. leiocalyx* and 35.8% to 72.0% for *A. disparimma* (Bai et al. 2012). At S2, the BNF ranged from 75.6% to 86.2% for *A. leiocalyx* and from 51.5% to 80.6% for *A. disparimma* (Bai et al. 2012). Our results showed that the BNF after 6-14 years was higher in the winter season than those in spring and autumn. The range in our study was much narrower for both species, and this variability may have been due to the within treatment variation caused by spatial variation of mutualistic organisms (Boddey et al. 1997, 2000) or non-homogenized N and water availability in the soil (Stevenson et al. 1995).

We assessed the BNF for young and old plants of acacia species (acacia ages) growing at suburban Toohey Forest sites subjected to 6-14 years of prescribed burns. We found that BNF numbers for younger plants peaked after 6 years of prescribed burning at S4 compared with those of the older understory plants at sites of S1, S2 and S3 after 9-14 years of prescribed burning. Since young acacia plants between 6 and 8 years were more active, the roots would be longer and healthier than those of the older plants. Conversely, this study highlighted that BNF for older acacia plants were lower at sites of S1, S2 and S3 after 9-14 years because the older plant become less active, but longer and more established root system would help older acacia plants to absorb mineral N from the soil. The current study estimated % Ndfa, based on

^{15}N natural abundance technique, for young and old plants of *A. leiocalyx* and *A.disparimma* at S2 and S3. Nonetheless, we found that BNF values for older plants were lower at sites of S2 and S3 after 12-14 years of prescribed burning compared with those of the same sites for younger plants of *A. leiocalyx* and *A.disparimma* after 1-3 years of prescribed burning reported by Bai et al. (2012) and after 3-6 years reported by Reverchon et al. (2020) who found that BNF peaked between 1 and 6 years for both sites of S2 and S3.

Our result showed that there were significant differences in foliar $\delta^{15}N$ between *A. leiocalyx* and *A. disparimma* at sites of S1-B 9 and S3-B14 after 9-14 years of prescribed burning, but not at sites of S2-B12 and S4-B6 after 6-12 years of prescribed burning, which is similar to the findings from Bai et al. (2012) who have found foliar $\delta^{15}N$ is significant higher for *A.leiocalyx* than that of *A. disparimma* at S1 in Toohey Forest, but these results are not consistent with the findings of Ma et al. (2015) and Reverchon et al. (2020) who have reported that there are no significant differences in $\delta^{15}N$ between the two acacia species at S1. Again, this might be attributed to the temporal and spatial variations in foliar $\delta^{15}N$ of understory acacia species in response to the prescribed burning events.

3.5.2 $\delta^{13}C$ and WUE

The current study showed that foliar $\delta^{13}C$ was higher for August 2019 (winter-dry season) for all study sites compared with those in October 2018 (spring) and May 2019 (autumn). Our result showed that there were no significant differences in foliar $\delta^{13}C$ between *A. leiocalyx* and *A. disparimma* at sites of S1-B 9 and S3-B14 and S4 after 9-14 and 6 years of prescribed burning, while there were significant differences in foliar $\delta^{13}C$ between both acacia species for August 2019 at S2, which is consistent with the finding by Bai et al. (2013) and Reverchon et al. (2020) who have found that no significant difference in foliar $\delta^{13}C$ between both *A.leiocalyx* and *A. disparimma* at S1 in Toohey Forest, but these results were not consistent with the finding of Bai et al. (2013) who have reported that there are significant differences in $\delta^{13}C$

between the two acacia species at S2. Our result showed that $\delta^{13}C$ of *A. disparimma* was significantly higher in August 2019 at sites of S2, S3 and S4 respectively than those of *A. leiocalyx*, but not at S1. Again, the temporal and spatial variations in foliar $\delta^{13}C$ might account for these differences in response to the prescribed burning events.

We assessed the WUE of two acacia species growing at suburban Toohey Forest sites subjected to prescribed burns. We found that 6-14 years after prescribed burning, *A. disparimma* had higher WUE than that of *A. leiocalyx* for sites of S2, S3, and S4, but not at S1. This contradicts the findings of Bai et al. (2012) and Ma et al. (2015). This might be due to the temporal and spatial variations in foliar $\delta^{13}C$ between the species, as foliar $\delta^{13}C$ has been reported to change with water and light availability (Bai et al. 2017).

Our study reported that foliar WUE was higher in winter season of August 2019 at the all study sites of S1, S2, S3, and S4 after 6-14 years of prescribed burning. In general, this study demonstrates that *A. disparimma* for all sites S1, S2, S3, and S4 had relatively higher WUE than *A. leiocalyx* in winter season sampling of August 2019. Higher WUE may suggest that *A. disparimma and A. leiocalyx* would be able to alter the strategy of water use with drought stress as argued by Otieno et al. (2005), using this factor as a tool of adaptation to climate change. Increased WUE in winter August 2019 may be due to increasing N availability and water limitation in the dry winter season, therefore increased BNF in both acacia species may have played an important role to increase WUE.

3.6 Conclusions

Both acacia species were highly active in BNF, which could be beneficial to the N cycle in the suburban native forest ecosystem subjected to the prescribed burning in the burning interval of 4-10 years. The younger acacia plants were more active in BNF than the older plants. Hence, the understory acacia BNF seemed to peak between 6 and 8 years after prescribed burning.

Conversely, the BNF of understory acacia plant was lower after 9-14 years of prescribed burning. There were significant and positive relationship between foliar $\delta^{13}C$ and $\delta^{15}N$, highlighting the role of BNF in improving plant WUE and subsequently tree growth in the suburban native forest ecosystem.

3.7 Reference

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CHAPTER 4 Impact of biochar addition on water use efficiency, biological nitrogen fixation and growth of understory acacia as *Acacia leiocalyx* and *Acacia disparimma* in Toohey Forest of subtropical Australia

4.1 Abstract

Biochar is a stabilised, carbon-rich by-product derived from pyrolysis of biomass. Recently, biochar has received extensive attentions because of its multi-functionality for agricultural and environmental applications. The current study examined short-term response of biochar amendment on water use efficiency (WUE), biological nitrogen fixation (BNF) and growth of understory as *A. leiocalyx* and *A. disparimma* in typical Toohey Forest. Although, our study showed that different biochar application rates improve foliar total N, total C, $\delta^{13}C$, $\delta^{15}N$, BNF and growth of understory acacia species in the first 9 months of biochar application compared with those of the control. Our study concluded that not all potential impacts are evident at 0- 9 months before and after biochar application, and hence specific questions may require long-term experiments to attain answers. Therefore, this experiment needs to be undertaken for the longer periods on the full potential of using biochar to understand that effects on the biochar-soil system in the long-term (above 3 years) and their effects on BNF, WUE, and growth of understory acacia species as well as soil microbial, soil C and N pools in the suburban native forest ecosystems.

Although our study covered the short-term response period relative to a typical forest rotation length, it is an important first step in evaluating the impact of potential biochar application on BNF, WUE, and plant growth of the understory acacia species and eucalypt species. The

present study indicated that the biochar treatment of 10 t ha^{-1} improved foliar total N, BNF, and $\delta^{15}N$ of understory acacia species for the all sampling months, but not at month 2 (July 2019). However, our study showed that the biochar applied at 5 t ha^{-1} contributed to an increase total C, $\delta^{13}C$ and WUE, but did not differ from those of the 10 t ha^{-1} in the Toohey Forest ecosystem of subtropical Australia. Foliar total N concentrations of *A. leiocalyx* was higher in the autumn of March 2020 after 9 months of biochar application, also that foliar total N concentrations were significantly higher (P < 0.05) in the treatment of biochar applied at 10 t ha^{-1} in the autumn of March 2020 compared with those of the 5 t ha^{-1} and control. Both species still depended upon BNF for their N supply, with a higher dependence in the winter than in the spring and autumn. This study demonstrated that *A. disparimma* had relative higher BNF in the winter sampling than those of *A. leiocalyx*, and also the current study showed that BNF was increased after two months of biochar applied at 5 t ha^{-1} , which did not differ than those of biochar applied at 10 t ha^{-1}. The BNF ranged from 42.2% to 80.7% for *A. leiocalyx* and from 62.4% to 93.4% for *A. disparimma* at for all foliar samples before and after biochar application during the autumn and winter of April and July 2019. The estimated BNF varied from 30.8% to 77.9% for the control during the autumn and winter of April and July 2019 and from 50.6% to 93.2% for the biochar applied at 5 t ha^{-1} during the spring and winter of November and July 2019, As for 10 t ha^{-1}, the BNF ranged from 63.9% to 90.0%.

There were positive relationships among $\delta^{13}C$, $\delta^{15}N$ and TN (R^2= 36.6% and R^2= 13.3%, P<0.05) at month 0 and 6 for autumn and spring of April and November 2019 respectively. Meanwhile, there were positive relationships among $\delta^{13}C$, $\delta^{15}N$ and TN (R^2=15.2%, R^2= 22.8 and R^2= 19.9, P < 0.05) at 1-2 months after biochar applied for winter of June and July 2019. Positive relationship was found also between $\delta^{13}C$ and $\delta^{15}N$ (R^2= 6.1% and R^2= 10.1%, P<0.05) in the autumn and winter sampling of April and June 2019. Meanwhile, there was a positive relationship between TN and $\delta^{15}N$ (R^2=19.6% and 10.5%, P<0.05) at month 2 and 6 for winter and spring of July and November 2019, and between $\delta^{13}C$ and TN (R^2= 24.6%, R^2= 2.1%, R^2= 15.7% and R^2= 8.35%) at months 0, 1, 3 and 9 for autumn, winter and spring sampling.

There was no significant difference in foliar WUE among the biochar treatments. Foliar WUE was significantly higher ($P < 0.05$) in the treatment of biochar applied at 5 t ha^{-1} compared with those of the 10 t ha^{-1} and control. *A. disparimma* had higher WUE than that of *A. leiocalyx* before biochar application, while we found that short-term responses of biochar amendment decreased WUE after 9 months of biochar applied in autumn of March 2020. Higher WUE in April 2019 may suggest that *A. disparimma* and *A. leiocalyx* would be able to alter the strategy of water use with drought stress. There were no significant differences in plant growth for both tree height and diameter at ground level between *A. leiocalyx* and *A. disparimma* at S7-B3. However, plant growth for both tree height and diameter at ground level was higher in biochar treatments than those of the control at S7-B3 in the Toohey Forest.

We concluded that different biochar application rates led to different effects on BNF and WUE due to the high variation in biochar properties, highlighting the role of BNF in improving plant WUE and subsequently tree growth after biochar was applied in the suburban Toohey Forest.

4.2 Introduction

Biochar is a carbon-rich black solid material that is a by-product of energy generation during biomass pyrolysis intended specifically for soil application (Lehmann & Joseph, 2015; Lehmann et al., 2011). Biochar, a product formed by the incomplete combustion of biomass, has been shown to improve soil quality and increase crop growth (Adams et al., 2013). Several studies have shown that the biochar produced via pyrolysis of various biomass materials in a low-oxygen atmosphere has emerged as the most promising product for improving soil nitrogen (N) retention and thus retarding environmentally harmful N fluxes (Robertson & Groffman 2007; Nguyen et al. 2017). Lehmann et al. (2006) observed that biochar is produced through pyrolysis of a wide range of biomass sources, a process that may improve soil water storage through altering the portion of pore size distribution related to improved aggregation or water storage in biochar pores (Chen et al., 2010).

Biochar plays an important role as a soil additive in reducing N loss and improving physical and chemical soil fertility (Joseph et al., 2013; Joseph & Lehmann, 2009). The promoted benefits of biochar application to soil include increased plant productivity and reduced nutrient leaching (Biederman & Harpole, 2013). Some reports have shown that biochar improves both chemical and physical properties of soils, which increases soil productivity (Glaser et al., 2002; Lehmann, 2007; Lehmann et al., 2006). Biochar also may alter nutrient cycles through the addition of nutrient, including N, phosphorous (P), potassium (K), calcium (Ca), and magnesium (Mg) to the soil and by influencing exchange of nutrients with the addition of reactive surfaces (Gundale & DeLuca, 2006).

Biochar may also alter nutrient cycles by adding nutrients (N, P, K, Ca, and Mg) to the soil and by influencing exchange of nutrients through the addition of reactive surfaces (Gundale & DeLuca, 2006). However, studies have shown that specific combinations of biochar and soils lead to decreases in crop productivity (Van Zwieten et al., 2010). One interesting finding is that the mixing of biochar into soils frequently stimulates the microbial population and activates dormant soil microorganisms (Hilscher et al., 2009; Smith et al., 2010), thus leading to significant increases in microbial respiration.

Several reports have shown that the biochar has attracted much attention due to its promising role in many environmental management issues (Lehmann et al., 2006; Kumar et al., 2011). According to the result reported by Hammes and Schmidt (2009), pyrolysis above 900 °C causes deformation that results in a widening of the micropores due to the destruction of walls between adjacent pores that increase in surface area. Moreover, higher temperatures reduce volatile matter as well as decrease biochar particle size, leading to a higher ordering of graphene layers that, in turn, increase the density of the solid (Hammes and Schmidt, 2009).

According to the results reported by Nessa et al. (2021) for all pyrolysis temperatures (e.g., 500–850°C), cumulative nitrification increased initially, peaked at ca. 600°C and decreased thereafter. Several reports have shown that the pyrolysis is an alternative solution to the management of large amounts of manure, meanwhile producing renewable and sustainable energy by converting biomass into high value products such as biochar, chemicals, and syngas in the absence of oxygen at high temperatures (300-800°C) (Fernandez et al. 2015; Xu & Chen. 2013).

Biochar improves soil moisture and pH, and hence stimulates N mineralisation and nitrification, leading to improved plant uptake (Saarnio et al., 2013). Biochar pH increased significantly with increasing pyrolysis temperature. The total N concentration of biochar decreased with increased pyrolysis temperature; however, $\delta^{15}N$ of biochar increased with increased pyrolysis temperature (Nessa et al., 2021). According to the result reported by Nessa et al. (2021), biochar total N decreased with increasing pyrolysis temperature. On the other hand, in a study, total N of wood biochar does not change with increasing pyrolysis temperatures (e.g., 300–900°C) (Zhang et al. 2017b). One study has found that increased pyrolysis temperature of biochar (e.g., 600°C) reduces nitrate leaching via adsorption of NO_3^--N compared to low temperature biochar (e.g., 350 and 400°C) (Yao et al.2012). The cumulative N mineralization of the biochar-amended soils was lower than those of the control soils. Similar to nitrification, cumulative N mineralization responded nonlinearly with increasing pyrolysis temperature of biochars, increasing initially but peaking between 600 and 700°C and decreasing with the pyrolysis temperature thereafter (Nessa et al., 2021). Free-living diazotrophs (e.g. Azotobacter sp. and Azospirillum) are stimulated in biochar treatments due to an enlarged habitat with limited oxygen (O_2) inside biochar pores. As mentioned in the literature review, the overall reduction of approximately 10% of soil inorganic N (SIN) after biochar addition occurs regardless of experimental conditions. Another important finding in the literature review was that the main implications of this reduction are a decrease in SIN leaching, and reduced SIN availability for crops. Furthermore, the current review indicated that biochar decreased SIN when it stayed in the soil for a short term (Nguyen et al., 2017).

Biological nitrogen fixation (BNF) is a natural process through which inert N as N_2 gas, is transformed into non-inert N, for example, ammonium. It is an important biological process that moves N from the atmosphere into terrestrial ecosystems (Bohlool et al., 1992). Sullivan et al. (2014) showed that BNF represents a primary source of new N input into terrestrial ecosystems. Also, Sullivan et al. (2014) showed that the rate of BNF, at both regional and global scales, has decreased during recent decades, as a result of an increasing atmospheric N deposition due to human activities (Sullivan et al., 2014).

Researchers studying forest ecosystems at the tropical plantation of Heshan National Field Research Station, Guangzhou, China, found that forest type is an important factor regulating the effects of external N input on BNF, and that elevated atmospheric N deposition in recent decades has suppressed BNF in mature forests with legume trees (Zheng et al., 2016). However, BNF differs in relation to the given ecosystems and unique composition compartment, and BNF appears to be sensitive to the total N input of that whole ecosystem (Reed et al., 2011).

It has been suggested that biochar application influences N_2-fixing bacteria (diazotrophs), including both free-living and symbiotic soil bacteria; and, thus, that biochar influences BNF, which is the conversion of atmospheric N_2 to ammonia (NH_3)(Giller, 2001; Thies et al., 2015). Rondon et al. (2007) have also observed that increased biochar application rate leads to increased nodulation of rhizobia due to increased availability of boron (B), molybdenum (Mo), K, P, and Ca. In addition, biochar application improves plant uptake of soil inorganic N through different mechanisms, which is an important issue for future research (Chan et al., 2008).

The ^{15}N natural abundance in soil and plant is considered as an indicator of N cycling for plants at different locations, on a local, regional, or global scale, and is used to examine extractable soil mineral N pools (NH_4 and NO_3) and soil N turnover rates (Kahmen et al., 2008; Craine et al. 2009). Although, many of the mechanisms driving the variability of foliar and soil $\delta^{15}N$ values within and across ecosystems are still unclear, ^{15}N natural abundance in plants and soil is a simple tool for assessing ecosystem N dynamics (Cheng et al. 2010; Houlton et al. 2008; Kahmen et al., 2008). Emmett et al. (1998) suggested that the ^{15}N signal of vegetation may provide a useful tool in evaluating the past and current N status of forested ecosystems.

Water use efficiency (WUE) is considered the link between wood production and forest water usage in forest ecosystems (Cernusak et al, 2007). It has been found that WUE is related to the optimum water use of a plant and enhances plant responses to the competition for water under xeric conditions (Robinson, 2001; Schulze and Drevon, 2005). Previous studies have noted the relation between stable carbon isotope compositions ($\delta^{13}C$) of plants and WUE linearly, which is used to investigate WUE of plants (Farquhar et al, 1982; Succarie et al., 2020; Sun et al., 2010; Taresh et al., 2021). The higher $\delta^{13}C$ value generally means higher WUE , and this technique has been used in different forest ecosystems of subtropical Australia (Bai et al, 2014a; Bai et al, 2014b; Huang et al, 2008a; Huang et al, 2008b; Tutua et al, 2014). Water use efficiency (WUE) or stomatal conductance is considered useful information for photosynthesis and plant ecophysiology. Foliar C isotope composition can be useful index for plant WUE because this method enables researchers to assess long-term physiological processes (Huang et al, 2008a). Bai et al. (2013) observed no significant difference in long-term WUE, as shown by foliar $\delta^{13}C$, between the two species of *A. leiocalyx* and *A. disprimma,* regardless of measuring times. Adams et al. (2013) found that biochar application improved soil quality and increased crop growth. Biochar, produced through pyrolysis of a wide range of biomass sources, may improve soil water availability.

The current study aimed to examine the effect of wood biochar addition on understory acacia species of *A. leiocalyx* and *A. disparimma* with respect to their BNF, WUE and tree growth in Toohey Forest, Brisbane, Australia. The addition of biochar to soil may offer a chance to mitigate climate change by increasing soil carbon (C) stocks, improving soil fertility and enhancing plant growth. Our specific objectives were to quantify whether biochar addition could reduce N loss and improve biological, physical and chemical properties of surface soils; and to examine the short-term effect of biochar addition on BNF, WUE and tree growth of understory acacia species in the Toohey Forest of southeast Queensland, Australia.

4.3 Material and methods:

4.3.1 Biochar characterisation

Biochar was produced from a slow pyrolysis at 600°C. In this experiment, pine wood (*Pinus radiata*) was used to produce the biochar material through slow pyrolysis at temperature 600°C. The biochar produced was released via a jacketed cooling screw. Biochar chemical properties are summarised in Table 4.1.

Table 4.1 Biochar physical and chemical properties.

pH H_2O (1:2)	TC (%)	TN(%)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
7.27 (± 0.06)	79.44 (±0.41)	0.145 (±0.014)	-27.52 (±0.02)	1.81 (± 0.84)

4.3.2 Site description

A series of studies has been conducted within Toohey Forest which is located 10 km south of Brisbane, in South-East Queensland of subtropical Australia. This dry sclerophyll forest is approximately 400 ha and is dominated by different species of acacia and eucalyptus. About half of this ecosystem is a local government conservation reserve-surrounded by suburbans areas (Catterall et al., 2001; Farmer et al., 2004). The climate of the region is subtropical, with annual rainfall about 613.4 mm for 2019 (Fig. 4.1). Annual minimum and maximum temperatures ranged between 17.1 and 27.8°C in 2019 (Fig. 4.1). In general, summer is hot and wet, whereas winter is cool and dry (Catterall and Wallace, 1987; Stock et al., 1987).

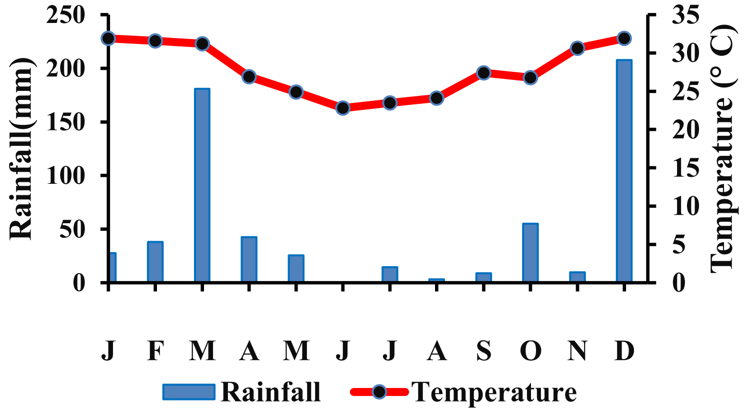

Figure 4.1 Monthly air temperatures and rainfall during the study period of 2019 in Toohey Forest.

Toohey Forest soil type is a combination of lithosols, podzolic and alluvial (Catterall & Wallace, 1987). The lithosols soil, which contains both stony components and loam, to silty loam, may be rich with humus. Whereas, the podzols are mainly less than 60 cm deep in the soil and are low in nutrients. Horizon-B is sandy clay, while horizon-A is sandy loam with coarser particles (Catterall & Wallace, 1987; Stock et al., 1987). Soil properties before and 3 years after prescribed burning are described in Table 4.2.

Table 4.2 Soil characteristics at S7 (B0) (shortly before burning), and S7-B3 (3 years after burning) in Toohey Forest. Samples were collected from experimental plots at soil depths of 0-5 cm, 5-10 cm, and 10-20 cm in September 2016, and April 2019 respectively.

Soil depths	Soil chemical properties before burning (September 2016)				After burning 29 April 2019			
	TC (%)	TN (%)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	TC (%)	TN (%)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
0-5 cm	6.08 (0.63) a	0.24 (0.02) a	-25.86 (0.11) b	0.10 (0.25) b	7.6 (0.70) a	0.3 (0.01) a	-26.9 (0.20) b	0.9 (0.10) c
5-10 cm	3.93 (0.52) b	0.15 (0.01) b	-25.34 (0.12) a	0.44(0.32) ab	5.2 (0.30) b	0.2 (0.02) a	-25.8 (0.10) a	1.7 (0.10) b
10-20 cm	3.58 (0.38) b	0.13 (0.01) b	-25.15 (0.04) a	1.49 (0.21) a	4.0 (0.16) b	0.2 (0.02) a	-25.4 (0.11) a	3.0 (0.15) a

Soil chemical properties include soil total C (TC), total N (TN), C isotope composition (δ^{13} C), and N isotope composition (δ^{15} N). Numbers in the brackets denote standard errors.

The trial plots were randomly located within one site (S7-B3) and contained a mixture of understorey legumes such as *A. leiocalyx* and *A. disparimma,* and overstorey such as *E. psammitica,* which is typical of the area. In each plot, there are 6 plants of *A. leiocalyx,* 6 plant of *A. disparimma* and one plant of *E. psammitica* as reference plants. Each plant area was defined by four steel pegs, providing an area of two square metres for each. Biochar treatment was applied in May 13th, 2019 at the pyrolysis temperature (600°C). Three biochar treatments (control 0, 5 t ha^{-1}, and 10 t ha^{-1}) were applied in two square meters. Soil samples were collected before and immediately after biochar application by 1, 2, 3, 6, and 9 months within two square metres of each plant. Foliar samples were taken from the upper part of the two species of acacia (*A. leiocalyx*, *A. disparimma*) and *E. psammitica* from each plot within the study site for sampling months of April, June, July, August, November for 2019, and March for 2020. These four circular plots with a radius of 12.62 m, giving an internal area of 500 m^2, were randomly located within each site, giving a total sampling area of one-fifth of a hectare. A randomised complete block design was used in this study within the main study site.

Figure 4.2 Picture showing the biochar application at the two square meters collection area in 13 May 2019.

4.3.3 Foliar sampling and analysis

Three leaves per plant (upper canopy position) were collected from two species of acacia and eucalyptus as reference plants within one study site (S7) and kept in separate paper bags and transferred to the laboratory to represent a comprehensive measurement of BNF, WUE and plant growth. There were four replicated plots at the study site, with foliar samples collected from two individuals of *A. leiocalyx*, and *A. disparimma* for each of the 3 biochar rates (0, 5 and 10 t ha^{-1}) and one reference plant of *E. psammitica* (with no biochar added). These foliar samples were collected at six sampling months (April, June, July, August and November of 2019 and March of 2019). In summary, the sampling regime consisted of six *A. leiocalyx,* six *A. disparimma* and one *E. psammitica* from each plot of the one site over 9 months. Overall, 52 foliar samples were collected for each sampling month (*A. leiocalyx* = 24, *A. disparimma* = 24), as well as 4 samples of *E. psammitica* and 312 foliar samples were collected over 6 sampling months (52 × 6 sampling month = 312).

Three leaves per plant were collected and kept in separate paper bags and transferred to the laboratory, then oven-dried at 60°C to a constant weight and ground to a fine powder by a Rocklabs™ ring grinder (Guinto et al. 2000). The homogenised powder was weighed and placed in 8×5 mm tin capsules for analysis using an isotope ratio mass spectrometer (GV Isoprime Manchester, UK). Total N (TN), total C (TC), C and N isotope composition ($\delta^{13}C$ and $\delta^{15}N$) were assessed using an isotope ratio mass spectrometer, as described by Prasolova et al. (2000) and Xu et al., (2003), 6-7 mg plant material was packed into tin capsule to determine foliar total C and N as well as $\delta^{13}C$ and $\delta^{15}N$ by isotope ratio mass spectrometer with Eurovector Elemental Analyser (Isoprime-EuroEA 3000, Milan, Italy) (Sollins et al., 1999; Molero et al., 2011), $\delta^{13}C$ or $\delta^{15}N$ was determined by $^{13}C/^{12}C$ ratio, and $^{15}N/^{14}N$ ratio as:

$$\delta^{13}C \text{ or } \delta^{15}N\ (‰) = \left(\frac{R\ sample}{R\ std} - 1\right) \times 1000 \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \textbf{(4.1)}$$

BNF was estimated by ^{15}N natural abundance for two acacia species. Also, one reference non-N fixing plant of *E. psammitica* was identified for all estimations which could minimise the potential error. The value of $\delta^{15}N$ was determined using the following equation:

$\delta^{15}N = 1000 \times [(R_{sample} - R_{std}) / R_{std}]$ **…………………………………. (4.2)**

Where

R_{sample} is the isotopic ratio $^{15}N/^{14}N$ of sample and R_{std} is $^{15}N/^{14}N$ ratio of the atmospheric N_2.

The percentage of N derived from atmospheric N_2 (%Ndfa) was calculated using the following equation (Shearer & Kohl, 1986):

%Ndfa = $[\delta^{15}N_{ref} - \delta\,^{15}N_{acacia}) / (\delta^{15}N_{ref} - B_{value})] \times 100$ **……………… (4.3)**

Where:

$\delta\,^{15}N_{ref}$: The $\delta^{15}N$ value of the reference plants

$\delta^{15}N_{acacia}$: The $\delta^{15}N$ value of the Acacia spp.

B value: The relative isotopic abundance of Acacia species. growing under a N-free nutrient condition.

The present study used different *B* values varying from -0.3‰ to 1.0‰ in this study to determine an applicable *B* value for acacia species to estimate BNF at the one site. In our study, using *B* value of -0.3‰ resulted in an estimation of %Ndfa that was a better and more reasonable than that of 1.0 ‰ for the study site.

Water use efficiency (WUE) was calculated based on expression of C isotope composition ($\delta^{13}C$) (Farquhar et al. 1982; McCarroll and Loader, 2004). WUE was defined as the ratio of CO_2 assimilation (A) to stomatal conductance for water vapor (gw):

$A/gw = (C_a-C_i) /1.6$ …………………………………. **(4.4)**

The relation between the isotope ratios in plants ($\delta^{13}Cp$) and the source of CO_2 for photosynthesis and the atmospheric CO_2 ($\delta^{13}Ca$) is described by the equation:

$\delta^{13}Cp = \delta^{13}C_a - a - (b-a) C_i / C_a$ …………………… **(4.5)**

Xu et al. (2014) calculated WUE by using the equation:

$$A/gw = C_a (\delta^{13}C_p - \delta^{13}C_a + b) / (b-a) /1.6$$

Where

C_a and C_i are the CO_2 concentrations in the atmosphere and the leaf intercellular space, respectively.

a = (4.4‰) refers to the enrichment during CO_2 diffusion, and b = (27‰) is the fractionation by Rubisco against $^{13}CO_2$. Historical annual atmospheric CO_2 concentrations were obtained by using the direct measurements at the South Pole. As for the trend in $\delta^{13}C_a$, the exponential functions adjusted for the compiled data by Feng (1998) were used.

4.3.4 Soil sampling and analysis

Soil samples was taken after identifying each area. Each plant area was defined by four steel pegs providing an area of two square metres. Soil sampling was carried out before and 1, 2, 3, 6, and 9 months immediately after biochar application within the two square metres for each plant. The biochar was applied on 13 May 2019 within the three biochar treatments (control 0, 5 t ha^{-1}, and 10 t ha^{-1}) in each of the two square meters for 6 plants of *A. leiocalyx* and 6 plants of *A. disparimma*. Two soil cores were randomly collected within 2 m^2 for each plant in each plot within the study site, at three depths (0-5, 5-10, 10-20 cm) before, 3, 6 and 9 months, while 8 soil cores were collected randomly from each plant after 1 and 2 months of biochar application at the one depth (0-5 cm), using a 2.5 cm diameter auger. The soil samples were kept in separate plastic bags and transferred to the laboratory to measurement of soil properties.

4.3.5 Plant growth measurement

Plant height (H) and diameter at ground level (D) were measured in August 2019 and November 2019 at one site (S7). All understory plants such as acacia species, as well as eucalyptus species for all plots were included for growth measurements, in total, 4 plots containing an average of 52 plants, 24 plants for each *Acacia* species. (*A. leiocalyx* = 24, *A. disparimma* = 24) and 4 plants of *E. psammitica*.

Diameter (D) at the ground level was measured at the one site (S7). The D and height (H) measurement were determined in August 2019 and November 2019 at S7 by using diameter tape and height measurements hypsometer (Vertex IV).

4.3.6 Statistical analysis

Three-way analysis of variance (ANOVA) was conducted to evaluate the differences in BNF, WUE and plant growth with respect to the biochar application rates, understory acacia species and sampling time after biochar application. Statistical analyses were conducted with Statistix software (version 8). Differences among means of all variables over time were determined with post-hoc pairwise comparison using the Tukey HSD test at $p < 0.05$ level (Idol et al. 2002; Schafer and Mack 2010; Köster et al. 2014). Multiple regressions were carried out to quantify the relationships among plant properties such as $\delta^{13}C$, $\delta^{15}N$, and total TN across any given site

4.4 Results

4.4.1 Foliar total N, BNF and $\delta^{15}N$

Foliar total N was significantly higher ($P < 0.05$) for *A. leiocalyx* after 9 months (March 2020) (Fig 4.3), while foliar total N concentrations was significantly lower at months 6 after biochar application (November 2019) for *A. disparimma* (Fig 4.3). Foliar total N concentrations of *A. leiocalyx* and *A. disparimma* were significantly different (P<0.05) between the two species before and 9 months after biochar application. There were no significant differences in foliar total N concentrations between the two acacia species in 1, 2, 3 and 6 months after biochar was applied

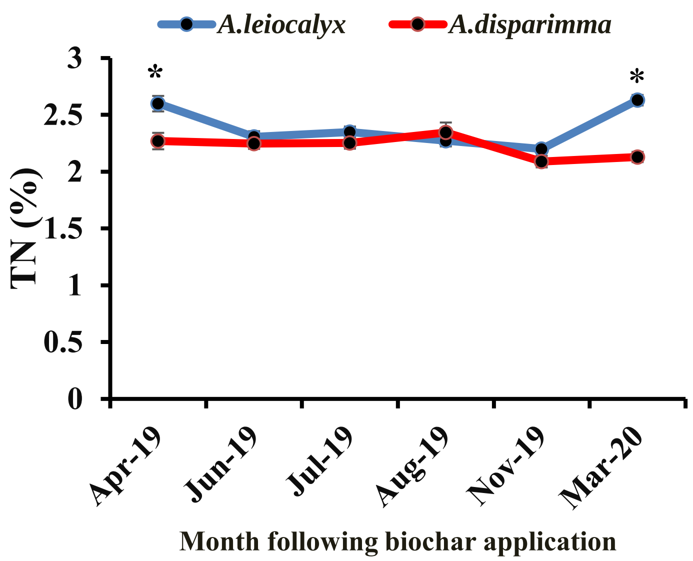

Figure 4.3 Impact of biochar treatments on total nitrogen (TN) for *A. leiocalyx* and *A. disparimma* at S7-B3 before and 1, 2, 3, 6 and 9 months after biochar application. Asterisk indicates significant differences between *A. leiocalyx* and *A. disparimma* at $P < 0.05$.

There were significant differences in foliar total N among the biochar treatments in November 2019 after 6 months of biochar application. Foliar total N concentrations were significantly higher ($P < 0.05$) in the treatment of 10 t ha^{-1} at March 2020 compared with those of the 5 t ha^{-1} and control. Foliar total N concentrations were significantly lower at month 6 (November 2019) for the treatment of 5 t ha^{-1} biochar applied (Fig 4.4).

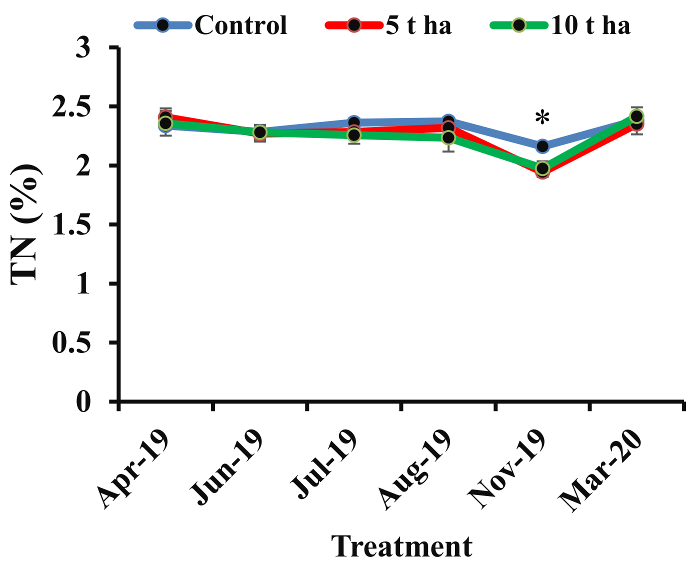

Figure 4.4 Impact of biochar treatments on total nitrogen (TN) for each sampling period at S7-B3 before and 1, 2, 3, 6 and 9 months after biochar application. Asterisk indicates significant differences among biochar treatments at $P < 0.05$.

Meanwhile, there were significant differences ($P < 0.05$) in foliar total N among sampling months, with the lowest foliar total N at month 6 (November 2019) (Table 4.5). The highest foliar total N was found in autumn sampling (March 2020) or 9 months after biochar application.

There were significantly differences ($P < 0.05$) in BNF between *A. leiocalyx* and *A. disparimma* in all foliar sampling times, but not at month 9 (March 2020). Our results showed that BNF of *A. disparimma* was significantly higher in month 2 (July 2019) than those of the *A. leiocalyx* (Fig. 4.5), while the lower BNF was found in *A.leiocalyx* before biochar applied (Fig. 4.5). The BNF ranged from 42.2% to 80.7% for *A. leiocalyx* and from 62.4% to 93.4% for *A. disparimma* for all foliar samples before and after biochar application for the autumn and winter seasons of April and July 2019.

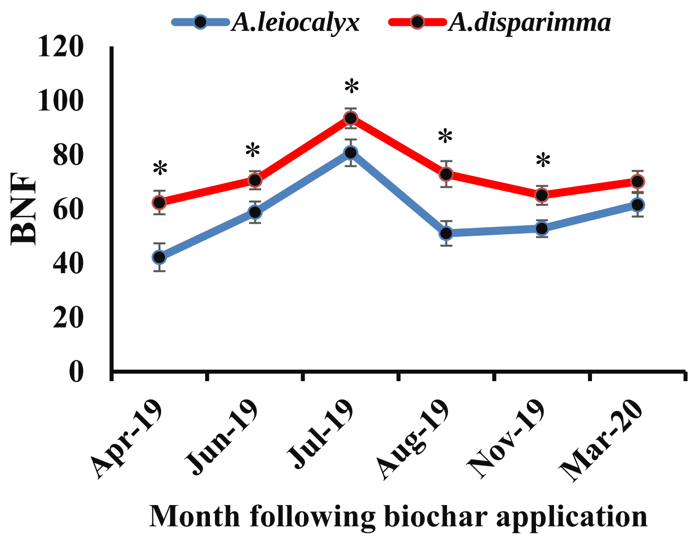

Figure 4.5 Biological nitrogen fixation (as assessed through Ndfa, N derived from atmosphere) by understorey *A. leiocalyx* and *A. disparimma* at S7-B3 before and 1, 2, 3, 6 and 9 months after biochar application. Asterisk indicates significant differences between BNF of *A. leiocalyx* and that of *A. disparimma* at $P < 0.05$.

Meanwhile, there were significant difference in BNF ($P < 0.05$) among biochar treatments before and 1, 9 months after biochar applied (April and June 2019, March 2020) respectively. The current study also indicated that BNF was significantly higher for the treatment of biochar applied at 10 t ha^{-1} than those of the 5 t ha^{-1} and the control (Fig. 4.6), while foliar BNF was significantly lower before biochar applied was found in control (Fig. 4.6).

The current study showed also that foliar BNF was significant different among the sampling periods. BNF was significantly higher in the winter sampling (July 2019) compared with those of the spring and autumn sampling (Table 4.5).

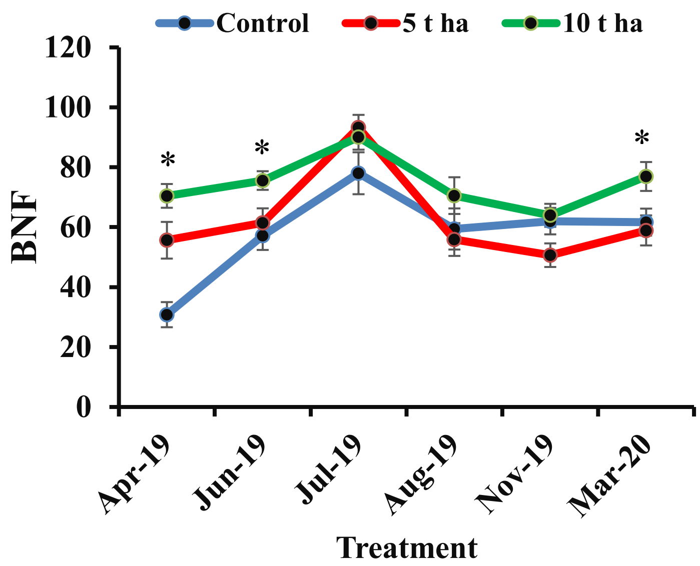

Figure 4.6 Impact of biochar treatments on BNF for each sampling period at S7-B3 before and 1, 2, 3, 6 and 9 months after biochar application. Asterisk indicates significant differences among biochar treatments at $P < 0.05$.

There were significant differences in foliar $\delta^{15}N$ between *A. leiocalyx* and *A. disparimma* for all sampling periods (0- 6 months), but not at month 9 (March 2020) (Fig 4.7). The current study showed that $\delta^{15}N$ values of *A. disparimma* was significantly higher in July 2019 (winter-dry season), 2 months after biochar application, than those of the *A. leiocalyx* (Fig. 4.7). Foliar $\delta^{15}N$ of *A. leiocalyx* was significantly lower in November 2019 (spring season), 6 months after biochar application.

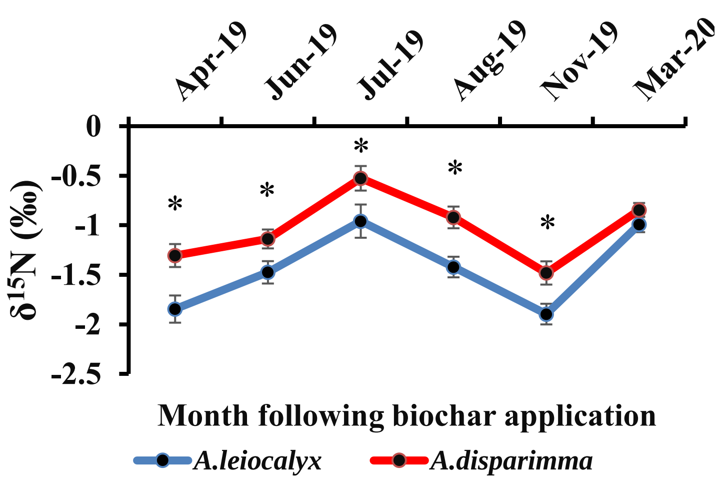

Figure 4.7 Foliar $\delta^{15}N$ of *A. leiocalyx* and *A. disparimma* for site of S7-B3 before and 1, 2, 3, 6 and 9 months after biochar application. Asterisk indicates significant differences between $\delta^{15}N$ of *A. leiocalyx* and *A. disparimma* at the level $P < 0.05$.

There were significant differences ($P < 0.05$) in foliar $\delta^{15}N$ among the biochar treatments before and 1, 9 months after biochar application. Our result showed that $\delta^{15}N$ values of treatment of 5 t ha^{-1} was significantly higher for July 2019 (winter-dry season) than those of the 10 t ha^{-1} and the control. Foliar $\delta^{15}N$ was significantly lower before biochar application in the control (April 2019) (Fig. 4.8). There were also significant differences ($P < 0.05$) in foliar $\delta^{15}N$ among sampling periods. Foliar $\delta^{15}N$ was significantly higher in July 2019 (winter-dry season), while the lower foliar $\delta^{15}N$ was found in autumn sampling (April 2019) (Tab 4.5).

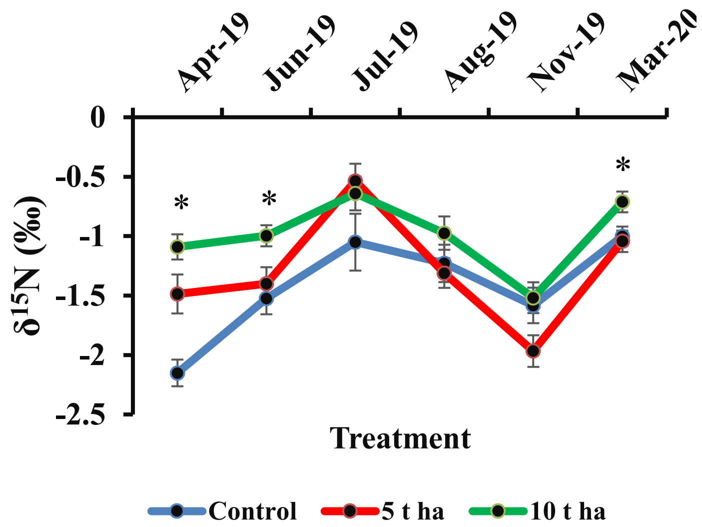

Figure 4.8 Impact of biochar treatments rates on $\delta^{15}N$ for each samples period at S7-B3 before and 1, 2, 3, 6 and 9 months after biochar application. Asterisk indicates significant differences among biochar treatments at $P < 0.05$.

4.4.2 Foliar total C, $\delta^{13}C$ and WUE

Foliar total C of *A. leiocalyx* and *A. disparimma* were significantly different ($P < 0.05$) between the two species before and 1, 2 and 9 months after biochar application. While, there was no significant difference in foliar total C between both acacia species 3 and 6 months after biochar was applied (Fig 4.9). Foliar total C was significantly higher ($P < 0.05$) for *A. disparimma* in month 3 (August 2019) after biochar application, while foliar total C was significantly lower at month 9 (March 2020) for *A. leiocalyx* (Fig. 4.9).

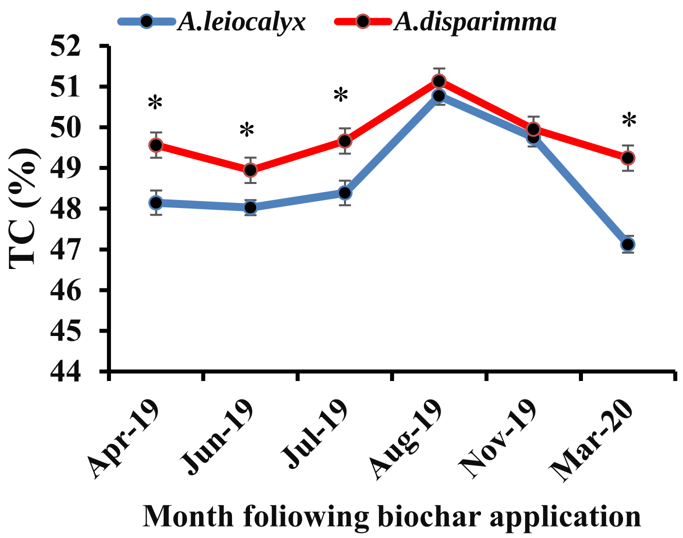

Figure 4.9 Total carbon (TC) of *A. leiocalyx* and *A. disparimma* at study site of S7-B3 before and 1, 2, 3, 6 and 9 months after biochar application. Asterisk indicates significant differences between *A. leiocalyx* and *A. disparimma* at $P < 0.05$.

There were significant differences in foliar total C among the biochar treatments 2 months after biochar applied (July 2019). Foliar total C was significant higher in month 3 (August 2019) for the treatment of 5 t ha^{-1} compared with those of the 10 t ha^{-1} and the control, while the lowest foliar total C was found in month 9 (March 2020) in the control (Fig. 4.10). Meanwhile, there were significant differences ($P < 0.05$) in foliar total C among the sampling periods. Foliar total C was significantly higher in winter sampling (August 2019), while the lowest foliar total C was found in autumn sampling (March 2020) (Table 4.5).

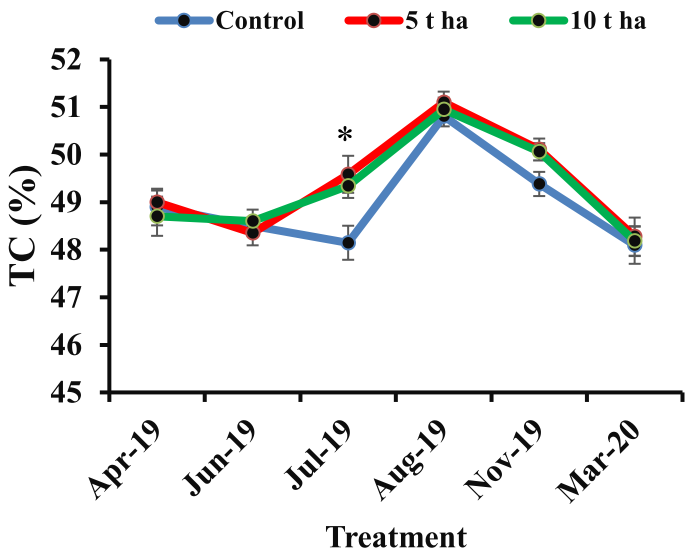

Figure 4.10 Impact of biochar treatments on total C (TC) for each sampling period at S7-B3 before and 1, 2, 3, 6 and 9 months after biochar application. Asterisk indicates significant differences among biochar treatments at $P < 0.05$.

Foliar $\delta^{13}C$ values of *A. leiocalyx* and *A. disparimma* were significantly different ($P < 0.05$) between the two species before and after 1, 2 and 9 months of biochar application (Fig. 4.11). While, there were no significant differences in foliar $\delta^{13}C$ between acacia species 3 and 6 months after biochar was applied. Foliar $\delta^{13}C$ was significantly higher ($P < 0.05$) for *A. disparimma* shortly before biochar application (Fig 4.11), while foliar $\delta^{13}C$ was significantly lower in months 9 (March 2020) for *A. leiocalyx* (Fig 4.11).

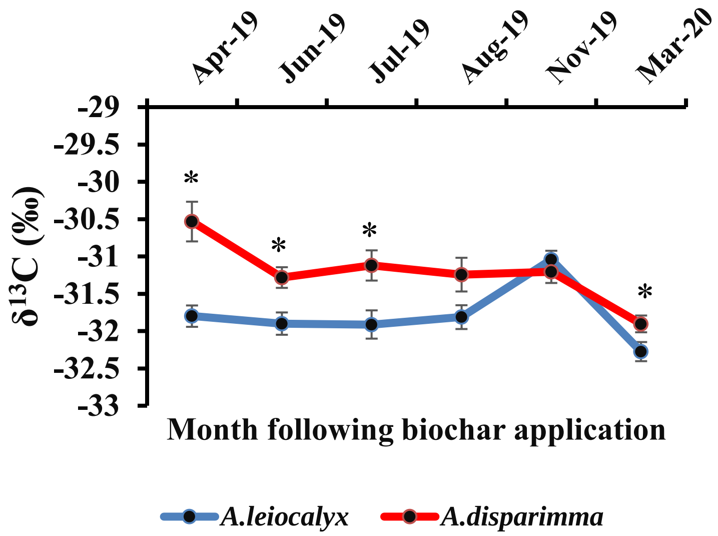

Figure 4.11 Carbon (C) isotope composition of *A. leiocalyx* and *A. disparimma* at study site of S7-B3 before and 1, 2, 3, 6 and 9 months after biochar application. Asterisk indicates significant differences between *A. leiocalyx* and *A. disparimma* at $P < 0.05$.

No significant difference in foliar $\delta^{13}C$ was found among the biochar treatments (Fig 4.12). Meanwhile, there were significant differences in foliar $\delta^{13}C$ among the sampling periods, except for March 2020 (Table 4.5). Foliar $\delta^{13}C$ was significantly higher ($P < 0.05$) in spring sampling period (November 2019), while the lowest foliar $\delta^{13}C$ was found in autumn sampling (March 2020) (Table 4.5).

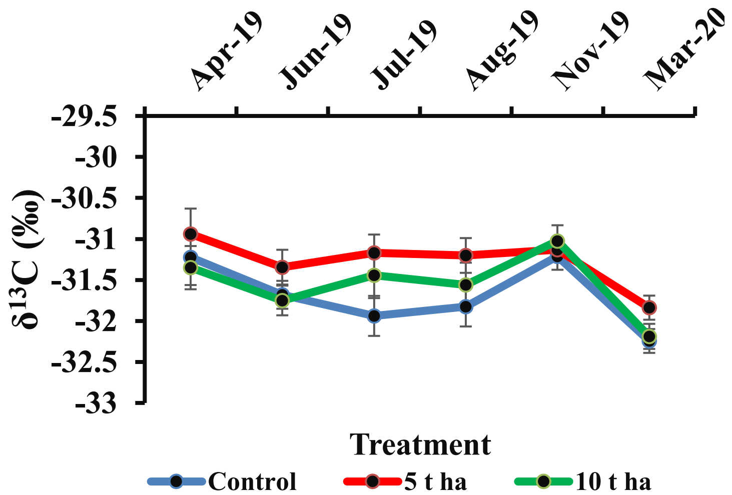

Figure 4.12 Impact of biochar treatments on foliar $\delta^{13}C$ for each sampling period at S7-B3 before and 1, 2, 3, 6 and 9 months after biochar application.

4.4.3 Relationship between plant physiological variables

Multiple regression analyses were performed for both acacia species (*A. leiocalyx* and *A. disparimma*) at S7 (3 years after burning) to find out the relationships between foliar $\delta^{13}C$ and $\delta^{15}N$ or total N, between foliar total N and $\delta^{13}C$, between foliar $\delta^{13}C$ and $\delta^{15}N$, and between total N and $\delta^{15}N$.

For the autumn sampling in April 2019, 36.7% of the variation in foliar $\delta^{13}C$ (Table 4.3) of both acacia species was explained by foliar $\delta^{15}N$ (6.1%) (Fig. 4.14a) and total N (24.6%) (Table 4.3), For the winter sampling of June 2019, 15.2% of the variation in foliar $\delta^{13}C$ (Table 4.3) of both acacia species in month 1 was explained by the corresponding foliar $\delta^{15}N$ (10.1%) (Fig. 4.14b) and total N (2.1%) (Tab 4.3). while 22.8% and 19.9 % of the variation in foliar $\delta^{13}C$ of both winter sampling (July and August 2019) were explained by the corresponding foliar $\delta^{15}N$ and total N (Tables 4.3 and 4.4). For the winter and spring sampling of July and November 2019, there were 19.6% and 10.5% of the variation in foliar total N of both acacia species at the month 2 and 6 respectively explained by the corresponding foliar $\delta^{15}N$ (Fig. 4.13a, b). For spring sampling, 13.3% of the variation in foliar $\delta^{13}C$ of acacia species at month 6 was explained by $\delta^{15}N$ and total N (Table 4.4).

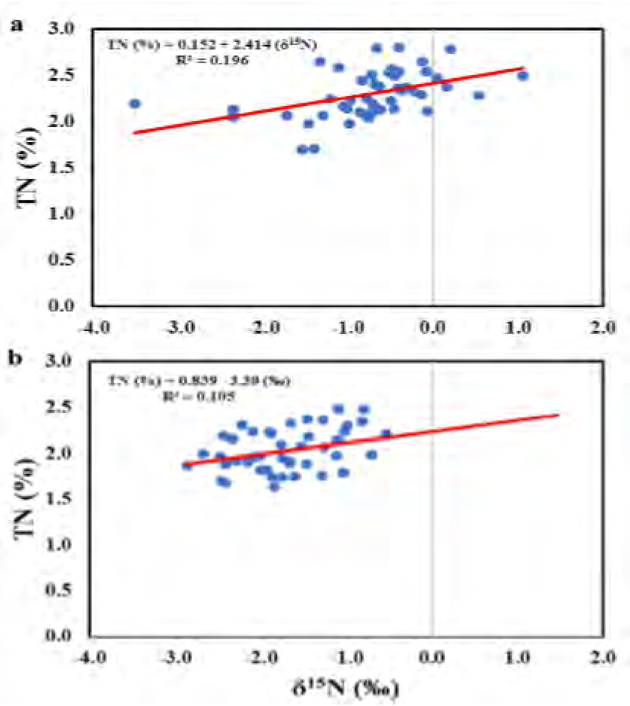

Figure 4.13 Relationships between foliar TN and δ^{15}N of both acacia species (a) for the winter sampling in June 2019; and (b) for the spring sampling in November 2019 at S7-B3 in Toohey Forest south-east Queensland.

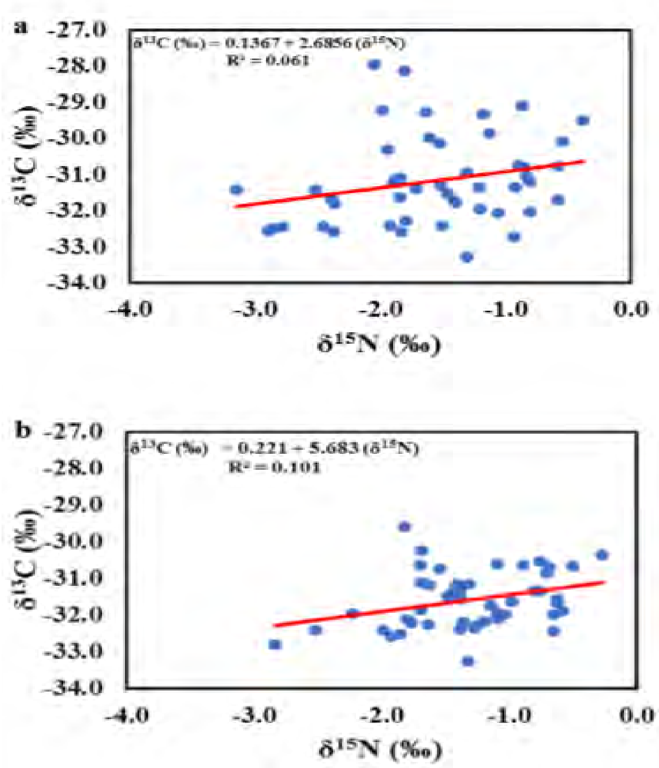

Figure 4.14 Relationships between foliar δ^{13}C and δ^{15}N of both acacia species (a) for the autumn sampling in April 2019; and (b) for the winter sampling in June 2019 at S7-B3 in Toohey Forest south-east Queensland.

Table 4.3 Relationships between foliar $\delta^{13}C$, total N (TN) and $\delta^{15}N$, and between foliar $\delta^{13}C$ and TN of both acacia species for the autumn sampling before biochar applied in April 2019 ; and for the winter sampling in June and July 2019 after 1 and 2 months of biochar application at S7-B3 in Toohey Forest south-east Queensland.

Month 0 April 2019	Month 1 June 2019	Month 2 July 2019
$\delta^{13}C$ (‰) = -25.81 + 0.634 ($\delta^{15}N$) - 1.78 (TN) (R^2 = 0. 367, n = 48, $P < 0.05$)	$\delta^{13}C$ (‰) = -29.07 + 0.547 ($\delta^{15}N$) – 0.791 (TN) (R^2= 0.152, N = 48, $P < 0.05$)	$\delta^{13}C$ (‰) = -26.82 + 0.627 ($\delta^{15}N$) – 1.84 (TN) (R^2= 0.228, N = 48, $P < 0.05$)
$\delta^{13}C$ (‰) = -27.30 - 1.58 (TN) (R^2 = 0. 246, n = 48, $P < 0.05$)	$\delta^{13}C$ (‰) = -30.54 – 0.459 (TN) (R^2 = 0. 021, n = 48, $P > 0.05$)	ns

Table 4.4 Relationships between foliar $\delta^{13}C$, total N (TN) and $\delta^{15}N$, and between foliar $\delta^{13}C$ and TN of both acacia species for the winter sampling 3 months after biochar applied in August 2019 ; spring sampling in November 2019 after 6 months of biochar applied, and for the autumn sampling in March 2020 after 9 months of biochar application at S7-B3 in Toohey Forest south-east Queensland.

Month 3 August 2019	Month 6 November 2019	Month 9 March 2020
$\delta^{13}C$ (‰) = -28.30 + 0.357 ($\delta^{15}N$) - 1.22 (TN) (R^2 = 0. 199, n = 48, $P < 0.05$)	$\delta^{13}C$ (‰) = -28.95 + 0.401 ($\delta^{15}N$) – 0.734 (TN) (R^2 = 0. 133, n = 48, $P < 0.05$)	ns
$\delta^{13}C$ (‰) = -28.87 - 1.15 (TN) (R^2 = 0. 157, n = 48, $P < 0.05$)	ns	$\delta^{13}C$ (‰) = -30.857 - 0.518 (TN) (R^2 = 0.083 , n = 48, $P < 0.05$)

Our results showed that there were significant differences in WUE (P < 0.05) between *A. leiocalyx* and *A. disparimma* before, 1 and 2 months after biochar application, while there were no significant difference in WUE between the two acacia species 3, 6 and 9 months after biochar application (August 2019, November 2019 and March 2020) respectively. Foliar WUE was significantly higher (P < 0.05) of *A. disparimma* before biochar application at month 0 shortly before biochar application (April 2019) (Fig. 4.15), while foliar WUE was significantly lower in months 9 (March 2020) for *A. leiocalyx* (Fig. 4.15).

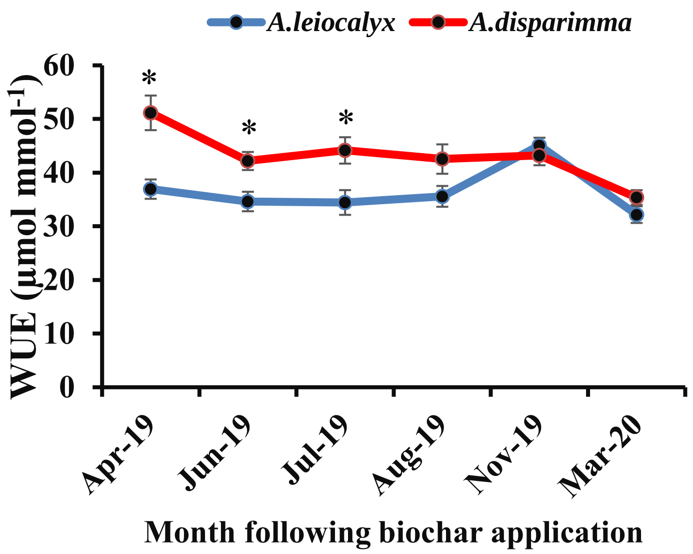

Figure 4.15 Foliar WUE of *A. leiocalyx* and *A. disparimma* at study site of S7-B3 before and 1, 2, 3, 6 and 9 months after biochar application. Asterisk indicates significant differences between *A. leiocalyx* and *A. disparimma* at P < 0.05.

No significant difference was found in foliar WUE among the biochar treatments (Fig. 4.16). Meanwhile, there were significant difference in foliar WUE among the sampling periods. Foliar WUE was significantly higher (P < 0.05) in the autumn sampling period of April 2019, while the lowest foliar WUE was found in the autumn sampling of March 2020 (Table 4.5).

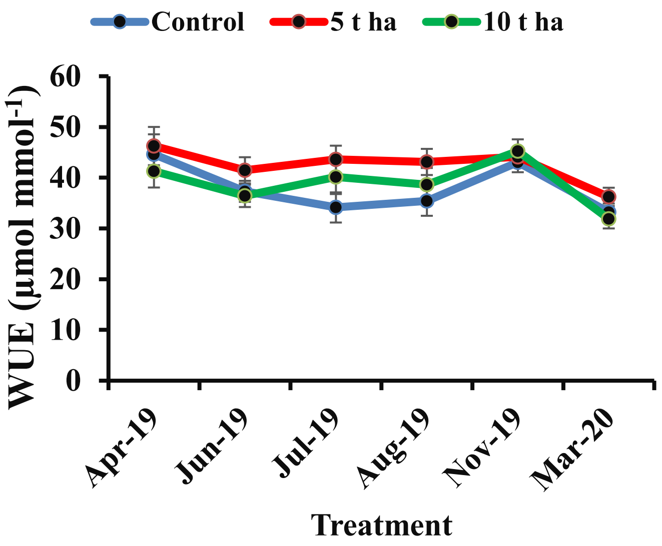

Figure 4.16 Impact of biochar treatments on foliar WUE for each sampling period at S7-B3 before and 1, 2, 3, 6 and 9 months after biochar application.

Table 4.5 Impact of biochar applied sampling period at site 7- B3 on foliar TC, TN, $\delta^{13}C$, $\delta^{15}N$, BNF and WUE within Toohey Forest, south-east Queensland. Different letters indicate significant differences among biochar application period at $P < 0.05$.

Sampling time	TC (%)	TN (%)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	BNF (%)	WUE (µmol mmol^{-1})
April 2019	48.85 (0.20) cd	2.00 (0.05) a	-31.17 (0.18) a	-1.58 (0.09) e	52.29 (3.65) c	44.04 (2.11) a
June 2019	48.48 (0.10) cd	2.28 (0.03) a	-31.58 (0.11) ab	-1.31 (0.08) cd	64.69 (2.72) bc	38.39 (1.35) ab
July 2019	49.02 (0.20) c	2.30 (0.04) a	-31.51 (0.15) a	-0.74 (0.11) a	87.08 (3.18) a	39.29 (1.82) ab
August 2019	50.95 (0.13) a	2.31 (0.05) a	-31.53 (0.14) a	-1.17 (0.08) bc	61.94 (3.64) bc	39.06 (1.75) ab
November 2019	49.85 (0.14) b	2.03 (0.03) b	-31.11 (0.10) a	-1.69 (0.08) de	58.88 (2.48) bc	44.14 (1.17) a
March 2020	48.18 (0.21) d	2.38 (0.05) a	-32.09 (0.09) b	-0.92 (0.05) ab	65.84 (2.96) b	33.77 (1.06) b

4.4.4 Plant growth

The heights of *A. leiocalyx* were significantly higher than those of *A. disparimma* (P < 0.05; Table 4.6). Our study indicated that the highest height was observed in the treatment 5 t ha^{-1}, while the lowest height was found in the control (Table 4.7). Overall, our hypothesis that short-term effect after biochar application may alter carbon (C) and N pools, thus it could influence plant growth. This study showed that short-term (1-9 months) after biochar application increased foliar total N and N availability as reflected in foliar $\delta^{15}N$ which play an important role in improving plant growth.

Table 4.6 Height and diameter at ground level (DGL) of *A. leiocalyx* and *A. disparimma* at site7-B3 before and 1, 2, 3, 6 and 9 months after biochar application in Toohey Forest south-east Queensland. Different letters indicate significant differences in Heights and DGL between the acacia species at P < 0.05.

Species	Height (cm)	DGL (cm)
A. leiocalyx	12.55 (1.7) a	3.30 (0.2) a
A. disparimma	10.07 (0.7) b	3.15 (0.1) a

Table 4.7 Height and diameter at ground level (DGL) among biochar treatments at site7-B3 study sites. Different letters indicate significant differences in Heights and DGL among the biochar treatments at P < 0.05.

Treatment	Height (cm)	DGL (cm)
Control	8.50 (0.8) b	2.93 (0.2) b
5 t/ha^{-1}	12.73 (1.5) a	3.11 (0.1) b
10 t/ha^{-1}	12.70 (2.1) a	3.65 (0.2) a

4.5 Discussion

4.5.1 Foliar total N, BNF and $\delta^{15}N$

Our study showed that the foliar total N was significantly different (P < 0.05) among the biochar treatments in the autumn of March 2020, but this result was not consistent with the findings from Bai et al. (2015); Sun et al. (2020), Asadyar et al. (2021), and Hannet et al. (2021) who found no significant difference in foliar total N between the control and the biochar treatments. We found that foliar total N was reduced in the first month after biochar was applied, followed with an increased in foliar total N for both treatments of 5 t ha^{-1} and control at months 2 and 3 for winter of July and August 2019, after that the foliar total N decreased at month 6 for spring of November 2019. There were no significant differences in foliar total N among the biochar treatments in the autumn of March 2020 after 9 months of biochar application. We found that biochar applied at 10 t ha^{-1} had higher total N than 5 t ha^{-1} and control in the autumn of March 2020 after 9 months of biochar application, which is similar to the findings from Zhang et al. (2017a and 2017b). This is consistent with the finding from Fan et al. (2020) who found that foliar total N was significantly increased after 6 years of biochar application.

This study showed that significant differences in foliar total N were found between *A.leiocalyx* and *A.disparimma* shortly before and 9 months after biochar application. This study demonstrates that *A. leiocalyx* had relative higher total N than *A. disparimma* in the autumn of March 2020. This is similar to the findings of Bai et al. (2012) and Ma et al. (2015) who found that foliar total N at S1 was significantly greater in *A. leiocalyx* than that of *A. disparrima*. The temporal and spatial variations in foliar total N concentrations might account for these differences in response to the prescribed burning events (Bai et al. 2017). Therefore, *A. leiocalyx* may recover more quickly after 9 months of biochar application and may also make a greater contribution to the N recovery because N is the main component of Rubisco, the enzyme facilitating C fixation at the carboxylation site (Wilson et al. 2000), and our foliar total N values were within the range of foliar total N for acacia species. According to the report by Bai et al. (2012) that microbial functional gene abundance and N cycling may be altered by biochar application, as a result of changing soil properties. There were no significant effects of biochar treatments and sampling time on foliar total N and no significant interaction between the treatment and sampling times (Bai et al. 2012). Foliar total N increased with biochar addition at 6 t ha^{-1} compared to the those of 0 and 1 t ha^{-1} (Drake et al. 2015). Zhang et al. (2017a) found that biochar addition, regardless of pyrolysis temperature, significantly increased total N across all plant species. According to the results reported by Asadyar et al. (2021) biochar application may have altered N fractionations but did not affect soil total N after 2 years of biochar application in a macadamia orchard.

We assessed the BNF among the biochar treatments and between two acacia species growing in suburban Toohey Forest. We found that even 3 years after the prescribed burning as well as biochar treatments, both acacia species still depended upon BNF for their N supply. The current study showed that there was significant difference in BNF among biochar application treatments, which is similar to the findings from Mia et al. (2014) who found that BNF did not significantly differ between the treatments. This contradicts the findings from De Assumpção. (2017); and Güereña et al. (2015) who found no significant difference in BNF was found among the application treatments after biochar amendment on the soil of a boreal forest. This study demonstrated that BNF was higher in the biochar applied at 5 t ha^{-1} compared with those of the 10 t ha^{-1} and the control in the winter of July 2019 after 2 months of biochar application, but BNF was decreased in the winter and spring of August and November 2019 and autumn of March 2020, which is consistent with the finding by Hiama et al. (2019) who found that increasing biochar rate beyond 5 t ha^{-1} decreased BNF and nutrients uptake in cowpea in the short term. We found that BNF was gradually reduced until Spring of November 2019 for month 6, thereafter we observed that BNF was gradually increased again for all biochar treatments in the autumn of March 2020 after 9 months of biochar application. The decrease in nodulation and BNF beyond the 5 t ha^{-1} biochar was possibly a result of increased C: N ratio (Quilliam et al., 2012), which reduced nutrient uptake for BNF. According to the result reported from Mia et al. (2018) who found that aged biochar treatment significant reduced BNF compared with those of the control. Meanwhile, Mia et al. (2014) observed that fresh biochar amendment contributed to increases in BNF and BNF has been reported with fresh biochar amendment by Van de Voorde et al. (2014). BNF was significantly increased after biochar was applied at 10 t ha^{-1} , leading to the highest rate of BNF compared with those of the biochar application rate at 0, 50 and 120 t ha^{-1} (Mia et al. 2014).

We assessed the BNF rates of two acacia species growing at suburban Toohey Forest site. There were significant differences in BNF between *A. leiocalyx* and *A. disparimma* at all sampling months, but not in the autumn of March 2020. This study also demonstrated that *A. disparimma* had higher BNF in the winter of July 2019 than those of *A. leiocalyx*. This contradicts the findings from Ma et al. (2015) and Reverchon et al. (2020). The seasonal differences in BNF were found for both *A. disparrima* and *A. leiocalyx* (Reverchon et al. 2020). However, both Ma et al. (2015) and Reverchon et al. (2020) reported the BNF for the short and medium- term impact of prescribed burning at the relevant sites.

The estimated BNF based on ^{15}N natural abundance technique for *A. leiocalyx* and *A. disparimma* at S7-B3 agreed with those for other acacia species reported in Australia. We found that the BNF ranged from 42.2% to 80.7% for *A. leiocalyx* and from 62.4% to 93.4% for *A. disparimma* at for all foliar samples before and after biochar application during the autumn and winter of April and July 2019. Our results showed that the BNF was higher in the winter of July 2019 than those in spring and autumn. The range in our study was much narrower for both species, and this variability may have been due to the within treatment variation caused by spatial variation of mutualistic organisms (Boddey et al. 2000) or non-homogenized N and water availability in the soil (Stevenson et al. 1995).

Overall, the current study was designed to determine the effect of short-term biochar application on BNF of leguminous plants (N_2-fixing species) mixed with non-N fixing trees, such as acacia species mixed with eucalypt species. Our study concluded that not all potential impacts are evident at 0- 9 months before and after biochar application, and hence specific questions may require long-term experiments to attain answers. Whereby, an implication of this is the possibility that effects on the biochar-soil system in the long-term (above 3 years) will be different than those found in the short-term (Gul et al. 2015).

Our results showed that there were significant differences in foliar $\delta^{15}N$ among the biochar treatments and between *A. leiocalyx* and *A. disparimma* at sites of S7-B3. This might be attributed to the temporal and spatial variations in foliar $\delta^{15}N$ of understory acacia species in response to the prescribed burning events. We found that foliar $\delta^{15}N$ was increased at the first two months for winter of July 2019 after biochar was applied, followed with an decrease in foliar $\delta^{15}N$ for all biochar treatments at months 3 and 6 for winter and spring of August and November 2019, due to biochar amendment has reduced the leaching of a ^{15}N depleted N (Menke, 2017), which is consistent with the findings by Asadyar et al. (2021); and Menke (2017) who found that $\delta^{15}N$ was decreased after biochar applied. However, after that our study demonstrated that all biochar treatments slowly increased foliar ^{15}N in the autumn of March 2020 after 9 months of biochar application. Biochar application showed reduced foliar $\delta^{15}N$ values combined with increased plant total N values due to reduction in the leaching of $\delta^{15}N$ depleted N source (Menke, 2017). The treatment of 5 t ha^{-1} had significantly higher foliar $\delta^{15}N$ in the winter of July 2019 after 2 months of biochar application compared with those of the 10 t ha^{-1} and the control but did not differ from that of 10 t ha^{-1}. Short term effect of biochar application (2 years after biochar application) had depleted foliar $\delta^{15}N$ in macadamia orchard

(Zhang et al., 2018). Our complementary study was undertaken in S7-B3 after 3 years of prescribed burning and short term of biochar application, and we found depleted foliar $\delta^{15}N$ after biochar application even in a short period of time at months 3 and 6. The findings by Bai et al. (2017) and Nguyen et al. (2017) showed that foliar $\delta^{15}N$ was linked up with plant uptake from N sources with deficient ^{15}N. Seedlings grown with biochar from pyrolysis and gasification had no significant difference with leaf and stem ^{15}N, while that was significantly lower than those of the control seedlings (Sun et al. 2020).

4.5.2 Foliar total C, $\delta^{13}C$ and WUE

Our study showed that there were significant differences in foliar total C among biochar treatments for winter of July 2019 compared with those of control. This contradicts the findings of Asadyar et al. (2021) who found no significant difference in total C between the biochar treatment and control, 2 years after biochar application in a macadamia orchard. We found that foliar total C was significantly higher in the winter of August 2019 after 3 months of biochar applied for the treatment of 5 t ha^{-1} compared with those of the 10 t ha^{-1} and the control, which is similar to the findings from Reverchon et al. (2015) and Fan et al. (2020). Chen et al. (2018) observed that organic C increased after organic amendments application and the size of increase was shown to be mainly driven by type of organic amendment. Conversely, this study highlighted that foliar total C decreased in the spring and autumn of November 2019 and March 2020 after 6 and 9 months respectively. This contradicts the findings from Menke. (2017) reported that biochar treatments increased foliar total C than those of the control in months 6 and 11.

This study showed also that significant difference was found in foliar total C between *A. leiocalyx* and *A. disparimma* for autumn and winter of April, June and July 2019 and for autumn of March 2020.

Our study showed that there were no significant differences in $\delta^{13}C$ among the biochar treatments for all sampling periods, which is consistent with the findings from Reverchon et al. (2015). We found that foliar $\delta^{13}C$ was significantly higher in the autumn of April 2019 before biochar application. This study also demonstrated that foliar $\delta^{13}C$ was reduced in the winter of June 2019 after 1 month of biochar application for all treatments , and then foliar $\delta^{13}C$ was increased for both treatments of 5 t ha^{-1} and 10 t ha^{-1} in the winter and spring of August and November 2019 after 2 and 6 months of biochar application. Thereafter, foliar $\delta^{13}C$ decreased in the autumn of March 2020 after 9 months of biochar application for all treatments, which is consistent with findings by Menke. (2017) who found that $\delta^{13}C$ was reduced after biochar application. The current study also indicated significant difference between both *A. leiocalyx* and *A. disparrima* before and 1, 2 months after biochar application, which is consistent with the finding by Bai et al. (2013) and Reverchon et al. (2020) who have found that there were no significant differences in foliar $\delta^{13}C$ between both *A. leiocalyx* and *A. disparimma* at S1 in Toohey Forest, but these results were not consistent with the finding of Bai et al. (2013) who have reported that there were significant differences in $\delta^{13}C$ between the two acacia species at S2. Before biochar application, foliar $\delta^{13}C$ of *A.disparimma* was significantly higher than *A.leiocalyx*. Shortly after the biochar was applied foliar $\delta^{13}C$ was decreased in the winter-dry season for both *A,leiocalyx* and *A. disparimma*. Furthermore, the current study showed that foliar $\delta^{13}C$ was increased in the spring of November 2019 6 months of biochar application for *A. leiocalyx*. Thereafter, foliar $\delta^{13}C$ was decreased in the autumn of March 2020 for both *A. leiocalyx* and *A. disparimma*.

We assessed the WUE of two acacia species growing in suburban Toohey Forest subjected to the biochar application. Our study showed no significant difference in WUE among the treatments before and 1-9 months after biochar application in Toohey Forest. However, our results indicated that the WUE was influenced by biochar application among the biochar treatments.

The current study indicated significant difference in WUE between both *A. leiocalyx* and *A. disparrima* in the autumn season of April 2019 and winter of June and July 2019. We found that before and 1-9 months after biochar application, *A. disparimma* had higher WUE than that of *A. leiocalyx* in the autumn of April 2019, but not in the spring of November 2019. This contradicts the findings of Bai et al. (2012) and Ma et al. (2015). This might be due to the temporal and spatial variations in foliar $\delta^{13}C$ between the species, as foliar $\delta^{13}C$ has been reported to change with water and light availability (Bai et al. 2017). However, we found that WUE was gradually reduced in the winter season of June, July and August 2019 until the autumn season of March 2020 for both *A. disprimma* and *A. leiocalyx,* but not at spring of November 2019 because the biochar addition may increase the soil water holding capacity (Ajayi et al. 2016; Fischer et al. 2019). In general, foliar $\delta^{13}C$ values and soil moisture were found to be affected by biochar amendment, indicating changes in water availability (Saarnio et al., 2013; Lehmann et al. (2006).

4.5.3 Plant height and diameter at ground level

A positive response in plant growth by biochar application was observed in our study. Although, the current study indicated no significant difference for height and diameters at ground level (DGL) between both *A. leiocalyx* and *A. disparimma*. The heights of *A. leiocalyx* were significantly higher than those of *A. disparimma* at S7. Meanwhile, the current study also showed that the biochar treatment increased height and DGL, which is similar to the findings by Zheng et al. (2013) who found that crops production increased after biochar and N fertilizer were applied (Van Zwieten et al., 2010; Liu et al., 2017).

4.6 Conclusion

Biochar application contributed to improve foliar total N, BNF and growth of understory acacia species in the first 9 months of biochar application in Toohey Forest. Both acacia species were improved after biochar application at the first three months, with the increased foliar total N, total C, BNF, $\delta^{13}C$ and $\delta^{15}N$, compared with the control. There were significant and positive relationship between foliar $\delta^{13}C$ and $\delta^{15}N$, highlighting the role of BNF in improving plant WUE and subsequently tree growth of understory acacia species in the suburban native forest ecosystem subjected to the repeated prescribed burning.

4.7 References

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CHAPTER 5 Long-term impacts of prescribed burning on litter floor recovery and soil total C, total N, and C and N isotope compositions in a Suburban Forest Ecosystem of Subtropical Australia

5.1 Abstract

Prescribed burning has been a tool used in Toohey forest management systems and has played an important role in the development of plant communities in Australia. This study aimed to examine the long-term impacts of prescribed burning on litter floor recovery, soil carbon (C) and nitrogen (N) pools and their dynamics at three sites (S1, S2, and S3) during 9-14 years of prescribed burning in a suburban Forest ecosystem of subtropical Australia. The S1-B9 (9 years after prescribed burning), S2-B12 (12 years after prescribed burning), and S3-B14 (14 years after prescribed burning), were sampled for litter floors and 0-20 cm soil profile in August 2019. Soil samples were collected at 0-5, 5-10 and 10-20 cm depths from each of the four plots at each of the three sites (S1, S2, and S3) for total C, total N, C isotope composition ($\delta^{13}C$) and N isotope composition ($\delta^{15}N$) 9-14 years following the last prescribed burning to examine the long term impacts of prescribed burning on soil C and N pools (total C, total N, $\delta^{13}C$ and $\delta^{15}N$) and their dynamics. The litter floor samples were also collected from each plot of the three sites (S1, S2, and S3) for total C, total N, $\delta^{13}C$ and $\delta^{15}N$ 9-14 years after prescribed burning, which would be expected to be burnt in the next 1-2 years. The soil total C, total N, $\delta^{13}C$ and $\delta^{15}N$ in the soil depths of 0-5, 5-10 and 10-20 cm at Site 1 after 9 years following the last prescribed burning were also compared with those of the corresponding properties shortly before the last prescribed burning in June 2011. Similarly, litter floor total C, total N, $\delta^{13}C$ and $\delta^{15}N$ as well as total C and N content at Site 1 after 9 years of prescribed burning would be compared with

those of the corresponding properties in the forest litter floor at Site 1 shortly before prescribed burning in June 2011 to quantify the recovery of C and N pools in the top 20 cm soil and litter floor 9 years after prescribed burning. Overall, after 9 years of prescribed burning at S1, soil total C and total N concentration at 0-5, 5-10 and 10-20 cm soil have recovered and indeed been higher than those of the corresponding soil depths shortly before the burning. Conversely, litter floor (leave and stem) total C and N contents have not recovered after 9 years of the mild prescribed burning at Site 1. This highlights that the forest litter floor would be more N limiting, with much less N losses or released into the soil via litter decomposition since litter floor $\delta^{15}N$ was much less 9 years after prescribed burning at Site 1 than that collected shortly before prescribed burning. Forest litter floor $\delta^{13}C$ was significantly higher 9 years later than that shortly before prescribed burning, suggesting that the vegetation / forest WUE would be higher, partly due to increasing water limitation under climate change. Soil total C and total N at Sites 2 and 3 were also significantly higher 9 years later than those of soil samples collected about 9 years ago. Both soil $\delta^{13}C$ and $\delta^{15}N$ at Sites 1, 2 and 3 (9, 12 and 14 years after prescribed burning) were also significantly lower 9 years later than those of soil samples collected 9 years ago, highlighting increasing N limitation and decreasing N availability as well as increasing organic C inputs into the soil in the suburban forest ecosystems. In conclusion, soil total C and total N at the three study sites were recovered after 9-14 years of prescribed burning. However, soil $\delta^{13}C$ and $\delta^{15}N$ decreased at the three sites in the soil profile (0-5, 5-10 and 10-20 cm) after 9-14 years of prescribed burning. Forest floor leaf and twig total C and total N, soil $\delta^{13}C$ and $\delta^{15}N$ were not fully recovered at S1 before and 9 years after prescribed burning, but litter leaf $\delta^{13}C$ was recovered after 9 years of prescribed burning.

5.2 Introduction

Fire plays an important role in the development of plant communities in Australia (Hamilton et al., 1991). Prescribed burning can be used as an effective tool for protecting both native and plantation forests from the risk of damaging wildfires (Guinto et al. 1999 a, b; 2000; Reverchon et al. 2012). Fire can increase the short-term availability of some nutrients (including nitrogen (N)), which may benefit regenerating plants shortly after the fire (Carter and Foster 2004). Prescribed burning has clear effects on nutrient balance and soil characteristics because of the volatilization of some nutrients at high temperature; particulate (ash) movement by wind or erosion from burned areas and deposition at the burned site or on adjacent site (Boerner, 1982; Raison et al., 1985a; Raison et al., 1985b; Mills and Fey, 2004); and leaching of ions through precipitation which can dissolve the ash on the soil surface and cause leaching beyond the root zone (Yildiz et al., 2010).

The nutrients may be available temporarily after the fire, which can benefit plants, although leaching could further contribute to nutrient losses (Yildiz et al., 2010). Fire regimes influence terrestrial ecosystems and processes, which include vegetation distribution and structure, thus the effect of fire on carbon (C) and N cycling and climate (Hurteau and Brooks, 2011; Bai et al., 2012; Bai et al., 2015). Litter floor plays an important role in nutrient cycling within forest ecosystems and litter production constitutes an important process controlling nutrient cycling within forest ecosystems and is considered to be the main mechanism of transfer of dead organic matter and nutrients from the living biomass to soils (Bansal et al., 2014; Tutua et al., 2014). The study quantified the long-term impacts of prescribed burning on the quantity and quality of forest litter floor, which can lead to loss of an important source of nutrients (Guinto et al., 1999a; Guinto et al., 1999b; Guinto et al., 2001; Tuininga et al., 2002; Penman and York, 2010). Nutrient cycling and carbon (C) dynamics are important for ecosystem sustainability, which may be significantly affected by frequent burning used as a forest management practice

(Bauhus et al., 1993; Bauhus et al., 2002; Alexis et al., 2007). Thus, there is a need to evaluate the long-term impacts of prescribed burning and the recovery of soil C and N pools following the fire. The nutrients may be available for temporarily time after fire, which can benefit plants, although the post fire leaching can contribute to nutrient losses (Yildiz et al., 2010).

Several reports have shown that stable C and N isotope composition ($\delta^{13}C$ and $\delta^{15}N$) techniques can be useful to acquire a better understanding of C and N cycling processes in forest ecosystems (Guinto et al., 2000; Pan et al., 2008; Xu et al., 2009; Rivas et al., 2012; Bai et al., 2015). Other studies by Rumpel et al (2006), Rumpel et al (2007) and Saito et al (2007) have found that the fire may lead to a selective loss of lighter isotopes in soil organic matter compared with the heavy isotopes, but this selective loss be substantial. Several studies (Bai et al. 2012; Bai et al. 2013; Huang et al. 2013; Ma et al. 2015) have reported that the suburban Toohey Forest ecosystem of subtropical Australia has been managed with a repeated fuel reduction fire over the last 20 years to protect surrounding built-up areas from the danger of wildfires, which have caused the changes in different ecosystem functions. Several Soil organic C, total N, $\delta^{13}C$ and $\delta^{15}N$, and soil organic matter composition can be affected by prescribed burning in different ecosystems (Martí-Roura et al., 2013; Ando et al., 2014; Roaldson et al., 2014; Grande et al., 2015; Jones et al., 2015; Rhoades et al., 2015). Other study indicated that $\delta^{15}N$ values decreased with increasing soil depth, which was probably caused by ^{15}N-depletion of NO_3 leaching followed by N immobilisation at greater depths (Cheng et al., 2010). Cheng et al. (2010) also noted that $\delta^{15}N$ values in surface organic and mineral soils did not always decrease with an increase in soil depth. Previous studies have noted that the effect of fire intensity has played an important role in the change of soil organic matter composition after fire (Guinto et al., 1999a; Knicker et al., 2006; Cécillon et al., 2009; Guénon et al., 2011). However, several reports have shown that the low intensity fire led to increased soil C content, as a result of partly charred material or litter from decaying trees (Knicker et al., 2006; Guénon et al., 2011). Meanwhile, the result of study by Knicker et al. (2006) observed that 1 to 2 years after light fire intensity in continental Mediterranean ecosystems, Spain, led to greatest enrichment of aromatic carbon (C).

The objectives of this study were to examine the recovery of forest litter floor and total C, total N, C and N isotope composition ($\delta^{13}C$ and $\delta^{15}N$) in the 0-5, 5-10, and 10-20 cm soil at Site 1 (S1, 9 years after burning), Site 2 (S2, 12 years after burning) and Site 3 (S3, 14 years after burning) in the suburban Toohey Forest ecosystem of subtropical Australia; and to evaluate the role of forest litter floor in the nutrient cycling and forest litter floor impacts on soil C and N pools and their dynamics in the periods of 9-14 years after prescribed burning in the suburban Toohey Forest ecosystem. It was hypothesised that soil C and N pools as well as forest litter floor would gradually be recovered after 9-14 years of prescribed burning in the suburban Toohey Forest ecosystem; and that forest litterfall and litter floor recovery would play important roles in the recovery process of soil C and N pools after 9-14 year of prescribed burning.

5.3 Material and methods

5.3.1 Site description and experimental design

A series of studies was conducted within Toohey Forest which is located 10 km south of Brisbane, in South-East Queensland, Australia "27°30′S" & "135°E". The climate of the region is subtropical, with annual rainfall 613.4 mm for 2019 (Fig. 1). Annual minimum and maximum temperatures ranged between 17.1 and 27.8°C in 2019 (Fig. 1). In general, summer is hot and wet, whereas winter is cool and dry (Catterall and Wallace, 1987).

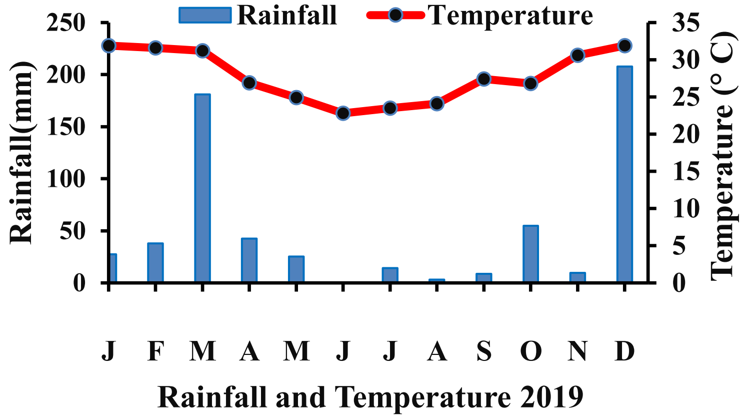

Figure 5.1 Monthly air temperatures and rainfall during the study period of 2019 in Toohey Forest.

Toohey Forest consists of four soil types, which are different from the other regions in the same forest, whereby these soil types consist of lithosols, red yellow podzolic, red earth, and alluvial. The lithosols soil, which contains both stony components and loam to silty loam, may be rich with humus. Whereas, the podzols are mainly less than 60 cm deep in soil, and are low in nutrients, and horizon-B is sandy clay, while horizon-A is sandy loam with coarser particles (Catterall and Wallace 1987; Stock et al., 1987).

Before 1950s, the history of burning in Toohey Forest is unknown. During the period between 1950s and the early 1970s, individual fires might have burned across a large proportion of the area. In the early 1970's individual fires were confined to more localised areas, and also created a spatially heterogeneous landscape. In general, fires usually occur at the end of the dry season in September-October. From 1993, prescribed burning has been applied in the forests, including Toohey Forest, whereby there is an establishment of 27 blocks within the forest. Prescribed burning has been applied in frequencies between 4-5 years and 8-10 years (Catterall and Wallace 1987).

Three sites within Toohey Forest were used in this study. Each site had a different history of prescribed burns. Site 1 in Block 17B was last burned in August 2011 (9 years after prescribed burning), and earlier in March 2003. Site 2 in Block 24 of Toohey Forest was last burnt in April 2008 (12 years after prescribed burning), and earlier in September 1999. Site 3 in Block 17 was last burnt in May 2006 (14 years after prescribed burning), and earlier in August 1995. Four circular plots with a radius of 12.62 m, giving an internal area of 500 m^2 were randomly located within each site giving a total sampling area of 1/5 of hectare. Location of the plots were randomised within the trial area; there was a mixture of understory acacia species and overstory eucalyptus species that would be typical of the area. There are four quadrants for each plot and each quadrant was defined by four pegs driven into the soil marking out an area of 1 square meter had one litter floor collection area. The experimental design was a randomised block design with 4 replications.

5.3.2 Litter floor sample collection and analysis

The trial plots were randomly located within three sites (S1, S2 and S3). There were four replicated plots at each study site, and within each plot there were four quadrants. The litter floor samples were collected from each quadrant in each plot at each study site of S1-B9 (9 years after prescribed burning), S2-B12 (12 years after prescribed burning), and S3-B14 (14 years after prescribed burning). Each litter floor sampling area was defined by four pegs driven into the soil marking out an area of 1 square meter. In August 2019, litter floor collection was done by using a metal square that was placed over the marker pegs and defined the edges of the sampling area. All litter deposited within the square was collected in paper bags for subsequent drying and sorting. The ground was swept clear at the start of the experiment. The four collection areas were distributed randomly, one within each quadrant of the circular plot. Sample collection was undertaken just at Site 1 (9 years after burning), Site 2 (12 years after burning), and at Site 3 (14 years after burning), while the litter floor (leaf and twigs) collection was just once on 23 August 2019 at S1-B9 S2-B12 and S3-B14 following the last burning. In summary, the sampling regime consisted of litter floor samples (leaves and twigs) in August

2019 from each quadrant within each plot at each of the three sites. Overall, 16 litter floor samples (leaves and twigs) were collected for each study site (16 × 3 sites = 48).

The litter floor were kept in separate paper bags and transferred to the laboratory, and then these samples were oven-dried at 60°C for 7 days to a constant weight and ground to a fine powder by a Rocklabs™ ring grinder (Guinto et al., 2000). The homogenised powder was weighed into 8 mm × 5 mm tin capsules for analysis using an isotope ratio mass spectrometer (GV Isoprime Manchester, UK). Total N, total C, $\delta^{13}C$ and $\delta^{15}N$ were assessed using an isotope ratio mass spectrometer. As described by Prasolova et al. (2000) and Xu et al. (2003), 6-7 mg plant material was packed into tin capsule to determine foliar total C and N as well as $\delta^{13}C$ and $\delta^{15}N$ by isotope ratio mass spectrometer with a Eurovector Elemental Analyser (Isoprime-EuroEA 3000, Milan, Italy) (Sollins et al., 1999; Molero et al., 2011), $\delta^{13}C$ or $\delta^{15}N$ was determined by $^{13}C/^{12}C$ ratio, and $^{15}N/^{14}N$ ratio as:

$$\delta^{13}C \text{ or } \delta^{15}N\ (‰) = (R_{sample} / R_{std} - 1) \times 1000$$

where R_{sample} is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio of the samples and R_{std} is the $^{13}C/^{12}C$ ratio of the international PeeDee Belemnite (PDB) standard or $^{15}N/^{14}N$ ratio of N_2 gas (Xu et al., 2000; Xu et al., 2003; Molero et al., 2011).

$\delta^{15}N$ and $\delta^{13}C$ are calculated based on the following formulae:

$$\delta^{15}N \text{ or, and } \delta^{13}C\ (‰) = (R_{sample} / R_{std} - 1) \text{ x } 1000.$$

5.3.3 Soil sample collection and analysis

Three soil cores were randomly collected from each quarter in each plot within each site. Three depths were sampled using a 2.5 cm diameter auger: 0-5, 5-10, and 10-20 cm. The soil samples were kept in separate plastic bags and transferred to the laboratory. The air-dried samples were ground (< 150 μm) and stored at room temperature. In the laboratory, soil samples were passed through 2 mm sieve. A sub-sample of the soil was air-dried, and the rest was refrigerated at 4°C and processed shortly after sampling. Total C, total N, $\delta^{13}C$ and $\delta^{15}N$ were determined using mass spectrometer (GV Isoprime Manchester, UK), as described by (Prasolova et al., 2000; Xu et al., 2000, 2003).

Total C and N were analysed by isotope ratio mass spectrometer with a Eurovector Elemental Analyser (Isoprime-EuroEA 3000, Milan, Italy). The 40-50 mg soil material was packed into tin capsule for both 0-5 and 5-10cm depths, and 50-60 mg soil material was packed into tin capsule for 10-20cm depth which were loaded into the auto-sampler that dropped the capsule and sample into the combustion column. Samples were determined for total C and N, $^{13}C/^{12}C$ ratio, and $^{15}N/^{14}N$ ratio which were used to calculate $\delta^{13}C$ or $\delta^{15}N$ (‰) as:

$\delta^{13}C$ or $\delta^{15}N$ (‰) = $(R_{sample} / R_{std} - 1) \times 1000$

where R_{sample} is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio of the samples and R_{std} is the $^{13}C/^{12}C$ ratio of the international PeeDee Belemnite (PDB) standard or $^{15}N/^{14}N$ ratio of N_2 gas (Xu et al., 2000; Xu et al., 2003; Molero et al., 2011).

$\delta^{15}N$ and $\delta^{13}C$ are calculated based on the following formulae:

$\delta^{15}N$ or, and $\delta^{13}C$ (‰) = $(R_{sample} / R_{std} - 1)$ x 1000.

5.3.4 Statistical analysis

Two-way repeated analysis of variance (ANOVA) was conducted to detect the differences in total N, total C, $\delta^{13}C$ and $\delta^{15}N$ with respect to the sampling times and soil depths. Statistical analyses were conducted with Statistix software (version 8). Direct comparison among experimental sites was not possible due to the fact that they had different properties and plant cover, so each site has been independently assessed. Differences among means of all variables over the time were determined with post-hoc pairwise comparison using the Tukey HSD test at $P< 0.05$ level (Idol et al., 2002; Schafer and Mack, 2010; Köster et al., 2014). Multiple regressions were carried out to quantify the relationships among plant properties such as $\delta^{13}C$, $\delta^{15}N$, and total N across each of the four sites.

5.4 Results

5.4.1 Litter floor properties

5.4.1.1 Total N, total C, $\delta^{13}C$ and $\delta^{15}N$

Total N and total C in leaf litter were significantly different ($P < 0.05$) shortly before and after 9 years of prescribed burning at S1. Similarly, total N and total C in twig litter were significantly different shortly before and after 9 years of prescribed burning at S1(Table 5.1). The current study indicated that leaf total N and total C were significantly higher shortly before prescribed burning at S1 compared with those of the corresponding variables 9 years after prescribed burning. Meanwhile, our study indicated that twigs total N and total C were significantly higher shortly before prescribed burning than those of the corresponding variables after 9 years of prescribed burning (Table 5.1).

Litter floor leaf $\delta^{13}C$ and $\delta^{15}N$ were significantly different ($P < 0.05$) between those collected shortly before and 9 years after prescribed burning at S1. The present study indicated that leaf $\delta^{13}C$ was significantly higher 9 years after prescribed burning at site of S1 compared with those of the corresponding variables shortly before the last prescribed burning in June 2011(Table 5.1) highlighting that there was increasing water limitation in the last 9 years under intensifying climate change . However, both leaf and twigs $\delta^{15}N$ were significantly higher shortly before prescribed burning compared with those of the corresponding variables 9 years after the last prescribed burning in August 2019 (Table 5.1). This suggests that there were also increasing N limitations as indicated by the lower $\delta^{15}N$ in leaf and twigs $\delta^{15}N$ in the Toohey Forest subject to the prescribed burning in the context of intensifying climate change.

At S2 and S3, there were significant differences ($P < 0.05$) in leaf and twigs total C and N 12-14 years after prescribed burning compared with those of the 3-5 years of prescribed burning (Table 5.1). Similarly, leaf $\delta^{13}C$ and $\delta^{15}N$ were significantly different ($P < 0.05$) after 12-14 years of prescribed burning at sites of S2 and S3 (Table 5.1). On the other hand, there were significant differences in twigs $\delta^{13}C$ after 5 and 14 years of prescribed burning at S3 (Table 5.1). The current study indicated that leaf $\delta^{13}C$ was significantly higher at sites of S2 and S3 after 12-14 years of prescribed burning than those of the 3 and 5 years after the last prescribed burning in June 2011(Table 5.1). However, leaf $\delta^{15}N$ was decreased after 12-14 years of prescribed burning compared with those of 3 and 5 years after prescribed burning (Table 5.1). Meanwhile, twigs $\delta^{13}C$ was significant different ($P < 0.05$) after 14 years of prescribed burning, while no significantly difference was found at S2, 12 years after prescribed burning. However, there were no significant differences in twigs $\delta^{15}N$ after 12-14 years of prescribed burning (Table 5.1).

The current study indicated that twigs $\delta^{13}C$ was significantly higher at S2 after 12 years of prescribed burning than those of the 3 years after the last prescribed burning in June 2011. The present study showed that twigs $\delta^{15}N$ was significantly higher after 12-14 years of prescribed burning compared with those of 3 and 5 years after prescribed burning (Table 5.1). Overall, leaf $\delta^{13}C$ was fully recovered at sites of S2 and S3 after 12-14 years of prescribed burning, while leaf $\delta^{15}N$ was not yet recovered after 12-14 years of prescribed burning. However, twigs $\delta^{13}C$ and $\delta^{15}N$ were fully recovered at sites of S2 and S3 after 12-14 years of prescribed burning, but twigs $\delta^{13}C$ was not recovered at S3 after 14 years of prescribed burning (Table 5.1).

Table 5.1 Leaves and twigs litter floor total carbon (TC), total nitrogen (TN), C isotope composition ($\delta^{13}C$) and N isotope composition ($\delta^{15}N$) at sites of S1- B9 (9 years after burning), Site 2- B12 (12 years after burning), and Site 3 (14 years after burning) located at Toohey Forest. Different letters indicate significant differences at $P < 0.05$.

Site	Litter floor (Leaves)					Litter floor (Twigs)				
	TC (%)	TN (%)	C/N	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	TC (%)	TN (%)	C/N	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
S1-B0	52.83 a	0.96 a	55.00 b	-30.34 b	-1.54 a	48.63 a	0.56 a	86.8 b	-27.41 a	-2.59 a
S1-B9	49.33 b	0.68 b	72.54 a	-29.04 a	-3.14 b	47.29 b	0.36 b	131.4 a	-27.62 a	-2.90 a
S2-B3	48.76 b	0.39 b	125.0 a	-29.60 b	-4.33 a	45.31 b	0.40 a	113.3 a	-28.67 a	-4.73 a
S2-B12	49.79 a	0.46 a	108.2 b	-28.48 a	-4.53 a	46.73 a	0.43 a	108.7 a	-28.31 a	-4.61 a
S3-B5	48.30 b	0.56 a	86.3 a	-29.33 b	-3.81 a	45.28 b	0.42 b	107.8 a	-27.82 a	-4.35 a
S3-B14	50.64 a	0.55 a	92.1 a	-28.43 a	-4.51 b	46.84 a	0.49 a	95.6 b	-29.95 b	-3.94 a

- **B0, means shortly before burning   B9, means 9 years after burning (August 2019)**
- **B3, means 3 years after burning   B12, means 12 years after burning (April 2008)**
- **B5, means 5 years after burning   B14, means 14 years after burning (May 2006)**

5.4.1.2 Litter Floor C and N pools

Our study showed there was no significantly difference in leaf litter floor dry matter (DM) shortly before and 9 years after prescribed burning at S1, while significant difference ($P < 0.05$) was found in litter floor twigs between shortly before and 9 years after prescribed burning at S1 (Table 5.2). The present study indicated that leaf litter floor was significantly higher shortly before prescribed burning at site of S1 compared with those of the corresponding variables 9 years after prescribed burning. Similarly, our study showed that highest leaf amount was found shortly before prescribed burning in June 2011 compared with those of the corresponding variables 9 years after prescribed burning at site of S1(Table 5.2).

The present study indicated there was significant difference ($P<0.05$) for both leaf and twigs litter floor after 9-14 years of prescribed burning (Table 5.3). The current study showed that leaf was significantly higher after 9 years of prescribed burning. Similarly, twigs litter floor was found significantly higher 9 years after prescribed burning (Table 5.3).

Table 5.2 Litter floor (leaves and twigs) dry matter production (g/ t ha^{-1}) at site 1- B0 shortly before burning, and B9 - 9 years after burning.

Site	Litterfall amount/ Leaves (g/ t ha^{-1})	Litterfall amount/ Twigs (g/ t ha^{-1})	Total DM (t/ ha^{-1})
S1-B0	4.29	4.25	5.90
S1-B9	3.96	1.61	7.86

- **B0, means shortly before prescribed burning (August 2011)**
- **B9, means 9 years after prescribed burning (August 2011)**

Table 5.3 Litter floor (leaves and twigs) dry matter production (g/ t ha^{-1}) at site 1- B9 9 years after burning, site 2, 12 years after burning and site3 14 years after burning.

Site	Litterfall amount/ Leaves (g/ t ha^{-1})	Litterfall amount/ Twigs (g/ t ha^{-1})	Total DM (t/ ha^{-1})
S1-B9	3.96	4.25	7.86
S2-B12	2.86	3.60	6.46
S3-B14	1.68	2.84	4.52

- **B9, means 9 years after burning (August 2011)**
- **B12, means 12 years after burning (April 2008)**
- **B14, means 14 years after burning (May 2006)**

5.4.2 Soil total C, total N, $\delta^{13}C$ and $\delta^{15}N$

5.4.2.1 Soil total N and total C

Soil total N was significantly different ($P < 0.05$) in the 0-5 cm soil after 9-12 years of prescribed burning at sites of S1 and S2 respectively compared with those of the corresponding variables shortly before and 3 years after the last prescribed burning in June 2011 (Fig. 5.2a, b), while there were no significant differences in total N for all soil depths (0-5, 5-10 and 10-20 cm) at S3 14 years after prescribed burning (Fig. 5.2c).

The current study indicated that total N were significantly higher in soil depth 0-5 cm at sites of S1, S2 and S3 9-14 years after prescribed burning than those of the shortly before and 3 and 5 years after the last prescribed burning in June 2011(Fig. 5.2). At S1, there were no significant differences in total N of 5-10 and 10-20 cm soil, and 10-20 cm depth for S2. Overall, total N in the soil profile (0-5, 5-10 and10-20 cm) were either fully recovered or higher for all the three sites after 9-14 years of prescribed burning (Fig. 5.2).

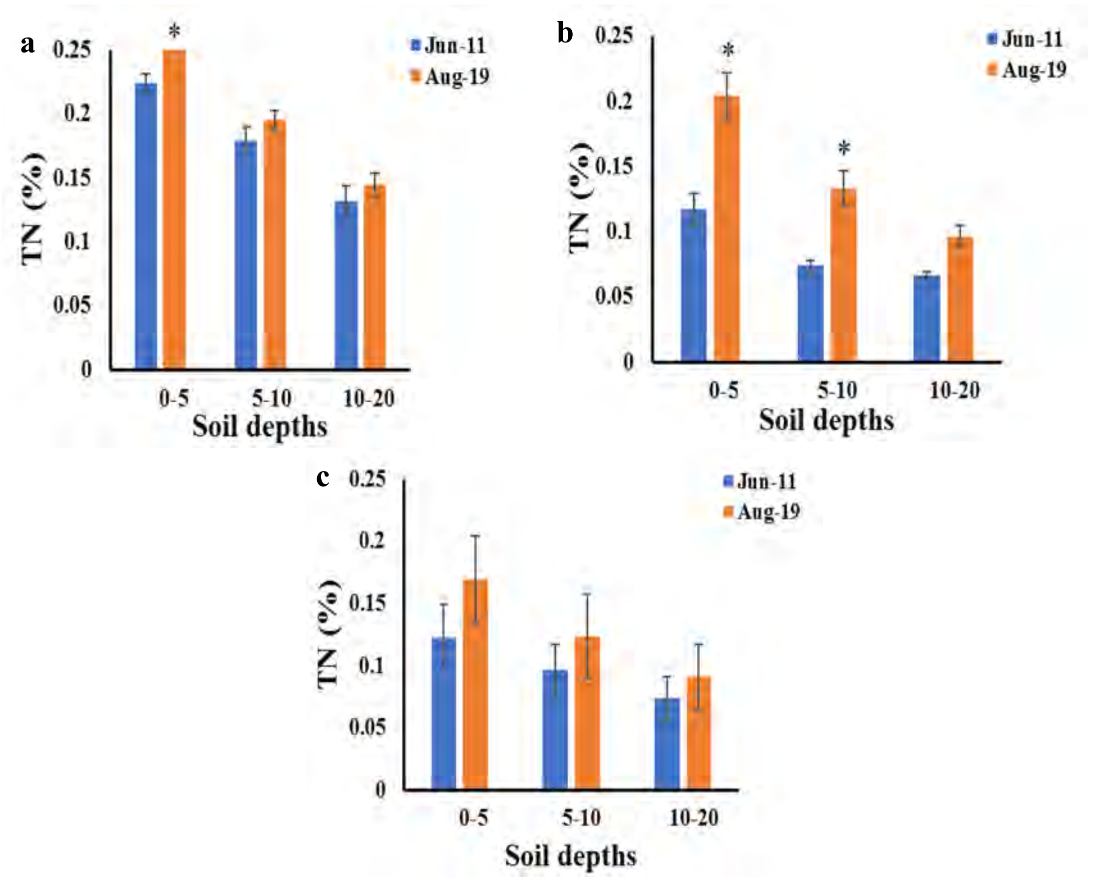

Figure 5.2 Soil total nitrogen (TN) at all study sites: (a) S1-B9 (before and 9 years after burning), (b) S2-B12 (3 and 12 years after burning); and (c) S3-B14 (5 and 14 years after burning) for 3 soil depths (cm). Asterisk indicates significant differences between soil depths for each site at $P < 0.05$. Bars are means ± standard errors.

Soil total C was significantly different ($P < 0.05$) for all soil depth (0-5, 5-10 and 10-20 cm) after 9-12 years of prescribed burning at sites of S1 and S2 respectively compared with those of the corresponding variables shortly before and 3 years after the last prescribed burning in June 2011 (Fig. 5.3a, b), while there were no significant differences in total C for all soil depths (0-5, 5-10 and 10-20 cm) at S3 14 years after prescribed burning (Fig. 5.3c).

The current study indicated that total C was significantly higher in the soil at 0-5 cm at sites of S1, S2 and S3 9-14 years after prescribed burning than those of the shortly before and 3 and 5 years after the last prescribed burning in June 2011(Fig. 5.3). Overall, total C values in the soil profile (0-5, 5-10 and10-20 cm) were either fully recovered or higher for all the three sites after 9-14 years of prescribed burning (Fig. 5.3).

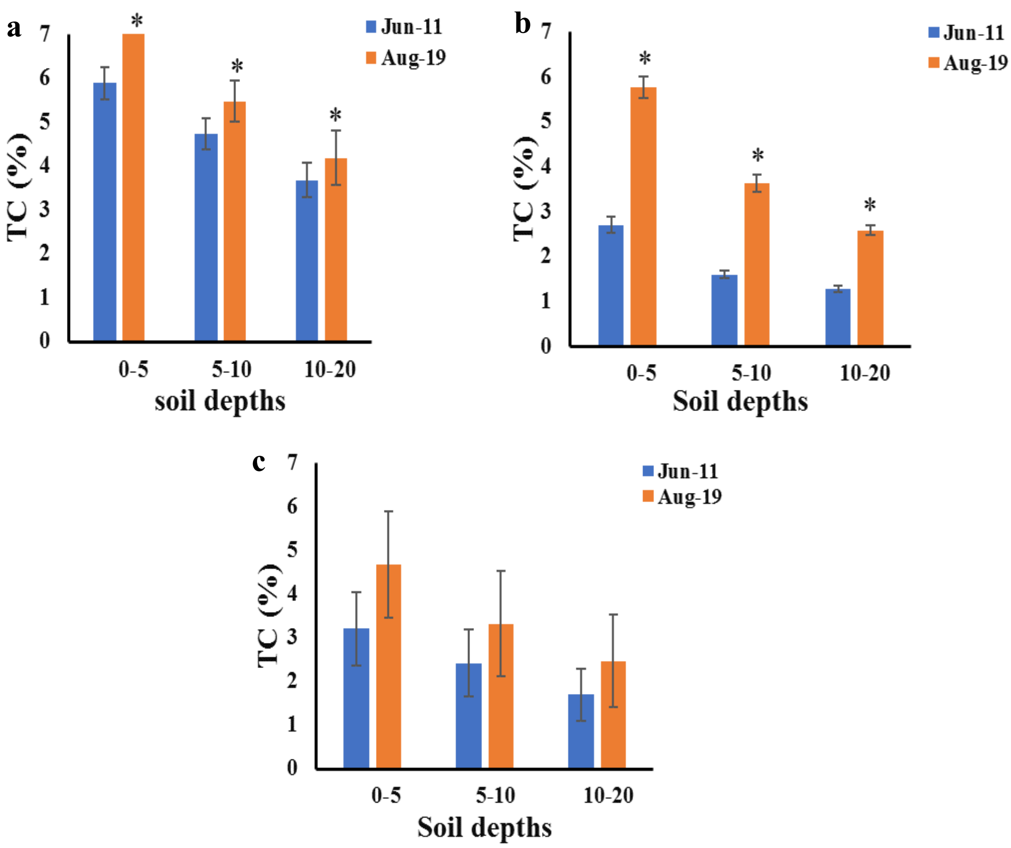

Figure 5.3 Soil total carbon (TC) at all study sites: (a) S1-B9 (before and 9 years after burning), (b) S2-B12 (3 and 12 years after burning); and (c) S3-B14 (5 and 14 years after burning) for 3 soil depths (cm). Asterisk indicates significant differences between soil depths for each site at $P < 0.05$. Bars are means ± standard errors.

5.4.2.2 Soil $\delta^{13}C$ and $\delta^{15}N$

At S1, soil $\delta^{13}C$ was significantly different ($P < 0.05$) in the soil at 5-10 and 10-20 cm shortly before prescribed burning compared with those of the corresponding variables 9 years after the last prescribed burning in August 2019 (Fig. 5.4a), while there were no significant differences in soil $\delta^{13}C$ for all soil at 0-5 cm at S1 between shortly and after 9 years of prescribed burning (Fig. 5.4a).

At S2 and S3, soil $\delta^{13}C$ values were significantly different ($P < 0.05$) for all soil depths (0-5, 5-10 and 10-20 cm) after 12-14 years of prescribed burning, compared with those of the corresponding variables 3 and 5 years after the last prescribed burning in June 2011 (Fig. 5.4b and c).

The current study indicated that $\delta^{13}C$ was significantly higher in soil depth 10-20 cm at sites of shortly before and after 3 and 5 years of prescribed burning than those of the 9-14 years after the last prescribed burning in August 2019 (Fig. 5.4). Overall, soil $\delta^{13}C$ in the soil profile (0-5, 5-10 and10-20 cm) was not fully recovered or higher for all the three sites after 9-14 years of prescribed burning (Fig. 5.4).

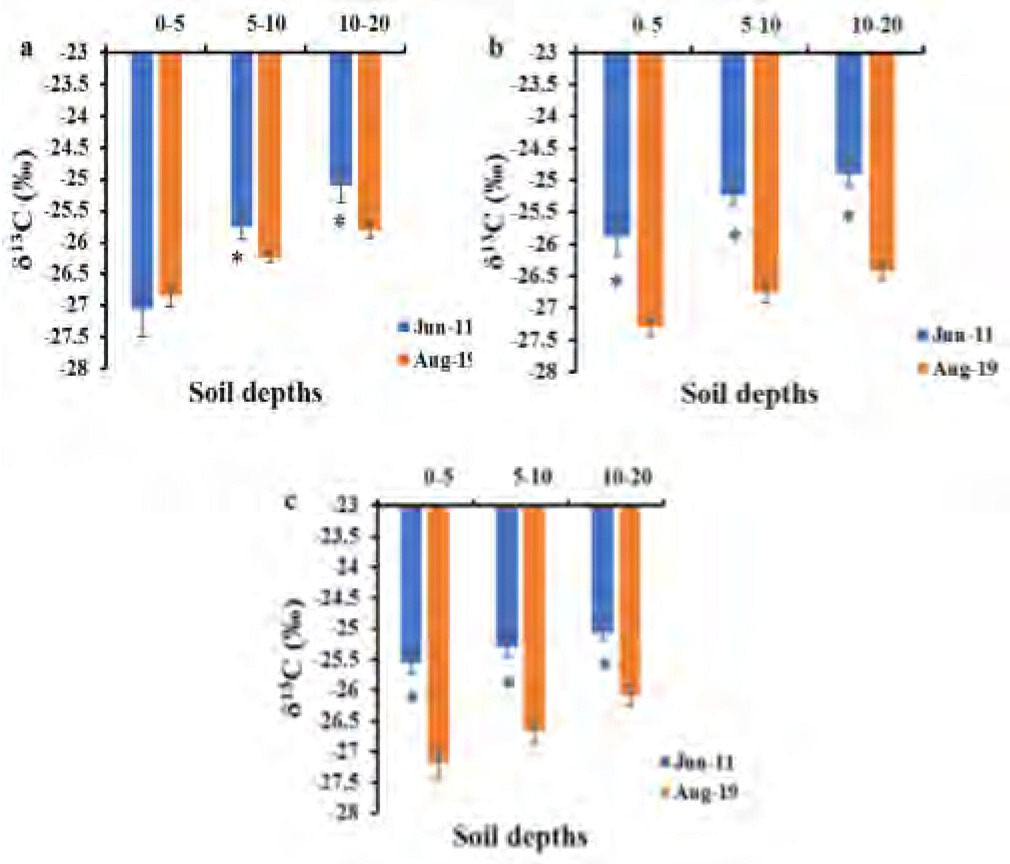

Figure 5.4 Soil C isotope composition ($\delta^{13}C$) at all study sites: (a) S1-B9 (before and 9 years after burning), (b) S2-B12 (3 and 12 years after burning); and (c) S3-B14 (5 and 14 years after burning) for 3 soil depths (cm). Asterisk indicates significant differences between soil depths for each site at $P < 0.05$. Bars are means ± standard errors.

At S1, soil $\delta^{15}N$ was significantly different ($P < 0.05$) in the soil at 10-20 cm shortly before prescribed burning compared with those of the corresponding variables 9 years ago after the last prescribed burning in August 2019 (Fig. 5.5a). However, there were no significant differences in soil $\delta^{15}N$ in the soil at 0-5 and 5-10 cm at S1 between shortly and after 9 years of prescribed burning (Fig. 5.5a).

At S2 and S3, soil $\delta^{15}N$ were significantly different ($P < 0.05$) for all soil depths (0-5, 5-10 and 10-20 cm) after 3 and 5 years of prescribed burning compared with those of the corresponding variables after 12-14 years of the last prescribed burning (Fig. 5.5b and c), except for the 0-5 cm soil at S3,where soil $\delta^{15}N$ were no significantly differences for soil depth 0-5 cm between 5 and 14 years of prescribed burning (Fig. 5.5c).

The current study indicated that $\delta^{15}N$ was significantly higher in the soil at 10-20 cm at sites of shortly before and after 3 and 5 years of prescribed burning than those of the 9-14 years after the last prescribed burning in August 2019 (Fig. 5.5). Overall, soil $\delta^{15}N$ in the soil profile (0-5, 5-10 and10-20 cm) was not fully recovered or higher for all the three sites after 9-14 years of prescribed burning (Fig. 5.5).

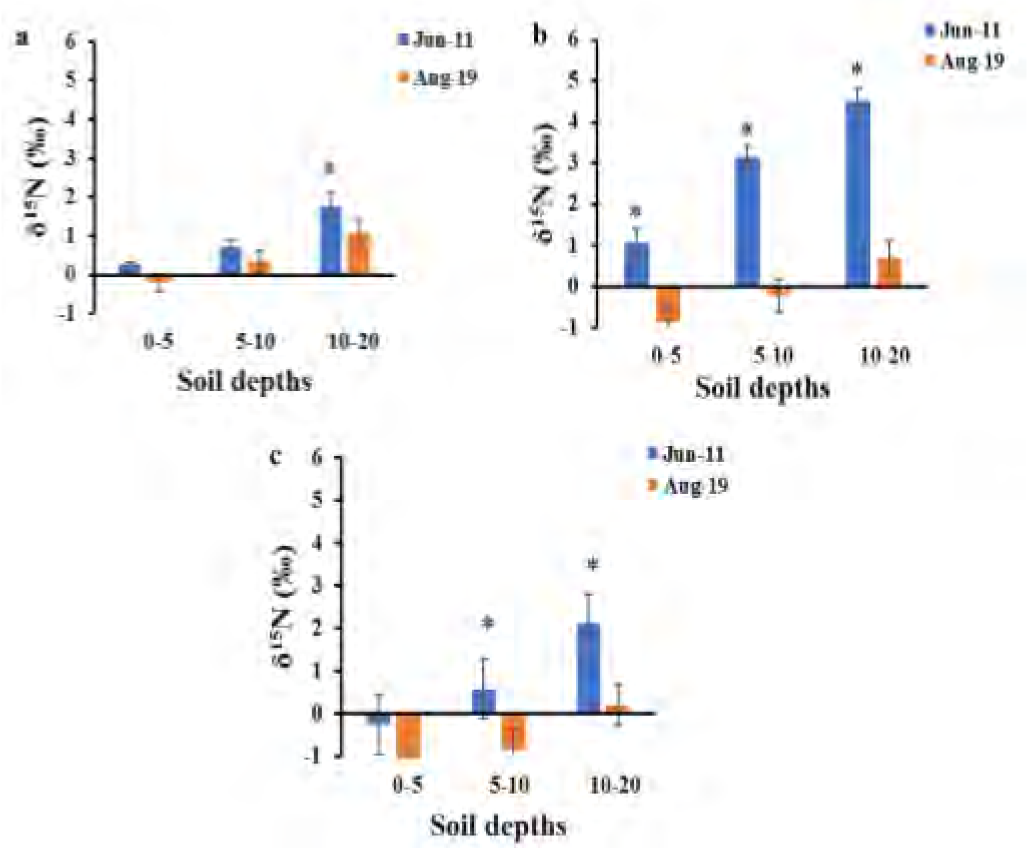

Figure 5.5 Soil N isotope composition ($\delta^{15}N$) at all study sites: (a) S1-B9 (before and 9 years after burning), (b) S2-B12 (3 and 12 years after burning); and (c) S3-B14 (5 and 14 years after burning) for 3 soil depths (cm). Asterisk indicates significant differences between soil depths for each site at $P < 0.05$. Bars are means ± standard errors.

5.4.3 Relationship between soil and litter variables

For the winter sampling in August 2019 between soil variables at different soil depths, 48.3% of the variation in soil $\delta^{13}C$ (Table 5.4) at 10-20 cm depth, was explained by the corresponding soil total N (42.3%) shortly before prescribed burning, while 72.1% and 42.2% of soil $\delta^{13}C$ at 0-5 and 5-10 cm depth respectively was explained by soil $\delta^{15}N$ (70.6%) and (35.5%) respectively at S1-B9 after 9 years of prescribed burning (Table 5.4).

For the winter sampling of August 2019, there were 68.4%, 71.3% and 68.5% of the variation in soil $\delta^{13}C$ of 0-5, 5-10 and 10-20cm at S3-B14 respectively explained by the corresponding soil $\delta^{15}N$ (68.2%), (69.9%) and (67.0%) at 0-5, 5-10 and 10-20 cm respectively (Table 5.4). About 56.5%, 60.1% and 26.8% of the variation in soil $\delta^{13}C$ of 0-5, 5-10 and 10-20 cm at S3-B14 respectively explained by the corresponding soil total N (Table 5.4). There were 78.7%, 93.0% and 53.5% at S3 for 0-5, 5-10 and 10-20 cm respectively of the variation in soil total N were explained by the corresponding soil $\delta^{15}N$ values respectively (Table 5.4).

Meanwhile, there was 37.1% of the variation in litter floor twigs total N at S1-B0 shortly before burning, which was explained by the corresponding litter floor leaf $\delta^{15}N$ values respectively (Table 5.5). However, 48.0% and 34.7% of the variation in soil $\delta^{13}C$ (Table 5.5) of litter floor twigs was explained by the corresponding soil $\delta^{15}N$ values at site of S3 after 14 years of prescribed burning (Table 5.5).

Table 5.4 Relationships among soil $\delta^{13}C$, total N (TN) and $\delta^{15}N$; between $\delta^{13}C$ and $\delta^{15}N$; between $\delta^{13}C$ and TN; and between TN and $\delta^{15}N$ for the winter sampling in August 2019 at sites of S1-B9, S2-B12, and S3-B14 in Toohey Forest of south-east Queensland.

Soil depths (cm)	S1-B0	S1-B9	S2-B12	S3-B14
0-5	ns	$\delta^{13}C$ (‰) = -26.32 + 0.728 ($\delta^{15}N$) -1.51 (TN) (R^2 = 0.721, n = 16, $P < 0.05$)	ns	$\delta^{13}C$ (‰) = -26.79 + 0.365 ($\delta^{15}N$) - 0.708 (TN) (R^2 = 0.684, n = 16, $P < 0.05$)
5-10	ns	$\delta^{13}C$ (‰) = -25.86 + 0.460 ($\delta^{15}N$) -2.87 (TN) (R^2 = 0.422, n = 16, $P < 0.05$)	ns	$\delta^{13}C$ (‰) = -25.73+ 0.586 ($\delta^{15}N$) -3.63 (TN) (R^2 = 0.713, n = 16, $P < 0.05$)
10-20	$\delta^{13}C$ (‰) = -23.96 + 0.999 ($\delta^{15}N$) - 4.14 (TN) (R^2 = 0.483, n = 16, $P < 0.05$)	ns	ns	$\delta^{13}C$ (‰) = -26.00 + 0.428 ($\delta^{15}N$) -1.92 (TN) (R^2 = 0.685, n = 16, $P < 0.05$)
0-5	ns	$\delta^{13}C$ (‰) = -26.72 + 0.778 ($\delta^{15}N$) (R^2= 0.706, n = 16, $P < 0.05$)	ns	$\delta^{13}C$ (‰) = -26.61 + 0.404 ($\delta^{15}N$) (R^2=0.682 , n = 16, $P < 0.05$)
5-10	ns	$\delta^{13}C$ (‰) = -26.38 + 0.374 ($\delta^{15}N$) (R^2= 0.355, n = 16, $P < 0.05$)	ns	$\delta^{13}C$ (‰) = -26.35+ 0.386 ($\delta^{15}N$) (R^2=0.699, n = 16, $P < 0.05$)
10-20	ns	ns	ns	$\delta^{13}C$ (‰) = -26.17 + 0.370 ($\delta^{15}N$) (R^2= 0.670, n = 16, $P < 0.05$)
0-5	ns	ns	ns	$\delta^{13}C$ (‰) = -28.21- 5.99 (TN) (R^2= 0.565, n = 16, $P < 0.05$)
5-10	ns	ns	ns	$\delta^{13}C$ (‰) = -27.45 - 6.29 (TN) (R^2= 0.601, n = 16, $P < 0.05$)
10-20	$\delta^{13}C$ (‰) = -23.86 - 9.62 (TN) (R^2= 0.423, n = 16, $P < 0.05$)	ns	ns	$\delta^{13}C$ (‰) = -26.61 - 5.71 (TN) (R^2= 0.268, n = 16, $P < 0.05$)
0-5	ns	ns	ns	TN (%) = 0.248 + 0.055 ($\delta^{15}N$) (R^2= 0.787, n = 16, $P < 0.05$)
5-10	ns	ns	ns	TN (%) = 0.169 + 0.055 ($\delta^{15}N$) (R^2= 0.930, n = 16, $P < 0.05$)

10-20	ns	ns	ns	TN (%) = 0.085+ 0.029 ($\delta^{15}N$) (R^2= 0.535, n = 16, P < 0.05)

Table 5.5 Relationships among litter floor leaves and twigs $\delta^{13}C$, total N (TN) and $\delta^{15}N$; between $\delta^{13}C$ and $\delta^{15}N$; and between TN and $\delta^{15}N$ for the winter sampling in August 2019 at sites of S1-B9, S2-B12, and S3-B14 in Toohey Forest of south-east Queensland.

Litter floor components	S1-B0	S1-B9	S2-B12	S3-B14
Leaf	ns	ns	ns	ns
Twigs	ns	ns	ns	$\delta^{13}C$ (‰) = -23.96 + 0.999 ($\delta^{15}N$) - 4.14 (TN) (R^2 = 0.480, n = 16, P < 0.05)
Leaf	ns	ns	ns	ns
Twigs	ns	ns	ns	$\delta^{13}C$ (‰) = -26.21 + 0.949 ($\delta^{15}N$) (R^2= 0.347, n = 16, P < 0.05)
Leaf	ns	ns	ns	ns
Twigs	TN (%) = 1.87 + 0.515 ($\delta^{15}N$) (R^2 = 0.371, n = 16, P < 0.05)	ns	ns	ns

5.5 Discussion

5.5.1 Litter floor leaves and twigs

5.5.1.1 Total N and total C

The present study indicated that litter floor leaf total N and total C were not fully recovered after 9 years of prescribed burning at S1. Similarity, litter floor twigs total N and total C were not fully recovered at S1 after 9 years of prescribed burning, which is consistent with the findings from Abdullah (2016) who found that the effect of the time after fire (TSF) accounted for 45% of the variation in litterfall leaf total N over the short-term sampling time. Other studies have shown that prescribed burning may affect the quantity and quality of litter floor, as a result of combustion of the litter layer, which would result in losses of plant nutrients (Guinto et al., 1999a; Guinto et al., 1999b; Guinto et al., 2001; Tuininga et al., 2002; Penman and York, 2010). On the other hand, our results showed that the litter floor leaf total N and total C were fully recovered after 12-14 years than those of the corresponding values 3 and 5 years after the last prescribed burning in June 2011 at S2 and S3. This is similar to the findings of the Abdullah. (2016) who found that the sampling time at S2, litterfall quality changed significantly for leaves. The reason of increased litterfall leaf N may have been caused by the increasing of N availability after fire (Schafer and Mack, 2010). However, the current study indicated that litter floor leaf total N was not recovered at S3 after 14 years of prescribed burning than those of the 5 years after the last prescribed burning in June 2011.

Meanwhile, the current study showed that litter floor twigs total N and total C were fully recovered after 12-14 years than those of the corresponding variables 3-5 years after the last prescribed burning in June 2011 at S2 and S3 respectively, which is consistent with the findings from Abdullah (2016) who found that the time since fire (TSF) accounted for 45% of the variation in litterfall leaf total N over the short-term sampling time.

The changes in N availability can be attributed to the relationship between N mineralization and soil organic composition, which can be affected significantly by fire, because frequent fires decreased the ratio of alkyl C and O-alkyl C and the amounts of carbohydrates relative to waxes, which is an index of organic matter decomposition (Guinto et al., 1999a).

This is similar to the findings of Abdullah. (2016) who found that TSF accounted for 45% of the variation in litterfall leaf total N and total C (TC) over the short-term sampling time. Other studies have shown that the low intensity fire led to increased soil C content, as a result of partly charred material or litter from decaying trees (Knicker et al., 2006; Guénon et al., 2011).

5.5.1.2 Litter floor leaf and twigs $\delta^{13}C$ and $\delta^{15}N$

The present study indicated that litter floor leaf $\delta^{13}C$ was higher for August 2019 (winter-dry season) at S1 after 9 years of prescribed burning than those of the shortly before prescribed burning in June 2011. This is consistent with the finding of Wang et al. (2020) who found that leaf litter $\delta^{13}C$ increased over the study period. Similarity, litter floor leaf $\delta^{13}C$ was recovered after 12-14 years of prescribed burning than those of the 3-5 years of the last prescribed burning in June 2011 at S2 and S3 respectively, which is consistent with the finding of Wang et al. (2020). The temporal and spatial variations in litter floor leaf $\delta^{13}C$ might account for those differences in response to the prescribed burning events. The N availability condition after prescribed burning as well as climatic factors, including air temperature and rainfall (water availability) were the main driving factors of litterfall $\delta^{13}C$ (Tahmasbian et al. 2019). Conversely, our result showed that there were no significant differences in litter floor twigs $\delta^{13}C$ between shortly before and 9 years after prescribed burning at S1. The current study indicated that litter floor twigs $\delta^{13}C$ was higher shortly before burning in June 2011 at S1 than those of the 9 years after prescribed burning in August 2019. Overall, litter floor leaf $\delta^{13}C$ in August 2019 or 9, 12 and 14 years after their last burning were significantly higher at the three sites of S1, S2 and S3 than those of corresponding litter floor leaf samples collected shortly before , 3 and 5 years after their last burning. This highlights that there would be increasing water limitation in the last 9 years at these sites due to the intensifying climate change as reported by Sun et al. (2010), Xu et al. (2014) and Succarie et al. (2020).

The current study indicated that litter floor leaf and twigs $\delta^{15}N$ were not fully recovered after 9 years of prescribed burning than those of the shortly before the last burning in July 2011 at S1, which is consistent with the findings from Abdullah (2016); Tahmasbian et al. (2019); and Wang et al. (2020). Similarity, litter floor leaf $\delta^{15}N$ were not recovered after 12-14 years of prescribed burning than those of the 3-5 years of the last prescribed burning in June 2011 at S2 and S3 respectively, which is consistent with the findings from Abdullah (2016); Tahmasbian et al. (2019); and Wang et al. (2020) who found that leaf $\delta^{15}N$ decreased after long-term prescribed burning for both sites of S2 and S3. The decreasing litter floor leaf $\delta^{15}N$ at the three sites of S1, S2 and S3 in August 2019 or 9, 12 and 14 years after their last prescribed burning, compared with those of corresponding samples collected shortly before, 3 and 5 years after the prescribed burning, may be partly due to the N deposition in the suburban areas and partly due to the decreasing N availability (lower $\delta^{15}N$) in the native forest ecosystem under intensifying climate change (Sun et al. 2010; Xu et al. 2014; Fu et al. 2020; Succarie et al. 2020; Liu et al. 2021).

5.5.2 Soil total N, total C, $\delta^{13}C$ and $\delta^{15}N$

5.5.2.1 Total N and total C

The current study indicated that soil total C was significantly different after 9-12 years of prescribed burning among all soil depths sampled at both study sites (S1 and S2), which is consistent with the findings from Abdullah (2016) and Wang et al. (2020). Meanwhile, our current study indicated that total C was significantly higher in the soil depth of 0-5 cm after 9-

14 years of prescribed burning than those of the shortly before and 3 and 5 years after the last burning in June 2011 at site of S1, S2 and S3. This is similar to the findings of Abdullah (2016) who found that soil total C at all study sites of S1, S2 and S3 were significantly greater in 0-5 cm soil depth. The reason may be the litterfall which may make a significant contribution to the variation in soil total C over the time since fire (TSF) and this contribution varied among the soil sampling depths or could be attributed to the soil properties and the climatic variables over the time since fire for all soil sampling depths (Abdullah 2016).

The current study indicated that the soil total N was significantly different after 9-12 years of prescribed burning in the soil at 0-5 cm sampled at both study sites (S1 and S2), which is consistent with the findings from Abdullah (2016) and Wang et al. (2020). Meanwhile, our current study indicated that total N was significantly higher in the soil at 0-5 cm after 9-14 years of prescribed burning than those of the shortly before, 3 and 5 years after the last burning in June 2011 at sites of S1, S2 and S3. This is similar to the findings of Abdullah. (2016) who found that soil total N at all study sites of S1, S2 and S3 were significantly greater in 0-5 cm soil. Overall, our study demonstrates that total C and total N in the soil profile (0-5, 5-10 and10-20 cm) were either fully recovered or higher for all the three sites after 9-14 years of prescribed burning.

5.5.2.2 Soil $\delta^{13}C$ and $\delta^{15}N$

At S1, soil $\delta^{13}C$ was significantly different ($P < 0.05$) in the soil at 5-10 and 10-20 cm after 9 year of prescribed burning compared with those of the corresponding variables shortly before last prescribed burning in June 2011(Fig. 6.4 and 6.5). This is similar to the findings from Abdullah (2016) who found that values of soil $\delta^{13}C$ was significantly affected by TSF at S1. The present study indicated that soil $\delta^{13}C$ was not recovered after 9 years of prescribed burning in August 2019 (winter-dry season) than those of the corresponding variables shortly before the last prescribed burning in June 2011 at S1, which is consistent with the findings from Abdullah (2016) and Tahmasbian et al. (2019). The current study showed that soil $\delta^{13}C$ was significantly higher in 10-20 cm depth than those of 0-5 and 5-10 cm depths at S1, which is consistent with the findings from Abdullah (2016), Tahsambian et al. (2019); and Wang et al. (2020) who found that soil $\delta^{13}C$ was significantly higher at 10-20 cm depth. Meanwhile, there were significant differences in soil $\delta^{13}C$ after 12-14 years after prescribed burning for all soil depths at S2 and S3 respectively. This is similar to the findings from Abdullah (2016) who found that soil $\delta^{13}C$ was significantly affected by TSF for all study sites (S1, S2 and S3). The current study showed that soil $\delta^{13}C$ was significantly higher in 10-20 cm depth than those of 0-5 and 5-10 cm depths at all study sites, which is consistent with the findings from Abdullah (2016), Tahsambian et al. (2019) and Wang et al. (2020).

In this study, soil $\delta^{15}N$ was significantly different ($P < 0.05$) in the soil at 10-20 cm after 9 years of prescribed burning at S1, which is consistent with the findings by Abdullah (2016). However, the present study indicated that soil $\delta^{15}N$ was not recovered after 9 years of prescribed burning than those of the corresponding variables shortly before the last prescribed burning in June 2011 at S1, which is consistent with the findings from Abdullah (2016) and Tahmasbian et al. (2019). The current study showed that soil $\delta^{15}N$ was significantly higher in 10-20 cm depth than those of 0-5 and 5-10 cm depths at S1, which is consistent with the findings from Abdullah (2016); Tahsambian et al. (2019); and Wang et al. (2020).

At S2 and S3, soil $\delta^{15}N$ was significantly different ($P < 0.05$) in the soil profile (0-5, 5-10 and 10-20cm) after 9 year of prescribed burning compared with those of the corresponding variables 3 and 5 years after the last prescribed burning. This is similar to the findings from Abdullah (2016) who found that soil $\delta^{15}N$ was significantly affected by TSF at S2 and S3. However, the present study indicated that soil $\delta^{15}N$ was not fully recovered 12-14 years after prescribed burning than those of the corresponding variables 3 and 5 years after the last prescribed burning at S2 and S3, which is consistent with the findings from Abdullah (2016) and Tahmasbian et al. (2019). Overall, soil $\delta^{15}N$ at the three sites was consistently lower 9, 12 and 14 years shortly before, 3 and 5 years after the prescribed burning compared with those of the corresponding samples collected about 9 years ago. This is consistent with our litter floor leaf $\delta^{15}N$ at the three sites, which is partly due to the N deposition in the suburban areas and partly due to the decreasing N availability in the forest ecosystem under intensifying climate change (Sun et al. 2010; Xu et al. 2014; Fu et al. 2020; Succarie et al. 2020; Liu et al. 2021). Overall, soil total N in the soil profile (0-5, 5-10 and10-20 cm) at all study sites of S1, S2 and S3 was fully recovered, even after 9-14 years of prescribed burning.

Multiple regression analyses were performed to investigate relationships among $\delta^{13}C$, $\delta^{15}N$, and TN at all study sites of S1 (9 years after burning); S2 (12 years after burning); and S3 (14 years after burning). Meanwhile, linear regression was positive correlation in the soil depth between $\delta^{13}C$ and $\delta^{15}N$, between $\delta^{13}C$ and TN at S1. Linear regression also showed a positive relationship in the soil profile (0-5, 5-10 and 10-20 cm) between $\delta^{13}C$ and TN, between $\delta^{13}C$ and $\delta^{15}N$ and between TN and $\delta^{15}N$ at S3.

5.6 Conclusion

The prescribed burning significantly affected total N and total C for all soil depths after 9-14 years of prescribed burning. Soil total C and total N were fully recovered after 9-14 years of prescribed burning, as well as litter floor leaf and twigs total C and total N were fully recovered 12-14 years after prescribed burning in the suburban Toohey forest ecosystem of subtropical Australia. Litter floor leaf and twigs total C and total N were not fully recovered 9 years after the prescribed burning at S1, compared with those of corresponding samples collected shortly before the last burn in June 2011. Litter floor leaf and twigs $\delta^{13}C$ 9, 12 and 14 years after prescribed burning at sites of S1, S2 and S3 were significantly higher compared with those of corresponding samples collected shortly before, 3 and 5 years after the prescribed burning, which would be partly due to the N deposition in the last 9 years as shown with lower $\delta^{15}N$ in litter floor leaf at all three sites and partly due to the decreasing N availability as shown in the lower soil $\delta^{15}N$. Overall, soil total C and total N were fully recovered 9-14 years after prescribed burning in Toohey Forest of subtropical native forest ecosystems.

5.7 Reference

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CHAPTER 6 Long-term impact of prescribed burning on responses of soil labile organic carbon and nitrogen pools in a Suburban Forest Ecosystem of Subtropical Australia

6.1 Abstract

Soil labile organic carbon (C) and nitrogen (N) pools play an important role in nutrient cycling, while fire can be a key driver of biogeochemical cycle, shaping ecosystem structure and functioning. However, how soil labile organic C and N pools respond to the long-term repeated prescribed burning is largely unknown. Prescribed burning can be an effective tool used in Toohey Forest management systems and played an important role in the development of plant communities in Australia. This study aimed to examine the long-term impact of prescribed burning on water extractable organic C (WEOC) and total N (WETN), hot water extractable organic C (HWEOC) and total N (HWETN), microbial biomass C (MBC) and N (MBN) at three sites (S1, S2, and S3) after 9-14 years of prescribed burning in a suburban forest ecosystem of subtropical Australia.

Three study sites were established in Toohey Forest, Queensland, Australia. The S1-B9 (9 years after prescribed burning), S2-B12 (12 years after prescribed burning), and S3-B14 (14 years after prescribed burning), were sampled for 0-20 cm soil profile in August 2019. Soil samples were collected at 0-5, 5-10 and 10-20 cm depths from each of the four plots at each of the three sites (S1, S2, and S3) to determine WEOC, WETN, HWEOC, HWETN, MBC and MBN, for the 9-14 years following the prescribed burning.

The soil WEOC and WETN, HWEOC and HWETN, MBC and MBN at the 0-5, 5-10 and 10-20 cm soil depths at Site 1 after 9 years of prescribed burning were also compared with those of the corresponding variables shortly before the last prescribed burning in June 2011.

Overall, after 9 years of prescribed burning at Site 1, HWEOC and HWETN at 0-5, 5-10 and 10-20 cm soil were recovered and indeed been higher than those of the corresponding soil depths shortly before the last burning. Similar findings of both HWEOC and HWETN were also noted for both sites of S2 and S3 after 12-14 years of prescribed burning. However, soil WEOC and WETN, and MBN were not recovered at three study sites (S1, S2, and S3) 9-14 years after prescribed burning. At S2, soil MBC was recovered at 5-10 and 10-20 cm depths after 12 years of prescribed burning, but not at soil depth of 0-5 cm. Nonetheless, after 14 years of prescribed burning at Site 3, MBC at 0-5, 5-10 and 10-20 cm soil was recovered and indeed higher than those of the corresponding soil depths 9 years after the last burning.

In conclusion, HWEOC, HWETN and MBC at the three study sites were recovered after 9 -14 years of prescribed burning, but WEOC, WETN and MBN were not fully recovered after 9 - 14 years of prescribed burning. This study highlights that under the current burning cycle of 8-12 years, there would be decreases in WEOC and WETN as well as MBN. With the intensifying climate change of warmer and drier futures, there would be accelerating decreases in the most labile C and N pools of WEOC and WETN in the Toohey forest ecosystems subjected to prescribed burning.

6.2 Introduction

Prescribed burning can be used as an effective tool for protecting both native and plantation forests from the risk of damaging wildfires (Guinto et al. 1999 a, b; 2000; Reverchon et al. 2012). The nutrients may be available temporarily after the fire, which can benefit plants, although nutrient leaching could also contribute to nutrient losses (Yildiz et al., 2010). Fire regimes influence terrestrial ecosystems and processes, which include vegetation distribution and structure, thus there would be the effect of fire on carbon (C) and nitrogen (N) cycling and climate (Hurteau and Brooks, 2011; Bai et al., 2012; Bai et al., 2015). Prescribed burning significantly affected soil dissolved organic C and N contents such as hot water extractable organic C and total N (HWEOC, HWEON), water extractable organic C and total N (WEOC and WEON), as well as soil microbial biomass C and N (MBC and MBN). Dissolved organic C and N pools have been measured in various extracts (cold water, hot water) to represent labile organic C and N pools (Chen et al., 2005). According to the results reported by Richards et al. (2012), WETN was increased in the soil of burnt plots in Australian tropical savannas than those of the unburnt plots, although the time since fire (TSF) did not have a significant impact on the soil N pools. Most researchers investigating fire impact on labile C and N have found that water soluble organic C and N were decreased after fire (Palese et al., 2004; Tokareva et al., 2011; Vergnoux et al., 2011). Other studies indicated that soil type, vegetation cover, climate, soil microbes, and analytical methods used played an important role in the variations in water soluble organic C and N after the fire (Hishi et al., 2004; Chen et al., 2005; Lu et al., 2011). According to the results reported by Muqaddas et al. (2019), the short- term impact of prescribed burning (2 years after burning) had significantly lower soil dissolved C and N in both hot and cold water and K_2SO_4 extracts, compared with those of the 4 years after burning and control treatments, but this was only significant for hot water and K_2SO_4-extracts, indicating high frequency fire (prescribed burning every 2 years) led to the reduction of soil labile C and N pools. Prescribed burning significantly affected dissolved organic C and N contents in all soil extracts except for dissolved organic C and total soluble N in cold water extracts after medium-term burning (Muqaddas et al., 2019).

Soil C and N pools can change and be affected after the frequent burning due to changing C and N inputs and outputs (Mastrolonardo et al., 2015; Näthe et al., 2018). Litter decomposition and nutrient recycling play an important role in regulating soil labile organic C and N pools (González-Perez et al., 2004; Li et al., 2017). These labile pools are very sensitive to the environmental changes and management practices compared with the total C and N pools due to the high background values of the total pools and great heterogeneity of soils (Goidts & van

Wesemael, 2007). Zhou et al. (2013) found that hot water extraction was preferred to predict mineralizable-C and mineralizable-N that have been measured in various extracts (cold water and hot water) to represent labile organic C and N pools (Chen et al., 2005; Ghani et al., 2003). Some studies indicated that soil properties were considered factors influencing soil microbial activities. It has been reported that lower soil moisture would limit soil microbial activities and might cause microbial death, further reducing soil N transformations (Aponte et al., 2010; Wang et al., 2013)

The reduction in soil MBC and MBN abundance shortly (2 weeks) after the fire (B0 site) in comparison to the pre-fire results observed in the present study may be due to microbial mortality by heating (Abdullah., 2016). According to the results reported by Abdullah. (2016), labile C and N pools increased in the first two weeks after the fire; therefore, the decline in soil microbial biomass could be mainly caused by the direct killing by heating from the fire. According to the results reported by Wang et al. (2012), a meta-analysis on the responses of soil properties to fire showed that soil total organic C and total N were reduced, but microbial biomass C and N increased after wildfire. Labile C and N pools play an important role in microbially mediated C and nutrient cycling processes during their role as energy source for microbes and are sensitive to changes in environmental conditions and management practices (Ghani et al., 2003).

The objective of this study aimed to quantify the long-term impacts of prescribed burning (9-14 years) on soil HWEOC and HWETN, WEOC and WETN, as well as MBC and MBN at the three sites in the Toohey Forest ecosystems of subtropical Australia, subjected to the periodically prescribed burning at an interval of ca. 8-10 years.

6.3 Material and methods

6.3.1 Site description:

A series of studies was conducted within Toohey Forest which is located 10 km south of the Brisbane, in South-East, Queensland, Australia (27°30′S, 135°E). The climate of the region is subtropical, with annual rainfall 859.2 mm and 613.4 mm for 2018 and 2019, respectively (Fig. 6.1). Annual minimum and maximum temperatures ranged between 16.5 and 27.0 °C in 2018, and between 17.1 and 27.8 °C in 2019 (Fig. 6.1). In general, summer is hot and wet, whereas winter is cool and dry (Catterall and Wallace, 1987).

Toohey Forest consists of four soil types, which are different from the other regions in the same forest, whereby these soil types consist of lithosols, red yellow podzolic, red earth, and alluvial. The lithosols soil, which contains both stony components and loam to silty loam, may be rich with humus. Whereas, the podzols are mainly less than 60 cm deep in soil, and are low in nutrients, and horizon-B is sandy clay, while horizon-A is sandy loam with coarser particles (Catterall & Wallace, 1987; Stock et al., 1987).

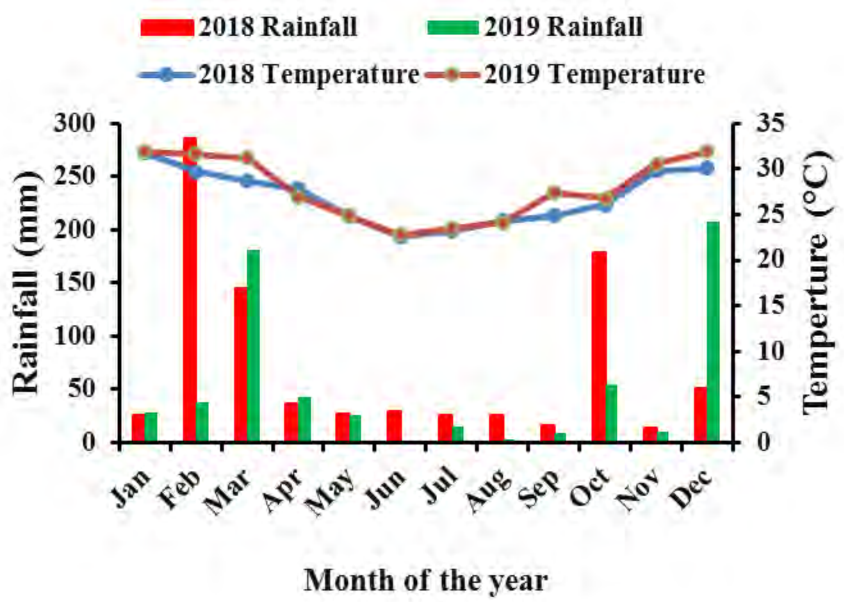

Figure 6.1 Monthly air temperatures and rainfall during the study period of 2018-2019 in Toohey Forest.

Before 1950s, the history of burning in Toohey Forest is unknown. During the period between 1950s and the early 1970s, individual fires might have burned across a large proportion of the area. In the early 1970's individual fires were confined to more localised areas, and also created a spatially heterogeneous landscape. In general, fires usually occur at the end of the dry season in September-October. From 1993, prescribed burning has been applied in the forests, including Toohey Forest, whereby there is an establishment of 27 blocks within the forest. Prescribed burning has been applied in frequencies between 4-5 years and 8-10 years (Catterall and Wallace 1987).

Three sites within Toohey Forest were used in this study. Each site had a different history of prescribed burns. Site 1 in Block 17B was last burned in August 2011 (9 years after prescribed burning), and earlier in March 2003. Site 2 in Block 24 of Toohey Forest was last burnt in April 2008 (12 years after prescribed burning), and earlier in September 1999. Site 3 in Block 17 was last burnt in May 2006 (14 years after prescribed burning), and earlier in August 1995.

6.3.2 Soil sampling

Three soil samples were randomly collected from each quarter in each plot at each study site. Three depths were sampled using a 2.5 cm diameter auger: 0-5, 5-10, and 10-20 cm. The soil samples were kept in separate plastic bags and transferred to the laboratory. The air-dried samples were ground (< 150 µm) and stored at room temperature. All samples were bulked from each quarter to make one sample at each depth per plot. In the laboratory, soil samples were passed through a 2-mm sieve. A sub-sample of the soil was air-dried, and the rest was refrigerated at 4°C and processed shortly after sampling. The concentrations of organic C and total N in the filtered solutions for hot and cold water, as well as MBC and MBN were determined using Shimadzu TOC-VCSH/CSN TOC/N analyser (Chen and Xu, 2005).

6.3.3 Soil analyses

6.3.3.1 Labile C and N pools

Labile C and N pools were measured as HWEOC and HWETN, WSOC and WSTN. HWEOC and HWETN were extracted by adding 25 ml water to 5.0 g of air-dried soil and the samples were incubated in a capped test tube at 70°C for 18 h. After the incubation, the samples were shaken by an end-to-end shaker for 1 hour followed by centrifuging at 10,000 rpm for 10 min. The suspensions were filtered through a Whatman 42 paper, then were filtered by a 33-mm Millex syringe driven 0.45-µm filter membrane. WSOC and WSTN were extracted using only cold water for air-dried soil: water ratio (1:5) and followed the same steps used for hot water extraction. The concentrations of organic C and total N in the filtered solutions were determined using Shimadzu TOC-VCSH/CSN TOC/N analyser (Chen and Xu, 2005).

6.3.3.2 MBC and MBN

The fumigation-extraction method was used on fresh soil and it is now widely used in heated soil because of its simplicity and accuracy (Brookes et al., 1985; Vance et al., 1987; Diaz-Raviña et al., 1992). Briefly, 10 g of fresh soil was fumigated with purified chloroform $CHCl_3$. Both fumigated and non-fumigated soils were extracted with 40 ml of 0.5 M K_2SO_4. After adding K_2SO_4 to soil samples, all were shaken for 30 min. The samples were then filtered through a Whatman 42 paper, then were filtered by a 33-mm Millex syringe driven 0.45-µm filter membrane. A Shimadzu TOC-VCPH/CPN analyser was used to determine total organic C (TOC) and total soluble N (TSN) in the fumigated and non- fumigated samples (Chen and Xu, 2005). The MBC and MBN were calculated as described by Vance et al. (1987), Brookes et al. (1985), and Jenkinson (1988).

6.3.4 Statistical analysis

Two-way repeated analysis of variance (ANOVA) was conducted to detect the differences in HWEOC, HWETN, WEOC, WEON, MBC and MBN with respect to the sampling times and soil depths. Statistical analyses were conducted with Statistix software (version 8). Direct comparison among experimental sites was not possible due to the fact that they had different properties and plant cover, so each site has been independently assessed. Differences among means of all variables over time were determined with post-hoc pairwise comparison using the Tukey HSD test at $P < 0.05$ level (Idol et al., 2002; Schafer and Mack, 2010; Köster et al., 2014).

6.4 Results

6.4.1 Long-term impact of prescribed burning on soil labile C and N pools

6.4.1.1 HWEOC and HWETN

HWEOC and HWETN were significantly different ($P < 0.05$) in the soil depth 0-5 cm after 9-14 years of prescribed burning at sites of S1, S2 and S3 respectively compared with those of the corresponding variables shortly before and 3 and 5 years after the last prescribed burning (Fig. 6.2 and 6.3), while there were no significant differences in HWETN in all soil depths (0-5, 5-10 and 10-20 cm) at S1 before and 9 years after prescribed burning .

The current study indicated that HWEOC and HWETN were significantly higher in soil depth 0-5 cm at sites of S1, S2 and S3, 9-14 years after prescribed burning than those of the shortly before and 3 and 5 years after the last prescribed burning (Fig. 6.2 and 6.3). At S1, there were no significant differences in HWEOC of 5-10 and 10-20 cm soil. Overall, HWEOC and HWETN in the soil profile (0-5, 5-10 and10-20 cm) were either fully recovered or higher for all the three sites after 9-14 years of prescribed burning (Fig. 6.2 and 6.3).

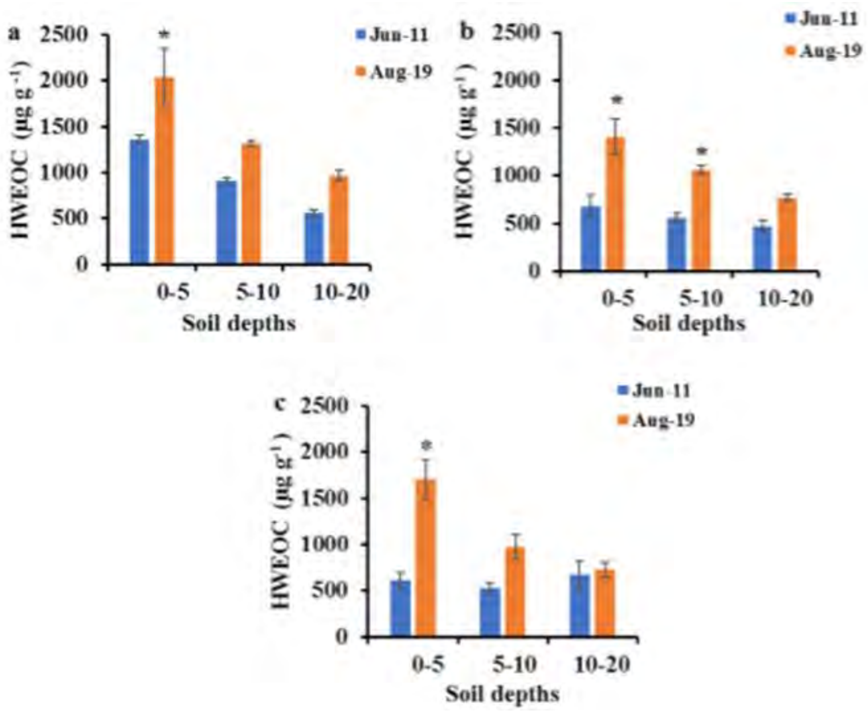

Figure 6.2 Long-term effects of prescribed burning on soil HWEOC shortly before the burning and 9 years after the burning at S1 (a), 3-12 years after the burning at S2 (b), and 5-14 years after the burning at S3 (c), for 3 soil depths (cm). Asterisk indicates significant differences between soil depths (cm) for each site at $P < 0.05$. Bars are means ± standard errors.

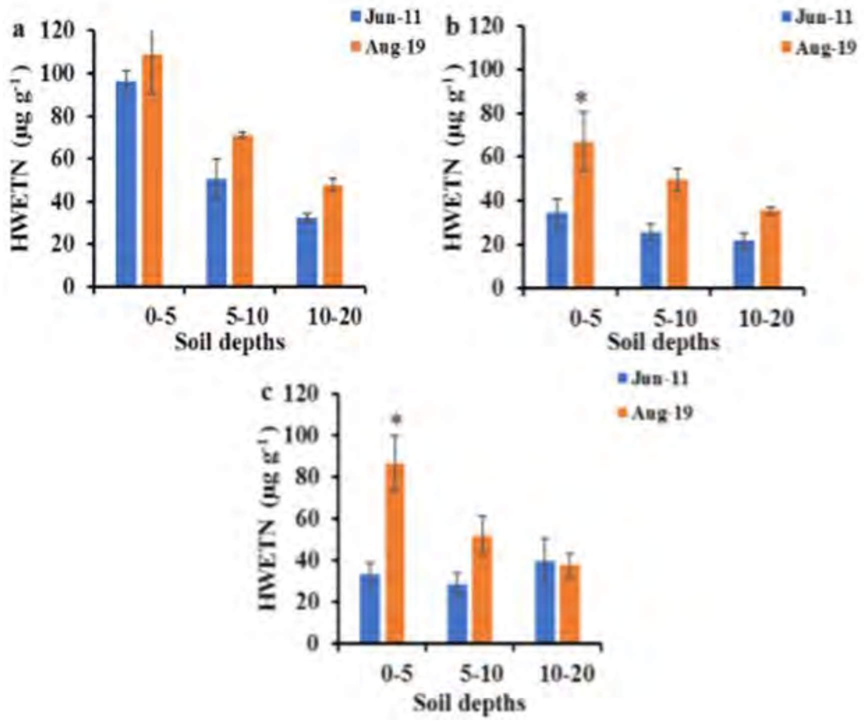

Figure 6.3 Long-term effects of prescribed burning on soil HWETN shortly before the burning and 9 years after the burning at S1 (a), 3-12 years after the burning at S2 (b), and 5-14 years after the burning at S3 (c), for 3 soil depths (cm). Asterisk indicates significant differences between soil depths (cm) for each site at $P < 0.05$. Bars are means ± standard errors.

6.4.1.2 WEOC and WETN

At S1, WEOC and WETN were significantly lower ($P < 0.05$) in the soil profile (0-5, 5-10 and 10-20cm) after 9 year of prescribed burning compared with those of the corresponding variables shortly before last prescribed burning in June 2011(Fig. 6.4 and 6.5). However, there were no significant differences in WEOC and WETN in the soil profile (0-5, 5-10 and 10-20cm) at S2, 3 and 12 years after prescribed burning. On the other hand, WEOC and WETN were significantly different ($P < 0.05$) only in soil depth of 10-20 cm after 5 and 14 years of prescribed burning at S3 (Fig. 6.4 and 6.5).

Overall, WEOC and WETN in the soil profile (0-5, 5-10 and10-20 cm) at S1 did not recover, even after 9 years of prescribed burning, and also those of 10-20 cm soil at S3 did not recover even after 14 years of prescribed burning (Figures 6.4 and 6.5).

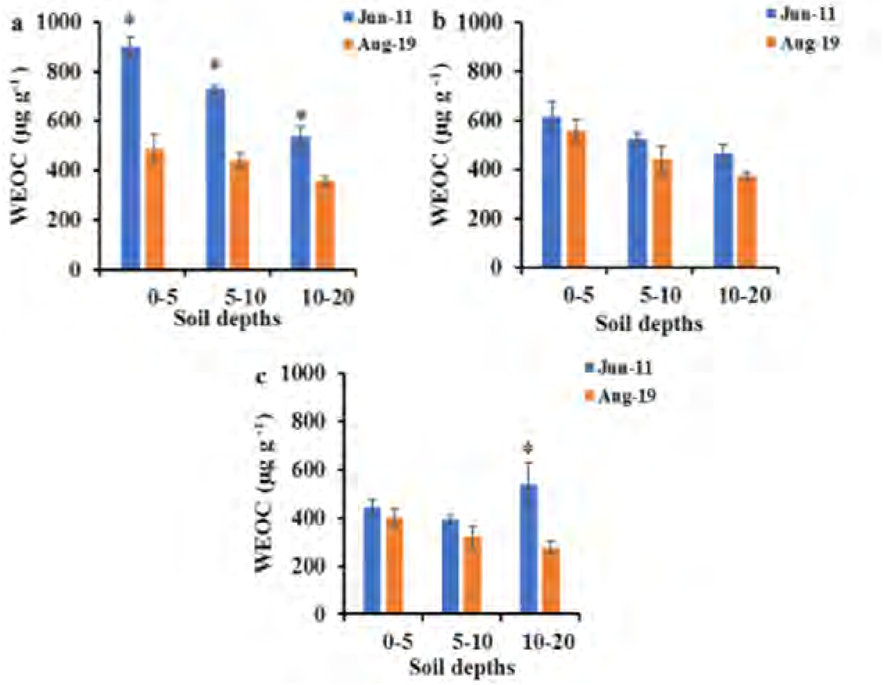

Figure 6.4 Long-term effects of prescribed burning on soil WEOC shortly before burning and 9 years after the burning at S1 (a), 3-12 years after the burning at S2 (b), and 5-14 years after the burning at S3 (c), for 3 soil depths (cm). Asterisk indicates significant differences between soil depths (cm) for each site at $P < 0.05$. Bars are means ± standard errors.

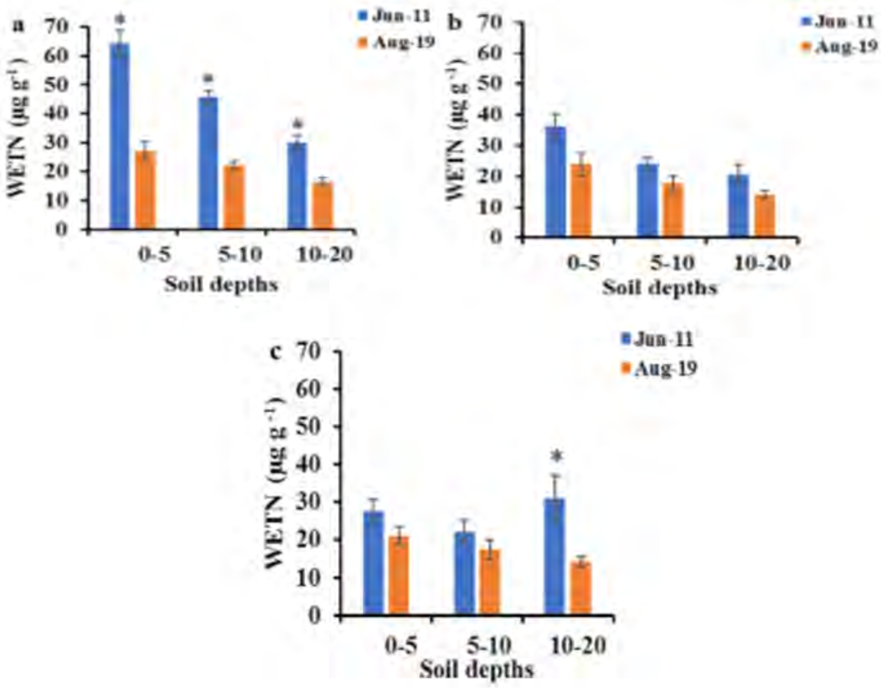

Figure 6.5 Long-term effects of prescribed burning on soil WETN shortly before burning and 9 years after the burning at S1 (a), 3-12 years after the burning at S2 (b), and 5-14 years after the burning at S3 (c), for 3 soil depths (cm). Asterisk indicates significant differences between soil depths (cm) for each site at P < 0.05. Bars are means ± standard errors.

6.4.2 Long-term impact of prescribed burning on soil MBC and MBN

There were no significant differences in MBC in the soil profile (0-5, 5-10 and 10-20cm) for all the three sites of S1, S2 and S3 after 9-14 years of prescribed burning, compared with those of the corresponding variables shortly before and 3-5 years after the last prescribed burning (Fig. 6.6).

However, there were significant differences (P < 0.05) in MBN in the soil depths of 0-5 and 5-10 cm after 9-12 years of prescribed burning at S1 and S2 respectively, compared with those of the corresponding variables shortly before and 3 years after last prescribed burning in June 2011(Fig. 6.7). No significant differences were found in MBN in the soil profile (0-5, 5-10 and 10-20 cm) at S3, 5 and 14 years after prescribed burning (Fig. 6.7).

Overall, soil MBC was fully recovered at sites of S1 and S2 in the soil depths of 0-5 and 5-10 cm and for all soil depths for S3 after 9-14 years of prescribed burning, while MBN in the 0-5 and 5-10 cm soil at S1 and MBN in the 0-5 cm soil at S2 were not yet recovered after 9-12 years of prescribed burning. However, MBN in the soil profile (0-5, 5-10 and 10-20 cm) at S3 did not differ between 5 and 14 years after prescribed burning.

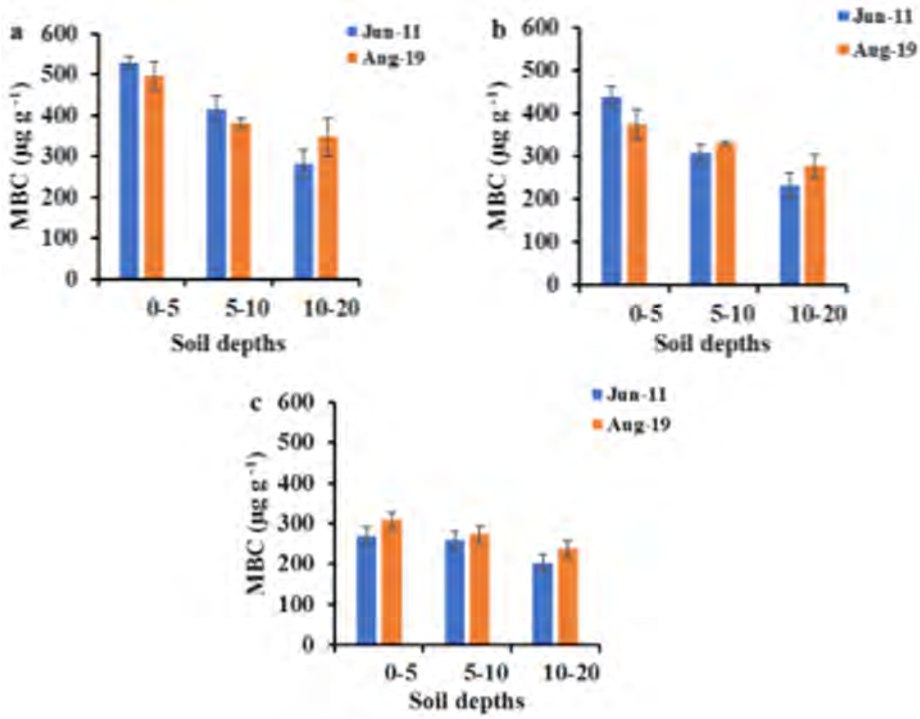

Figure 6.6 Long-term effects of prescribed burning on microbial biomass C (MBC) shortly before burning and 9 years after the burning at S1 (a), 3-12 years after the burning at S2 (b), and 5-14 years after the burning at S3 (c), for 3 soil depths (cm).

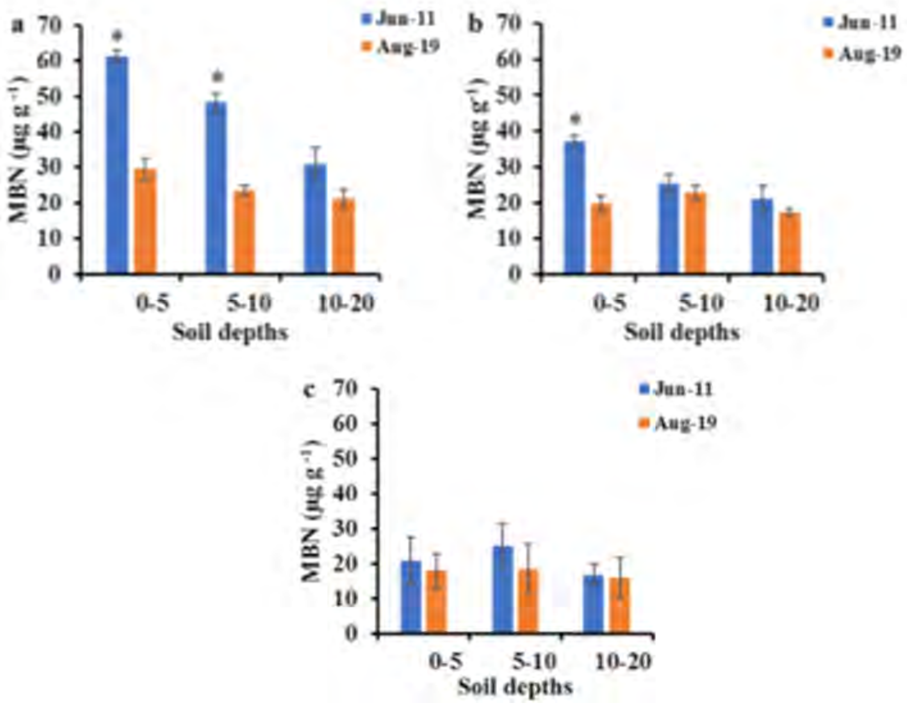

Figure 6.7 Long-term effects of prescribed burning on microbial biomass N (MBN) shortly before burning and 9 years after the burning at S1 (a), 3-12 years after the burning at S2 (b), and 5-14 years after the burning at S3 (c), for 3 soil depths (cm). Asterisk indicates significant differences between soil depths (cm) for each site at $P < 0.05$. Bars are means ± standard errors.

6.5 Discussion

6.5.1 Long-term impact of prescribed burning on the sizes of soil labile C and N pools

Soil labile C and N pools, such as HWEOC, HWETN, WEOC, WETN, MBC and MBN were reliable and sensitive indicators of soil organic matter (SOM) pools in response to the prescribed burning. Hot water extraction methods were used in this study to determine the easy water dissolved C and N pools over the time since fire (TSF). In the present study, the impacts of the prescribed burning on soil labile C and N pools persisted for up to 9-14 years for all the three soil depths, although the effect decreased with increasing soil depth. After 9 years of prescribed burning at S1, HWEOC and HWETN at 0-5, 5-10 and 10-20 cm soil were recovered and indeed higher than those of the corresponding soil depths shortly before the last burning reported by Abdullah (2016). Similar findings of both HWEOC and HWETN were also noted for both sites of S2 and S3 after 12-14 years of prescribed burning compared with those reported by Abdullah (2016) and Wang (2020a), which is consistent with the findings from Ghani et al. (2003). The reason would be that the HWEOC and HWETN were extracted under higher temperature, which could include a broader spectrum of C and N compounds compared to room temperature water, their responses to burning could be less sensitive (Wang et al., 2020a). Therefore, determining the most suitable extraction method is important to predict the potential changes and dynamics of labile C and N pools under specific management practices in different ecosystems in a suburban forest of subtropical Australia. Some studies been explained that variations in soluble C and N pools following prescribed burning may be due to different soil types, vegetation covers, climates, soil microbes, and analytical methods used (Hishi et al., 2004; Lu et al., 2011; Guigue et al., 2015).

The present study indicated that WEOC and WETN were significantly lower in soil depth of 0-5 cm at sites of S1 and S2, 9-12 years after prescribed burning than those of shortly before and 3 years after the last prescribed burning, but not at site of S3. Soil WEOC and WETN were not recovered at three study sites (S1, S2, and S3) 9-14 years after prescribed burning than those of the shortly before and after 3 and 5 years of the prescribed burning, which is consistent with the findings from Muqaddas et al. (2015) who found that WEOC and WETN decreased due to decreases in the quantity and quality of litter floor (relatively low C and N contents) and greater forest floor consumption by fire maybe decrease C and N inputs into the soil in more frequently burnt plots. Our research findings that WEOC and WSTN in the soil profile (0-5, 5-10 and 10-20 cm) at S1 did not recover even 9 years after the prescribed burning. This is consistent with the findings of Wang et al. (2020 a) that WEOC and WSTN were the most sensitive and useful indicators of soil labile C and N pools in response to the long-term impact of prescribed burning in Toohey Forest ecosystems.

The current study indicated that the lowest WEOC and WETN varied across sites. However, WEOC and WETN decreased with increasing of the soil depth, and it is clearly noted in 10-20 cm of soil depth which occurred in winter for all sites of S1, S2, and S3. This is similar to the findings of Abdullah. (2016) who found that the impact of the time since fire on WEOC and WETN decreased with increasing soil depth at site 1(B0). The reason may be that WEOC and WETN in the surface mineral soil were closely associated with litter floor or an organic layer. Thus, the recovery of the water-extractable C and N pools would depend on the recovery of litter floor and forest productivity (Muqaddas et al., 2019; Wang et al., 2020b). Additionally, lower dissolved C and N may also be associated with higher leaching losses of dissolved C and nutrients (Muqaddas et al., 2019). Post burning C and N losses in soil could be ascribed to both accelerated mineralisation due to destabilisation of soil aggregate by heat (Jian et al., 2018) and lower dissolved organic C and dissolved total N associated with the diminished forest floor or organic layer caused by burning (Kawahigashi et al., 2011). However, WEOC and WETN were sensitive indicators of the prescribed burning impacts.

The lengthy recovery of soil WEOC and WETN indicated that prescribed burning had profound impacts on soil C and N cycling in Toohey Forest. The burning cycle probably needs to be extended to allow the full recovery of soil WEOC and WETN. In general, soil WEOC and WETN were not recovered at the three study sites (S1, S2, and S3) 9-14 years after prescribed burning. Meanwhile, HWEOC and HWETN were recovered at three study sites (S1, S2, and S3) 9-14 years after prescribed burning in the suburban Toohey Forest ecosystem of subtropical Australia. This has confirmed our hypotheses that the labile soil C and N pools could be affected 9-14 years after prescribed burning in the suburban Toohey Forest ecosystem of subtropical Australia.

6.5.2 Long-term impact of prescribed burning on soil microbial biomass carbon (MBC) and nitrogen (MBN)

Our study indicated that no significant differences were found in MBC for all soil depths at the three study sites of S1, S2 and S3, 9-14 years after prescribed burning, which is consistent with the findings from Abdullah (2016) who found there were no significant differences in MBC before and 3-5 years after prescribed burning at Sites of S1, S2 and S3. Conversely, after 12 years of prescribed burning at Site 2, MBC at 5-10 and 10-20 cm soil was recovered and indeed been higher than those of the corresponding soil depths after 3 years of the last burning. This is contradicting the findings from Dumontet et al. (1996) who found that MBC was decreasing in subsequent years up to 11 years. However, after 14 years of prescribed burning at Site 3, MBC at 0-5, 5-10 and 10-20 cm soil were recovered and indeed higher than those of the corresponding soil depths 5 years after the last burning.

The current study demonstrated that MBN was significantly lower in soil depths of 0-5 and 5-10 cm at S1, and 0-5 cm at S2. Meanwhile, there was no significant difference in MBN among all soil depths at S3, which is consistent with the findings from Abdullah (2016); and Wang et al. (2020) who found that no significant differences in MBN were found in all soil depths at S3. The current study, after 9 years of prescribed burning at Site 1, MBN at 0-5, 5-10 and 10-20 cm soil were not recovered compared with those of the corresponding soil depths shortly before the last burning reported by Abdullah (2016). Similar findings of MBN have also been noted for both sites of S2 and S3 after 12-14 years of prescribed burning compared with those reported by Abdullah (2016) and Wang (2020). We initially hypothesised that MBC and MBN could be affected 9-14 years after prescribed burning in the suburban Toohey Forest ecosystem of subtropical Australia. In support of this hypothesis, we found that time since the fire has a significant impact on MBC after 12- 14 years, but not at S1.

6.6 Conclusion

Soil HWEOC and HWETN were recovered after 9-14 years of prescribed burning. MBN of 0-5 and 5-10 cm soil at S1 did not recover even 9 years after the prescribed burning. Both WEOC and WETN in the surface soil were the most sensitive indicators of fire disturbances on soil labile C and N pools in this natural forest, compared with those of HWEOC, HWETN, MBC and MBN. Both WEOC and WETN did not recover even 9 years after prescribed burning at S1. Similar trends of WEOC and WETN were also noted for sites of S2 and S3 12-14 years after prescribed burning in Toohey Forest ecosystem of subtropical Australia.

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CHAPTER 7 General Conclusions and Future Studies

7.1 General Conclusions

7.1.1 BNF, WUE and plant growth of acacia species after long-term of prescribed burning

In Study 1, the long-term impacts of prescribed burning on biological nitrogen fixation (BNF), water use efficiency (WUE) and plant growth of understory acacia species were investigated in a suburban native forest of subtropical Australia. Young acacia plants were more active in BNF than older plants, with the BNF peaked between 6 and 8 years after prescribed burning, hence highlighting the role of BNF in improving plant WUE and subsequently tree growth in the suburban native forest. The BNF of understory acacia species played an important role in the recovorey of soil carbon (C) and nitrogen (N) pools in the 6-14 years after the prescribed burning in the Toohey Forest ecosystems of subtropical Australia.

7.1.2 BNF, WUE and growth of understory acacia species after biochar addition

In Study 2, the fuel reduction prescribed burning is employed every 8-10 years in suburban native forest ecosystem of subtropical Australia creating large variations in the biochar distributions from nil biochar in the patches of unburnt areas to the biochar / ash hot spots (more than 10 t ha^{-1} biochar per ha) after each burning, but the biochar impacts on soil C and N pools as well as BNF, WUE and plant growth of understory acacia species are not well understood under the field settings. The short-term impacts of biochar application rates (0, 5 and 10 t ha^{-1}) on the BNF, WUE and plant growth of two understory acacia species (*A. leiocalyx* and *A. disparimma*) as well as soil C and N pools were studied 1, 2, 3, 6 and 9 months after the biochar application in the Toohey forest of subtropical Australia. Biochar application contributed to improve foliar total C and total N in winter August 2019 (3 months after biochar applied) and autumn March 2020 (6 months after biochar application), as well as foliar $\delta^{13}C$ in spring November 2019 compared with those of control 6 months after biochar application. Foliar $\delta^{15}N$ and BNF were increased in winter July 2019 and autumn March 2020 for biochar rates of 5 t ha^{-1} and 10 t ha^{-1} respectively compared with those of the control. Our study highlights the role of BNF in improving plant WUE and subsquatly tree growth in the short-term after biochar application to the understory acacia plant of *A. leiocalyx* and *A. disparimma* in the suburban native forest ecosystem.

7.1.3 Litter floor leaves and twigs and soil total C, total N, $\delta^{13}C$ and $\delta^{15}N$ recovery after long-term of prescribed burning

In Study 3, the long-term impacts of prescribed burning on forest litter floor and soil total C and total N, $\delta^{13}C$ and $\delta^{15}N$ in the soil profile of 0-5, 5-10, and 10-20 cm at Site 1 (S1, 9 years after burning), Site 2 (S2, 12 years after burning) and Site 3 (S3, 14 years after burning) in the suburban Toohey Forest ecosystem of subtropical Australia. In this suburban forest ecosystem, the environmental variables such as rainfall and temperature were significant drivers of litter floor leaves and twigs production in longer term after the fire. Firstly, the study concluded that litter floor leaves and twigs total C, total N and $\delta^{15}N$ were not recovered 9 years after prescribed burning at S1, but litter floor leaves and twigs total C, total N and $\delta^{15}N$ were recovered 12-14 years after prescribed burning at S2 and S3. Secondly, this study concluded that litter floor leaves and twigs $\delta^{13}C$ were recovered 9 years after prescribed burning. Finally, the time since fire (TSF) explained the soil total C, total N, $\delta^{13}C$ and $\delta^{15}N$ recovery 9-14 years after prescribed burnung. This is consistent with the our hypothesis that soil C and N pools as well as forest litter floor would gradually be recovered after 9-14 years of prescribed burning in the suburban Toohey Forest ecosystem; and that forest litterfall and litter floor recovery would play important roles in the recovery process of soil C and N pools after 9-14 year of prescribed burning.

7.1.4 Soil labile C and N pools, MBC and MBN after long-term prescribed burning

In Study 4, the long-term impacts of prescribed burning on soil labile C and N pools, such as HWEOC, HWETN, WEOC, WETN, MBC and MBN were evaluated 9-14 years after the prescribed burning at three sites in the Toohey forest of subtropical Australia. The key conclusions are:

(1) Soil HWEOC and HWETN were recovered after 9-14 years of prescribed burning; (2) Soil HWEOC, HWETN, WEOC and WETN in the surface soil were the most sensitive indicators of soil labile C and N pools in response to the prescribed burning; (3) WEOC and WETN did not recover even 9-14 years after prescribed burning at the three sites; (4) MBC was fully recovered in the soil depths of 0-5, 5-10 and 10-20 cm 12-14 years after prescribed burning at S2 and S3; and (5) soil MBN was not fully recovered after 9-14 years of prescribed burning.

7.2 Future Studies

This study has developed a better understanding of the effect of prescribed burning on BNF, WUE, tree growth, C and N pools and their dynamics in the suburban forest ecosystem of subtropical Australia. The information could help the managers of this ecosystem to review the fire regimes implemented in this ecosystem. The following future studies are highly recommended:

- Field trials need to be undertaken for the longer periods on the full potential of using biochar to improve the BNF, WUE, and growth of understory acacia species as well as soil microbial, soil C and N pools in the suburban native forest ecosystems;
- This study extends the knowledge of BNF, WUE and tree growth of understory acacia species in response to the biochar rates in Toohey Forest after long term and will serve as a base for future studies concerning applying biochar on Toohey Forest;

- Further studies need to be undertaken for evaluating the interactive impacts of intensifying climate change with a warmer and drier climate as well as increasing climate extremes and prescribed burning with increasing intensify on the BNF, WUE and growth of understory acacia species as well as soil C and N pools in the suburban native forest ecosystem of subtropical Australia;
- Quantification of long-term impacts of prescribed and unburnt N deposition on C and N cycling in the context of intensifying climate change and increasing bushfires in the suburban native forest ecosystems of subtropical Australia.